Subglottic host defence in prolonged intubation

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Abstract

Ventilator-associated pneumonia (VAP) is pneumonia developing 48-hours or more after tracheal tube insertion and mechanical ventilation. VAP is associated with significant morbidity, mortality and costs. The excessive use of antimicrobial agents in VAP treatment and prevention is contributing to antibiotic resistance. Introduction of the tracheal tube is thought to be a key contributing factor in VAP development, providing a conduit for invasion of the airways with virulent microorganisms. The subglottis (the region located immediately below the vocal cords, and directly above the tracheal tube cuff) is key to VAP development. The subglottis in intubated ICU patients has not previously been characterised, and therefore therapeutic targets for preventing VAP are limited.

I have demonstrated the presence of a diverse population of oropharyngeal commensal bacteria in the subglottic mucus of newly intubated, non critically ill control patients. In a cohort of ICU patients, intubated for four or more days, there was a less diverse population of more virulent bacterial and fungal organisms.

I have also established that the subglottic mucus of long-term ventilated ICU patients, compared to newly intubated controls, is more viscous, has increased neutrophil counts, and increased concentrations of mucins, cytokines and neutrophil proteases.

I was able to demonstrate *in vitro* that neutrophils isolated from the blood of healthy volunteers, when incubated with purified ICU-derived mucin, showed impaired chemotaxis, phagocytosis and bacterial killing, which was reversible upon treatment with a mucolytic agent. These effects were concentration dependent and only found at mucin levels found in ICU patients, not controls.

Finally I describe the establishment of a successful method to culture primary human subglottic epithelial cells, at air-liquid interface (ALI). This provides the first *in vitro* model of this region of the airway. This model suggests that high concentrations of purified ICU-derived mucin applied to ALI cultures, may induce bacterial growth and invasion of the epithelial membrane.

Dedication

I would like to dedicate this work to my wife and to my parents.

Acknowledgements

I would like to acknowledge the help and support of my supervisors, Professor Janet Wilson, Dr Chris Ward, Professor Jeff Pearson and Professor John Simpson, for their continued support, research vision and mentorship.

I would also like to acknowledge the help of Dr Bernard Verdon and the rest of the Pearson/Ward lab group who assisted me with primary cell cultures, Ussing chamber work and mucus purification. The Simpson lab group, in particular Dr Jon Scott who performed the blood derived neutrophil purification and Dr Michael Mather who assisted with several of the neutrophil assays. Dr Stephen Wright, Carmen Scott, Verity Calder and Craig Samson in the Newcastle Critical Care Research team for their help with ICU recruitment. Jane White in the surgical waiting list department for her help with theatre patient recruitment. Professor John Perry and his team at the Freeman Hospital microbiology lab, who performing the microbiological assessment of mucus samples. Professor Stephen Cummings and Dr Andrew Nelson, of Northumbria University, who performing the bacterial and fungal gene profiling. Dr Faye Cooles for assisting with the MSD assays. Kasim Jiwa of the William Leech lab, who performed the differential cell counts of mucus and blood samples.

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Declaration

The candidate (Jason Powell) confirms that the work submitted is his own work and that appropriate credit has been given where reference has been made to the work of others.

The work in this thesis was performed from August 2014 to July 2017. All work was performed within the Institute of Cellular Medicine, Institute for Cell and Molecular Biosciences, the Newcastle Upon Tyne Hospitals laboratories or at Northumbria University.

No part of this thesis has been submitted for the award of any other degree.

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List of abbreviations

ALI	air-liquid interface
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
BEGM	bronchial epithelial growth medium
CBA	cytometric Bead Array
CD	cluster of differentiation
CF	cystic fibrosis
CFU	colony forming units
COPD	chronic obstructive pulmonary disease
DAB	3,3'-diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPAS	diastase-resistant periodic acid-Schiff
dsDNA	double stranded DNA
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
fMLF	formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte-macrophage colony-stimulating factor
GP	general practitioner
HBSS	Hanks balanced salt solution
HBSS-	Hanks balanced salt solution without Ca^{2+} and Mg^{2+}

HBSS+	Hanks balanced salt solution with Ca^{2+} and Mg^{2+}
ICU	intensive care unit
IL	interleukin
IMDM	Iscove's modified dulbecco's medium
IMS	industrial methanol spirits
IR	infrared
ITS	internal transcribed spacer
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MFI	median fluorescence intensity
MRSA	methicillin resistant *S. aureus*
MSD	meso scale discovery
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in Research
NET	neutrophil extracellular trap
NGS	next generation sequencing
NHS	national health service
NMDS	non-metric multidimensional scaling
NSAIDs	non steroidal anti inflammatory drugs
OTU	operational taxonomic units
PA	*P. aeruginosa*
PAF	platelet activating factor
PAS	periodic acid-Schiff

PBS	Dulbecco's phosphate-buffered saline
PCR	polymerase chain reaction
PIS	patient information sheet
PMN	polymorphonuclear cells
PSEC	primary subglottic epithelial cells
RCT	Randomised control trial
REC	research ethics committee
RNA	ribonucleic acid
rRNA	ribosomal RNA
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute-1640
SEM	scanning electron microscopy
SSD	subglottic suction drainage
TBS	tris-buffered saline
TEM	transmission electron microscopy
TER	trans-epithelial resistance
VAP	ventilator associated pneumonia

Chapter 1: Introduction

1.1 Ventilator-associated pneumonia

1.1.1 Ventilator-associated pneumonia (VAP) overview

Tracheal intubation and mechanical ventilation is a life-saving procedure in critical illness and respiratory failure (1). An adverse consequence of this intervention is the development of ventilator-associated pneumonia (VAP) (2). VAP is defined as pneumonia developing 48 hours or more after tracheal intubation and mechanical ventilation (1). It is the most common hospital-acquired infection in the intensive care unit (ICU) (3). The reported incidence of VAP varies, depending on the diagnostic criteria, but between 9 and 27% of patients in the ICU develop VAP (4). It also remains the most commonly fatal infection in critical illness, with an overall attributable mortality of 13%, and is associated with a significantly increased length of ICU stay and cost (5, 6).

VAP is frequently associated with antibiotic-resistant pathogens (7-9), particularly late onset pneumonia, developing after four days (1, 9). Furthermore VAP is estimated to account for half of all antibiotics given in ICU (10). The individual organisms identified from VAP bronchoalveolar lavage samples are highly variable, depending on several factors, including the geographical location and patient group. Gram positive bacteria such as *Staphylococcus aureus*, including methicillin resistant *S. aureus* (MRSA), represent a large proportion of isolates in VAP. Other VAP-causing pathogens include aerobic Gram negative bacilli such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Escherichia coli, Enterobacter* species and *Acinetobacter* species (7-9, 11)

Prevention of VAP is focused around three key strategies; reduced colonisation of the aerodigestive tract with pathogenic bacteria; prevention of aspiration of infective contents into the alveolar regions of the lung; and limiting the duration of mechanical ventilation (1). Introduction of various combinations, or bundles, of interventions into international ICU guidelines has been associated with some recent reduction in VAP rates (12, 13).

1.1.2 The pathogenesis of VAP

The key causative factor in VAP development is the presence of the tracheal tube (endotracheal or tracheostomy tube) (Figure 1) (14). The tube provides a conduit for invasion of the lower airway with virulent microorganisms (1, 14, 15). It is postulated that infected secretions gravitationally pool above the tracheal tube cuff, in the subglottic region (16). These infected secretions, on or immediately above the tracheal cuff, can then be micro-aspirated around the tracheal cuff, or pass en masse into the lower airway when the cuff is deflated or removed (1, 14, 15). The tracheal cuff further facilitates lung infection through prevention of these aspirated secretions being cleared from the lower airways, acting both as a physical barrier and in preventing an effective cough reflex. These organisms are then at liberty to infect the lower airways of compromised ICU patients, culminating in VAP development (Figure 2) (1, 14, 15).

Longitudinal studies of ventilated ICU patients have previously demonstrated a strong correlation between oropharyngeal or tracheal microbiology samples and organisms subsequently found in the lungs (11, 17, 18). Berdal *et al* (11) in a small ICU cohort demonstrated correlation between the organisms recovered from oropharyngeal swabs, tracheal aspirates (below the cuff) and lung lavage samples at various intervals up to 14 days after intubation. In a further small series specifically looking at VAP derived microbiology, Gil-Perotin *et al* (17) compared the microbiology findings of ICU tracheal aspirates and found a strong correlation with VAP lavage cultures taken some days later. In a much larger study Pirracchio *et al* (18) demonstrated strong correlation between upper airway samples at admission and later VAP recovered microorganisms. All these studies have significant limitations, furthermore the correlation of organisms at these different airway subsites does not prove migration from the upper to the lower airway.

The origin of these VAP-causing organisms is much debated in the literature. Some suggest that deterioration of oral hygiene, with persistent mouth opening and poor oral care, leads to pathogenic oral colonisation. However interventions to prevent this have had variable effects (19). Reflux and aspiration of infected stomach contents has also been suggested. The extensive use of acid suppression therapy in critical illness, to prevent gastric ulcer formation, is thought to generate overgrowth of gastric bacteria,

which are subsequently aspirated into the airway (20). Other potential reservoirs that have been postulated include the oropharynx and paranasal sinuses (21). There is also the unknown contribution of medical devices and healthcare workers, introducing organisms into the instrumented airway (21). Biofilm formation is also frequently found on the tracheal tube cuffs of ventilated patients and likely contributes to infection (17, 22).

Over recent years numerous strategies have been introduced with the aim of preventing VAP. These include selective digestive decontamination, use of oral mouthwashes and tooth brushing, changes in body positioning, coated endotracheal tubes and subglottic suction drainage (SSD) (16, 19, 21, 23-25). The aim of all these interventions has been to reduce upper airway bacterial load (21, 25). The most effective of these interventions has been the use of SSD (24). In a recent meta-analysis of SSD and VAP incidence by Mao *et al* (24) the authors identified twenty randomised control trials (RCTs). Of these the authors acknowledged only four high-quality (low-bias) trials with 901 pooled participants (spread relatively equally between the studies). The studies included varying drainage techniques (continuous or intermittent), and had slightly differing criteria for VAP diagnosis. The participants in all studies were from general ICU populations and did not specifically investigate high risk VAP patients (such as burns or trauma patients (2)). They demonstrated that, overall SSD was associated with a significantly reduced incidence of VAP (relative risk 0.54, 95 % confidence interval 0.40–0.74). However, interestingly, subgroup analysis demonstrated no significant differences in late onset VAP, ICU mortality, hospital mortality, or ICU length of stay. These secondary outcome measures were however analysed with the inclusion of all twenty studies (including high-bias studies), furthermore specific outcomes were only available in certain studies, introducing further potential bias.

1.1.3 The subglottic region and VAP development

The anatomical and clinical definitions of the laryngeal sub-sites vary in the literature, however the most commonly used definition of the subglottis is from the inferior arcuate line of the vocal folds to the lower boarder of the cricoid cartilage (26). The subglottis is the region immediately above the tracheal tube cuff in intubated patients.

Despite the fact that almost all studies implicate the subglottis in VAP development and identify SSD as an effective intervention to prevent VAP, very little is known about specifically how this part of the airway contributes to the pathophysiology of VAP (1, 14, 15, 24). SSD involves the use of specially designed tracheal tubes that have an additional port to allow aspiration of subglottic secretions by clinical staff. It is hypothesised that this reduces the burden of infected secretions above the endotracheal tube cuff, reducing the reservoir of pathogenic organisms for aspiration (14). Recent meta-analyses of large-scale trials have demonstrated that SSD use is associated with lower VAP rates (27, 28). However, these reviews were unable to demonstrate a reduction in the duration of mechanical ventilation, length of ICU stay, antibiotic usage or importantly mortality (27). There are also concerns regarding mucosal injury and subsequent negative outcomes with SSD, that require further investigation (28). It is likely that the limitations of VAP interventions are due to a lack of understanding of VAP pathophysiology, in particular the role of the subglottis and important host defences in this region. Given that the subglottic region is a respiratory epithelial sub-site, host defences are primarily the mucus layer, mucociliary clearance and resident and recruited immune cells (29).

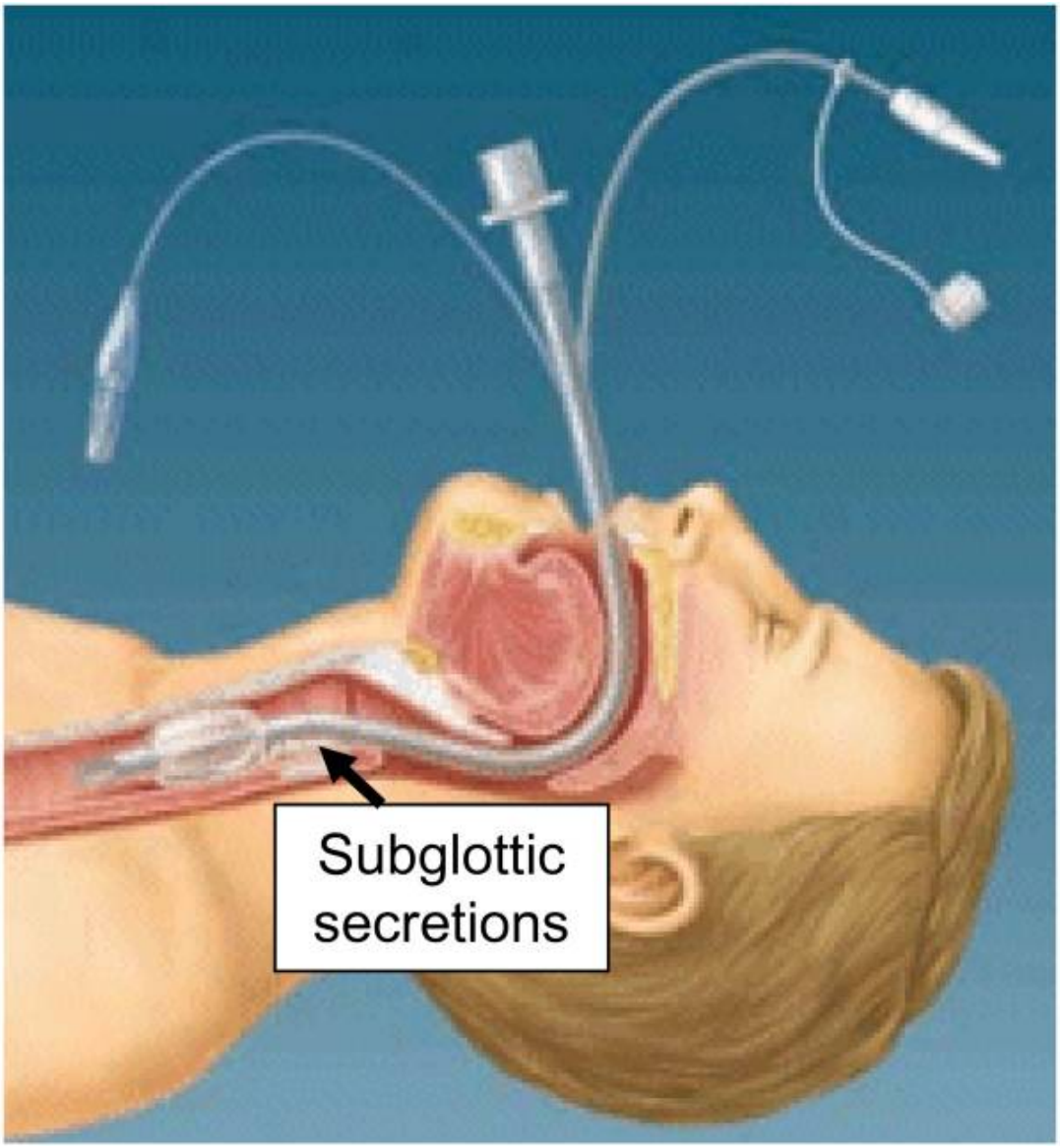

Figure 1.1 - Diagram of endotracheal tube placement.

Adapted from The tracheal tube: gateway to ventilator-associated pneumonia Zolfaghari *et al* 2011 (14).

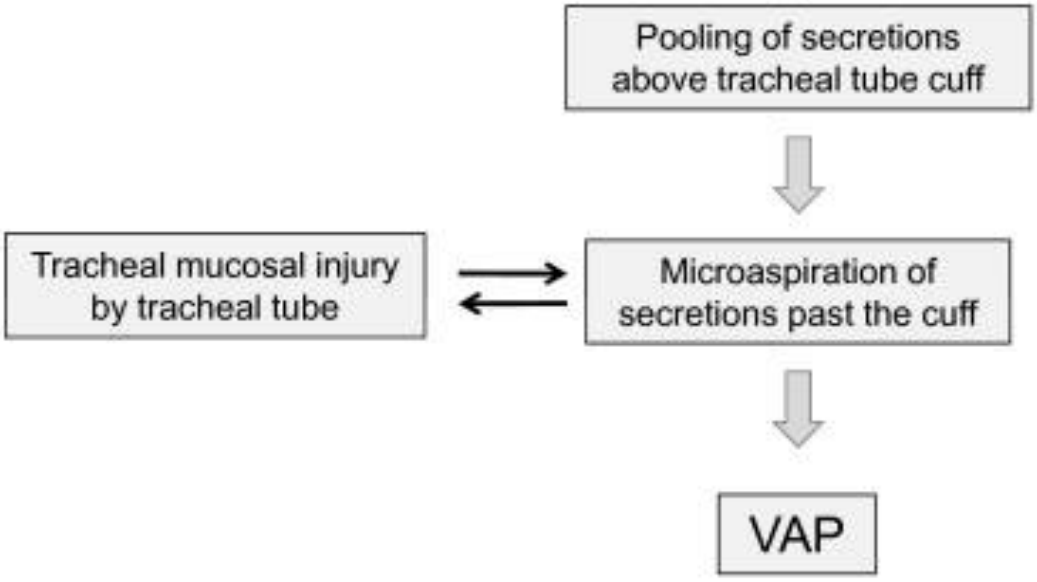

Figure 1.2 - The pathogenesis of VAP development.

Adapted from The tracheal tube: gateway to ventilator-associated pneumonia Zolfaghari *et al* 2011 (14).

1.2 Innate immunity

1.2.1 Innate immunity overview

The normal host defence response has been artificially divided into innate (non-specific) and adaptive (specific) immune responses. The innate immune system is an evolutionarily ancient part of the host defence mechanism (30). Its role is to protect the host from insults related to microbes or chemicals. It consists of structural defences, such as epithelial mucosal membranes, antimicrobial molecules, the complement system, as well as immune cells. These include neutrophils, monocytes, macrophages, eosinophils, mast cells, and natural killer cells (31).

1.2.2 Innate immunity in critical illness and VAP

Critical illness usually constitutes an acute illness or injury resulting in organ dysfunction and failure (32). Critically ill patients have a higher rate of hospital-acquired infection than other patient groups (10, 33). VAP is the most commonly fatal infection in critical illness (3, 5, 6). Various factors may contribute to this increased risk of infection, including the underlying illness (34). The high prevalence of secondary infections across all critical illness suggests a common underlying immune cause. Innate immune cells typically drive the initial inflammatory response to critical illness; these cells include principally neutrophils, monocytes, and macrophages (32, 35, 36). It is this initial inflammatory response that is responsible for many of the pathophysiologic features of critical illness (32). There is however also a compensatory anti-inflammatory response, including the amplification of anti-inflammatory mediators and impairment of innate immune cell function (35, 37, 38). In severe cases this can lead to immunoparalysis and markedly increased risks of secondary infection and death in the ICU. This immune suppression has been best described in sepsis, but can be present in any state of critical illness found in an ICU (39-41). The mechanism by which this immune suppression leads to infection and death is poorly understood. However, immune suppression has been demonstrated in most innate immune cell types in critical illness, including neutrophils, monocytes, macrophages and dendritic cells (41-44).

1.2.3 Innate immunity in tracheal intubation

There is limited understanding about the direct impact of tracheal intubation on local innate immunity in the airway. Animal models have previously demonstrated reduction of mucociliary clearance and an increase in local inflammation with tracheal intubation (45-49). It has previously been demonstrated that even short-term (median time 3 hours) tracheal intubation can result in significant tracheal mucosal inflammation in humans, with a significant migration of neutrophils, rise in cytokines interleukin (IL)-6 and IL-1β, and a significant increase in C5a concentration in a longitudinal theatre patient cohort (50).

1.3 Neutrophils

1.3.1 Neutrophil overview

Neutrophils are polymorphonuclear leukocytes that represent 50 – 70% of blood circulating leukocytes in humans (51). Neutrophils play a key role in acute inflammation and infection and are crucial to the clearance of bacterial and fungal pathogens (52, 53). This is best exemplified by the increased rate of infection in neutropenia or neutrophil dysfunction (54, 55). Neutrophil homeostasis requires not only a balance between production and destruction, but also margination, which refers to the prolonged transit of neutrophils through specific organs (56, 57). These marginalised pools are found in the spleen, liver and lung (56, 57). In physiological conditions, it is thought that neutrophils are mainly cleared from the circulation in the liver, spleen and bone marrow (58).

Inflammation can lead to rapid shifts in the location of these circulating and marginalised neutrophils (58). At inflammatory sites within tissues, bacterial-derived (such as lipopolysaccharide (LPS)) and host-produced (such as IL-1β, C5a and IL-8) inflammatory molecules are abundant (58). These compounds are detected by neutrophil surface receptors, via endothelial cell signalling, and allow neutrophils to migrate from the systemic circulation to the site of inflammation. This process is broadly called chemotaxis. Neutrophils are traditionally considered short lived with a circulating half-life of 6 – 8 hours. However during inflammation neutrophils become activated by various cytokines, growth factors and bacterial products, and their longevity can increase several fold (58, 59).

1.3.2 Neutrophils and the clearance of pathogens

Neutrophils can eliminate pathogens by both intra- and extracellular means (58). Their killing mechanism can be broadly divided into three groups; pathogen phagocytosis, degranulation and neutrophil extracellular traps (NETs) (Figure 3) (58). Phagocytosis involves the direct encapsulation of a pathogen in a phagosome. The pathogen is then killed through nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase-dependent mechanisms. NADPH oxidase generates superoxide, which forms hydrogen peroxide, and ultimately reactive oxygen species (ROS) (53).

Neutrophils are also able to generate antibacterial proteins, including cathepsins, defensins, lactoferrin and lysozyme (53). The antibacterial proteins are released from the neutrophil granules into either phagosomes or the extracellular space (53). NETs are composed of DNA, histones, proteins and enzymes (such as neutrophil elastase), which are released from neutrophil granules. NETs are through to immobilise pathogens, facilitating subsequent phagocytosis of trapped microorganisms (60, 61). They are also thought to directly kill pathogens by means of antimicrobial histones and proteases (60, 61).

While neutrophils are primarily responsible for the acute phase of inflammation, they also have a role in modifying the overall immune response (both innate and adaptive). This is achieved by communication with other immune cells (such as macrophages and dendritic cells) via direct cell-cell contact and release of mediators such as chemokines and cytokines (62).

Through the anti-microbial properties described, neutrophils are highly inflammatory and destructive cells and this raises the potential for neutrophils to cause damage to healthy tissues. This occurs through the release of pro-inflammatory cytokines and proteases, such as neutrophil elastase (52, 53, 63-67). This damage is evident in many inflammatory airway diseases, such as acute respiratory distress syndrome, cystic fibrosis (CF), asthma, chronic obstructive pulmonary disease (COPD) and bronchiectasis (63, 68-71).

1.3.3 Neutrophils in critical illness

The neutrophil is the key innate immune cell involved in the clearance of bacterial pathogens, which are often responsible for secondary infection in critical illness (52, 53). Conversely, organ dysfunction in critical illness is driven, to a large part, by neutrophils (72). Neutrophils from critically ill patients demonstrate an activated phenotype, with enhanced release of proteolytic enzymes and impaired transmigration and chemotaxis (72, 73). This hyper inflammatory state is however paralleled by profound neutrophil dysfunction in key roles, such as phagocytosis of pathogens (41, 42, 72, 73). A number of mechanisms of dysfunction have been hypothesised in critical illness. One of the best-described mechanisms of neutrophil dysfunction is the role of the anaphylatoxin C5a (39-41). C5a is derived from complement C5 and

released in large quantities in critical illness (74, 75). It has previously been proposed that high levels of C5a may inhibit RhoA activation, preventing actin polymerisation and phagocytosis by systemic neutrophils in critical illness (39-41). The authors were however unable to identify any underlying mechanism responsible for the demonstrated dysfunction of airway mucosa neutrophils.

1.3.4 Neutrophil-mucus interactions

In health, the number of neutrophils in airway mucus membranes is relatively small (58). During inflammation, neutrophils are rapidly recruited from the systemic circulation to the epithelium due to a chemoattractant gradient (58). At this stage, the interactions between neutrophils and mucus membranes are crucial to the appropriate function of both these parts of the innate immune system. A number of respiratory diseases demonstrate corresponding mucus hyperviscosity, neutrophil accumulation and tissue damage, most evidently demonstrated in CF disease progression (63). Little is known, however, about the interaction between these two key pillars of innate immunity, mucus and neutrophils. It has previously been suggested in one *in vitro* study that viscous mucus, with high mucin concentrations, may adversely affect neutrophils' ability to migrate and kill bacteria (76).

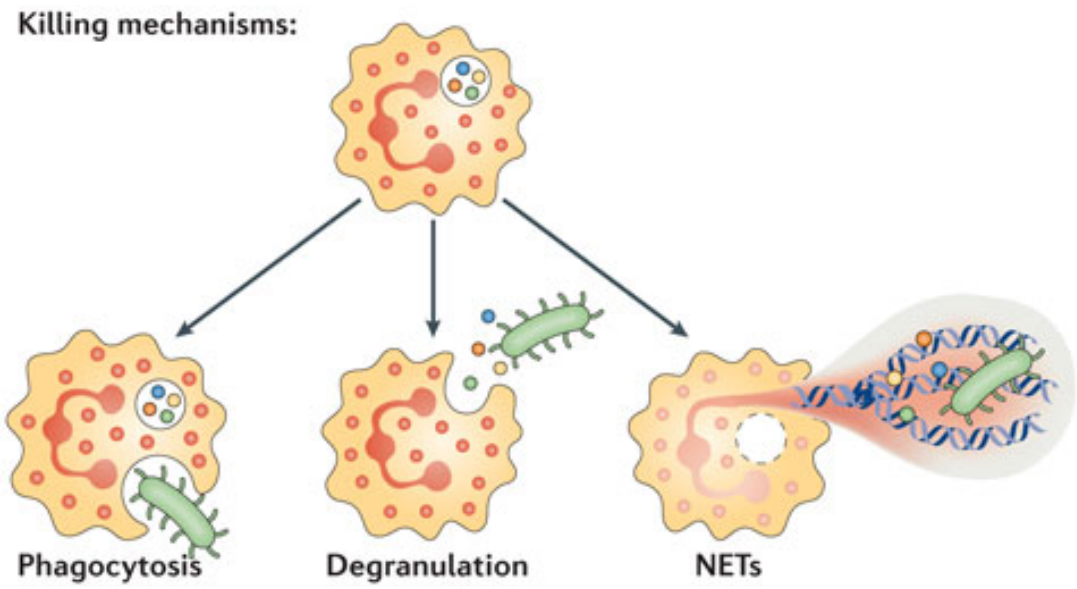

Figure 1.3 - Neutrophil killing mechanisms.

Adapted from Neutrophil recruitment and function in health and inflammation Kolaczkowska *et al* 2013 (58).

1.4 Airway mucus and mucins

1.4.1 Mucus and mucins overview

Airway epithelial cells are continually exposed to pathogens, toxins and particulate matter (77, 78). A crucial early host defence against these challenges is mucus, which covers all epithelial linings, including the respiratory tract (79-81).

Mucus consists mainly mucin, which are large molecular weight biopolymers, and water (77, 81). Furthermore, mucus contains a multitude of other constituents, such as DNA, lipids, ions, proteins, cells and cellular debris (77, 81). Its physical behaviour is complex (non-Newtonian), with highly variable properties that are best described as representing those of a viscous liquid and an elastic solid (81). Changes in the rheological properties of mucus may greatly affect its ability to function as a lubricant, selective barrier, and the body's first line of defence against infection (81).

Mucins are a family of high molecular weight (2–20 x 10^5 Da) glycoproteins with a high carbohydrate content (50 - 90% by weight) (77). Airway mucins are secreted by both goblet cells and the seromucinous glands of the lamina propria at the apical epithelium (82). Once mucins are produced they are stored in secretory granules within the epithelium ready for release onto the apical surface in response to mucin secretagogues (in the case of secreted mucins) or integrated into the membrane (in the case of membrane-tethered mucins) (82). More than 20 mucins are described, however only a subset occurs in the airway, MUC1, MUC4 and MUC13, which are membrane bound, and MUC2, MUC5AC, MUC5B, MUC7 and MUC19, which are secreted (83). By far the most predominant secreted (and gel-forming) mucins within the airway are MUC5AC and MUC5B (77, 84).

Mucin molecules contain a MUC protein backbone (encoded by the *MUC* gene) that has a high number of tandem repeats, a characteristic that distinguishes mucins from other glycoproteins (77, 85). The specific number of tandem repeats in turn distinguishes different mucin molecules. This area also provides numerous sites for glycosylation, resulting in the high carbohydrate content of mucins (79). Most mucin glycoproteins also have a high sialic acid and sulphate content, which leads to a strongly negatively charged surface that increases the rigidity of the polymer via charge repulsion (Figure 4) (81).

1.4.2 Secreted mucins in airway disease

In health, mucus is a key constituent of the innate immune system (77, 78). The physical properties of mucus, attributed primarily to mucins, produce a chemical barrier against harmful agents, protecting the airway epithelium beneath (78, 79, 84, 86). Mucus is able to trap particulate matter and infectious agents (78, 79). These trapped particles are then cleared by ciliary movement that propels the mucus up to the oropharynx for swallowing, or expectoration, allowing clearance from the airway (78, 79).

Airway inflammatory factors, including bacterial-derived (such as LPS), and host-derived (such as tumour necrosis factor-α, IL-1β, IL-6, IL-13, IL-17, C3a and neutrophil elastase) and environmental factors (such as cigarette smoke and allergens) have all been shown to stimulate hypersecretion of various mucins (87, 88). Acute challenges to the respiratory tract will induce rapid release of mucin granules and mucin hypersecretion. More sustained challenge will result in increased expression of the *MUC* gene and increased glycosyltransferase activity and gene expression. In chronic diseases, such as asthma and COPD, this persistent mucin stimulation leads to goblet and glandular cell hyperplasia and persistent over-secretion (77). Acute mucin hypersecretion is thought to be a protective mechanism, however it is poorly understood on a molecular level. Prolonged mucin hypersecretion has been implicated in many respiratory disease processes, such as asthma, COPD and CF, through small airway obstruction and poor pulmonary clearance of pathogens, due to hyperviscous mucus (77, 78, 88). Additionally, in response to acute and chronic stimulation, mucins are not only over produced, but also tend to undergo further sulphation and glycosylation, resulting in further increased mucus viscosity (77, 79, 82, 89).

It has previously been suggested that mucins may play more than a passive role in pathogen propagation in the airway. Mucins have been implicated in aiding bacterial biofilm formation through acting as binding sites for pathogens, (90-92). Furthermore, previous studies have proposed that VAP-causing bacteria, such as *P aeruginosa,* may have mucin sulphatase activity, allowing them to overcome mucins' repulsive forces (89, 93). This would potentially allow bacteria to expose the mucin carbohydrate and protein cores to bacterial glycosidases and proteinases. It is hypothesised that this

could then lead to mucins being digested by bacterial pathogens, potentially promoting bacterial growth (89, 93-95).

1.4.3 Secreted mucins in VAP development

Despite the increasing focus on mucins in a multitude of airway diseases, very little is known about mucin in VAP development. One study has previously investigated the lower airway mucin concentrations in patients with VAP, intubated ICU patients and a cohort of healthy controls (96). The authors demonstrated that patients with VAP had eight-fold higher mucin concentrations in lower airway lavages, compared with healthy outpatient bronchoscopy controls. No significant difference was found between the intubated ICU patients and outpatient controls. The study was well designed and included reasonably large patients cohorts. Unfortunately no correction was made for the lavage samples dilution factor (97). Therefore direct correlation of absolute mucin values with other studies is not possible. The importance of mucins in the subglottic region and in further VAP development is unknown.

Figure 1.4 - Diagram representing a secretory mucin glycoprotein.

The tandem repeat domains are depicted in yellow and may be of varying length. Adapted from Rose *et al,* 2006 (77).

1.5 Models of VAP development

1.5.1 Animal models

A number of animal models of tracheal tube placement, ventilation and VAP development have been described, these include murine, porcine, primate, dog and cat models (98-105). These models have studied the local impacts of intubation on mucociliary clearance and innate immunity (45-49), of ventilation on the lungs (106, 107) and of instillation of pathogens in the ventilated airway, as a model of VAP (108-111). Pig models in particular are able to best approximate the human airway physiology, however mice have also been extensively used due to their size and practicality (100, 112). The obvious strength of animal models is that they allow whole body *in vivo* study of disease pathogenesis, or the evaluation of novel therapeutic strategies (100, 113). These investigations or interventions would, in many cases, not be technically or ethically possible in humans. There are, however, a number of limitations to animal models, including important inter-species differences in innate immunity, the need for different ventilation and anaesthetic techniques from humans, and the absence of co-morbidities, common in VAP patients (108-111). Furthermore there are limited antibodies designed for animal testing, for examples inflammatory cytokines or mucins, restricting experimental use of many particularly non-murine animals. There are also the additional costs associated with animal husbandry, chiefly with larger animals such as pigs or primates (108-111).

1.5.2 in vitro models

The airway epithelium is the first line of defence against potentially pathogenic microorganisms. Its importance goes far beyond its role as a barrier and it is an important component of the innate immune system, initiating signalling to activate and recruit immune cells to the airways (114-116). It has been possible to culture human airway epithelial cells *in vitro* for a number of decades (117). Human primary cell cultures and immortalised cell lines have been established from respiratory epithelial sub-sites, including the trachea and small airways of the lung (118-120). Cells may be cultured under submerged conditions on plastic petri dishes or flasks, however these assume a poorly differentiated phenotype (121). Advanced culture techniques, such as an air-liquid interface culture system (Figure 5), where cells are

cultured on semi-permeable supports, enable cellular polarisation and a cell phenotype which more closely recapitulates that of the normal *in vivo* airway (121). The limitation of these models is the lack of a whole body system; conversely they do utilise relevant, human-derived tissue that should better represent human responses to stimuli or interventions (121).

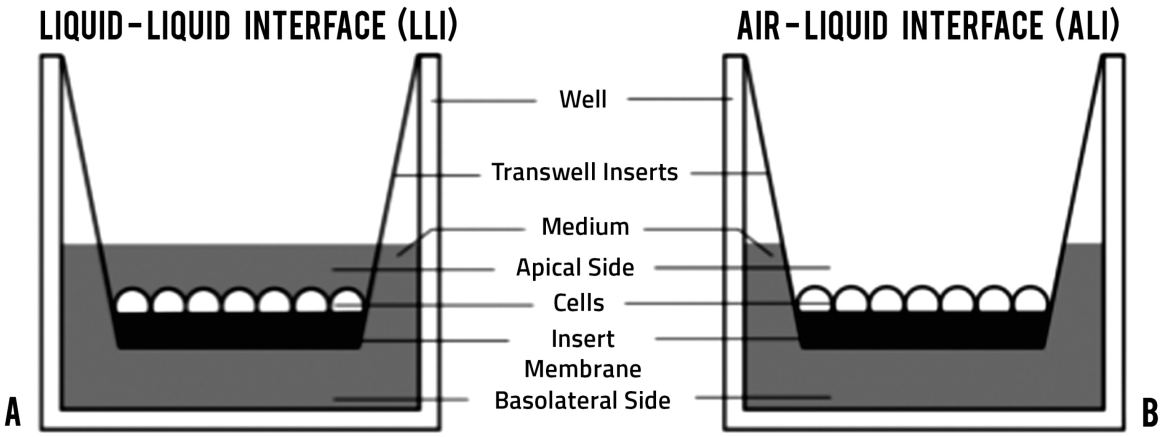

Figure 1.5 - Graphical depiction of submerged cultures and air-liquid interface (ALI) cultures.

Epithelial cells are initially cultured in submerged conditions (A) prior to the apical fluid being removed (B). In these air-liquid interface (ALI) conditions the respiratory epithelial cells will often differentiate into a pseudo-stratified respiratory epithelium. Adapted from Lee *et al* 2016 (122).

1.6 Hypothesis

That the tracheal tube induces local deregulation of subglottic host defences, resulting in detrimental microbiological shifts within the mucus layer:

To address this hypothesis my PhD was divided into the following areas of study:

1. Microbiology of mucus derived from the subglottis.
2. Subglottic mucosal host defences.
3. The effect of mucin on neutrophil function and epithelial-pathogen interactions.
4. Development of subglottic epithelial cell cultures.

Chapter 2: Materials and methods

2.1 Recruitment

2.1.1 Intensive care unit patients

Intensive care unit (ICU) study participants were identified through screening of inpatients in the Newcastle upon Tyne Hospitals ICUs from November 2016 – May 2017. Patients who met the inclusion criteria (Table 2.2) and were likely to be intubated and mechanically ventilated for 4 or more days were identified. Agreement to approach the family/next of kin was obtained from the patient's clinical team. The study research nurse approached the family/next of kin initially. Family members were given at least 24 hours to consider the patient information sheet (PIS) prior to consent being taken by me. Sampling was then performed, usually within 24 hours of consent being given. Clinical and demographic details were collected by the research nurse, who also recorded the patient's subsequent clinical course. A letter was sent to the patient's general practitioner (GP) informing him/her about the patient's participation in the study.

2.1.2 Theatre attenders

Patients listed for upper airway examination under general anaesthesia were screened for inclusion in the study after being listed at the Department of Otolaryngology-Head and Neck Surgery, Freeman Hospital, Newcastle upon Tyne. This cohort was a control group to the ICU patients for mucus sampling and epithelial brushings (Table 2.1). Patients were approached if they were having a planned laryngeal examination in theatre under general anaesthesia. Patients who met the inclusion criteria (Table 2.3) were identified through theatre booking lists and screening of clinic letters. Potentially eligible patients were those deemed unlikely to have structural laryngeal abnormalities and who had no history of upper aerodigestive tract malignancy. Recruitment packs were sent by post, including a patient invitation letter and PIS. Patients had at least two days prior to their theatre attendance to consider participation in the study. Patients were approached on the day of theatre attendance and consented by me. Demographic details were collected, including age, sex, medication use and co-morbidities. A letter was sent to the patient's GP informing him/her about the patient's participation in the study.

2.1.3 Healthy volunteers

Healthy volunteers with no personal history of autoimmunity, infection or other significant medical conditions (Table 2.4) were recruited from Newcastle University (staff and students) and the general population through flyer advertisements. Demographic details (age and gender) were again collected. This cohort was a control group to the ICU blood samples (Table 2.1). A separate group of healthy volunteers was also recruited to provide blood in order to generate healthy neutrophils for *in vitro* experiments.

2.1.4 Ethical approval and consent

The subglottic samples collected from theatre attenders came from one of two Research Ethics Committee (REC) approvals. The first submission was approved by the South East Scotland 01 REC, reference number 14/SS/1015, based on an application submitted by Professor Janet Wilson and I. A further submission approved by the West Midlands REC, reference number 15/WM/0349, based on a further application submitted by Professor Janet Wilson and I. Ethical approval was obtained for the collection of samples from incapacitated ICU subjects from the Newcastle and North Tyneside 2 REC, reference number 15/NE/0323, based on an application by Dr Stephen Wright and I. Healthy volunteers' blood samples were used based on an approval from the County Durham & Tees Valley REC, reference number 12/NE/0121 (principal investigator Professor John Simpson). All submissions were also approved by the Research and Development Department of the Newcastle upon Tyne Hospitals NHS Foundation Trust. Ethical approval forms and associated documents are listed in Appendix B. Inclusion and exclusion criteria for all cohorts are listed in Tables 2.2, 2.3 and 2.4.

Consent was obtained from participants prior to sampling in the theatre attender and blood volunteer cohorts. Personal consultee consent was obtained from the next of kin or a family member in the ICU cohort due to loss of capacity of the person being sampled. Retrospective consent was taken from the sampled patient in cases where capacity was regained.

Group	Blood samples	Mucus aspirates	Epithelial brushings
ICU patients	✔	✔	✔
Theatre attenders (controls)	✖	✔	✔
Healthy blood volunteers (controls)	✔	✖	✖

Table 2.1 - Sampling approvals in place for each of the three study cohorts

Inclusion criteria	***Exclusion criteria***
Age 18+ years of age	Known current or previous malignancy of the subglottic region
Participant expected to be intubated and mechanically ventilated for 4 or more days on the ICUs within the Newcastle upon Tyne Hospitals NHS Foundation Trust	Known current or previous radiotherapy to the subglottic region
	Patients who the clinical team judge would not tolerate the procedure (i.e. delirium)
	No personal legal representative (i.e. relative/friend/carers) to give consent
	PaO_2<8kPa on FiO_2>0.7
	Positive end-expiratory pressure >15cmH_2O
	Peak airway pressure >30 cmH_2O
	Heart rate >140 bpm
	Mean arterial pressure <60mmHg
	Bleeding diathesis (including platelet count <20x10^9 per litre of blood or Prothrombin time or activated partial thromboplastin time greater than 1.5 time the reference range)
	Poorly controlled intracranial pressure (>20mmHg), if measured
	ICU consultant deems procedure not to be safe
	Allergic to local anaesthetic agents

Table 2.2 – ICU cohort inclusion and exclusion criteria

Inclusion criteria	***Exclusion criteria***
Age 18+ years of age	Known current or previous malignancy of the upper aerodigestive tract.
Having a planned laryngeal examination under general anaesthetic	Known current or previous chemo-radiotherapy of the upper aerodigestive tract.
Laryngeal mucosal appearances at both clinic appointment and general anaesthetic examination demonstrate no clinically apparent malignancy (variations of normal such as mild injection or benign looking cysts will be allowed).	Any patient in whom such an increase in GA duration of 10 to 15 minutes is deemed in appropriate by the anaesthetist for whatever reason

Table 2.3 – Theatre attender controls inclusion and exclusion criteria

Inclusion criteria	*Exclusion criteria*
Age 18+ years of age	Donated blood (e.g. to the Blood Transfusion Service or to research studies) in the previous 90 days.
	Donated >1 litre of blood in the previous year (this equates to around 3 donations to the Blood Transfusion Service in the past year).
	Anaemia in the past year.
	Taking any regular, prescribed medication (the oral contraceptive pill is permissible in female participants).

Table 2.4 – Healthy blood volunteers inclusion and exclusion criteria

2.2 Collection of subglottic mucus samples

2.2.1 Subglottic mucus sampling

Subglottic mucus aspirates were collected from ICU patients either 1) directly from the subglottic suction drain (SSD) attached to the endotracheal (ET) cuff, or 2) if a SSD was not in place mucus was collected directly from the subglottic region, via a bronchoscope, at the time of epithelial brushing. In both cases samples were trapped in a sterile system using a mucus trap (MST-3000, Pennine Healthcare, Derby, UK). Subglottic mucus aspirates were collected from theatre attenders (controls), via a sterile theatre suction catheter and mucus trap, at the time of epithelial brushing. In all cases the mucus sample was immediately placed in a cooled container and transported to the laboratory for processing (transit time less than 30 minutes).

2.3 Microbiological assessment of subglottic mucus samples

2.3.1 Mucus sampling and processing for microbiological assessment

Mucus samples were aseptically collected as described in section 2.2.1. In both the ICU and theatre attender cohorts a sterile microbiology swab (MW172P, Medical Wire & Equipment, Swindon, UK) was immersed in the mucus immediately after collection, rotated 5 - 10 times, and sheathed.

2.3.2 Semi-quantitative microbiology culture

Semi-quantitative microbiology culture was performed by Professor John Perry, Department of Microbiology, Freeman Hospital, following protocols based on standard NHS culture techniques. In brief, samples were mixed with 5 mL 0.1% DTT solution and incubated for 30 minutes at room temperature, with repeated vortexing. Serial dilutions were then performed in 0.85% saline. Samples were then plated onto the following media and cultured at 37°C for 48 hours:

1) Chocolate agar incubated in air plus 5% CO_2. This allows growth of the vast majority of aerobic and capnophilic bacteria.

2) Fastidious anaerobe agar incubated anaerobically. This allows growth of the majority of anaerobic bacteria.

3) Sabouraud agar incubated in air. This allows growth of the majority of yeasts or fungi likely to be encountered.

All different colony types were then quantified and identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

2.3.3 Bacterial and fungal DNA isolation and gene profiling

DNA extraction

Microbiology swab samples were placed in a cold tissue transportation container and transported immediately to the laboratory for storage in a -80°C freezer. Not more than 6 months later samples were defrosted fully and processed using a PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio laboratories, CA, USA). The swab was

vigorously vortexed with bead solution (Mo Bio laboratories, CA, USA) to extract the sample from the swab and then the solution was placed in glass bead tubes (Mo Bio laboratories, CA, USA). Samples were then processed according the manufacturer's instructions. A NanoDrop 1000 spectrophotometer (Thermofisher, MA, USA) was used to quantify DNA yield and purity. 1 µL of each sample was loaded onto the spectrophotometer, zeroed against the DNA diluent (ultra pure water). Measurement of absorbance was taken at 260 nm to estimate DNA yield and ratios of 260/280 nm and 260/230 nm to estimate levels of contamination. Extracted DNA was then stored in a -80°C freezer for later analysis.

16S rRNA bacterial and ITS fungal profiling

Dr Andrew Nelson at NU-OMICS, Northumbria University, performed the bacterial and fungal profiling and community analysis. In brief, polymerase chain reaction (PCR) amplification of the V4 region of the bacterial 16S rRNA gene was performed with primers targeting the region as previously described (123). Fungal communities were amplified with primers targeting the internal transcribed spacer (ITS) 1 region as described previously (124). Libraries were normalised using the Sequel normalisation kit (Invitrogen, CA, USA) and quantified using KAPA Library Quantification Kits for NGS (KAPA Biosystems, MA, USA). The libraries were sequenced using a V3 600 cycle kit on the MiSeq system (Illumina, CA, USA).

Bacterial community analysis

Fastq files generated for bacterial communities were trimmed to 250bp using Cutadapt before being processed in Mothur according to the MiSeq SOP (123, 125). Paired reads were merged using the make.contigs command and processed to remove sequences containing ambiguous bases, homopolymers >8bp and sequences with a length >275bp. Sequences were aligned to the SILVA database and chimeras were removed using the vsearch algorithm. The remaining sequences were classified using the RDP database and sequences not identified as bacteria were removed from the downstream analysis. Operational taxonomic units (OTUs) present in PCR negative controls were removed using the remove.otulabels command and the positive control was removed using the remove groups command. Reads were normalised by subsampling to 1000 reads per sample, which resulted in exclusion of samples with low coverage.

Fungal community analysis

Fungal fastq files were trimmed to remove adapter sequences using cutadapt from 5' and 3' ends of the sequence before being processed using Mothur (125). Paired reads were assembled using the make.contigs command with trimoverlap set to true. Reads were quality filtered to remove sequences with ambiguous bases and homopolymers >8bp. Chimeric sequences were removed using vsearch and a distance matrix generated using the dist.seqs command to allow for varied sequence length. Sequences were classified against the UNITE database (126). Reads were normalised by subsampling to 100 reads per sample, which resulted in exclusion of samples with low coverage.

Statistical analysis

Subsampled BIOM files were imported into the phyloseq package which was used to generate diversity box plots and nonmetric multidimensional scaling (NMDS) analysis using Bray-Curtis distances (127). Bar plots of proportional taxa abundance and Kruskal-Wallis tests for difference between groups were performed as described previously (128). All *P* values were adjusted for multiple comparisons with the false discovery rate algorithm as previously described (129).

2.4 Extraction and processing of subglottic mucus-derived leukocytes

2.4.1 Extraction of subglottic mucus-derived leukocytes

Extraction of mucus derived leukocytes was performed using methods adapted from those previously described (130). All procedures were performed in a class II laminar flow cabinet. A proportion of the mucus sample was aliquoted into 2 mL microcentrifuge tubes (Sigma-Aldrich, Dorset, UK) and immediately placed in a -80°C freezer for later analysis. The remaining mucus was used for leukocyte extraction and quantification. This sample was mixed with 0.08% dithiothreitol (DTT) Sputolysin Reagent (Merck Millipore, MA, USA) in phosphate-buffered saline (PBS, Sigma-Aldrich, Dorset, UK), and gently agitated for 5 minutes at room temperature, followed by further dilution in an equal volume of PBS. The mucus was then filtered through a wet 30-micron filter (Miltenyi Biotec, Surrey, UK) and centrifuged (300g, at 10°C for 10 minutes).

2.4.2 Differential cell count

The pellet was re-suspended in 0.5 mL PBS. 10 µL of this suspension was then mixed with 5 µL of 0.4% Trypan Blue solution (Sigma-Aldrich, Dorset, UK). Cells were then counted using a light microscope using four quadrants of a haemocytometer (Sigma-Aldrich, Dorset, UK). Live cells were visualised through exclusion of Trypan Blue. An average number of leukocytes, both alive and dead, was thereby ascertained in each sample.

2.4.3 Cytospin preparation

The cell pellet was re-suspended in PBS. The final volume was adjusted to give a concentration of 0.5 million cells/mL. Cytospins were prepared on glass slides using 100 µL aliquots and then spun at 300 rpm for 3 minutes (Cytospin 3, Shandon, London, UK). The cytospins were then fixed in acetone (Sigma-Aldrich, Dorset, UK) at room temperature for 10 minutes and air-dried. Slides were finally treated with 10% Giemsa stain (Sigma-Aldrich, Dorset, UK) for 10 minutes, rinsed in distilled water and air-dried. Slides were counted by an expert pathology technician (Kasim

Jiwa, William Leech Lab, Freeman Hospital, Newcastle upon Tyne, UK) under a light microscope to elicit an average number of each cell type.

2.5 Collection and extraction of whole blood-derived leukocytes

2.5.1 Blood sampling

Whole blood samples were collected from ICU patients by aspiration from a central arterial or venous line already *in situ*. Aseptically, 10 mL of whole blood was aspirated and discarded. Then 36 mL of whole blood was aspirated and placed in a 50 mL centrifuge tube (Corning, NY, USA) with 4 mL citrate solution (Sigma-Aldrich, Dorset, UK), for immediate transportation to the laboratory. In the case of healthy volunteers, blood samples were aseptically taken via venepuncture of a peripheral vein and placed in a 50 mL centrifuge tube with 1 mL of citrate solution per 10 mL of blood, for immediate transportation to the laboratory.

2.5.2 Extraction of whole blood-derived leukocytes

All procedures were performed in a class II laminar flow cabinet. The whole blood-citrate mix was initially centrifuged at 300g for 20 minutes at room temperature. The platelet rich plasma was then removed to leave a cell pellet. The plasma was aliquoted into 2 mL microcentrifuge tubes and immediately stored in a -80°C freezer for later analysis. Serum was prepared by adding 220 μL of 1 M $CaCl_2$ solution (Sigma-Aldrich, Dorset, UK) per 10 mL of plasma and incubated in a 37°C water bath for 30 – 60 minutes. The cell pellet was then dextran-sedimented by addition of 2.5 mL of 37°C 6% dextran (Pharmacosmos, Holbæk, Denmark) per 10 mL of cell pellet. The total volume was made up to 40 mL with 37°C 0.9% NaCl saline (Sigma-Aldrich, Dorset, UK). The tube was mixed and allowed to sediment for 30 minutes at room temperature, leaving a leukocyte rich upper layer that was collected.

2.5.3 Differential cell count and cytospin preparation

Purity was confirmed via differential cell count of leukocytes with Trypan Blue exclusion and cytospin preparation as described in section 2.4.3/2.4.4.

2.6 Functional studies of subglottic mucus and whole blood-derived neutrophils

2.6.1 pHrodo S. aureus bioparticle assay

Measurement of neutrophil phagocytosis was performed using methods adapted from those previously described (131). Stock pHrodo® Green *S. aureus* Bioparticles® (Thermofisher, MA, USA) at 1 mg/mL were opsonised by incubation at a 1:10 dilution in 50% Iscove's Modified Dulbecco's Medium (IMDM, Thermofisher, MA, USA) / 50% serum for 30 minutes at 37°C in a shaking water bath, in the dark. Mixed leukocytes (50 µL, fresh from extraction, at 0.5 - 1 million cells per mL in IMDM) were then incubated with 5 µL of opsonised bioparticles in 2 mL round bottom microcentrifuge tubes for 90 minutes, in the dark. For each condition or sample three leukocyte tubes were prepared; a tube with particles incubated in a water bath at 37^{o}C, a control tube without bioparticles, and a second control tube with bioparticles kept on ice during the incubation. For the final 30 minutes of incubation all tubes were stained with a neutrophil marker (Pacific blue-conjugated anti-CD66b antibody) together with a viability marker (Near-IR Dead Cell Stain Kit) (Table 2.5). Mixed leukocytes were then washed using flow cytometry buffer (PBS containing 1% bovine serum albumin (BSA)) and centrifuged at 200g for 5 minutes, twice. Cell pellets were re-suspended in flow cytometry buffer at a final volume of 300 µL and data were acquired immediately on a calibrated FACSCanto II (Becton, Dickinson and Company (BD), NJ, USA) flow cytometer.

2.6.2 Annexin V and propidium iodide assay

Measurement of neutrophil viability and apoptosis was performed using an Annexin V and propidium iodide assay, utilising methods adapted from those previously described (132). Mixed leukocytes (50 µL, fresh from extraction, at 0.5 - 1 million cells per mL in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-)) were incubated for 30 minutes on ice with a neutrophil marker, Pacific blue-conjugated anti-CD66b antibody (Table 2.5). Mixed leukocytes were then washed as previously described and re-suspended in Annexin V buffer (Thermofisher, MA, USA) at a volume of 50 µL. Leukocytes were then incubated for 15 minutes on ice with Allophycocyanin (APC)-conjugated Annexin V and propidium iodide (Table 2.5). A control tube constituted unstained cells. Leukocytes were then re-suspended in

Annexin V buffer (Thermofisher, MA, USA) at a final volume of 300 µL. Data were acquired immediately on a calibrated FACSCanto II (BD, NJ, USA) flow cytometer.

2.6.3 Surface marker expression

Mixed leukocytes (50 µL, fresh from extraction, at 0.5 - 1 million cells per mL in HBSS-) were incubated for 30 minutes on ice with a panel of antibodies to cell surface markers: CD62L/CD66b/CD11b/CD88 (Table 2.5). Isotype controls were also prepared for each antibody (Biolegend, CA, USA). A viability marker, Near-IR Dead Cell Stain Kit, was also added to all samples. Mixed leukocytes were then washed as previously described and re-suspended in flow cytometry buffer at a final volume of 300 µL. Data were acquired immediately on a calibrated FACSCanto II (BD, NJ, USA) flow cytometer.

2.6.4 Flow cytometry analysis

Initial gating strategy

All data were analysed using FlowJo Version 8.7.1 (Treestar Inc., OR, USA). Cellular debris was excluded by its characteristic position on the forward and side scatter area. Dead cells were excluded by Near-IR Dead Cell Stain Kit, where positive uptake indicated cell death. Neutrophils were identified by positive staining with anti-CD66b antibody, relative to an isotype control (133).

Phagocytosis gating

Ingestion of pHrodo® Green *S. aureus* Bioparticles® by phagocytic cells was determined through cellular florescence measured at 509⁄533 nm excitation⁄emission on the flow cytometer. Control samples kept on ice were used to determine the gating threshold of background fluorescence. This allowed calculation of the percentage of cells involved in phagocytosis (by the percentage of positively fluorescent cells) (131).

Annexin V and propidium iodide gating

Viable, necrotic, early and late apoptotic neutrophils were identified via gating four quadrants against APC Annexin V and propidium iodide fluorescence, as previously described (132).

Marker	Fluorophore	Clone	Dilution factor	Isotype control	Company
CD66b	Pacific blue	G10F5	1/10	Pacific Blue Mouse IgM, κ, Clone MM30	Biolegend, CA, USA
CD62L	APC	DREG-56	1/5	APC Mouse IgG1, κ, clone MOPC-21	Biolegend, CA, USA
CD88	PE	S5/1	1/5	PE Mouse IgG2a, κ, Clone MOPC-173	Biolegend, CA, USA
CD11b	FITC	CBRM1/5	1/10	FITC Mouse IgG1, κ, clone MOPC-21	Biolegend, CA, USA
Annexin V	APC	-	1/50	-	Thermofisher, MA, USA
Propidium iodide	-	-	1/500	-	Thermofisher, MA, USA
LIVE/DEAD ® Fixable Near-IR Dead Cell Stain Kit	-	-	1/500	-	Thermofisher, MA, USA

Table 2.5 - Fluorophore labelled antibodies used in flow cytometry experiments.

2.7 ELISAs, fluorometric assays and cytometric bead arrays

2.7.1 MUC5AC indirect ELISA

In a 96 well microtitre plate (Thermofisher, MA, USA) standard concentrations were prepared in duplicate (100 µL) using MUC5AC (in house purified pig gastric mucin) diluted in PBS in the range of 0 - 10 µg/mL. Samples were mixed in PBS to a dilution of 1/10 and 1/20 or higher, and 100 µL placed in wells, in duplicate, and allowed to absorb overnight. The following day, the plate wells were completely emptied and washed three times with PBS containing 0.05% Tween20 (Sigma-Aldrich, Dorset, UK). This wash process was repeated after every step. Unoccupied binding sites were blocked with 300 µL of 1% casein (Sigma-Aldrich, Dorset, UK) in PBS for 2 hours. Then 100 µL of primary monoclonal antibody for MUC5AC (45M1 mouse anti-human MUC5AC (Abcam, MA, USA)), diluted 1/100 in antibody diluent (0.1% casein/0.05% Tween20 in PBS)) was added and incubated for 2 hours. 100 µL of secondary antibody was added and incubated for 2 hours (horseradish peroxidase-conjugated goat anti-mouse (Dako, CA, USA), diluted 1/5000 with antibody diluent). Finally 100 µL of peroxidase substrate ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) was added and the plate allowed to develop. Absorbance at 405 nm was measured after 5 - 10 minutes with a plate reader (M200, Infinite, Männedorf, Switzerland). Lower limit of detection 0.156 µg/mL.

2.7.2 MUC5B indirect ELISA

In a 96 well microtitre plate (Corning, NY, USA) standard concentrations were prepared in duplicate (100 µL) using MUC5B (in house purified human salivary mucin) diluted in PBS in the range of 0 - 5 µg/mL. Samples were diluted in PBS to a concentration of 1/2000 and 1/5000 or higher, and 100 µL placed in wells, in duplicate, and allowed to absorb overnight. The next day the plate wells were completely emptied and washed three times with PBS containing 0.05% Tween20. This wash process was repeated after every step. Unoccupied binding sites were blocked with 300 µL of 1% casein in PBS for 2 hours. 100 µL of primary monoclonal antibody for MUC5B (EPR6920, rabbit anti-human MUC5B (Abcam, MA, USA)), diluted 1/200 in antibody diluent (0.1% casein/0.05% Tween20 in PBS)) was added and incubated for 2 hours. 100 µL secondary antibody was then added and incubated

for 2 hours (horseradish peroxidase-conjugated goat anti-rabbit (Dako, CA, USA)), diluted 1/5000 with antibody diluent). Finally 100 µL of ABTS was added and the plate was left to develop. Absorbance at 405 nm was measured after 5 - 10 minutes with a plate reader (M200, Infinite, Männedorf, Switzerland). Lower limit of detection 0.078 µg/mL.

2.7.3 Human neutrophil elastase ELISA

Human neutrophil elastase was measured in mucus (diluted 1/500 and 1/1000) using Human PMN Elastase ELISA (ab119553, Abcam, MA, USA) as per the manufacturer's instructions. Lower limit of detection 1 ng/mL.

2.7.4 MSD multiplexing technology

Quantification of cytokines in plasma (diluted 1/2) and mucus supernatants (diluted 1/100) was performed by Meso Scale Discovery (MSD) technology using the V-PLEX Proinflammatory Panel 1 (human) kit (K15049D, Meso Scale Diagnostics, MD, USA), measuring interferon (IFN)-γ, interleukin (IL)-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, Tumour necrosis factor (TNF)-α, according to the manufacturer's instructions. Quantification of IL-8 in mucus supernatants (diluted 1/10,000) was performed using a U-PLEX (Human) IL-8 Kit (K151TYK, Meso Scale Diagnostics, USA), according to the manufacturer's instructions. Quantification of GM-CSF was measured in mucus supernatants (diluted 1/5) using a U-PLEX (Human) GM-CSF Kit (K151UMK, Meso Scale Diagnostics, USA), according to the manufacturer's instructions. Lower limits of quantification were as follows; IL-8 0.15 pg/mL, IL-6 1.58 pg/mL, IL-1β 2.14 pg/mL, IL-10 0.68 pg/mL, GM-CSF 0.12 pg/mL.

2.7.5 dsDNA quantification

dsDNA was measured from mucus supernatants (diluted 1/10) using a Quant-iT dsDNA fluorometric assay kit (Q33120, Thermofisher, MA, USA) as per the manufacturer's instructions. Lower limit of detection 0.2 ng/mL.

2.7.6 Complement quantification

The Cytometric Bead Array (CBA) Human Anaphylatoxin Kit (561418, BD, NJ, USA) was utilised to quantify C4a, C3a, and C5a, and their desArg forms. Quantification was performed on plasma (diluted 1/100) and mucus supernatants (diluted 1/100) according to the manufacturer's instructions. Analysis was performed on a calibrated FACSCanto II (BD Biosciences, USA) flow cytometer. Lower limit of detection 1.15 pg/mL.

2.8 Physical properties of subglottic mucus

2.8.1 Dry weight

The percentage dry weight of mucus was calculated by using a microbalance (Pioneer, OHAUS, Greifensee, Switzerland) to compare weight before and after drying the sample at 80°C overnight (134).

2.8.2 Rheology

Subglottic mucus was fully thawed and shear viscosity was rheologically analysed at 37°C using a Kinexus Pro (Malvern Instruments, Worcester, UK) with 60 mm parallel plates, used according to the manufacturer's instructions. A table of shear rates was produced over the range of 0 – 100 s^{-1}. Analysis was performed on rSpace software (version 1.6, Malvern Instruments, Worcester, UK) (135).

2.9 Neutrophil functional assays with ICU-derived mucin

2.9.1 Purification of ICU-derived subglottic mucus

Caesium chloride equilibrium density gradient ultracentrifugation and dialysis.

Subglottic mucus was collected from the ICU patient cohort, as described in section 2.2.1. The methods used to isolate and purify mucin from mucus are based on methods previously described (136-139). The individual ICU mucus samples were defrosted, pooled and mixed with an equal volume of an enzyme inhibitor buffer cocktail (pH 6.7 with NaOH) (Table 2.6), to inhibit any proteolytic activity. The sample was extensively homogenised and centrifuged at 10,000g for 1 hour at 4 °C to remove any debris. The supernatant was adjusted to a density of 1.42 g/mL (± 0.005g) by the addition of solid caesium chloride (CsCl) (Sigma-Aldrich, Dorset, UK). Ultracentrifugation was then performed at 100,000g, for 48 hours, at 4°C (T-1170, Centricon, USA), to generate a CsCl gradient. Eight fractions were then recovered from the gradient and individually dialysed against distilled water extensively for 72 hours. The proportion of DNA/protein in the fractions was calculated using the 260/280 nm ratios on a spectrophotometer (8625 UV/VIS, Unicam, NJ, USA).

Periodic Acid Schiff assay

The fractions were then subjected to Periodic Acid Schiff assay to determine glycoprotein concentrations (135). Samples were diluted 1:20 and 1:40 with PBS. Porcine mucin (Sigma-Aldrich, Dorset, UK) was used as a standard. The fractions and mucin standards were plated, in duplicates (200 µL), onto a 96 well microtitre plate. 20 µL of periodic/acetic acid solution (10 µL of 50% periodic acid solution (Sigma-Aldrich, Dorset, UK) to 5 mL of 7% acetic acid solution (Sigma-Aldrich, Dorset, UK)) was added to each well and incubated at 37°C for 45 minutes. This was followed by addition of 20 µL of Schiff's reagent (containing 17 mg/mL sodium metabisulphate (Sigma-Aldrich, Dorset, UK)). The reaction was allowed to develop for 30 minutes at room temperature. Readings were taken with a spectrophotometer read at 550 nm (8625 UV/VIS, Unicam, NJ, USA).

Lyophilisation

The glycoprotein-rich, protein-low fractions were pooled and lyophilised for 72 hours using a vacuum freeze dryer (Shanghai Bilon Instrument, Shanghai, China). Remaining fractions were discarded. The fractions of purified solid MUC5B/MUC5AC were then stored at -20°C for later use.

Constituent	Molar concentration (mM)
KH_2PO_4	42
$NA_2HPO_42H_2O$	25
α-anionohexanoic acid	100
Ethylenediamine tetra-acetic acid (EDTA)	10
Lodoacetamide	1
N-ethyl maleimide	10
Benzamide HCL	5
Phenylmethylsulfonyl fluoride (PMSF) (dissolved in 2 mL warm propan-2-ol)	1

Table 2.6 - Inhibitor buffer used to prevent mucus proteolytic activity (all supplied by Sigma-Aldrich, Dorset, UK)

2.9.2 Healthy volunteer blood-derived neutrophil collection and isolation

Whole blood samples were collected from healthy volunteers as described in section 2.5.1. All procedures were performed in a class II laminar flow cabinet. Neutrophils were isolated from whole blood by means of dextran sedimentation and fractionation over discontinuous Percoll gradients, as previously described (140, 141). The blood-citrate mix was initially centrifuged at 300g for 20 minutes at room temperature. The platelet rich plasma was then removed to leave a cell pellet. Serum was prepared by adding 220 µL of 1 M $CaCl_2$ (Sigma-Aldrich, Dorset, UK) per 10 mL of plasma and incubated in a 37°C water bath for 30 – 60 minutes. The cell pellet was then dextran sedimented using 2.5 mL of pre-warmed (37°C) 6% Dextran (Pharmacosmos, Holbæk, Denmark) per 10 mL of cell pellet. The volume was then made up to the original whole blood volume with 0.9% NaCl saline at 37°C (Sigma-Aldrich, Dorset, UK). The tube was mixed and allowed to sediment for 30 minutes at room temperature, leaving a leukocyte rich upper layer that was transferred to a new centrifuge tube and topped up with 50 mL of 0.9% saline at 37°C. The sample was then centrifuged at 200g for 5 minutes at room temperature. The supernatant was removed and the pellet re-suspended in 55% Percoll (GE Healthcare, IL, USA) in PBS. This was then overlaid onto a 70% Percoll layer on top of an 81% Percoll layer and centrifuged at 700g for 20 minutes at room temperature. The neutrophils were then extracted from the bottom band in the 70%/81% interface. The cells were then washed with HBSS- and centrifuged at 200g for 5 minutes at room temperature. Purity was confirmed via differential cell count of leukocytes with Trypan Blue exclusion as well as cytospin preparations as described in section 2.5.3.

2.9.3 pHrodo S. aureus bioparticle assay

Healthy volunteer whole blood derived neutrophils were resuspended in HBSS+ ($1x10^7$ cells per mL). 5 µL of the cell solution was mixed with 50 µL of purified mucin solution, containing 5 µL of serum opsonised pHrodo *S. aureus* bioparticles (as previously described), in round bottom 2 mL microcentrifuge tubes. Samples were incubated for 1 hour at 37°C in a shaking water bath, in the dark. For each experiment a control without bioparticles, and a second control with bioparticles kept on ice during the incubation, were also prepared. Cells were removed from the mucus

mixture by incubating for 5 minutes with 0.08% DTT. An equal volume of PBS was then added to dilute the DTT. The neutrophils were then pelleted by centrifugation at 300g for 5 minutes. All samples were then re-suspended in 50 µL HBSS+ and incubated with a neutrophil marker, Pacific blue-conjugated anti-CD66b antibody (Table 2.5), as well as a viability marker, Near-IR Dead Cell Stain Kit, for 30 minutes on ice. Cells were then washed twice in flow cytometry buffer (PBS containing 1% BSA) by centrifuging at 200g for 5 minutes. Cell pellets were re-suspended in flow cytometry buffer at a final volume of 300 µL and data were acquired immediately on a FACSCanto II (BD, CA, USA). Initial and phagocytosis-specific gating strategies are described in section 2.6.4.

2.9.4 Annexin V and propidium iodide assay

Healthy volunteer whole blood-derived neutrophils were resuspended in HBSS+ ($1x10^7$ cells per mL). 5 µL of the cell solution was mixed with 50 µL of purified mucin solution, in a round bottom 2 mL microcentrifuge tube. Samples were incubated for 1 hour at 37°C in a shaking water bath, in the dark. Cells were removed from the mucus mixture by incubating for 5 minutes with 0.08% DTT. An equal volume of PBS was then added to dilute the DTT. The neutrophils were then pelleted by centrifugation at 300g for 5 minutes. All samples were then re-suspended in 50 µL HBSS+ and incubated with a neutrophil marker, Pacific blue-conjugated anti-CD66b antibody (Table 2.5). Cells were then washed as previously described and re-suspended in Annexin V buffer (Thermofisher, MA, USA) at a volume of 50 µL. Neutrophils were then incubated for 15 minutes on ice with APC-conjugated Annexin V and propidium iodide (Table 2.5). A control sample constituted unstained cells. Cells were then re-suspended in Annexin V buffer (Thermofisher, MA, USA) at a final volume of 300 µL. Data were acquired immediately on a calibrated FACSCanto II (BD, CA, USA). Initial and Annexin V and propidium iodide-specific gating strategies are described in section 2.6.4.

2.9.5 Neutrophil killing of live bacteria

Measurement of neutrophil killing of live bacteria was performed utilising methods adapted from those previously described (42, 142). *P. aeruginosa* (lab strain PA01) was taken from frozen stock (ATCC, TX, USA), thawed and diluted 1:1000 in a 50 mL Falcon tube (Sigma-Aldrich, Dorset, UK) containing 10 mL Roswell Park Memorial Institute-1640 (RPMI) medium (Sigma-Aldrich, Dorset, UK) and incubated at 37°C in an orbital shaker overnight (Barnstead Max Q4000, Hyland Scientific, WA, USA). The next morning, optical density was measured at 600 nm using a spectrophotometer (8625 UV/VIS, Unicam, NJ, USA), zeroed against a control of RPMI without bacteria. The bacterial culture was then diluted with RPMI to an optical density of 0.2 (approximately $2x10^7$ colony forming units (CFU)/mL determined by previously plating out serial dilutions). Bacteria were opsonised with 10% autologous serum for 30 minutes, then 5 µl of opsonised PA01 was placed in a round bottom 2 mL Eppendorf tubes with 50 µl of purified mucin and mixed. Healthy volunteer whole blood-derived neutrophils were resuspended in HBSS+ ($1x10^7$ cells per mL). 5 µL of the cell solution was mixed with the mucin/bacteria solution and incubated for 30 minutes (37°C 5% CO_2). After co-culture the mixture was treated with 0.08% DTT containing 0.01% Triton X-100 (Sigma-Aldrich, Dorset, UK), in HBSS+, for 30 minutes at room temperature, with repeated vortexing. Bacterial viability was determined by serial dilution and incubation on agar plates (Lennox B agar (Thermofisher, MA, USA), diluted in distilled water and autoclaved). Plates were incubated overnight (37°C 5% CO_2) and CFU were counted. A control sample without neutrophils was used to determine neutrophil-specific bacterial killing.

2.9.6 Neutrophil chemotaxis

The methods used in this section are adapted from Matsui *et al* (76). Whole blood-derived healthy volunteer neutrophils were stained with carboxyfluorescein succinimidyl ester (CFSE, 1/1000, Thermofisher, MA, USA) for 30 minutes, protected from the light in a 37°C water bath, then washed and the cells re-suspended in HBSS+ ($1x10^7$ cells per mL). In the lower chamber of a 96-well transwell permeability support (3.0 µm pore size, Corning, NY, USA) 10 µL of 10,000 pg/mL reconstituted IL-8 solution (Sigma-Aldrich, Dorset, UK) was incubated for 1 hour at

room temperature. 2 µL of the neutrophil suspension ($1x10^7$ cells per mL) was then mixed with 22 µL of purified ICU mucin and placed in the upper chamber of the transwell permeable support. The lower chamber was then flooded with 117.5 µL warm HBSS+ and incubated at 37°C in a 5% CO_2 incubator for 3 hours. Imaging was performed with a confocal fluorescent camera (Axio Imager II, Zeiss, Oberkochen, Germany) to quantify the number of neutrophils in the lower chamber as a percentage of the total cells incubated in the upper chamber. A control sample comprised a well without IL-8-containing solution. Neutrophils in each chamber were quantified by an automated programme utilising the JOBS module of NIS Elements (Nikon, Tokyo, Japan).

2.10 Subglottic epithelial cell culture

2.10.1 Harvesting of subglottic epithelial cells

The surgeon operating on participants performed a full laryngeal examination and any further procedures as required. The surgeon then confirmed the absence of structural laryngeal abnormalities prior to subglottic brushing. I performed sampling in most cases though in a limited number of cases samples were obtained by the operating surgeon. Assessment of the subglottis was performed under direct vision using the operating surgeon's rigid laryngoscope. If the operating surgeon had the operating microscope set up for the procedure this was also utilised. Under direct vision a sheathed cytology brush (BC-202D-5010, Olympus, Tokyo, Japan) was passed through the vocal cords into the subglottis. The brush was unsheathed in the subglottis to reduce contamination from the rest of the airway. Vigorous brushing was performed with at least 5 - 10 strokes of the mucosal surface of the subglottis. The brush was removed and the tip cut off and placed in an individual 10 mL centrifuge tube (E1415-0500, Starlab, UK) containing 5 mL tissue culture medium, RPMI (Sigma-Aldrich, Dorset, UK), for immediate transport to the laboratory (transit time <30 minutes).

2.10.2 Differential cell count

Differential cell count was performed to ascertain the number of ciliated respiratory epithelial cells harvested from the subglottic brushing. All procedures were performed in a class II laminar flow cabinet. Samples were first gently manually agitated to separate cells from the brush head. The brushes were removed using forceps before centrifugation (200g at 10°C for 7 minutes). The pellet was then re-suspended in 0.5 mL PBS. 20 µL of this suspension was then mixed with 20µl of 0.4% Trypan Blue solution (Sigma-Aldrich, Dorset, UK) and placed in a haemocytometer. Cells were then counted under a light microscope using four quadrants on the haemocytometer. Live cells were identified by exclusion of Trypan Blue. An average number of ciliated respiratory epithelial cells (both alive and dead) was thereby ascertained for each sample.

2.10.3 Cytospin preparations

The cell pellet was re-suspended in PBS to give a final concentration of 0.5 million cells/mL. Cytospins were prepared on glass slides using 100 µL aliquots and then spun at 300 rpm for 3 minutes (Cytospin 3, Shandon, London, UK). The cytospins were fixed in acetone (Sigma-Aldrich, Dorset, UK) at room temperature for 10 minutes and then air-dried. The cytospins were next treated with 10% Giemsa stain (Sigma-Aldrich, Dorset, UK) for 10 minutes, rinsed in distilled water and air-dried.

2.10.4 Cell expansion and passage

Samples were gently agitated by hand to separate cells from the brush head. The brushes were removed using forceps before centrifugation (200g, at 10°C for 7 minutes). The pellet was then re-suspended in 2 mL fresh Bronchial Epithelial Growth Medium (BEGM, CC-4175, Lonza, Basel, Switzerland), warmed to 37°C, and supplemented with 100 µg/mL streptomycin (Sigma-Aldrich, Dorset, UK) and 100 U/mL penicillin (Sigma-Aldrich, Dorset, UK). The cell suspension was then transferred to a 25 cm^2 cell culture flask (Corning, NY, USA) pre-coated with 1% collagen (Thermofisher, MA, USA), containing a further 3 mL of medium. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every 2 - 3 days. Cells were observed daily to ensure satisfactory growth and to check for any evidence of infection. In the latter event the flask was immediately removed from the incubator and the contents sent for routine bacterial and fungal cultures to identify the infecting organism and relevant antimicrobial sensitivities.

Passage was performed when cells reached 70 - 80% confluence. The cells were removed from the culture surface with trypsin 0.05% in 0.02% EDTA. 5 mL of trypsin/EDTA was added to the flask and incubated at 37°C with 5% CO_2 for 2-4 minute. Trypsin was inactivated by addition of 5 mL RPMI medium supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Dorset, UK). This cell suspension was then centrifuged at 200g, at 10°C for 7 minutes. The supernatant was removed and the cell pellet re-suspended with fresh BEGM medium prior to transfer to another growth flask.

2.10.5 Cryopreservation

5 mL of trypsin/EDTA was added to the cell culture flask and incubated at 37°C with 5% CO_2 for 2-4 minute. When the cells were dislodged and in suspension, trypsin was inactivated using 5 mL RPMI medium supplemented with 10% FCS. The cells were transferred to a 15 mL tube and centrifuged at 200g, at 10°C for 7 minutes. The supernatant was discarded and the pellet re-suspended in 1 mL of freezing media (90% FCS and 10% Dimethyl sulfoxide (DMSO)). The cell suspension was then transferred into a cryovial and placed in the freezer at -80°C for 24 hours before being transferred to liquid nitrogen.

2.10.6 Reconstitution of cryopreserved cells

The samples were retrieved from liquid nitrogen and immediately thawed in a 37°C water bath. Once defrosted, the suspension was diluted in 10 mL BEGM and centrifuged at 200g, at 10°C for 7 minutes. The supernatant was discarded and the cell pellet immediately re-suspended in 2 mL pre-warmed BEGM. The cell suspension was then transferred to a 25 cm^2 cell culture flask (Corning, NY, USA) pre-coated with 1% collagen (Thermofisher, MA, USA), containing a further 3 mL of medium.

2.10.7 Air-liquid interface (ALI) culture

In a class II laminar flow cabinet 12 µm polyester transwell inserts (0.4 µm pore, Corning, NY, USA) were pre-coated with 1% collagen (Thermofisher, MA, USA) and seeded with 0.5 mL cell suspension at approximately 200,000 cells per mL. At this density cells were 80-100% confluent after adherence. The lower chamber was filled with 1.5 mL of BEGM medium supplemented with 100 µg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich, Dorset, UK). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. At 24 hours the apical medium was replaced to remove cell debris. Once at 100% confluence, the apical medium was removed so that the cells were exposed to air at the apical membrane. At this stage the medium in the basolateral chamber was substituted with 1.5 mL ALI medium (Table 2.7), changed every 2 - 3 days.

Constituent	Volume or concentration
Bronchial epithelial basal medium (Lonza, Basel, Switzerland)	250 mL
Dulbecco's Modified Eagle's Medium (DMEM), high glucose (Thermofisher, MA, USA)	250 mL
1% Penicillin /Streptomycin (Sigma-Aldrich, Dorset, UK)	5 mL
Human epidermal growth factor (R&D systems, UK)	0.5 ng/mL
Retinoic acid (Sigma-Aldrich, Dorset, UK)	100 ng/mL
$CaCl_2$ (Sigma-Aldrich, Dorset, UK)	1mM
Bovine pituitary extract	2mL*
Insulin	500µL*
Hydrocortisone	500 µL*
Transferrin	500 µL*
Epinephrine	500 µL*
Tri-iodothyronine	500 µL*

Table 2.7 - Air-liquid interface culture medium

**concentrations not published by the manufacturer (available as product number CC-4175 from Lonza, Basel, Switzerland).*

2.11 Characterisation of the epithelial cell cultures

2.11.1 Scanning electron microscopy and transmission electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed by the Electron Microscopy Research Services at Newcastle University. In brief, air-liquid interface (ALI) cultures of primary subglottic epithelial cells (PSECs) were fixed by treatment with glutaraldehyde followed by osmium tetroxide. This was followed by dehydration through graded alcohols and embedding in resin. SEM was performed using a Cambridge Steroscan 240 electron microscope and TEM with a Philips CM100, both with digital image capture.

2.11.2 Immunohistochemistry for pan-cytokeratin

The transwell epithelial membrane was washed extensively three times, both apically and basolaterally with 37°C PBS to ensure thorough removal of the mucus layer. Cells were then fixed and permeabilised with 100% methanol at -20°C for 10 minutes. The wash step was repeated and the cells stained for 3 hours at room temperature with 5 µg/mL (diluted in 5% FBS/PBS) monoclonal anti-cytokeratin (pan-reactive) antibody (raised in mouse), conjugated with Alexa Fluor® 647 (Biolegend, CA, USA). This pan-cytokeratin recognises cytokeratins 4, 5, 6, 8, 10, 13, and 18. The wash step was then repeated. The transwell membrane supporting the epithelial cells was then entirely removed from the insert with a scalpel blade and fixed onto a glass slide below and glass cover slip above using an aqueous mounting medium containing DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) for nuclear counterstaining (using Vectashield with DAPI, Vector Labs, Cambridgeshire, UK). Imaging was performed with a confocal fluorescent camera (Axio Imager II, Zeiss, Oberkochen, Germany). A control sample compromised an epithelial membrane, processed in parallel, without anti-cytokeratin antibody staining.

2.11.3 Preparation of paraffin-embedded blocks and sections

The transwell epithelial membrane was washed apically and basolaterally with 37°C PBS. The wash and aspiration process was repeated 3 times to ensure thorough

removal of the mucus layer. Cells were then fixed with 4% paraformaldehyde for 20 minutes at room temperature. The transwell membrane was then entirely removed from the insert using a scalpel blade and placed in cassettes (Simport, Beloeil, Canada). The cassettes were then dehydrated through graded alcohols. This was performed by immersion for 10 minutes in 70% industrial methanol spirits (IMS), 70% IMS, 80% IMS, 80% IMS, 95% IMS, 95% IMS, 74 over proof (op) IMS, 74op IMS and 74op IMS respectively. Finally the sample was submerged in xylene (VWR, Lutterworth, UK) for 20 minutes, twice, and then embedded in paraffin for 1 hour at 60°C. Sections of the paraffin blocks were then cut to 5 μm thickness using a microtome (Thermofisher, MA, USA).

2.11.4 Haematoxylin and eosin staining

The paraffin-embedded sections were pre-heated at 60°C for 1 hour before de-waxing. De-waxing was performed by soaking the sections in xylene for 5 minutes, twice, and then rehydrating through graded alcohols, for 1 minute each in 95% IMS, 99% IMS and 99% IMS. The sections were then washed in water and stained in freshly filtered Carrazzi's Haematoxylin (Thermofisher, MA, USA) for 1 minute. The staining process was assessed dynamically under the light microscope. The sections were then washed in running tap water for 2 - 3 minutes before being placed in 0.1% acid alcohol (Sigma-Aldrich, Dorset, UK) and washed in running tap water until the nuclei were appropriately blue. The sections were then counterstained with Eosin Y (Fisher Scientific, UK) for 2 minutes. Finally the sections were washed in tap water and dehydrated back through graded alcohols and xylene. The sections were then mounted with DPX (VWR, Lutterworth, UK).

2.11.5 Diastaseresistant periodic acid-Schiff staining

Diastase-resistant periodic acid-Schiff (DPAS) staining was used as a stain for mucin glycoproteins in the paraffin-embedded epithelial section. The DPAS staining was performed by the Department of Histopathology, Royal Victoria Infirmary, Newcastle upon Tyne. In brief, the sections were de-waxed and rehydrated via an automated

process, then stained for DPAS and counter-stained with Haematoxylin and Eosin Y, also via an automated process.

2.11.6 MUC5B immunohistochemistry

The sections were de-waxed as previously described. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes. Samples were washed for at least 10 minutes in gently running tap water, then rinsed in tris-buffered saline (TBS). The primary polyclonal antibody for human MUC5B (Tepa II, raised in rabbit, diluted 1 in 1,000 in antibody diluent (0.1% non-fat milk protein in TBS)) was then added to the sample in a bioassay-incubating tray containing moist tissue, at room temperature, and incubated for 30 minutes. The sample was then washed in TBS for 5 minutes twice. Secondary antibody was added for 30 minutes (horseradish peroxidase-conjugated goat anti-rabbit, diluted 1 in 200 with antibody diluent) in a bioassay incubating tray. The sample was then washed in TBS for 5 minutes twice. The sections were then treated with 3,3'-diaminobenzidine (DAB) for 5-10 minutes and then neutralised by chlorine bleach. Sections were then washed in running tap water. Counter-staining was performed with Carrazzi's Haematoxylin for 1 minute and samples were then washed in tap water until blue. Finally the sections were washed in tap water and dehydrated back through graded alcohols and xylene, as described in section 2.11.3. The sections were then mounted with DPX (VWR, Lutterworth, UK). A control was also tested in place of the primary antibody (Rabbit IgG 1:40,000, Sigma-Aldrich, Dorset, UK). Human tonsil tissue was also used as a negative tissue control.

2.11.7 MUC5AC immunohistochemistry

The sections were de-waxed as previously described. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 30 minutes. Samples were washed for at least 10 minutes in gently running tap water, then rinsed in TBS. The primary monoclonal antibody for MUC5AC (45M1 epitope a, Thermofisher, MA, USA, diluted 1 in 100 in antibody diluent (0.1% non-fat milk protein in TBS)) was then added to the sample in a bioassay incubating tray containing moist tissue, at room temperature, and incubated for 30 minutes. The sample was then twice washed in TBS for 5 minutes. Secondary antibody was added for 30 minutes (horseradish peroxidase-conjugated goat anti-

mouse, diluted 1 in 100 with antibody diluent) in a bioassay incubating tray. The sample was then twice washed in TBS for 5 minutes. The sections were treated with 3,3'-diaminobenzidine (DAB) for 5-10 minutes and then neutralised by chlorine bleach. Counter-staining was performed with Carrazzi's Haematoxylin for 1 minute and samples were then washed in tap water until blue. Finally the sections were washed in tap water and dehydrated back through graded alcohols and xylene. The sections were then mounted with DPX (VWR, Lutterworth, UK). An isotype control was also tested in place of the primary antibody (Mouse IgG 1a 1:100, Sigma-Aldrich, Dorset, UK). Human tonsil tissue was also used as a negative tissue control.

2.11.8 Trans-epithelial resistance

Prior to trans-epithelial resistance (TER) measurement, 0.5 mL of pre-warmed BEGM was applied to the apical surface of ALI cultured PSECs and allowed to equilibrate for 20 minutes. TER measurements were performed using an epithelial volt-ohmmeter (World Precision Instruments, FL, USA) according to the manufacturer's instructions.

2.11.9 Ussing chamber electrophysiological studies

Experiments were performed using a static chamber system (World Precision Instruments, FL, USA) (143). Snapwell semi-permeable supports were mounted in the Ussing chamber to measure epithelial membrane properties. Apical and basolateral compartments were electrically isolated and separated by the polarised epithelial monolayer. A symmetrical Krebs solution was added to both compartments to eliminate potentially influential osmotic and chemical effects. Calomel voltage-sensing electrodes were placed on each side of the membrane. Trans-epithelial potential difference was clamped to 0 mV by current injection with silver-silver chloride electrodes to eliminate the voltage gradient. Under these conditions, the injected current, or short circuit current (I_{sc}), was a direct measure of net trans-epithelial ion transport. 3M potassium chloride salt bridges containing 3% agar were used to connect chambers to the electrodes. The chamber was maintained at 37°C and continuously gassed with 5%CO_2/95%O_2, pH 7.4. A 1 second 5 mV pulse was applied at 30-second intervals to monitor resistance changes, calculated by applying

Ohm's law. After 20 minutes of stabilisation, relevant ion channel inhibitors and activators were added and resultant I_{sc} responses recorded. Apical amiloride (Sigma-Aldrich, Dorset, UK) was added to inhibit epithelial sodium channel (ENaC). Apical forskolin (Sigma-Aldrich, Dorset, UK) was added to activate Cystic fibrosis transmembrane conductance regulator (CFTR)-mediated chloride transport through adenylate cyclase stimulation and intracellular cAMP increase. CFTR was inhibited by apical addition of CFTRinh172 (Sigma-Aldrich, Dorset, UK). The resultant analogue signal was digitised with a Powerlab 200 interface (AD instruments, Australia) and recorded by a computer with Scope 3 software (AD instruments, Australia).

2.11.10 Apical mucus characterisation

Mucus was harvested by direct aspiration from the apical aspect of ALI cultures and stored at -80°C for later analysis. Quantification of cytokines and mucins (MUC5B and MUC5AC) in the mucus layer produced by fully differentiated ALI epithelial cell cultures was performed via ELISA techniques described in section 2.7.1/2.7.2.

2.12 Bacterial-subglottic epithelial co-cultures

2.12.1 Pathogen-epithelial co-cultures

The methods used in this section are adapted from Garnett *et al* (144-146). PSECs cultured to differentiation at ALI, were placed in ALI medium (Table 2.7) without antibiotics for at least 24 hours prior to experiments. In a class II laminar flow cabinet the apical mucus layer was removed from the cultures by extensive washing with 37°C PBS. The mucus layer was then replaced with 50 µL of purified ICU-derived mucin (in HBSS+), or HBSS+ only control, and incubated (37°C 5% CO_2) for 1 hour. *P. aeruginosa* PA01 was prepared as previously described in section 2.9.5. Then 5 µL ($2x10^7$ CFU/mL determined previously by plating out serial dilutions) PA01 was applied apically onto the mucin solution and incubated for 7 hours (37°C 5% CO_2) (Figure 1). After co-culture the mucin layer was removed by repeated washing with 0.08% DTT (Merck Millipore, MA, USA) and 0.01% Triton X-100 (Sigma-Aldrich, Dorset, UK), in HBSS+. The epithelial layer was removed by scraping and repeated washing (0.08% DTT/0.01% Triton X-100 in HBSS+). The samples were incubated for 30 minutes and extensively homogenised by repeated pipetting. Bacterial growth in the mucin layer and epithelial homogenate was determined by serial dilution and incubation on agar plates (Lennox B agar (Thermofisher, MA, USA), diluted in distilled water and autoclaved). Plates were incubated overnight (37°C 5% CO_2) and CFUs counted (144-146).

Purified mucin solution (1 hour pre-incubation),

followed by addition of *P. aeruginosa* PA01 (7 hour incubation)

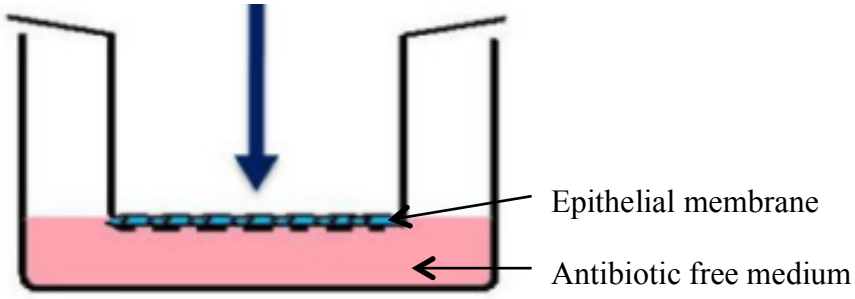

Figure 2.1 - Pictorial demonstration of pathogen-epithelial co-cultures performed on primary subglottic epithelial cells (PSECs) cultured at air-liquid interface.

2.13 Statistical methods

Statistical advice was sought as part of the design of this study and appropriate sample sizes obtained for a proof of concept/pilot study (147). Statistical analyses and preparation of graphs were performed using Prism 7.0a for Mac (GraphPad software, CA, USA). Non-parametric methods were used to tested test the null hypothesis by the most conservative approach possible. A P value <0.05 was considered to be statistically significant. Specific statistical tests used are stated in the figure legends.

Chapter 3: Microbiology of mucus derived from the subglottis

3.1 Introduction

Ventilator-associated pneumonia (VAP) is defined as pneumonia developing 48 hours or more after tracheal intubation and mechanical ventilation (1, 2). It is the most common hospital-acquired infection in the intensive care unit (ICU) (3). It also remains the most commonly fatal infection in critical illness, with an overall attributable mortality of 13%, and is associated with a significantly increased length of ICU stay and cost (5, 6). VAP is frequently associated with antibiotic-resistant pathogens (7-9), particularly late onset pneumonia, developing after four days (1, 9). Furthermore VAP is estimated to account for half of all antibiotics given in ICU (10).

The individual organisms identified from VAP lung lavage samples are highly variable, depending on several factors, including the geographical location and patient group. Gram positive bacteria such as *Staphylococcus aureus*, including methicillin resistant *S. aureus* (MRSA), represent a large proportion of isolates in VAP. Other VAP-causing pathogens include aerobic Gram negative bacilli such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Escherichia coli, Enterobacter* species, and *Acinetobacter* species (7-9, 11).

Subglottic suction drainage has been demonstrated to effectively prevent VAP (27). The rationale for this intervention is to remove infected subglottic secretions prior to their aspiration into the lungs (14, 15). This hypothesis is supported by previous studies that have demonstrated a strong correlation between organisms found in the oropharynx, on the tracheal tube cuff (after removal) or in the trachea (bellow the trachea cuff) prior to development of VAP, and organisms subsequently found in the lungs (11, 17, 18).

While these studies have investigated local microbiology, they have not examined the actual subglottic mucus that is thought responsible for VAP development. I therefore aimed to characterise the microbiology of subglottic mucus from long-term ventilated ICU patients and a control cohort of newly intubated theatre attenders.

3.2 Chapter aims

In this chapter I wished to answer the following questions:

1. What is the microbiology of subglottic mucus from long-term intubated ICU patients and newly intubated control patients, as assessed by microbiology cultures?
2. What is the microbiology of subglottic mucus from long-term intubated ICU patients and newly intubated control patients, as assessed by bacterial and fungal gene sequencing?

3.3 Results

3.3.1 Cohort demographic data

There were 24 patients in the ICU cohort and 27 in the control cohort, and their summarised demographic and clinical data are shown in Table 3.1. Briefly, ICU patients were a heterogeneous cohort, unified by being intubated, and mechanically ventilated, for four or more days. Controls comprised patients attending theatre for a general anaesthetic laryngeal examination, with no structural laryngeal abnormalities identified. Therefore the cohorts are described as long-term intubated ICU patients and newly intubated controls. There was no significant difference in the age or sex of the patients in the two cohorts (Table 3.1). The overall mortality during the subsequent ICU/hospital stay was in keeping with previous UK studies of ICU mortality (148). The rates of immunosuppressive and antibiotic use were also in keeping with previously published studies (Table 3.1) (149, 150). Detailed description of the ICU demographic and clinical data is listed in Table 3.2.

	ICU patients (n=24)	Newly intubated controls (n=27)	*P* Value
Median age, years (range)	66 (20 – 79)	58 (35 – 79)	0.43*
Percentage of patients male	63	59	>0.99†
Median (IQR) mechanically ventilated	5 (4 – 6) days	9 (5 – 10) minutes	
Mortality, %	42	0	
Percentage receiving systemic immunosuppressive drugs (including corticosteroids)	21	0	
Percentage receiving inhaled corticosteroids	0	15	
Percentage receiving antibiotics	83	0	
Percentage smokers	42	26	

Table 3.1 - Summary demographic and clinical data for patient and control groups

Definition of abbreviations: ICU = intensive care unit; IQR = interquartile range; * By Mann–Whitney U test, † By Chi Squared test.

Patient	ICU 1	ICU 2	ICU 3	ICU 4	ICU 5	ICU 6	ICU 7
Age (years)	67	73	62	65	26	58	73
Gender	F	F	M	F	M	F	M
Reason for admission to ICU	Post-op infection, sepsis	Community acquired pneumonia, sepsis	Community acquired pneumonia	Cerebrovascular accident	Hypoxic brain injury	Community acquired pneumonia, sepsis	Myocardial infarction
Days ventilated	5	5	4	6	4	13	4
Receiving immunosuppressive drugs	Y	N	N	N	N	Y	N
Receiving Antibiotics	Y	Y	Y	Y	Y	Y	Y
VAP diagnosed	N	N	N	N	N	Y	N
Outcome	Died	Died	Recovered	Long-term rehab	Long-term rehab	Died	Died

ICU 8	ICU 9	ICU 10	ICU 11	ICU 12	ICU 13	ICU 14	ICU 15
24	79	79	72	79	47	51	46
M	M	M	M	F	M	F	M
Head trauma	Community acquired pneumonia	Cerebrovascular accident	Head trauma	Pneumonitis	Community acquired pneumonia	Cerebrovascular accident	Encephalitis
9	5	6	4	7	4	4	4
N	N	N	N	Y	N	N	N
N	Y	Y	N	Y	Y	N	Y
N	N	N	Y	N	N	N	N
Long-term rehab	Recovered	Recovered	Long-term rehab	Died	Died	Long-term rehab	Recovered

ICU 16	ICU 17	ICU 18	ICU 19	ICU 20	ICU 21	ICU 22	ICU 23	ICU 24
35	72	67	48	79	74	48	71	20
F	M	M	M	M	F	M	M	F
Head trauma	Ruptured aortic aneurysm	Head trauma	Neurological condition	Head trauma	Community acquired pneumonia	Head trauma	Hospital acquired pneumonia	Hypoxic brain injury
6	16	6	4	5	4	5	5	
N	N	N	N	N	Y	N	Y	N
Y	Y	Y	Y	Y	Y	N	Y	Y
N	N	N	N	N	N	N	N	N
Recovered	Died	Long-term rehab	Recovered	Died	Died	Long-term rehab	Died	Recovered

Table 3.2 Extended clinical and demographic details for the ICU cohort

3.3.2 Semi-quantitative microbiology culture

A semi-quantitative culture technique was used to detect bacterial and fungal organisms in the subglottic mucus samples of long-term intubated ICU patients and newly intubated control patients (as described in Chapter 2 of this thesis).

I examined the organisms cultured from these two cohorts. Initial investigation of the number of individual organisms cultured demonstrated significantly fewer isolates in the ICU samples, compared with control samples (Figure 3.1).

I then went on to examine the organisms identified in each cohort. There were significant differences in the species identified between the two cohorts (see appendix A). Due to the vast array of organisms identified I grouped the species into genera. When grouped in this way there was a significantly greater proportion of samples containing *Streptococcus, Rothia* and *Actinomyces* in the newly intubated controls, compared to ICU patients. There were significantly more *Candida* and *Enterococcus* species isolated in the ICU samples, compared to controls. There was also a large number of different species, of varying genus, that were all in the family *Enterobacteriaceae.* These included important airway pathogenic organisms, such as *Escherichia, Enterobacter* and *Klebsiella.* When grouped there were significantly more organisms from the *Enterobacteriaceae* family recovered from the ICU cohort than controls mucus samples (Figure 3.2). A full list of organisms identified from both cohorts is detailed in Appendix A.

Next I investigated the quantity of each organism recovered from the mucus samples. Using the results of semi-quantitative culture I was able to demonstrate which organism was the most concentrated within each samples. This showed that the species/family with the highest colony forming units (CFU) recovered in each sample was significantly more often *Streptococci* in control samples and *Enterobacteriaceae* in ICU samples (Figure 3.3).

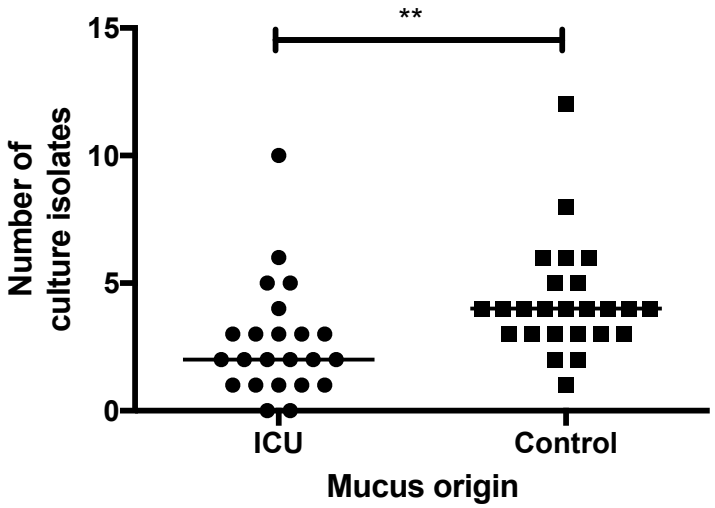

Figure 3.1 - Number of individual bacterial/fungal isolates from subglottic mucus samples.

Subglottic mucus samples were cultured and the individual number of isolates recovered recorded. Horizontal bars depict median values. Mann–Whitney U test, **p<0.01, ICU n = 23, control n = 25.

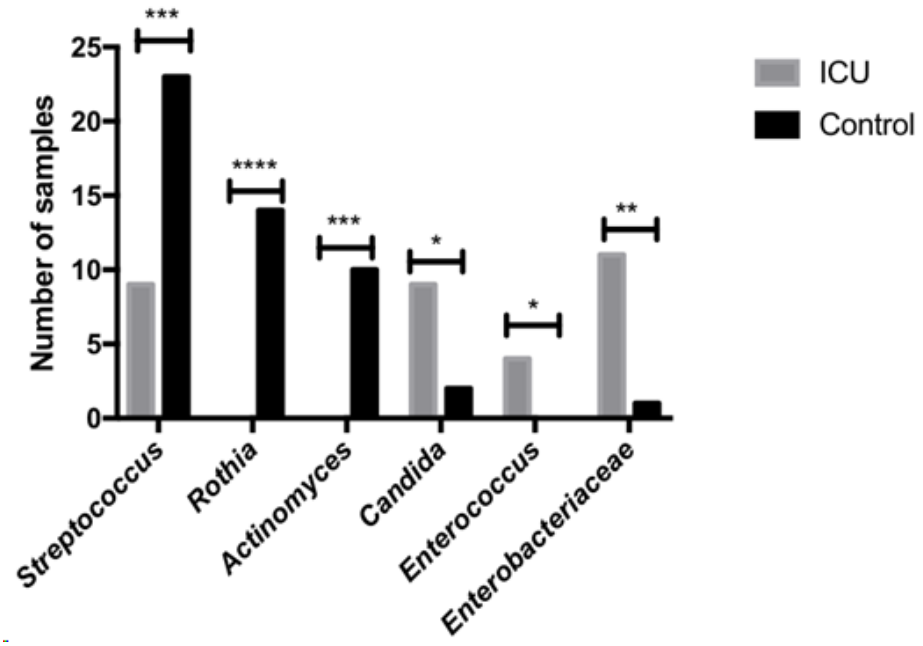

Figure 3.2 - Number of subglottic mucus samples yielding each species/family of bacteria/fungus.

Subglottic mucus samples were cultured and the number of samples containing each species/family was recorded. Significant results are demonstrated for each species (*Streptococcus, Rothia*, *Actinomyces*, *Candida*, *Enterococcus*). The family *Enterobacteriaceae* was also included due to the number of different individual species recovered. Horizontal bars depict median values. Fisher's exact test, *$p<0.05$, **$p<0.01$, ***$p<0.001$ ****$p<0.0001$, ICU n = 23, control n = 25.

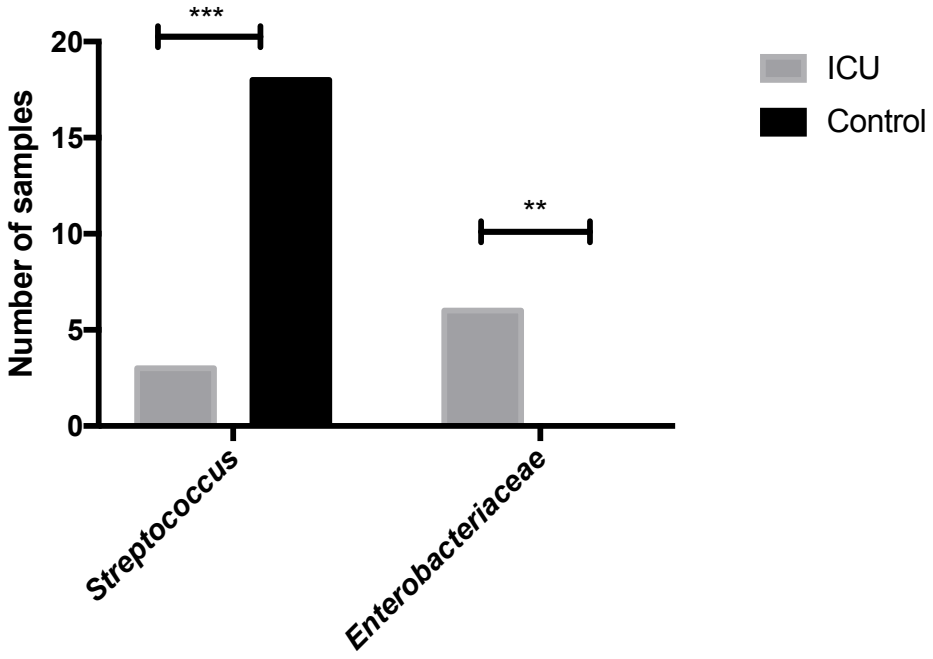

Figure 3.3 - Number of subglottic mucus samples yielding the highest colony forming units (CFU) for each species/family of bacteria/fungus

Subglottic mucus samples underwent semi-quantitative bacterial/fungal culture. The organism with the highest CFU count was recorded. Significant results are demonstrated for each species (*Streptococcus)* and family (*Enterobacteriaceae)*. Horizontal bars depict median values. Fisher's exact test, **p<0.01, ***p<0.001, ICU n = 19, control n = 24.

3.3.3 16S rRNA bacterial profiling

16S rRNA bacterial profiling was used to identify bacterial organisms in the subglottic mucus samples of long-term intubated ICU patients and newly intubated control patients (as described in Chapter 2 of this thesis).

I recruited 24 ICU patients and 27 control patients to the study. Of these 4 controls did not have enough mucus to extract DNA. A further 3 control samples were excluded at the gene sequencing stage due to low coverage, having less than 1000 reads per sample. This resulted in data being available for 24 ICU mucus samples and 20 control samples.

Firstly I examined the diversity of organism found within the subglottic mucus of the two cohorts. This demonstrated significantly less diversity of bacterial organisms in the ICU cohort compared to the controls (Figure 3.4). Next I investigated the individual taxonomic classification of bacterial reads as a measure of the organism identified. This indicated distinct differences between the two cohorts' microbiomes. There were 25 operational taxonomic units (OTUs) that were significantly more abundant between the two cohorts (Figure 3.5). In all but one case these were organisms found more frequently in the control cohort, included *Streptococcus, Rothia* and *Actinomyces*, but also a plethora of other mainly anaerobic organisms. There was significantly more *Mycoplasma* detected in the ICU cohort compared to controls. I then investigated the similarity of the microbial communities between samples using a nonmetric multidimensional scaling (NMDS) plot based on Bray-Curtis distances (Figure 3.6). This demonstrated similarity between the microbiomes of the individual control samples, with heterogeneity in the ICU samples.

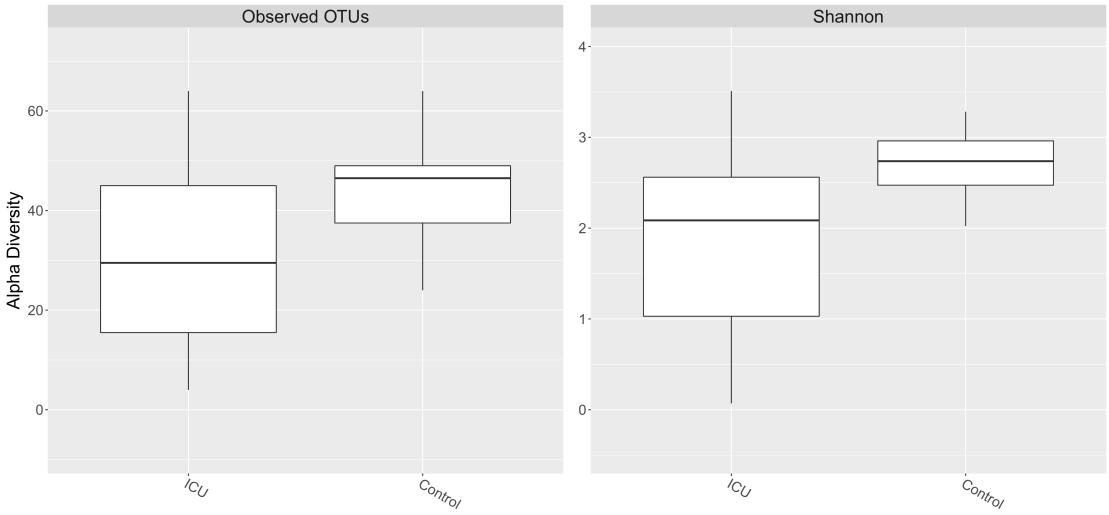

Figure 3.4 - Box plots of bacterial alpha diversity measured using observed operational taxonomic units (OTUs) and Shannon diversity.

Subglottic mucus samples were processed using 16S rRNA bacterial profiling. Observed OTU and Shannon alpha diversity are demonstrated. The boxplot shows the upper and lower quartiles and median, whiskers represent the range. Observed OTU diversity p = 0.018, Shannon diversity p = 0.003. Kruskal-Wallis test, ICU n = 24, control n = 20.

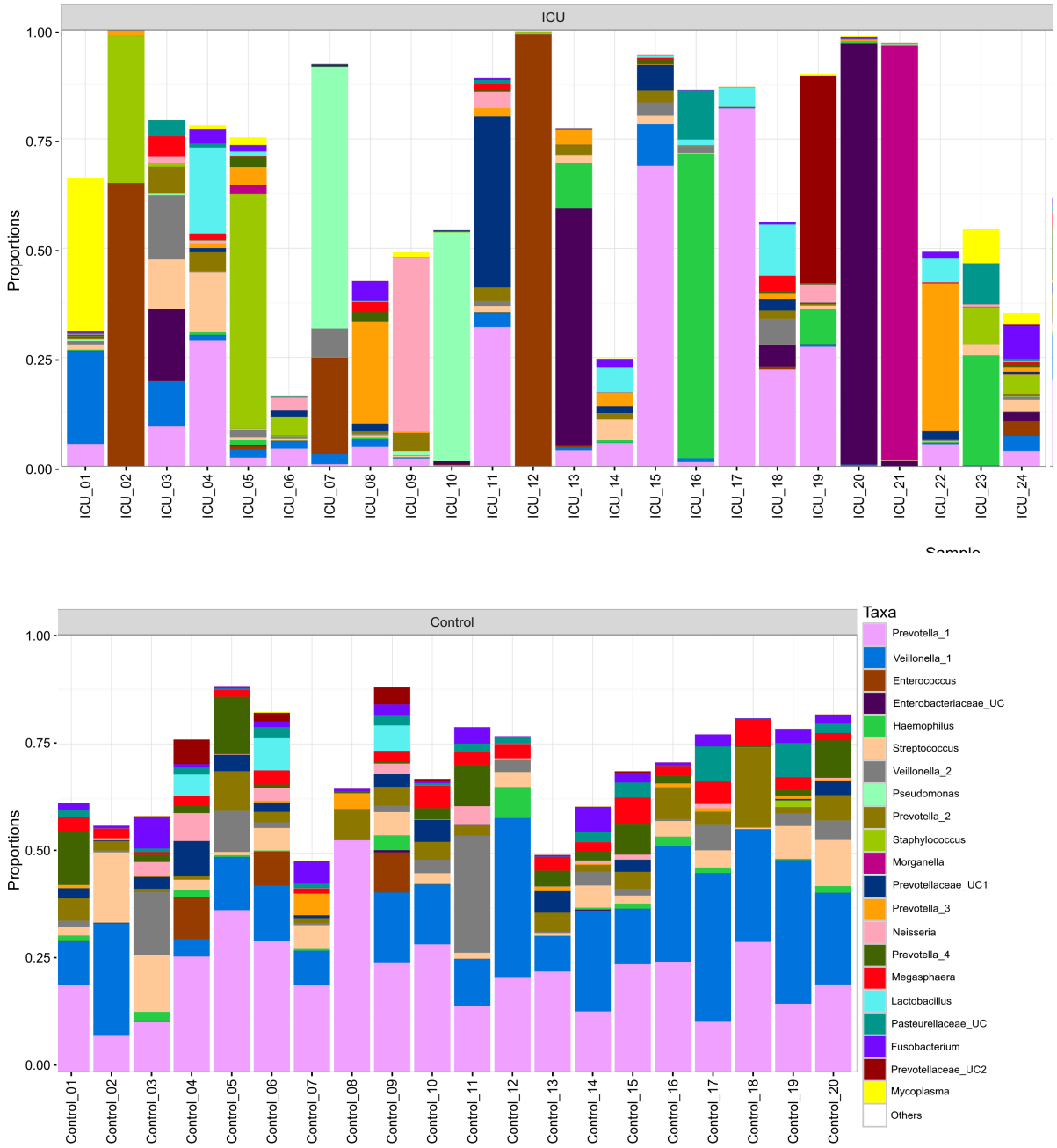

Figure 3.5 - Taxonomic classification of bacterial reads.

Subglottic mucus samples were processed using 16S rRNA bacterial profiling. Bacterial reads at operational taxonomic units (OTUs) level demonstrating the top 20 bacterial OTUs in each sample, ICU n = 24, control n = 20.

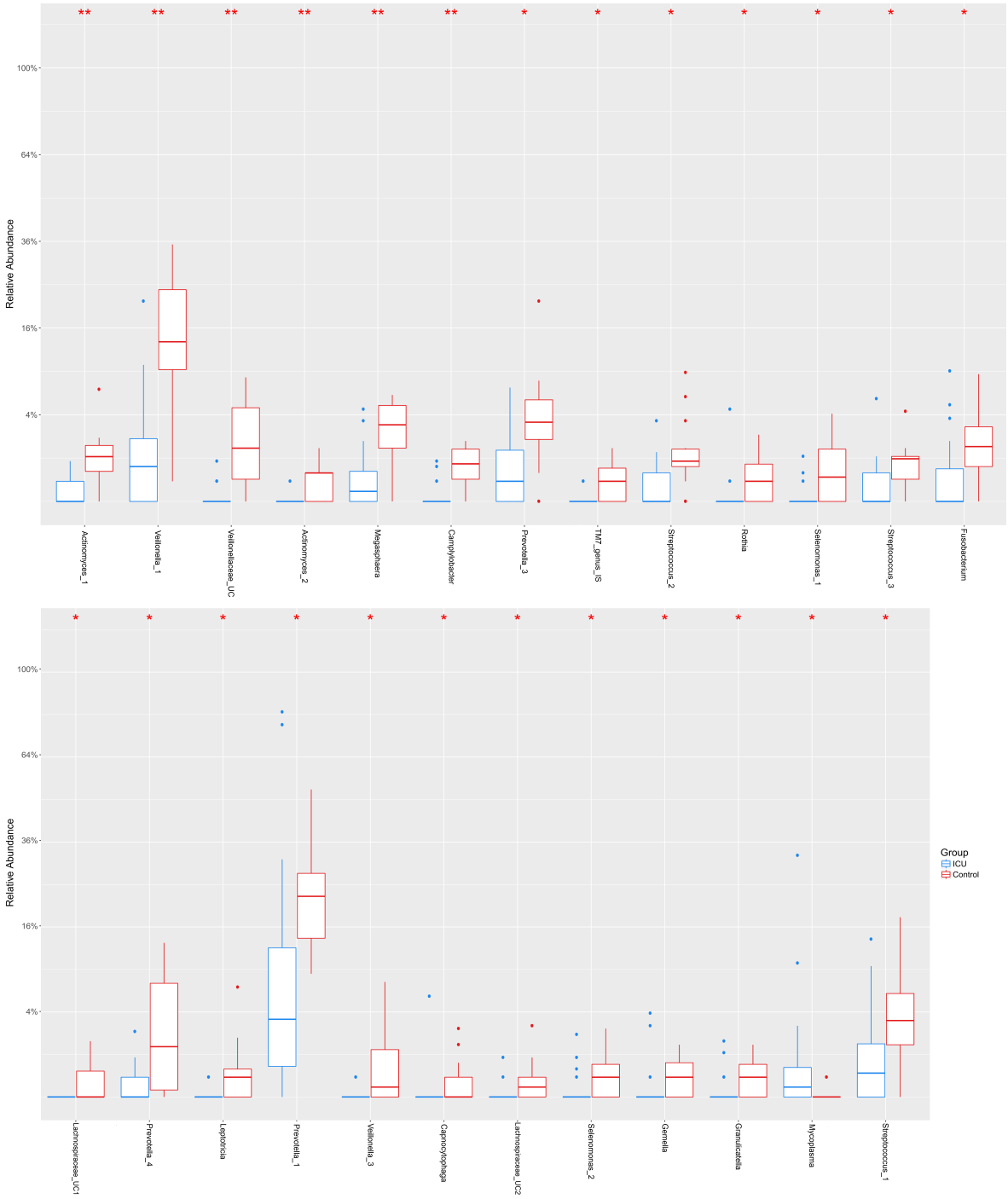

Figure 3.6 - Boxplot analyses of the significantly distinct bacterial operational taxonomic units (OTUs).

Subglottic mucus samples were processed using 16S rRNA bacterial profiling. Proportional taxa abundance is demonstrated. The boxplot shows the upper and lower quartiles and median. Mann–Whitney U test, ** $p<0.001$ *$p<0.05$, ICU n = 24, control n = 20.

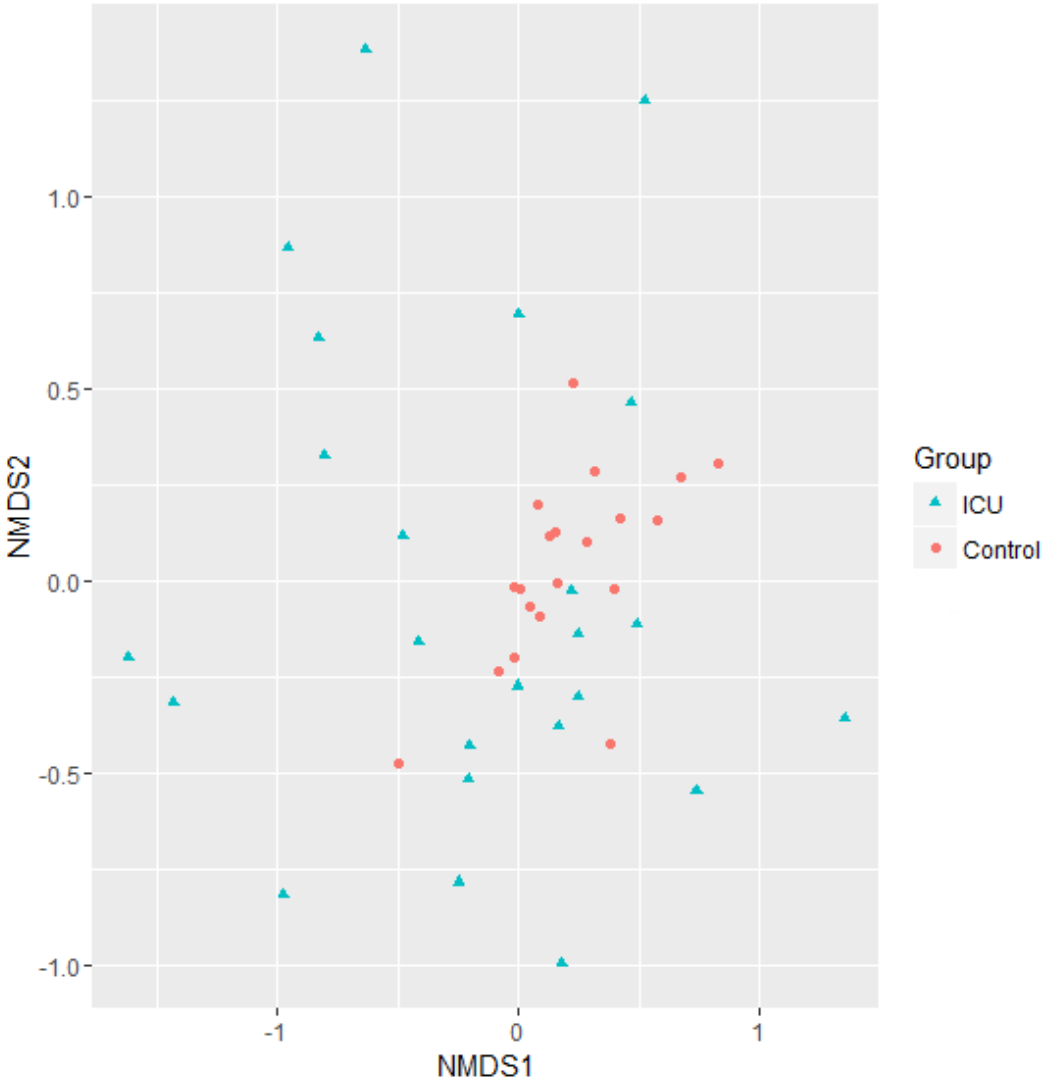

Figure 3.7 - Nonmetric multidimensional scaling (NMDS) plot.

Subglottic mucus samples were processed using 16S rRNA bacterial profiling. NMDS plot based on Bray-Curtis distances were generated using phyloseq package. The two most distinct taxonomic variables are plotted as correlations with control (red circles) and ICU (blue triangles) samples. ICU n = 24, control n = 20.

3.3.4 ITS fungal profiling

ITS fungal profiling was used to identify fungal organisms in the subglottic mucus samples of long-term intubated ICU patients and newly intubated control patients (as described in Chapter 2 of this thesis).

I recruited 24 ICU patients and 27 control patients to the study. Of these 4 controls did not have enough mucus to extract DNA. A further 15 ICU and 21 control samples were excluded at the gene sequencing stage due to low coverage, having less than 100 reads per sample. This resulted in data being available for 9 ICU mucus samples and 2 control samples. There was no significant difference in the diversity of fungal organisms in the ICU cohort compared to the controls (Figure 3.8). Next I investigated the individual taxonomic classification of fungal reads as a measure of the organism identified (Figure 3.9).

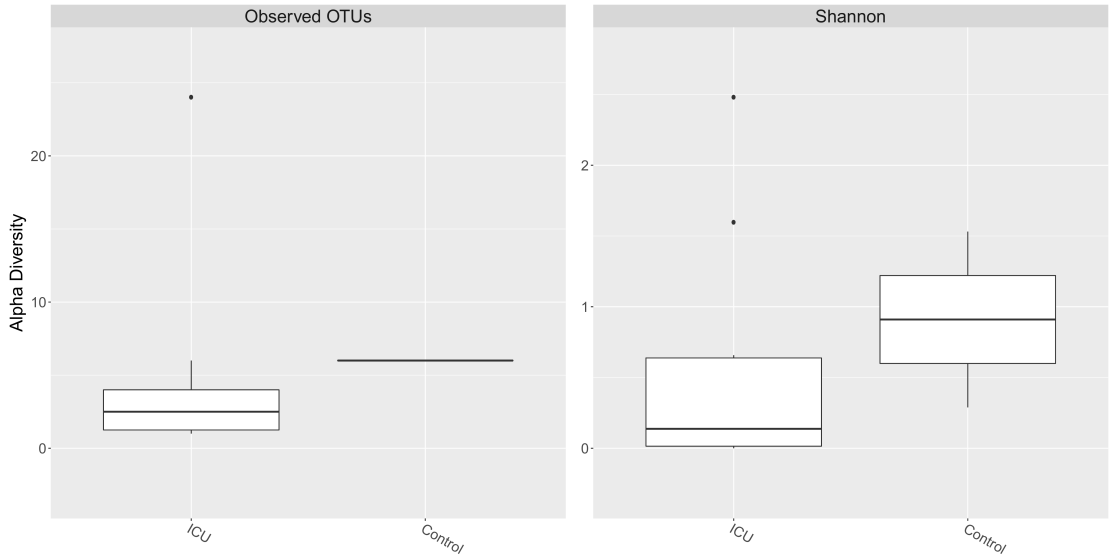

Figure 3.8 - Box plots of fungal alpha diversity measured using observed operational taxonomic units (OTUs) and Shannon diversity.

Subglottic mucus samples were processed using ITS bacterial profiling. Observed OTU and Shannon alpha diversity are demonstrated. The boxplot shows the upper and lower quartiles and median. Observed OTU diversity p = 0.16, Shannon diversity p = 0.45. Kruskal-Wallis test, ICU n = 9, control n = 2.

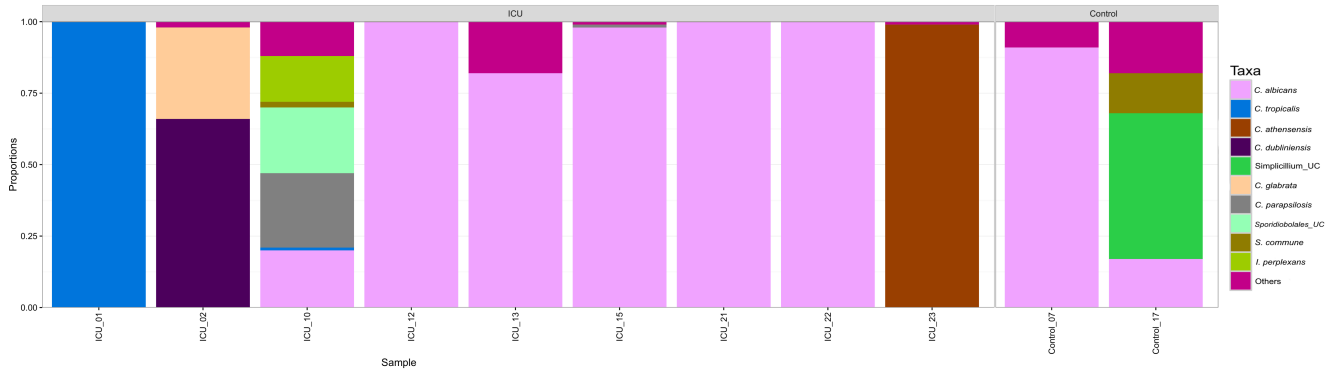

Figure 3.9 - Taxonomic classification of fungal reads.

Subglottic mucus samples were processed using ITS fungal profiling. Fungal reads at operational taxonomic units (OTUs) level demonstrating the top 20 fungal OTUs in each sample, ICU n = 9, control n = 2.

3.4 Discussion

In this chapter I sought to identify differences in the microbiology of subglottic mucus from long-term intubated ICU patients and newly intubated control patients.

I was able to demonstrate, by microbiology culture and gene sequencing, significantly reduced subglottic bacterial diversity in long-term intubated ICU patients, compared with newly intubated control patients. This is in keeping with previous studies of the lower airway and oropharynx of intubated ICU patients, which have shown reduced microbiological diversity over time (151-153). A key consideration of this finding is the potential cause of this reduced diversity - intubation and ventilation, critical illness, antibiotic administration or other factors (150). Zakharkina *et al* performed longitudinal bacterial sequencing of the lower airway of ICU patients. They demonstrated reduced microbiological diversity, however in subgroup analysis they were able to suggest that this was independent of antibiotic administration (151). Subgroup analysis of antibiotic or immunosuppressive use in my ICU cohort was not possible due to the small patient cohorts. Reduced microbial diversity has been extensively demonstrated in other inflammatory respiratory conditions, particularly cystic fibrosis, where reducing diversity correlates with disease progression in many studies (154-157).

The less diverse microbiology in the ICU cohort, compared to controls, was paralleled by distinct differences in the organisms identified. The bacterial gene profiling and microbiology culture data demonstrate that the control cohort consistently had a broad, heterogeneous microbiology, with characteristic oral commensal organisms (158, 159). It seems likely that these organisms have been introduced at the time of endotracheal intubation. Conversely, however, this may simply represent a normal flora of this sub-site. It has been repeatedly demonstrated that even areas previously considered sterile, such as the lung, still contains a distinct microbiome, largely indistinguishable from upper respiratory flora, just at a much lower density (160, 161). To my knowledge, while many other airway sub-sites have been studied, the microbiology of the subglottic airway has never been defined (162). Nevertheless, this cohort of newly intubated patients does provide an appropriate control group to the long-term intubated ICU patients.

There was little concordance between the microbiology of different patients' mucus samples in the ICU cohort, by culture or gene profiling. In many of the ICU patients one organism was dominant, in contrast to the more diverse microbiology found in the control cohort. These dominant organisms were often pathogenic organisms such as *Pseudomonas*, *Enterococcus* or *Enterobacteriaceae.* Semi-quantitative microbiology cultures also frequently confirmed these organisms to be the dominant in CFU counts. The individual organisms identified from VAP lung lavage samples are highly variable, depending on several factors, including the geographical location and patient group, however these organisms are commonly found in VAP (8, 9, 11). The origin of VAP-causing organisms is debated. Traditionally it has been suggested that the organisms migrate from the oral cavity or sinuses, or are introduced by healthcare workers (19, 21). Others suggest that these organisms are aspirated into the airway with infected stomach contents (20). It has also been postulated that the extensive use of acid suppression therapy in critical illness, to prevent ulcer formation, leads to overgrowth of gastric bacteria, which is subsequently aspirated into the airway (20). In microbiological culture I did identify a number of organisms in the ICU cohort that are associated with the gastrointestinal tract, such as *Enterococcus* and *Enterobacteriaceae* (163, 164). However it is impossible to confirm the origin of these organisms without doing multiple sub-site sampling. Another consideration is the role of biofilms, which have frequently been identified on the tracheal cuff of ICU patients (17, 165). Biofilms are of course important in terms of increasing antimicrobial resistance (166). Measurement of biofilm markers was not performed in this current work, but would be an area for potential further investigation.

Overall there was reasonable concordance between the standard cultures and gene sequencing in the ICU cohort. While direct comparisons are limited by the different methodologies and grouping of organisms there are nevertheless clear parallels. An interesting finding was the presence of significantly more *Mycoplasma* in the ICU mucus samples, which was identified by bacterial gene profiling. This was not detected on microbiology culture, as this would require specialised culture media and lengthy incubation periods that were not performed. A high incidence of *Mycoplasma* has recently been identified in a series of VAP lavage samples, and has been linked with immunomodulation (167, 168). While I only detected *Mycoplasma* in a limited number of samples it may be an area for further investigation. Routine microbiology

cultures performed in standard healthcare culture panels would in most cases not detect this organism. In the control cohort there was some concordance between the standard cultures and gene sequencing. *Streptococcus* was frequently identified by both techniques, however other organisms, such as *Veillonella,* were only identified on bacterial gene sequencing. This is probably because the microbiology culture techniques used were comparable with standard NHS culture techniques, which are biased towards the culture of pathogenic organism and are not exhaustive. They did not include specialised culture medium and lengthy incubation periods as would be required by some organisms, such as many anaerobes.

The presence of fungus, in particular *Candida,* in many of the ICU mucus samples on culture, was an intriguing finding. Fungus is identified in only a small proportion of VAP cases, however its importance in VAP is debated in the literature (8, 9, 11). Some studies suggest that fungal organisms alone are direct VAP causing agents, others that they act synergistically with pathogenic bacteria, and some suggest that fungal species are unrelated to VAP entirely (8, 9, 11, 169-171). Obvious potential contributors to the presence of fungus in the subglottic mucus samples would be the use antibiotic or systemic immunosuppressive agents in the ICU patients. Subgroup analysis of this was not possible in my ICU cohort due to the small sample sizes. Low reads meant that there were only a small number of results for the fungal gene sequencing. This may represent lack of fungal organisms, however low reads are also found in processing issues, and this difference is indistinguishable.

3.5 Conclusions

Long-term intubation has long been suggested as an independent causative agent in VAP development, providing a conduit for aspiration into the lower airway of virulent microorganisms (1, 14, 15). Subglottic mucus is gravitationally pooled above the cuff immediately above the lungs (1, 14, 15). The findings of this chapter identify a distinctly different microbiological environment in long-term intubated ICU patients compared to newly intubated controls. This is exemplified by the reduced diversity and increased proportion of pathogenic organisms in the ICU cohort, compared to controls (8, 9, 11). Subglottic suction drainage has repeatedly been demonstrated to effectively prevent VAP (27). I postulate that more effective, targeted subglottic drainage should remove this purulent material prior to aspiration and therefore prevent VAP. Chapter 4 of this thesis will investigate if there are host defence factors that may contribute to these microbiological differences.

3.6 Future work

Key considerations of the findings in this chapter are the cohorts used. I compared the organisms identified in two separate groups of patients. A longitudinal study in a group of newly intubated ICU patients (ideally followed up over several days), would demonstrate the changes in organisms identified. Furthermore it would more clearly be able to identify possible causes of microbiological changes, for example changes pre- and post-antibiotic therapy. These patients would however be difficult to identify and recruit for several logistical reasons, not least the consent process, which would need to include medical consent if next of kin cannot be identified acutely. However, based on these initial findings, this study would be a key next step.

If a longitudinal study were not possible it would be necessary to validate these findings in a larger, adequately powered cohort of ICU and control patients. Due to the patient number in the current cohort it was impossible to perform any meaningful subgroup analysis of the ICU patients. Investigation of clinical variables, such as VAP development, antibiotic and proton pump inhibitor use, presence of a subglottic suction drain and survival, would be highly valuable (172). Another important correlation would be with the lower airway microbiological findings in those patients developing VAP. Furthermore correlation with other host defence factors (described in chapter 4 of this thesis) may give additional insights into the reason for microbiological differences between the two cohorts. Due to the potential for reflux to be an aetiological factor in VAP development it would also be useful to measure subglottic markers of reflux, such as bile acids, pepsin and pH changes, in correlation with microbiology and host factors (173-175). It would also be helpful to sample multiple aerodigestive sub-sites, such as the oral cavity, pharynx, stomach, trachea (below the cuff) and lung to compare microbiological differences.

There are limitations in the ICU cohort that could be addressed in a future study. The main inclusion criterion was the presence of a tracheal tube for four or more days. This made for a highly heterogeneous ICU cohort, including patients intubated and ventilated for numerous reasons, from young patients with head injuries to elderly patients with hospital- or community-acquired pneumonia. Amongst the number of host differences that may contribute to the microbiological changes in this ICU cohort, it is likely that these patients would have different oropharyngeal

microbiology prior to intubation (176). This ICU cohort does however represent the heterogeneous nature of ICU patients (177). There are more homogeneous ICU cohorts, such as post-cardiac surgery patients, which could be utilised, however these findings would be less translatable to the wider ICU community at risk of VAP development (178, 179).

There are also limitations in the control group used. They had only just been intubated (<30 minutes), and the nature of their planned procedure allowed access to their upper airway for sampling, with only limited additional procedures. This cohort are, however, having an upper airway examination for 'persistent throat problems', and while the upper airway examination demonstrated a structurally normal airway, it is possible they may have chronic inflammatory conditions, such as laryngopharyngeal reflux, that may impact on their subglottic microbiology or chronic infections, such as chronic laryngitis (180, 181). Another consideration is that the introduction of an endotracheal tube is likely to be responsible for the oropharyngeal commensal organisms identified in the control cohort. An ideal control population would be awake non-intubated volunteers, but sampling from this region would be technically challenging. However, as an ICU patient at risk of VAP has an endotracheal tube introduced the present controls offer an acceptable comparator.

Another area for future work would be the use of more extensive and fully quantitative microbiology cultures. However, this would require extensive resources, such as cultures performed in triplicate and a wide range of selective culture media. The gene profiling could also be performed in a more quantitative manor to allow for measurement of organism density which would be an important factor in determining the potential pathogenicity of the subglottic mucus.

Chapter 4: Characterisation of subglottic mucosal host defences

4.1 Introduction

It has previously been demonstrated that even short-term tracheal intubation (i.e. for several hours) can cause significant mucosal inflammation around the tracheal cuff (49, 50). Furthermore, tracheal intubation can radically impede the function of the mucociliary escalator (45-48).

The mucus layer covering epithelial cells has a crucial role in maintenance of mucosal immune homeostasis (182). Mucus forms a physical barrier to protect the epithelium, helps to clear pathogens via the mucociliary escalator, and also has important interactions with cellular host defences (182). Mucus consists primarily of water and mucin (large molecular weight biopolymers), but also includes immune cells, DNA and anti-microbial proteinases, such as neutrophil elastase (77, 81). The most predominant secreted (and gel-forming) mucins in airway mucus are MUC5B and MUC5AC (77, 83, 84). Changes in the fractions of mucus constituents can have significant effects on the rheological properties of mucus and subsequently its function (81). A large number of inflammatory/immune response mediators have been shown to induce secretion of mucins by epithelial cells (77). These include common airway cytokines that have been shown to be increased in short-term tracheal intubation, such as IL-8, IL-6 and IL-1β (49, 50, 183-186).

Short-term intubation has also been shown to induce a rapid introduction to the local environment of immune cells, particularly granulocytes, such as neutrophils (49, 50, 187). This is likely due to the local pro-inflammatory response of the epithelium to tracheal intubation (49, 50). A number of these pro-inflammatory agents and chemotactic cytokines, such as IL-8, IL-6, IL-1β and C5a, can stimulate immune cell recruitment to the region (188). Neutrophils are predominantly pro-inflammatory cells and this recruitment is likely to have a further impact on local inflammation, through further immune cell recruitment, and release of pro-inflammatory cytokines and proteinases (58, 189, 190).

I therefore aimed to investigate the subglottic mucosal host defences in a group of long-term intubated ICU patents, intubated and mechanically ventilated for four or more days, and a cohort of newly intubated (<30 minutes) control patients.

4.2 Chapter aims

In this chapter I sought to answer the following questions:

1. What are the composition and physical properties of mucus derived from the subglottis of long-term intubated ICU patients and newly intubated control patients?
3. What are the immunological characteristics of mucus derived from the subglottis of long-term intubated ICU patients and newly intubated control patients?
4. How functional are neutrophils extracted from the subglottic mucus and whole blood of long-term intubated ICU patients and newly intubated control patients?

4.3 Results

4.3.1 Cohort demographic data

The demographic details of the newly intubated theatre attenders (control) cohort, in comparison with the ICU cohort, are described in section 3.3.1. Both groups provided the mucus samples described in this chapter. The ICU cohort also provided whole blood samples. An additional cohort of 16 healthy volunteers were recruited to provide only blood samples, and their demographic and clinical data are shown in Table 4.1 (in which the demographic details for the ICU cohort are repeated for comparative purposes). There was no significant difference in the age or gender of the ICU patients compared with either of the two control cohorts (Tables 3.1 and 4.1). Full inclusion and exclusion criteria for all groups are detailed in Tables 2.2 and 2.3.

	ICU patients (n=24)	Healthy blood volunteer controls (n=16)	*P* Value
Median age, years (range)	66 (20 – 79)	67 (23 – 89)	0.54*
Percentage of patients male	63	50	0.43†
Median (IQR) mechanically ventilated	5 (4 – 6)	0	
Mortality, %	42	0	
Percentage receiving immunosuppressive drugs (including corticosteroids)	21	0	
Percentage receiving antibiotics	83	0	

Table 4.1 - Demographic and clinical data for patient and control groups

Definition of abbreviations: ICU = intensive care unit; IQR = interquartile range; * By Mann–Whitney U test, † By Chi Squared test

4.3.2 Constituents of mucus derived from the subglottis

I designed experiments to determine the composition and properties of mucus derived from the subglottic region of long-term (four or more days) ventilated ICU patients and newly intubated control patients.

I assessed several factors that might contribute to the properties of the mucus samples, including the proportions of water to solids as measured via the dry weight. This demonstrated a significantly higher proportion of solids in the ICU samples, compared to controls (Figure 4.1). Next I investigated the concentration of the two most common gel-forming airway mucins MUC5B and MUC5AC in these mucus samples. This demonstrated significantly higher concentrations of MUC5B (median concentration ICU 21.7 mg/mL versus controls 3.5 mg/mL), but not MUC5AC (Figure 4.2). In addition, I demonstrated significantly higher levels of DNA in ICU patients' mucus compared to controls (Figure 4.3). One would expect dehydrated mucus with a high proportion of mucins and DNA to be more viscous (191). I therefore measured viscosity in a number of mucus samples using a rheometer. This demonstrated a significantly more viscous mucus gel in the ICU cohort compared with newly intubated control samples (Figure 4.4).

These results demonstrate a difference in the fraction of key mucus constituents and subsequent changes in the rheological properties of mucus from long-term intubated ICU patients compared with newly intubated control patients.

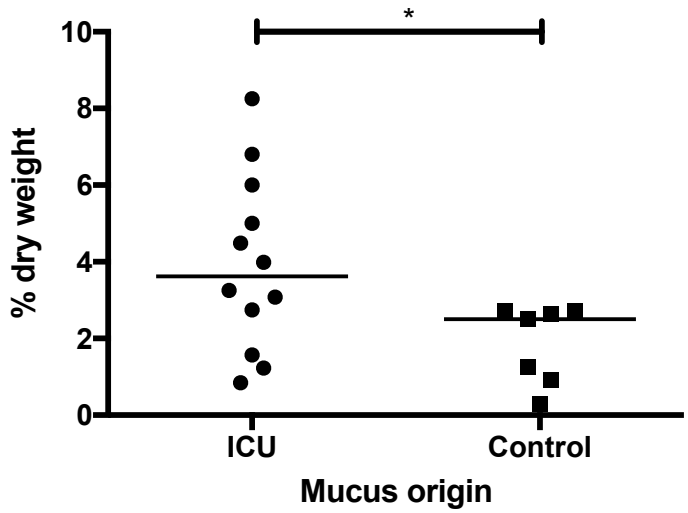

Figure 4.1 - Percentage dry weight of mucus samples.

Mucus samples were weighed before and after dehydration overnight at 80°C. The percentage solid was then calculated. Horizontal bars depict median values. Mann–Whitney U test, *$p < 0.05$, ICU n = 12, control n = 7.

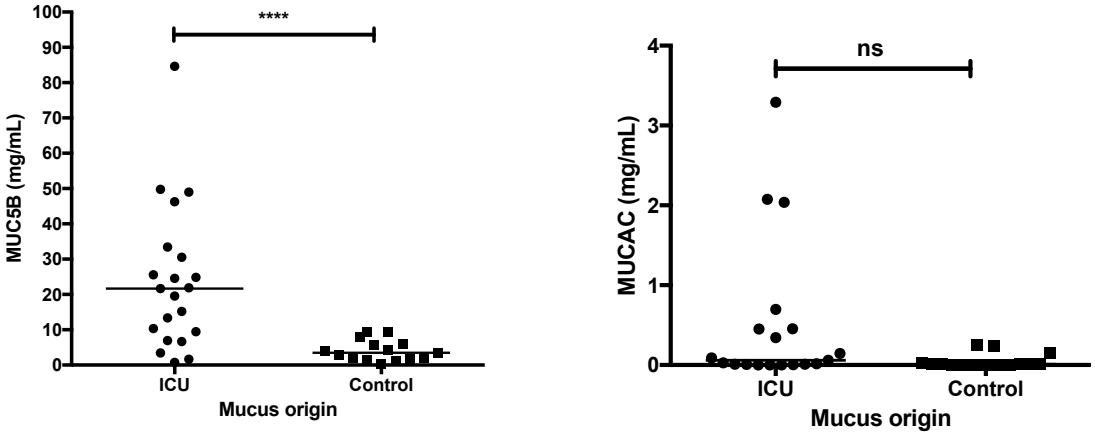

Figure 4.2 - MUC5B and MUC5AC concentrations in mucus samples.

Mucin MUC5B and MUC5AC concentrations were quantified in mucus samples by ELISA. Horizontal bars depict median values. Mann–Whitney U test. MUC5B ****$p<0.0001$, ICU n = 19, control n = 16. MUC5AC non-significant (ns), ICU = 21, control n = 15.

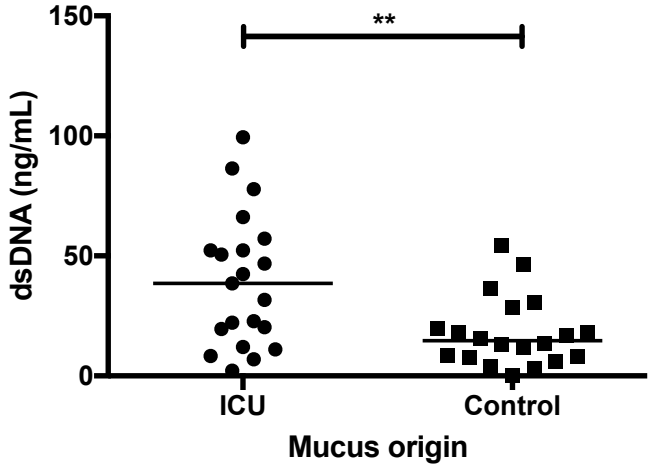

Figure 4.3 - Double stranded (ds)DNA content in mucus samples.

Mucus dsDNA concentration was quantified by Quant-i assay kit. Horizontal bars depict median values. Mann–Whitney U test, **$p<0.01$, ICU n = 21, control n = 20.

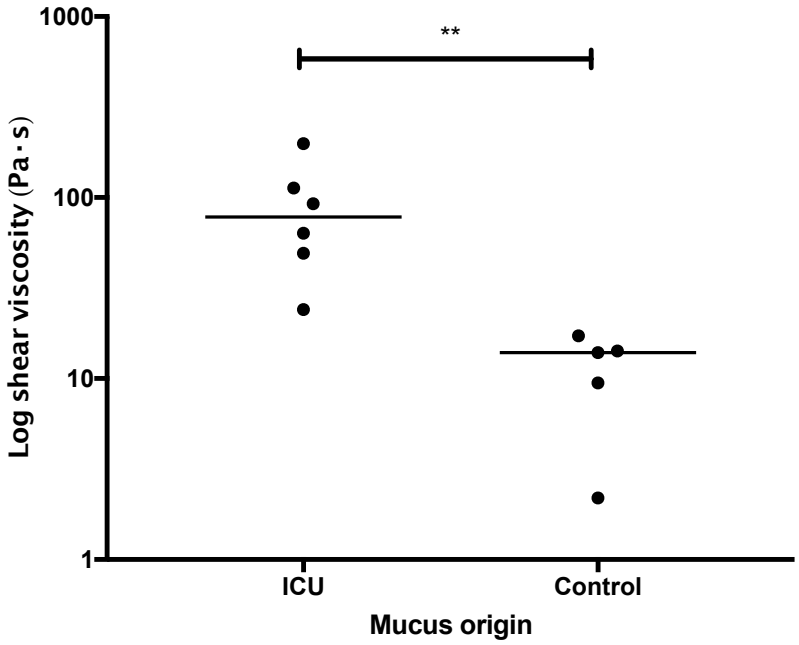

Figure 4.4 - Mucus viscosity measurements.

Mucus samples' shear viscosity were rheologically analysed at 37°C. Horizontal bars depict median values in Pascal-seconds (Pa·s) at a shear rate of 1 s^{-1}. Mann–Whitney U test, **p<0.01, ICU n = 6, control n = 5.

4.3.3 Immune profile of mucus derive from the subglottis

I further investigated the immunological properties of mucus derived from the subglottis. Firstly I found a significantly higher number of neutrophils in mucus derived from the subglottic region of long-term ventilated ICU patients compared to newly intubated control patients (Figure 4.5). There was no significant difference in the number of macrophages however. The percentage proportion of each cell type was significantly different (chi squared test $p<0.001$) (Figure 4.5). Given the large number of neutrophils found in the subglottic mucus of the ICU cohort I further investigated the viability of these cells using a flow cytometry-based assay on cells extracted from the mucus. This demonstrated that the majority of cells in both the ICU and control cohorts were viable, and that there was no significant difference in the proportion of viable, early/late apoptotic cells or necrotic cells (Figure 4.6). Neutrophils release a number of proteases, which are important in pathogen killing and mucosal immune homeostasis. I measured one of these key proteases, human neutrophil elastase. This was found to be at significantly higher concentrations in the mucus of ICU patients compared with controls (Figure 4.7).

To investigate the potential mechanisms behind the observed neutrophil recruitment, and other host defence changes, the levels of cytokines were examined in plasma and mucus samples. These were analysed using an MSD immunoassay, except anaphylatoxin C5a, which was examined using a Cytometric Bead Array (CBA) kit. Cytokines IL-8, IL-6, IL-1β and IL-10 were at significantly higher levels in the mucus samples (Figure 4.8), as well as the plasma samples (Figure 4.9) of ICU patients compared with controls. In both cohorts the relative concentration of each agent was higher in mucus samples compared to plasma (Figures 4.8 and 4.9). There was no correlation between mucus and plasma concentration in matched ICU mucus and plasma samples (data not shown). The concentration of C5a was significantly higher in the mucus samples of ICU patients compared to controls, but no difference was demonstrated between plasma samples (Figure 4.10). I also measured granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is an important immune modulator that has profound effects on the functional activities of various circulating leukocytes, including neutrophils (192). I found that concentrations were significantly higher in mucus samples of ICU patients compared to controls (Figure 4.11). Levels of GM-CSF were undetectable in plasma samples using this assay.

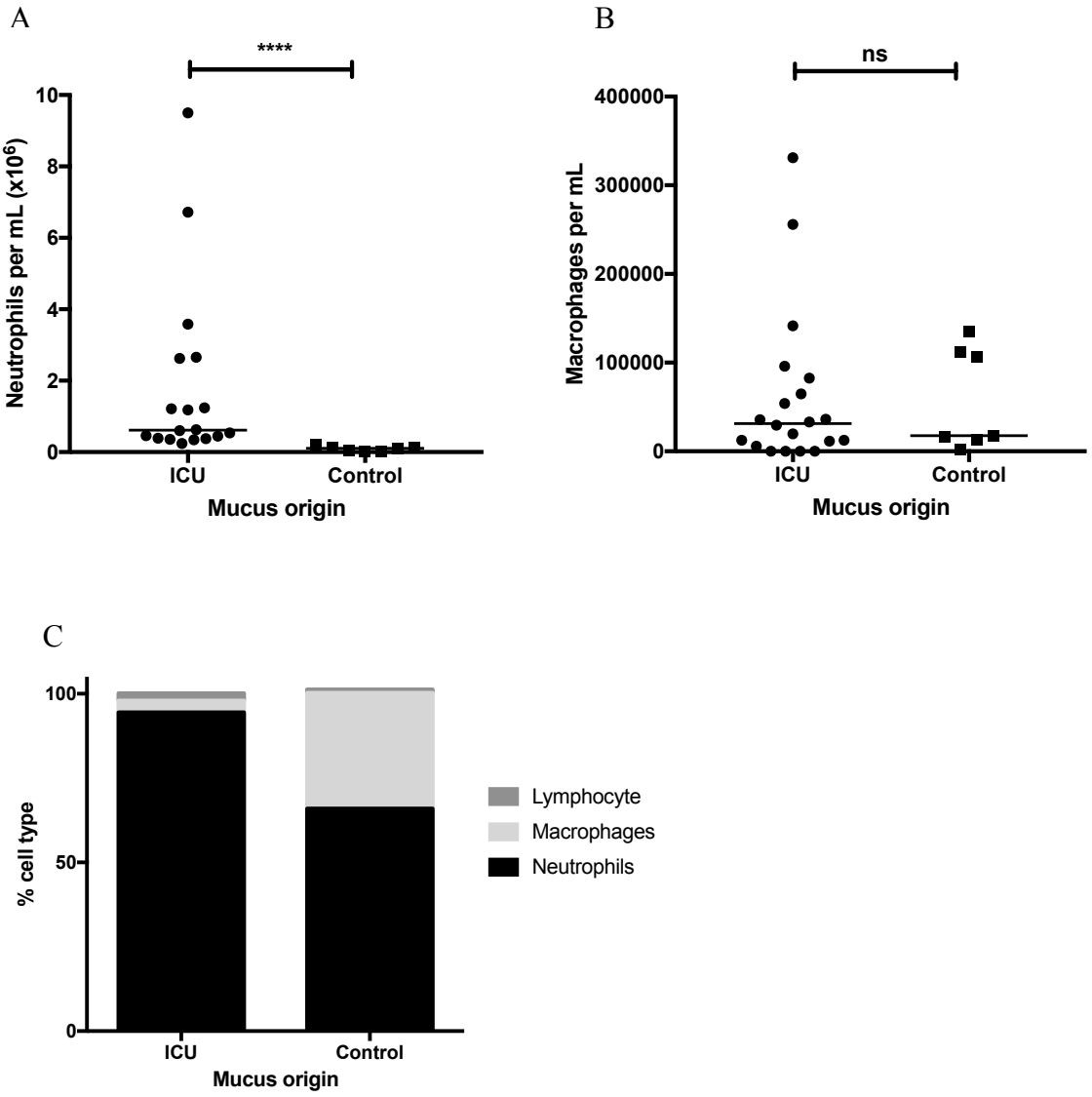

Figure 4.5 - Immune cell profile of subglottic mucus

Cells were extracted from the mucus of ICU patients or newly intubated control patients after treatment with 0.08% dithiothreitol. Differential cell count was then perfumed using a haemocytometer and cytospin preparations. The number of neutrophils (A) and macrophages (B) per mL is depicted. Horizontal bars depict median values. Mann–Whitney U test, ****$p<0.0001$. The percentage proportion of each cell type is also demonstrated (C), Chi squared test $p<0.001$. ICU n=18, control n=7.

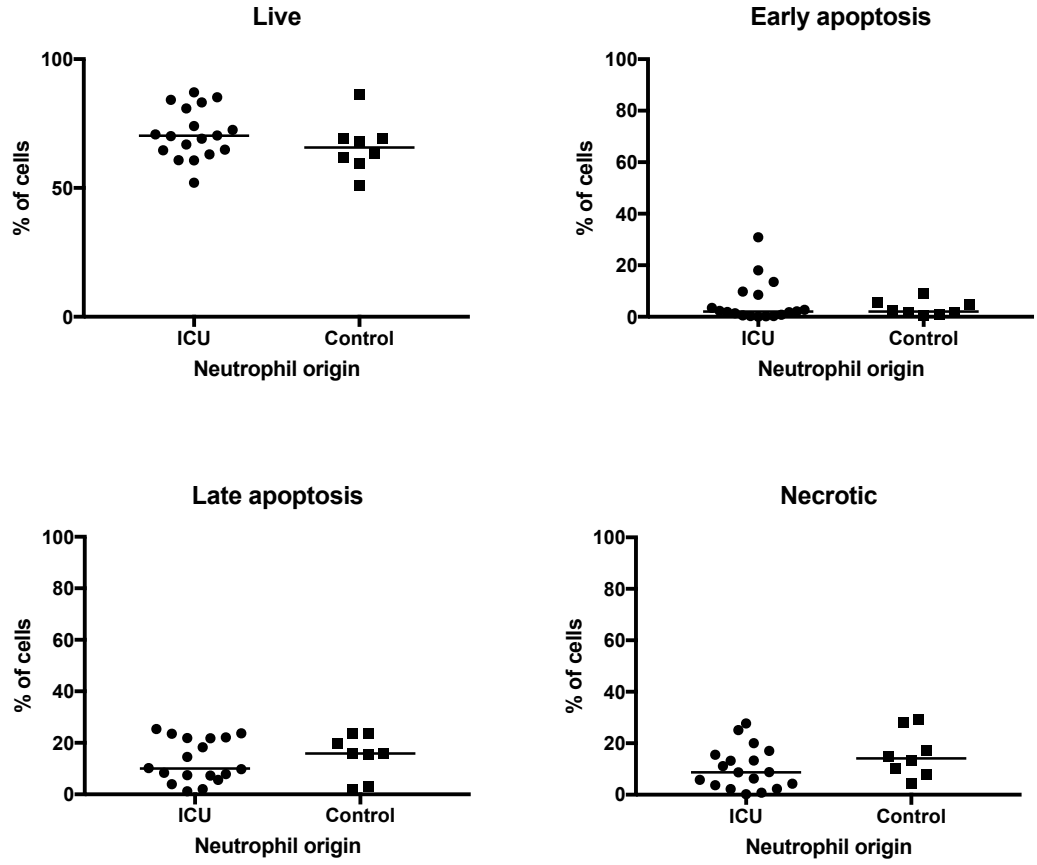

Figure 4.6 - Subglottic mucus-derived neutrophil viability.

Cells were extracted from the mucus of ICU patients or newly intubated control patients via treatment with 0.08% dithiothreitol. Cells were incubated with Annexin V and propidium iodide. Viability, apoptosis and necrosis were determined by flow cytometry gating of neutrophils with expression of these two agents. Horizontal bars depict median values. Mann–Whitney U test. No significant difference between the two cohorts was demonstrated, ICU n = 18, control n = 8.

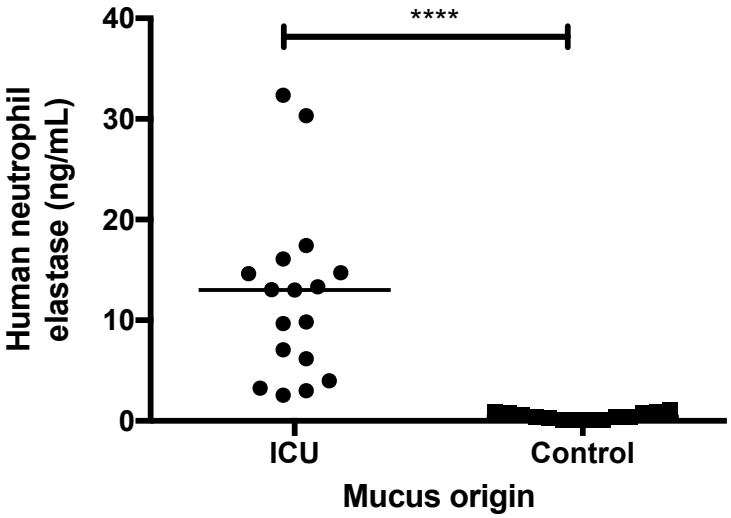

Figure 4.7 - Human neutrophil elastase content in subglottic mucus.

Human neutrophil elastase concentration in mucus was measured using ELISA. Horizontal bars depict median values. Mann–Whitney U test, ****$p<0.0001$, ICU n = 17, control n = 14.

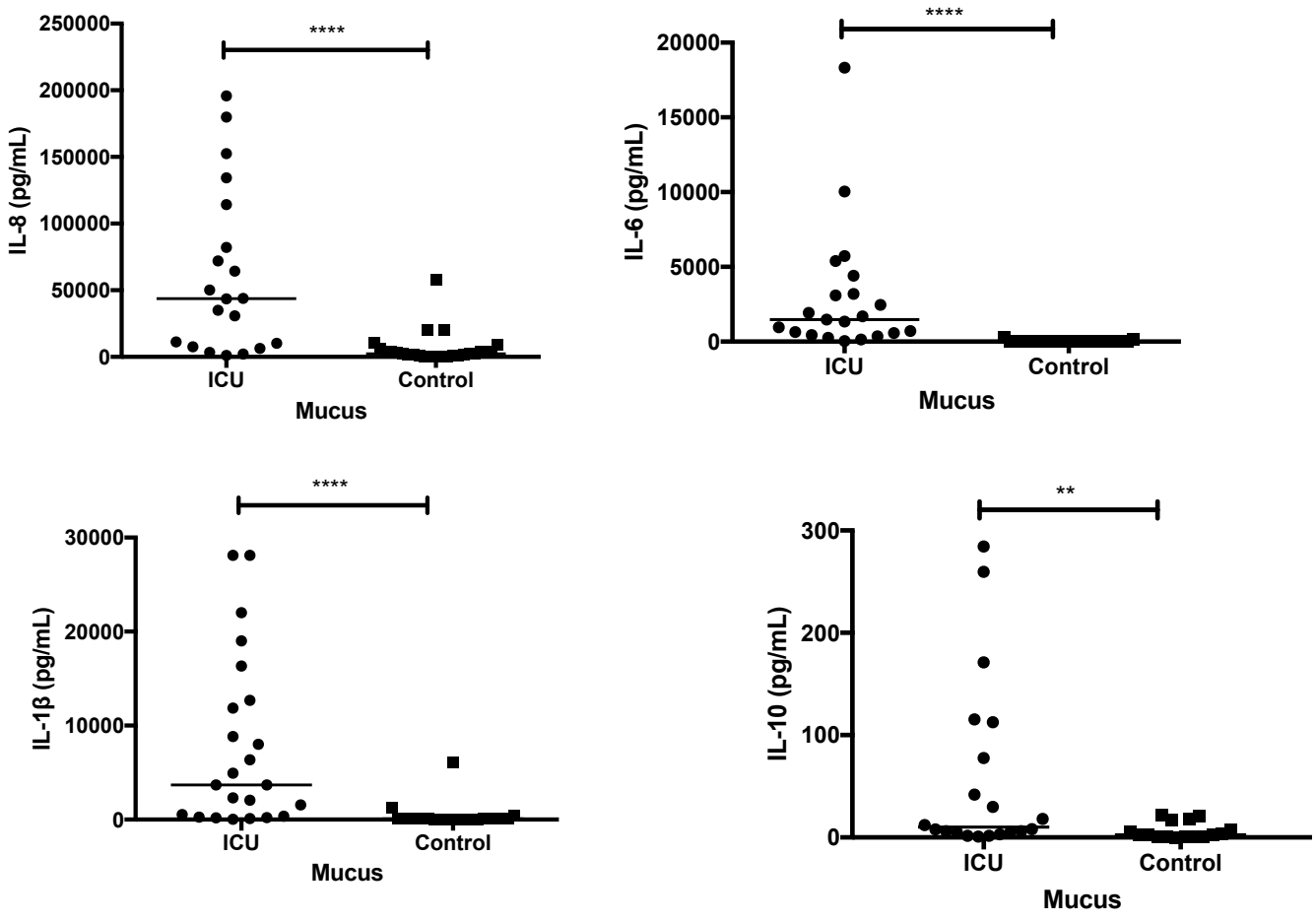

Figure 4.8 - Cytokine content in subglottic mucus.

Cytokines were measured using MSD immunoassay. Horizontal bars depict median values. Mann–Whitney U test, **p<0.01, ****p<0.0001, IL-8 ICU n = 20, control n = 26, IL-6 ICU n = 21, control n = 21, IL-1β ICU n = 23, control n = 22, IL-10 ICU n = 20, control n = 16.

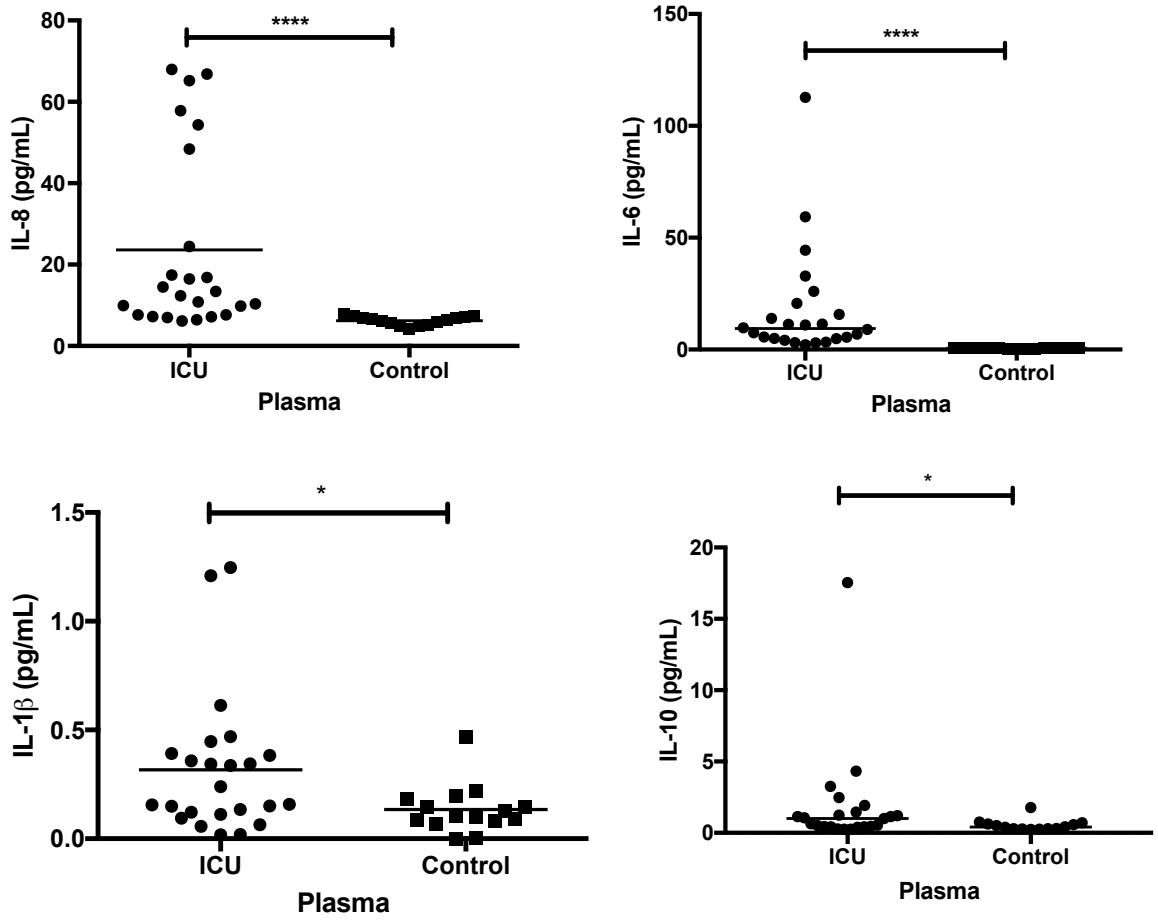

Figure 4.9 - Cytokine content in circulating plasma.

Cytokines were measured using MSD immunoassay. Horizontal bars depict median values. Mann–Whitney U test, *$p<0.05$, ****$p<0.0001$, *$p<0.05$, IL-8/IL-6/IL-1β ICU n = 24, control n = 15, IL-10 ICU n = 23, control n = 14.

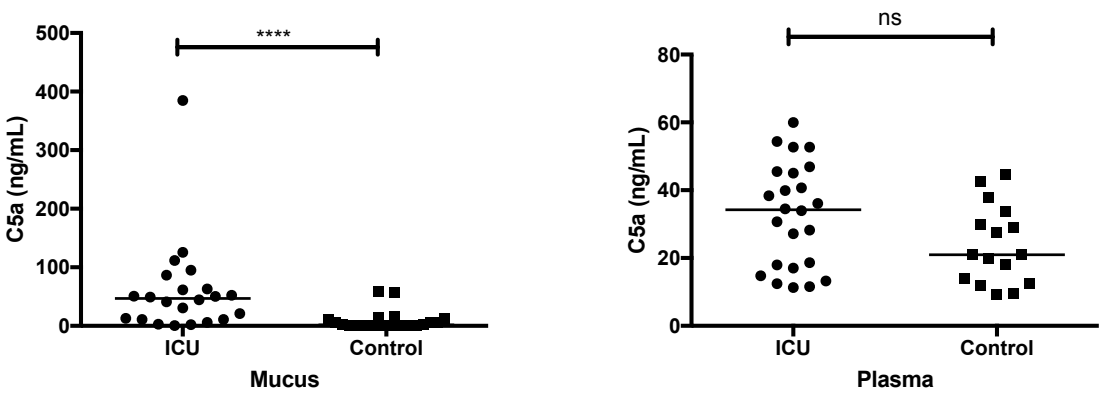

Figure 4.10 - C5a quantification in subglottic mucus and circulating plasma.

Anaphylatoxin C5a was measured using a Cytometric Bead Array (CBA) kit. Horizontal bars depict median values. Mann–Whitney U test, ****$p<0.0001$, mucus ICU n = 22, control n = 22, plasma ICU n = 24, control n = 16.

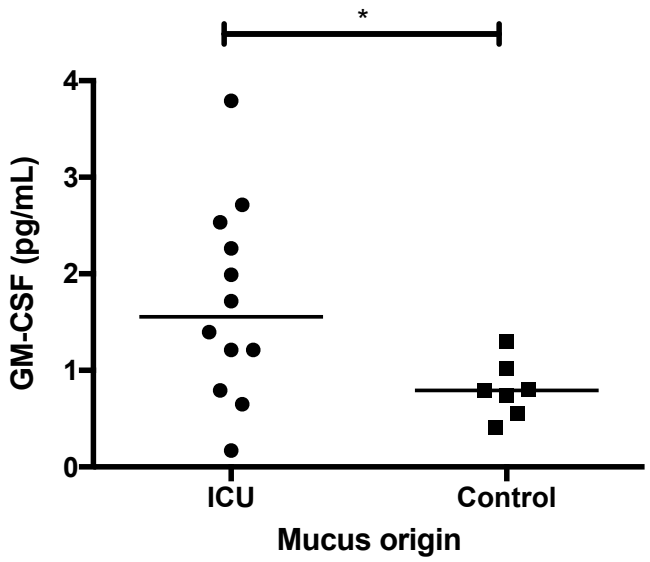

Figure 4.11 - GM-CSF content in subglottic mucus.

GM-CSF concentration in mucus was measured using MSD single-plex immunoassay. Horizontal bars depict median values. Mann–Whitney U test, *$p<0.05$, ICU n = 12, control n = 7.

4.3.4 Functional studies of neutrophils derived from subglottic mucus and whole blood

In light of the increased number of viable neutrophils found in the subglottic mucus of long-term-ventilated patients, I aimed to investigate the functional capacity of these cells. I also attempted to investigate the functional ability of neutrophils derived from the systemic circulation of the same ICU patients. The control group for the blood-derived neutrophils was a cohort of healthy volunteers (Table 4.1). The ability of neutrophils to phagocytose is a key function in the clearance of pathogens (52, 53). This was measured using serum-opsonised pHrodo® green *S. aureus* Bioparticles®. No overall difference was observed in the percentages of blood-derived neutrophils involved in phagocytosis from the ICU cohort, compared to the cohort of healthy volunteers (Figure 4.12). Equally, no significant difference was detected between the percentages of neutrophils involved in phagocytosis from the mucus-derived neutrophils of ICU patients compared to newly intubated control patients (Figure 4.12). The overall level of phagocytosis in the ICU mucus-derived neutrophils was however significantly lower than that in the ICU blood-derived neutrophils (Figure 4.12). There was however no correlation between mucus- and blood-derived neutrophil phagocytic function in paired ICU samples (data not shown).

I went on to investigate the surface marker expression of blood- and mucus-derived neutrophils. There was no significant difference in the expression of activation markers CD62L (shed with activation) or CD11b (increased with activation) in either mucus- (Figure 4.13) or blood-derived cells (Figure 4.14). I also investigated neutrophil expression of the C5a surface receptor CD88 (which is internalised in response to C5a binding (42)). There was a significantly higher surface expression of CD88 on ICU-mucus derived neutrophils, compared to controls (Figure 4.13). Despite my previous findings of higher levels of C5a in subglottic mucus derived from ICU patients compared to controls (Figure 4.10). Further to this, the blood-derived neutrophil expression of CD88 was significantly lower in ICU patients compared to controls (Figure 4.14). This is in contrast to my previous findings that demonstrated no significant difference in C5a levels in plasma derived from ICU patients or controls (Figure 4.10). There was no correlation between mucus- and blood-derived neutrophil phagocytic function and any surface marker expression (data not shown).

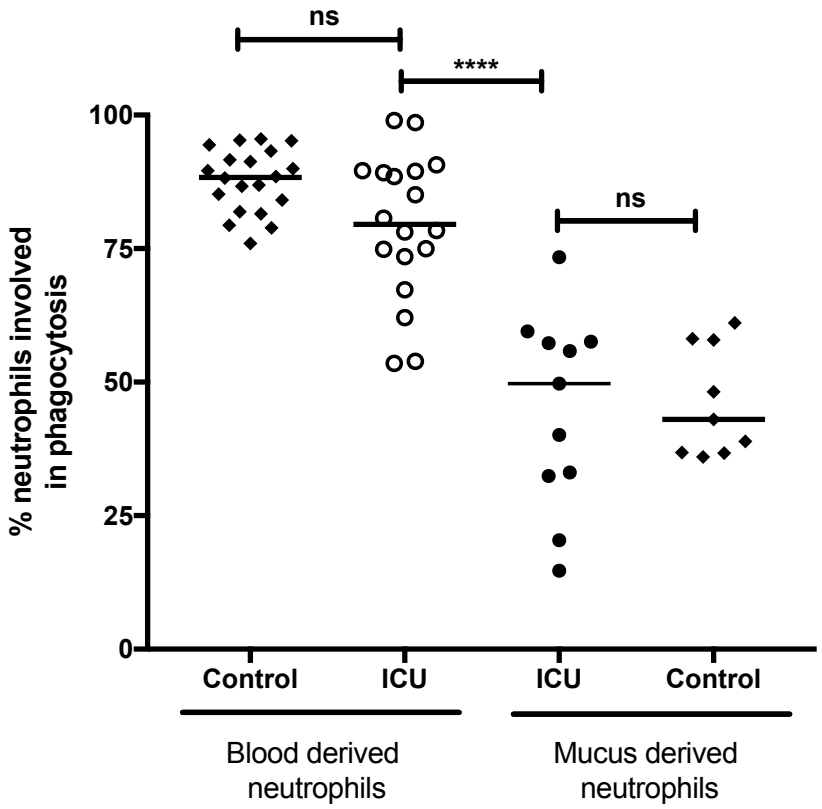

Figure 4.12 - Blood- and mucus-derived neutrophils' phagocytic function.

Cells were extracted from the whole blood of ICU patients or healthy volunteers using dextran sedimentation, or from the mucus of ICU patients or newly intubated control patients after treatment with 0.08% dithiothreitol. Cells were incubated at 37°C for 1.5 hours with serum-opsonised pHrodo® green *S. aureus* Bioparticles®. Phagocytosis was determined by flow cytometry gating of viable neutrophils with ingested particles, versus a control sample on ice. Horizontal bars depict median values. Kruskal-Wallis test and Dunn's post hoc test were used, ****$p<0.0001$, blood ICU n = 20, control n = 16, mucus ICU = 11, control n = 9.

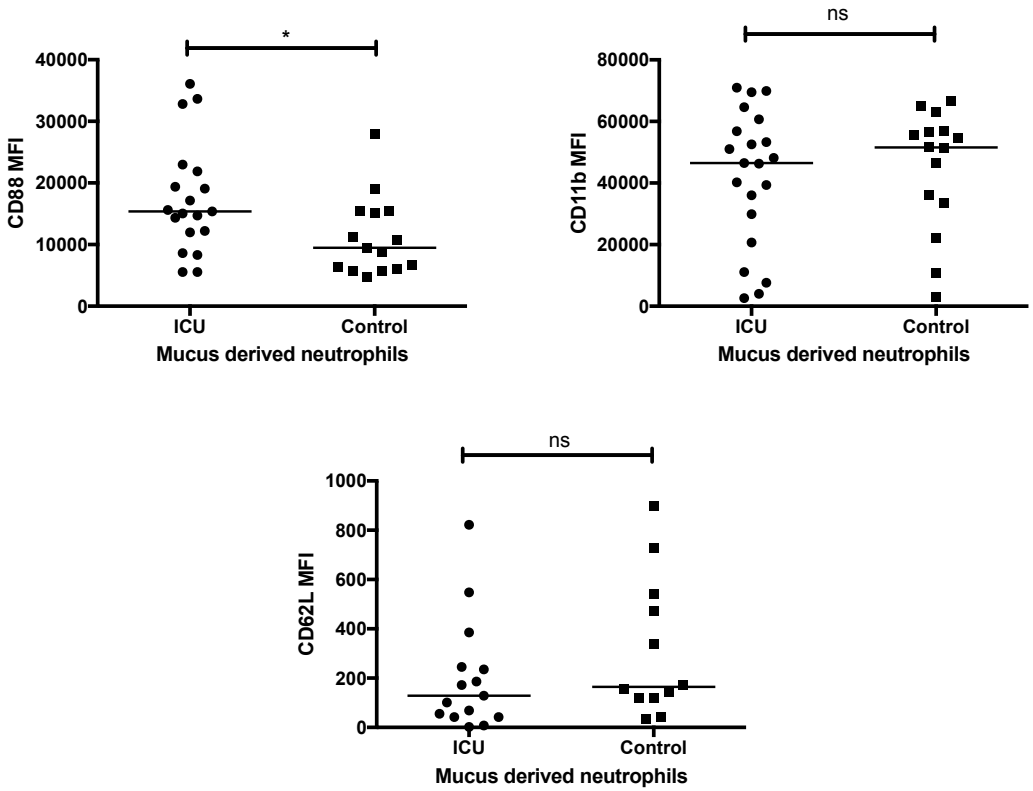

Figure 4.13 - Surface marker expression on subglottic mucus-derived neutrophils.

Cells were extracted from the mucus of ICU patients or newly intubated control patients via treatment with 0.08% dithiothreitol. Viable neutrophils were stained with antibodies to the C5a receptor CD88, and activation markers CD11b (increased with activation) and CD62L (reduced with activation). Horizontal bars depict median values. Mann–Whitney U test, *$p<0.05$, CD88 ICU n = 19, control n = 15, CD11b ICU n = 21, control n = 15, CD62L ICU n = 15, control n = 12. MFI = median fluorescent intensity.

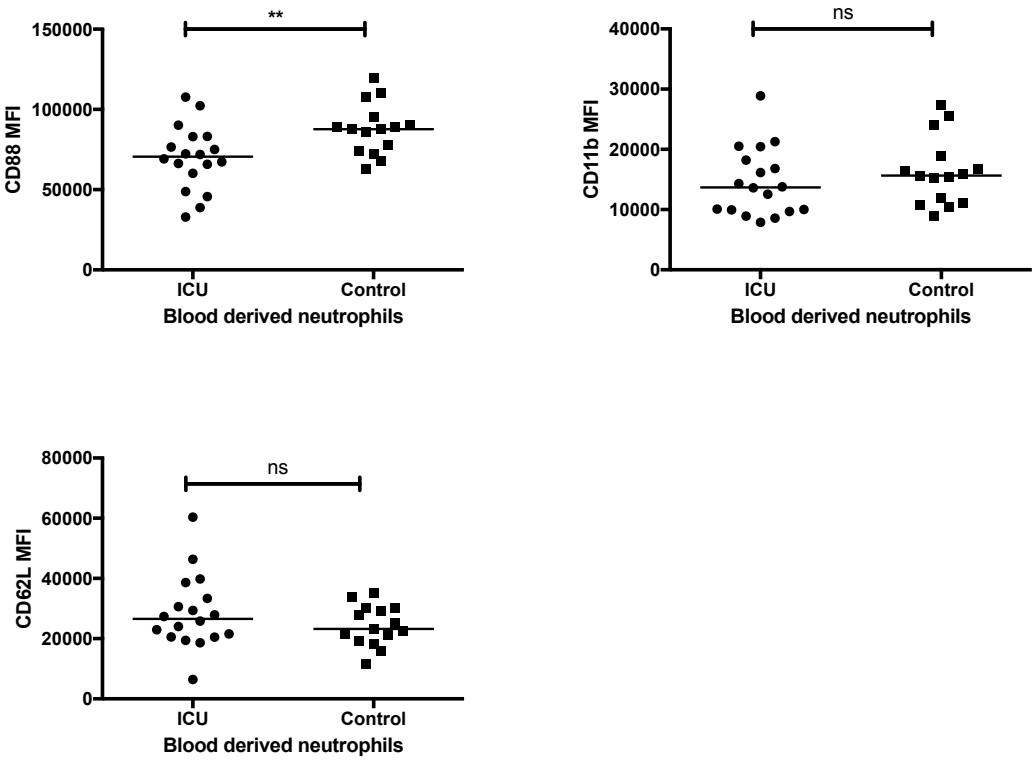

Figure 4.14 - Surface marker expression on blood-derived neutrophils.

Mixed leukocytes were extracted from the whole blood of ICU patients or healthy volunteers via dextran sedimentation. Viable neutrophils were stained with antibodies to C5a receptor CD88, and activation markers CD11b (increased with activation) and CD62L (reduced with activation). Horizontal bars depict median values. Mann–Whitney U test, **$p<0.001$, ICU n = 18, control n = 16. MFI = median flourecent intensity.

4.4 Discussion

In this chapter I sought to identify differences in subglottic host factors between long-term ventilated ICU patients and newly intubated control patients that may have contributed to the differences identified (in chapter 3 of this thesis) in the microbiology of the subglottic region in these two groups. I was able to demonstrate potentially important differences in the constituents and physical properties of the mucus derived from ICU patients compared to controls. Subglottic ICU mucus was shown to have a lower water content and higher concentration of MUC5B and DNA. These factors are likely to have contributed to the higher viscosity found with ICU subglottic mucus compared to control mucus samples. There was also evidence of local subglottic, as well as systemic, inflammation in the ICU cohort, compared to controls. There was also a significantly higher proportion, and concentration, of viable neutrophils in the subglottic mucus of ICU patients, compared to newly intubated controls. Finally there was no substantial difference in the phagocytic function of blood- or mucus-derived neutrophils, from ICU patients compared with controls.

Changes in the proportion of mucus constituents can have significant effects on the physical properties of mucus, influencing its ability to function as a lubricant and selective barrier (81). I was able to demonstrate significantly more viscous mucus in the ICU subglottic samples than in controls. A number of factors that I investigated could have contributed to this, including the ratio of liquid to solids. The percentage of solids in mucus in health has been reported as anywhere between 2 – 10%, depending on the sub-site (193). Reference values are not available for the subglottic region. However in CF, characterised by dehydrated airways, levels are reported as commonly above 8% solids (194). The proportion of solids in sputum from asthmatic patients has been reported in the region of 7 - 8% (195). Levels in chronic bronchitis and bronchiectasis have been reported as approximately 6% solids (194, 196). The median percentage solid in the ICU cohort was only 3.6, compared to 2.5 in newly intubated controls. While this does not approach the levels reported for these chronic diseases there was a broad distribution of values in the ICU cohort up to the levels found in CF. Even small differences in the concentrations of mucins may be sufficient to cause significant changes in the mucus viscosity (81, 197). I demonstrated a higher concentration of MUC5B in the subglottic mucus of long-term intubated ICU patients (median 21.7 mg/mL), compared to newly intubated controls (median 3.5 mg/mL). Reference values are not available for the subglottic region and reports from other respiratory sub-sites are variable and often based on older or non-quantifiable assays (81, 198, 199). Previous

comparable studies have suggested normal total mucin levels in a non-diseased airway of between 2 – 3 mg/mL (76, 96). An interesting study from Dennesen *et al* demonstrated total mucin levels in the lower airway of healthy controls as 1.9 mg/mL, ventilated patients without infection as 3.1 mg/mL and patient with VAP as 15.6 mg/mL (96). Mucus rheology is not simply determined by the concentration of mucins however. Mucin glycosylation and sulphation can affect viscosity also, which can change with multiple host and non-host factors, such as age or the presence of pathogens in the airway (77, 81). Furthermore the ratio of different mucins can affect viscosity. I have demonstrated that in the subglottic region MUC5B is the predominant mucin in both cohorts sampled. MUC5B and MUC5AC are by far the most predominant mucins in the airway, however their ratio can also vary depending on the airway sub-site (77, 83, 84). The importance of these mucin ratios are not understood, however changes in these ratios, due to mucin over or under production, has been demonstrated in many respiratory diseases, including pulmonary fibrosis, COPD and asthma (77, 200-202). DNA is another important factor in the viscosity of mucus, particularly in diseased airways (81). DNA can originate from epithelial shedding, but also neutrophil lysis, as is found in certain respiratory diseases like CF where DNase markedly reduces the viscosity of mucus (81). I was able to demonstrate significantly higher levels of DNA in the mucus of ICU patients, compared to controls.

Increased mucus viscosity is thought to aid innate immune defence by trapping particles that can subsequently be cleared by the mucociliary escalator (77, 84). However it has been previously demonstrated that tracheal intubation can reduce the function of the mucociliary escalator (45-48). Failure of the mucociliary escalator combined with prolonged, extensive or inappropriate mucin hypersecretion has been implicated in airway obstruction and poor clearance of pathogens in many respiratory diseases, such as asthma, COPD and CF (77, 78, 81, 88, 203).

It has previously been demonstrated that even short-term tracheal intubation can result in significant mucosal inflammation in humans and animal models (49, 50). Even at a median time of 3 hours of intubation Puyo *et al* demonstrated a significant rise in cytokines IL-6 and IL-1β, an increasing trend of IL-8 concentration and a significant increase in C5a concentrations in a longitudinal theatre patient cohort (50). There was also a ten-fold increase in the number of polymorphonuclear cells over the same time (50). Puyo's findings were however based on uncorrected lavage samples, which are known to be unreliable due to variable dilution factors (97). My results do however corroborate the findings of Puyo *et al,*

in that I was able to demonstrate significantly higher levels of IL-6 IL-1β, IL-8 and C5a in the mucus of long-term intubated ICU patients compared to controls. There are several methodological differences between the two studies, for example in the length of intubation, and also in the patient groups, theatre patients (in Puyo's study) are less unwell than ICU patients (in my study) in most cases. Further to Puyo's findings I was able to demonstrate significantly higher levels of GM-CSF and human neutrophil elastase in long-term intubated patients, when compared with controls.

There are a number of reasons why the demonstrated levels of pro-inflammatory agents in the subglottic mucus of long-term-ventilated patients may be important. Firstly this may explain a mechanism behind the higher levels of MUC5B. A large number of inflammatory/immune response mediators have been shown to induce secretion of mucins by epithelial cells (77). Secretagogues associated with MUC5AC have been investigated far more extensively than MUC5B (77). However IL-8, IL-6 and IL-1β have all been implicated in increased secretion of MUC5B by airway cells (183-186). Secondly these agents may influence the recruitment of immune cells, particularly the large number of neutrophils demonstrated in my results. IL-8 is a highly potent neutrophil chemokine, and IL-6, IL-1β, C5a and GM-CSF are all potent chemoattractants with roles in immune modulation (188, 204).

Neutrophils are crucial to the clearance of bacterial pathogens and are one of the key first lines of defence in the innate immune response (52, 53). However prolonged, extensive or inappropriate neutrophil presence in the airway is associated with propagation of a number of diseases in the airway, including CF, asthma, COPD and bronchiectasis (63, 69-71). Neutrophils are predominantly pro-inflammatory cells, and their role is to acutely clear pathogens, and then rapidly leave the site or apoptose, thereby resolving the inflammation (52, 53, 63, 189). The presence of a large number of neutrophils in the subglottic mucus of long-term ventilated ICU patients is an important finding. Neutrophils release a multitude of pro-inflammatory cytokines, such as IL-8, IL-6 and IL-1β, which will further precipitate the local inflammation, neutrophil migration and MUC5B secretion (205). Neutrophils also release proteases either through degranulation, cell necrosis or NETosis (52, 53). An important protease in inflammation and disease is neutrophil elastase (63). Although its physiological role is to degrade phagocytosed proteins, it can also cause significant damage to the airway. Neutrophil elastase has been implicated in directly damaging the epithelial membrane by degrading structural proteins, propagating inflammation through the epithelial production of cytokines, such as IL-8 (64-66). Also prolongation of the inflammatory process

by degrading complement and releasing C5a, a potent chemoattractant for neutrophils (67). Neutrophil elastase has also been shown to reduce ciliary beat frequency of the respiratory epithelium (65). An important consideration however is the role of antiproteases, which are crucial to counteracting the effects of proteases (206). Measurement of these agents in subglottic mucus would be an important future area of investigation. The presence of large numbers of neutrophils are also likely to contribute to the accumulation of DNA through cell necrosis or NETosis, further contributing to increased mucus viscosity.

An intriguing finding was the lack of increased neutrophil apoptosis in the ICU mucus-derived neutrophil cohort, compared to controls. Neutrophil apoptosis is crucial to the resolution of inflammation (207-210). The presence of several times more neutrophils and no greater proportion of apoptotic cells in the ICU cohort would likely lead to a precipitation of inflammation (63, 189). Several pro-inflammatory cytokines, including GM-CSF, prolong neutrophil survival (211, 212). I was able to demonstrate significantly higher concentrations of GM-CSF in the ICU cohort than controls, which may in part explain these findings.

I went on to investigate the functional abilities of neutrophils extracted from subglottic mucus. This involved extraction of cells from the mucus using DTT. DTT is a potent agent capable of splitting glycoprotein disulphide bonds, thus releasing cells from the mucin glycoprotein matrix. The literature is conflicting as to what degree DTT affects neutrophils function (213-216). DTT was used in both mucus-derived neutrophil cohorts, therefore reliable comparisons between neutrophil functional outcomes in these groups is possible. This demonstrated no difference in the percentage of cells involved in phagocytosis between ICU patients and newly intubated control patients. This therefore highlights that the large number of neutrophils in the subglottic mucus of ICU patients are as functionally capable as those from newly intubated controls. The neutrophils therefore appear to have intrinsic ability to phagocytose and clear pathogens. It should however be noted that by extraction from the mucus, the cells are removed from the environment in which they are expected to phagocytose, as described earlier in this chapter. The question therefore is: do these neutrophils still function as effectively if they were returned to the ICU or control mucus environment? This question is examined in chapter 5 of this thesis.

The proportion of mucus-derived neutrophils involved in phagocytosis was significantly lower than that of blood-derived neutrophils. Direct comparison between these measurements has to be considered with caution due to the different methodologies used to extract these

cells (dextran sedimentation for blood and DTT treatment for mucus samples). This data may therefore represent an artefact of processing. I was unable to demonstrate any overall difference in phagocytosis between the ICU blood-derived neutrophils and neutrophils from a cohort of healthy volunteers. It should however be noted that the ICU cohort was an extremely heterogeneous group, including elderly septic patients and young post-head injury patients (see table 3.2). This may explain the large range of phagocytic capacity demonstrated in the ICU cohort. Previous studies have determined that blood-derived neutrophils from critically ill ICU patients have phagocytic dysfunction, which is thought to be mediated through over-exposure to C5a (41, 42). My current ICU cohort was however very different from these previous studies. The inclusion criteria were primarily around length of intubation, and not severity of illness. However, I did demonstrate significantly lower expression of CD88 (which is internalised on exposure to C5a) in the whole blood sample of the ICU cohort compared to healthy volunteer blood samples. This was in contrast to the absence of significantly higher plasma concentration of C5a overall. Furthermore I was unable to demonstrate any correlation between C5a and its marker CD88 and neutrophil phagocytosis. In fact the results from mucus-derived neutrophils demonstrated the contrary, in that CD88 was actually expressed at a higher level on neutrophils in the ICU cohort, than in controls. This was despite significantly higher levels of C5a detected in subglottic mucus of ICU patients compared to controls. This does however corroborate previous studies that showed airway lavage samples derived from critically ill patients were able to inhibit neutrophil phagocytic function, but in a mechanism independent of C5a (42).

I did not observe any significant difference in neutrophil activation surface markers on ICU mucus- or blood-derived neutrophils, compared with control samples. It might be expected that with higher levels of neutrophil-relevant cytokines in the systemic circulation and locally, neutrophils would present a more activated phenotype (52, 53, 217). In the systemic circulation this could reflect the heterogeneous cohort of ICU patients, who in many cases did not have disseminated infection (72, 218). In the mucus samples this is less understandable and may be the result of DTT exposure, which has been suggested to affect expression of activation markers CD11b and CD62L (215, 216).

4.5 Conclusions

Subglottic mucus from long-term intubated ICU patients demonstrated distinct physical and immunological differences from that of newly intubated control patients. This may have contributed to the microbiological differences found in chapter 3 of this thesis. The presence of a tracheal tube for an extended period of time in long-term ventilated patients is likely to have led to the increased pro-inflammatory cytokine levels found in the subglottic mucus. IL-8, IL-6, IL-1β, C5a and GM-CSF are likely to have contributed to the recruitment, and persistence of, a vast numbers of viable neutrophils in the subglottic region of long-term intubated ICU patients. These neutrophils are likely to have had a further pro-inflammatory effect through release of cytokines and inflammatory proteases, such as neutrophil elastase. In parallel, non-resolving inflammation is liable to have contributed to the differences in the mucus constituents and physical properties of ICU-derived mucus, when compared to controls. In particular the effect of IL-8, IL-6 and IL-1β may have propagated the higher levels of MUC5B found in the subglottic mucus of ICU patients. Finally, when extracted from their mucus environment, the neutrophils found in the subglottic mucus of ICU patients had no defect of phagocytosis, compared to that of newly intubated controls. Chapter 5 of this thesis will investigate further if the mucus of ICU patients is capable of inhibiting neutrophil function.

4.6 Future work

Key considerations in interpreting the findings in this chapter include the cohorts used; this is discussed in section 3.6.

Specific issues relevant to this chapter include the lack of matched control blood and mucus samples. This was to prevent an additional procedures on this patient group. However, in the design of a new study, a control volunteer/patient cohort that would have comparable mucus and blood samples would be highly valuable. Another consideration is the anaesthesia applied to the theatre attender controls, which can have systemic inflammatory effects on the body (219, 220). An ideal control population would be awake non-intubated volunteers, however sampling from this region would be highly technically challenging.

It would be beneficial to increase the number of biological repeats of several key experiments. I was severely limited by the volume of mucus recovered from the control patients in particular. This limited the number of certain experiments I could do, particularly experiments such as viscosity measurements and phagocytosis assays, which require over 1 mL of mucus.

Further examination of mucus-derived neutrophil functions related to pathogen clearance would be useful (221). These would include: (a) neutrophil killing of live bacteria; (b) measurement of neutrophil oxidative burst, a key element of the neutrophil's killing machinery; and (c) investigation of actin polymerisation, crucial to neutrophil phagocytosis and chemotaxis, which may give valuable insights into neutrophils' functional abilities beyond just phagocytosis (52, 53).

A further consideration of the neutrophil phagocytosis experiments is that the bioparticles were incubated with a population of mixed leukocytes. There were differing ratios of leukocytes in the ICU and control samples and this may introduce some bias. It should be also noted that all results were based on flow cytometry-gated viable neutrophil populations only. However, ideally a population of pure neutrophils would be incubated with bioparticles in each experiment to prevent any bias from the presence of other immune cells. This was, however, representative of the population found in this sub-site. Furthermore, it was technically impossible to separate the neutrophils from other immune cells in the mucus samples, without inducing further activation or loss of viability from the extended processing. Neutrophils are inherently more phagocytic than macrophages (the second most common cell type), it is therefore unlikely that the presence of these cells will have confounded the readout

for neutrophil phagocytic function significantly (222). A further potential confounding factor was the timing of blood and mucus sampling. Samples were obtained when pragmatic, rather than at a fixed time of day. Several studies have suggested that neutrophils have a circadian rhythm with variable function throughout the day and night (223). It was logistically impossible to sample at a uniform time within or between any cohort groups. Sampling at a uniform time of day in future studies would reduce this potential bias.

The mucin ELISA used to measure MUC5B and MUC5AC were in-house ELISA kits developed within our research group. Optimisation of these ELISA techniques was performed within the research group. There are several limitations to the use of these types of assays. One key consideration is the use of standards, specifically purified pig gastric and human salivary mucin. Ideally the results of these ELISA would be confirmed by other techniques to confirm the validity of these important findings. Unfortunately there are limited antibodies available for these mucins and many other techniques rely on the same primary antibodies for detection.

There are a multitude of other factors that could be measured (by ELISA or other techniques) from the subglottic mucus samples, and may be relevant to local host defences. These include the level of other antimicrobial peptides, antiproteases and defensins and also constituents such as protein and lipids, which may have also impacted on viscosity. Finally, there may be future value in the assessment of glycosylation and sulphation of mucin molecules, which are important to mucin function and mucus viscosity, as previously discussed.

Chapter 5: The effect of mucin on neutrophil function and epithelial-pathogen interactions

5.1 Introduction

Mucus covers the epithelial lining of the respiratory tract (77, 84). Its role is in clearance of pathogens, through the mucocillary escalator, and as a chemical barrier protecting the epithelium beneath (78, 79, 84, 86). Mucus consists primarily of water and mucin (77, 81). More than 20 mucins are described, however by far the most predominant secreted (and gel-forming) mucins in the airway are MUC5B and MUC5AC (77, 83, 84). Changes in the proportion or properties of mucins can have significant effects on the rheological properties of mucus, influencing its ability to function as a lubricant and selective barrier (81). Acute mucin hypersecretion is thought to aid innate immune defence by trapping particles (77, 84). Nevertheless, prolonged, extensive or inappropriate mucin hypersecretion has been implicated in airway obstruction and poor clearance of pathogens in many respiratory diseases, such as asthma, COPD and CF (77, 78, 81, 88, 203). In chapter 4 of this thesis I demonstrated a higher concentration of mucin MUC5B in the subglottic mucus of long-term intubated ICU patients (median 21.7 mg/mL), compared to newly intubated controls (median 3.5 mg/mL). I therefore decided to investigate the impact of these higher mucin concentrations on aspects of innate immune defence relevant to the subglottic airway and potential VAP development.

The neutrophil is the key innate immune cell involved in the clearance of bacterial pathogens (52, 53). It has previously been suggested in one *in vitro* study that high mucin concentrations (31.2 mg/mL total mucin) may adversely affect neutrophils' capacity for chemotaxis and bacterial killing (76). These findings have, however, not been corroborated, nor have the mucin concentrations found in the subglottis of long-term intubated ICU patients been investigated. Mucins have also been implicated in promoting bacterial growth (89, 93). VAP-causing bacteria, such as *P. aeruginosa,* have previously demonstrated the capacity to digest the highly glycosylated portions of mucins, using them as a nutrient source (89, 93).

I therefore aimed to investigate the impact of relevant subglottic mucin concentrations on healthy volunteers' neutrophil function, and secondly the impact on bacterial growth.

5.2 Chapter aims

In this chapter I wished to answer the following questions:

1. What effect does mucin concentration have on neutrophil function?
2. What effect does mucin concentration have on bacterial-epithelial interactions?

5.3 Results

5.3.1 The impact of mucin concentration on neutrophil phagocytic function

I designed experiments to determine if mucin, purified from the mucus of long-term ventilated ICU patients, impacted on the function of neutrophils. The experiments described used healthy volunteer whole blood-derived neutrophils, and concentrations of mucin that were found in the subglottic region of; a) long-term ventilated ICU patients, and b) newly intubated control patients (demonstrated in chapter 4 of this thesis).

Firstly neutrophil viability was assessed at various concentrations of mucin. This demonstrated no significant reduction in viability of neutrophils incubated in any of the mucin concentrations tested, compared to a no mucin control sample (Figure 6.1). A key role of neutrophils is their ability to kill bacteria. This was assessed though co-culture of neutrophils with *P. aeruginosa* lab strain PA01. This demonstrated significant reduction in killing at 20 mg/mL mucin (Figure 6.2). I went on to investigate possible reasons for this dysfunction. Using a chemotaxis assay to measure the ability of neutrophils to migrate towards an attractant, a significant reduction in chemotaxis at 20 mg/mL mucin was observed (Figure 6.3).

In chapter 4 (Figure 4.12) of this thesis I demonstrated no significant difference in phagocytic function between neutrophils extracted from the subglottic mucus of long-term ventilated ICU patients (with high mucin levels) and newly intubated control patients (with lower mucin levels). I therefore investigated if mucin might induce a transient reduction in neutrophil function, impairing neutrophil function while in that high mucin environment, but reversible upon removal from it. Consequently I assessed the ability of neutrophils to phagocytose opsonised bacterial bioparticles while incubated in mucin, and after subsequent removal from the mucin, using a relevant mucolytic compound DTT (used to extract neutrophils derived from subglottic mucus samples). This assay demonstrated a significant phagocytic dysfunction in neutrophils cultured in 20mg/mL mucin, corroborating the bacterial killing results, but also demonstrated a significant restoration of this function after application of the mucolytic compound (Figure 6.4).

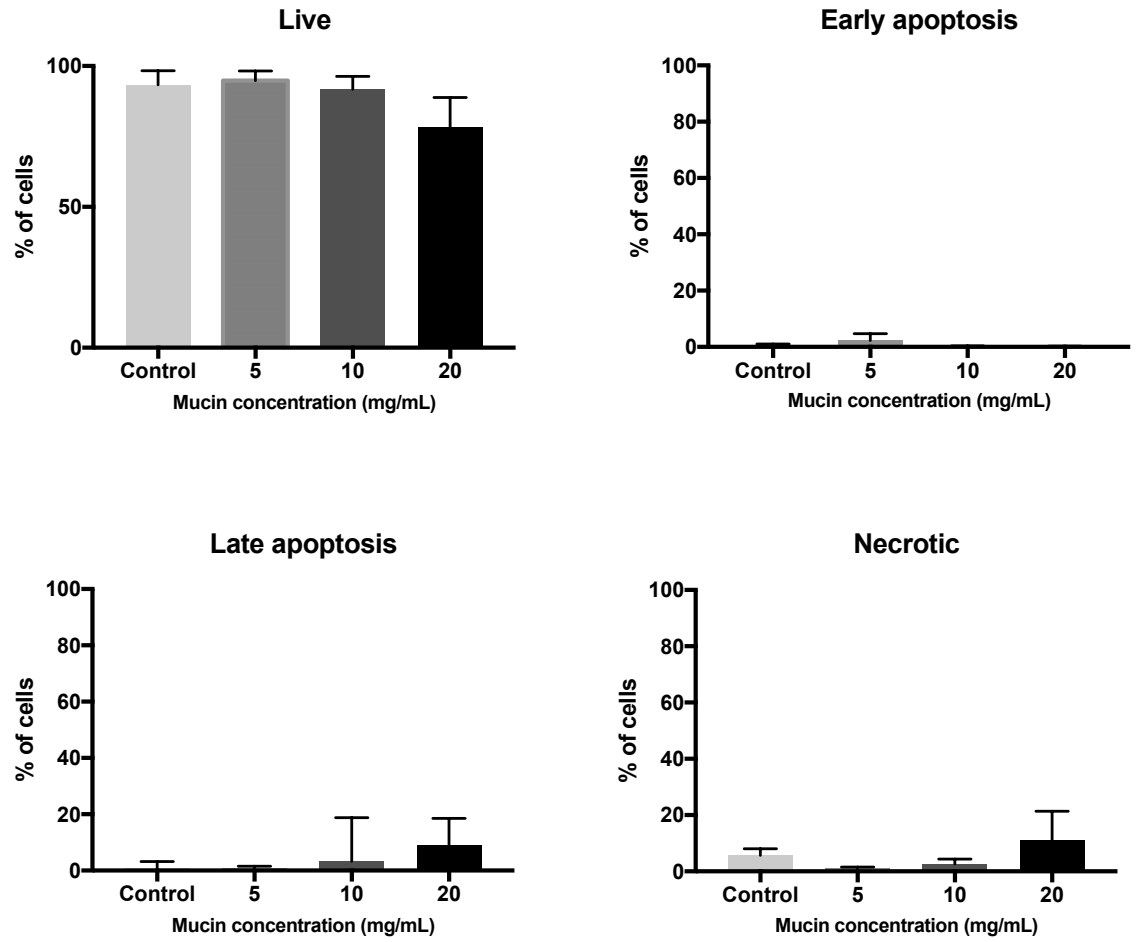

Figure 5.1 - Neutrophil viability in mucin.

Blood-derived neutrophils from healthy volunteers (n = 5) were incubated at 37°C in purified mucin (in HBSS with Ca^{2+} and Mg^{2+}) for 1 hour prior to flow cytometric analysis of viability, using Annexin V and propidium iodide. Data are expressed as median and 95% CI. Kruskal-Wallis test and Dunn's post hoc test were used to compare the differences against the control without mucin. No significant difference was found at any concentration.

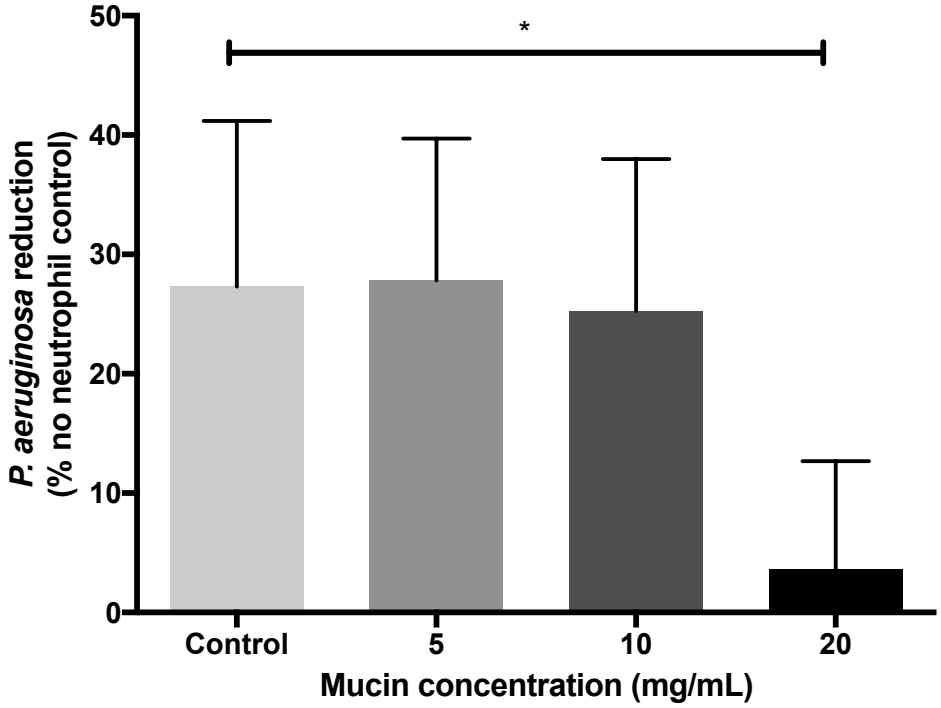

Figure 5.2 - Neutrophil killing of live bacteria in mucin.

Blood-derived neutrophils from healthy volunteers (n = 6) were incubated at 37°C in purified mucin (in HBSS with Ca^{2+} and Mg^{2+}) for 30 minutes with autologous serum-opsonised *P. aeruginosa* PA01. Bacterial killing was determined by serial dilution on LB agar plates, and colonies counted at 24 hours to give a percentage reduction in colony forming units (CFU), compared to a sample without neutrophils. Data are expressed as median and 95% CI. Kruskal-Wallis test and Dunn's post hoc test were used to compare the differences against the control without mucin, *$p<0.05$.

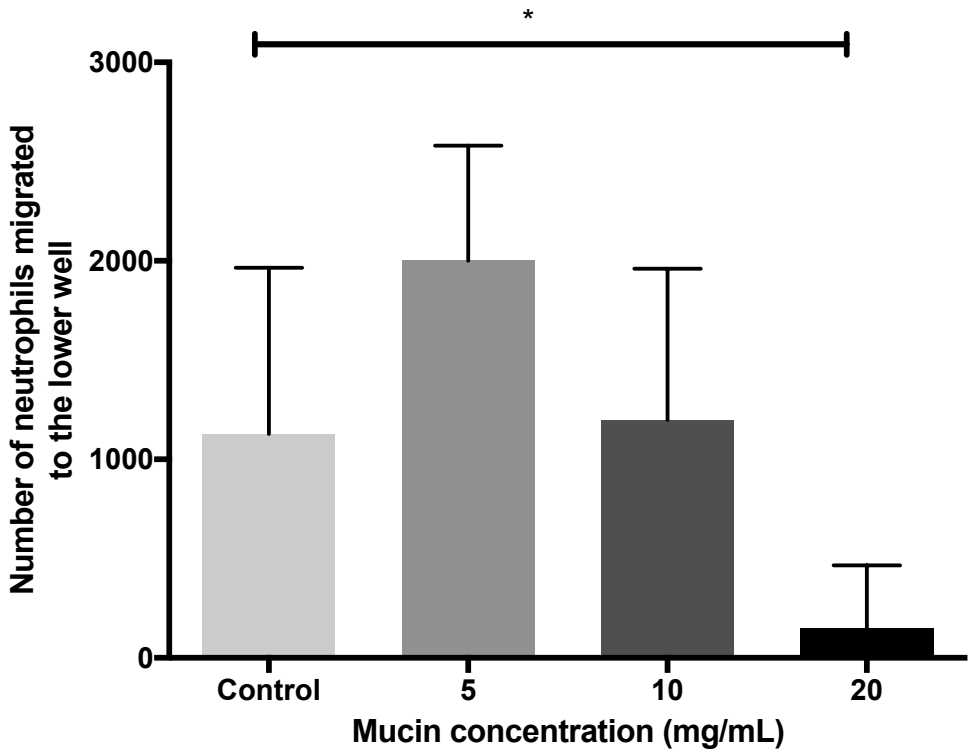

Figure 5.3 - Neutrophil chemotaxis in mucin.

Blood-derived neutrophils from healthy volunteers (n = 5) were fluorescently labelled with CFSE and incubated at 37°C in purified mucin (in HBSS with Ca^{2+} and Mg^{2+}) within a transwell insert (3.0 μm pore size). The number of neutrophils that migrated from the apical compartment to the lower compartment (containing 10,000 pg/mL IL-8) at 3 hours were counted using a confocal microscope and automated software. Data are expressed as median and 95% CI. Kruskal-Wallis test and Dunn's post hoc test were used to compare the differences against the control without mucin, *$p<0.05$.

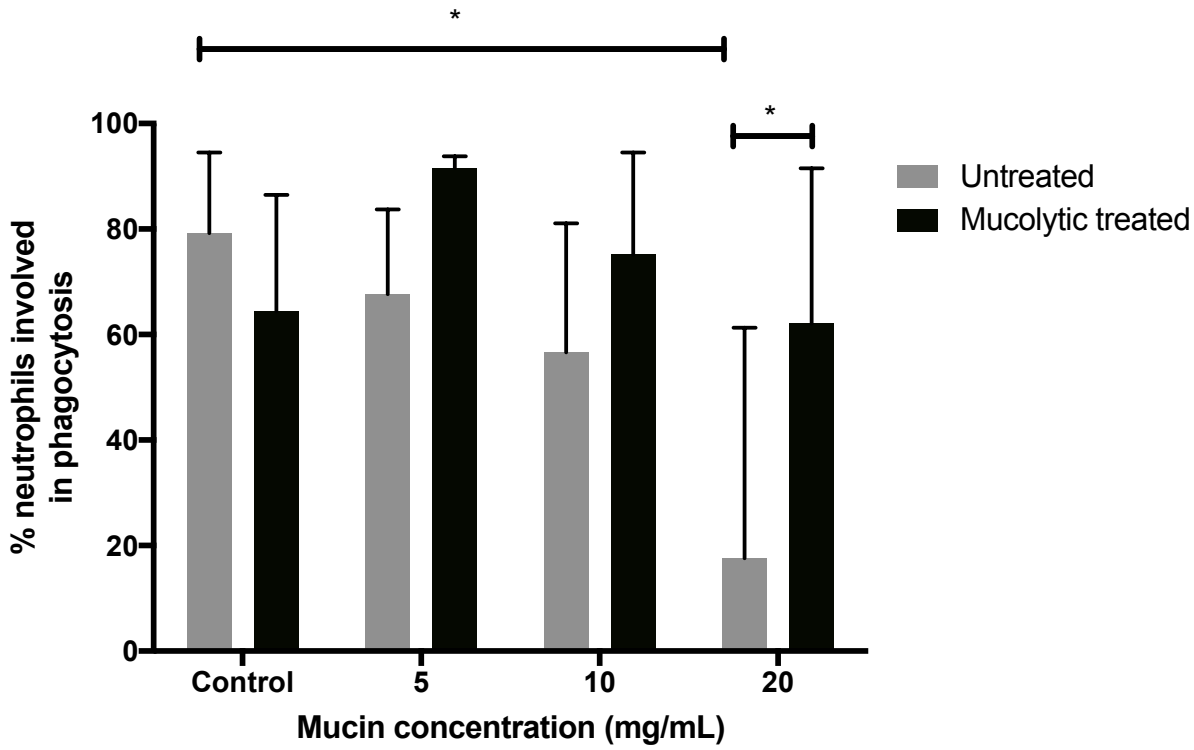

Figure 5.4 - Neutrophil phagocytic function in mucin and after extraction from mucin.

Untreated - Blood-derived neutrophils from healthy volunteers (n = 6) were incubated at 37°C in purified mucin (in HBSS with Ca^{2+} and Mg^{2+}) for 1 hour with autologous serum-opsonised pHrodo® green *S. aureus* Bioparticles®.

Mucolytic-treated - Blood-derived neutrophils from healthy volunteers (n = 6) were incubated at 37°C in purified mucin (in HBSS with Ca^{2+} and Mg^{2+}) for 30 minutes prior to treatment with 0.08% dithiothreitol for 5 minutes, centrifuged and re-suspended (in HBSS with Ca^{2+} and Mg^{2+}), containing no mucin. Neutrophils were then incubated at 37°C for 1 hour with autologous serum-opsonised pHrodo® green *S. aureus* Bioparticles®.

Phagocytosis was determined by flow cytometric gating of viable neutrophils with ingested particles versus a control sample on ice. Data are expressed as median and 95% CI. Kruskal-Wallis test and Dunn's post hoc test were used to compare the differences against the control without mucin. Mann–Whitney U tests were used to compare individual concentrations with and without mucolytic treatment, *$p<0.05$.

5.3.2 The impact of mucin concentration on bacterial-subglottic epithelial interactions.

I further sought to investigate the impact of mucin concentration on epithelial-bacterial interactions. Using *P. aeruginosa* lab strain PA01 and tissue relevant samples I performed bacterial-epithelial co-culture experiments. The development and characterisation of the differentiated subglottic epithelial cell cultures used are described in chapter 6 of this thesis. Bacterial-epithelial co-culture demonstrated that increased bacteria were present in the mucin layer and epithelial homogenate of cell cultures with an apical mucus layer of 20 mg/mL mucin (the highest tested concentration), compared with a no mucin control (Figure 6.5).

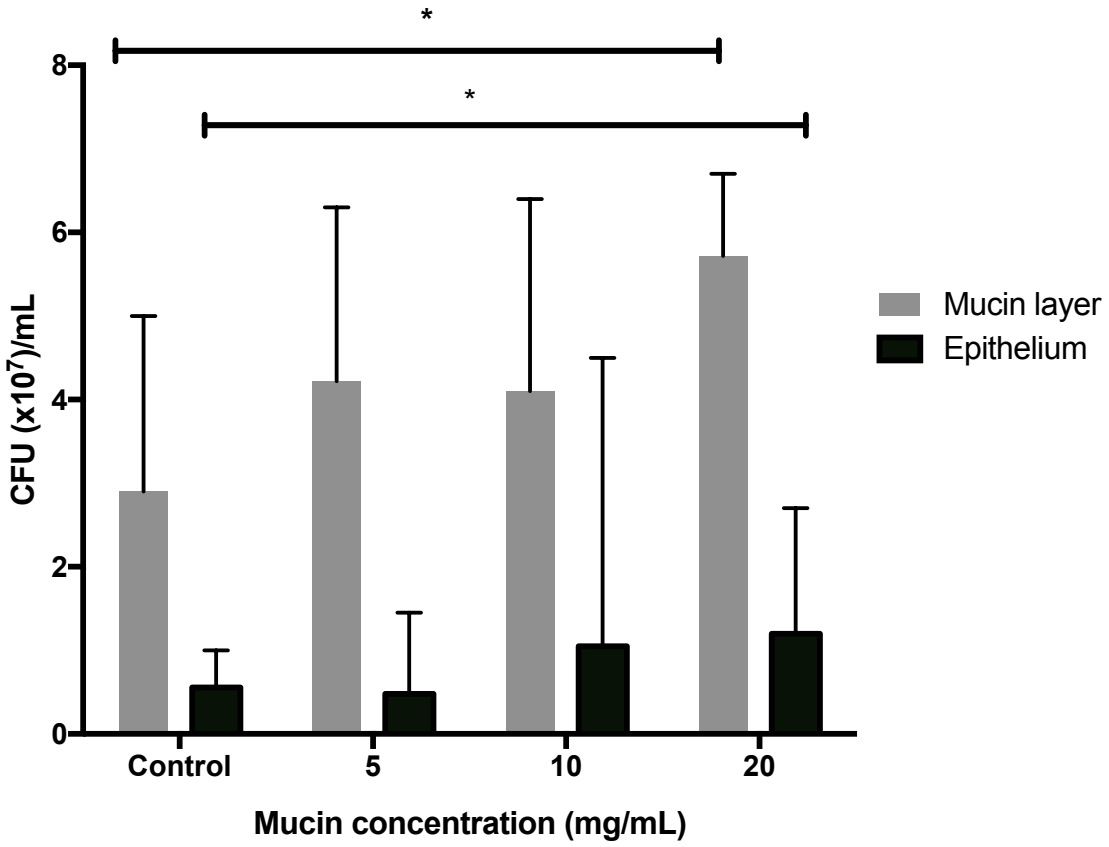

Figure 5.5 - Bacterial-mucin-epithelial interactions.

The mucus layer of primary subglottic epithelial cells (PSEC), cultured at ALI (n = 4), was removed and replaced with 50 µL of purified mucin (in HBSS with Ca^{2+} and Mg^{2+}), and incubated for 1 hour (37°C 5% CO_2). *P. aeruginosa* [5 µl ($2x10^7$ CFU/mL), PA01] was then applied apically and incubated for 7 hours. Bacterial growth in the mucin layer and epithelial homogenate was determined by serial dilution on LB agar plates, counted at 24 hours. Data are expressed as median and 95% CI. Kruskal-Wallis test and Dunn's post hoc test were used to compare the differences against the control without mucin, *$p<0.05$.

5.4 Discussion

In this chapter I sought to investigate the impact of mucin on both neutrophil function and bacterial-epithelial interactions, using concentrations of mucin found in the subglottis of both long-term intubated ICU patients and newly intubated controls (as described in chapter 4 of this thesis). Using purified mucin extracted from the subglottic region of long-term intubated ICU patients and neutrophils extracted from the whole blood of healthy volunteers I demonstrated that 20 mg/mL mucin (representative of long-term intubated ICU patients), but not lower concentrations (representing newly intubated control patients), had a significant negative impact on phagocytosis, bacterial killing and chemotaxis. However no condition tested had a significant impact on neutrophil viability. Furthermore I demonstrated in bacterial-epithelial co-culture experiments that mucin concentrations found in long-term intubated ICU patients were associated with increased bacterial growth *in vitro*.

This is the first time neutrophil function has been assessed in mucin concentrations found in the subglottis of long-term intubated ICU patients. Matsui *et al* (76), in the only other study investigating the impact of mucin on neutrophil phagocytic function, demonstrated reduced chemotaxis and bacterial killing at 31.2 mg/mL mucin, which they used as a model of CF. I was able to demonstrate similar dysfunction at 20 mg/mL, which is more relevant to the mucin concentration in the subglottis of long-term intubated ICU patients (median MUC5B concentration in long-term intubated ICU patients was 21.7 mg/mL, as described in chapter 4 of this thesis). Matsui *et al* (76) also used purified mucin derived from *in vitro* air-liquid interface cultures of primary CF and non-CF lower airway cells. There is evidence suggesting that *in vitro* cultures have variable mucin ratios and differing properties, compared with *in vivo* mucins (77, 79). It has also been demonstrated that mucin proportions and properties are variable depending on the location within the airway (77). I therefore used mucin derived from direct sampling of mucus from the subglottic region of long-term intubated ICU patients, providing location- and disease-relevant mucin for experimentation. I was also able to extend the findings of Matsui *et al* (76), by changing the concentration of mucin, and by further investigating dysfunction using a variety of neutrophil functional assays. Further to this I was able to provide the first demonstration of the reversibility of this neutrophil dysfunction with a mucolytic agent DTT, suggesting a potential translational opportunity for these findings.

I was also able to demonstrate reduced bacterial killing and ingestion of opsonised bacterial bioparticles at mucin concentrations found in the subglottis of long-term intubated ICU patients. In contrast no reduction in these functions were observed at lower concentrations representative of those found in mucus from the subglottis of newly intubated controls. The ability of neutrophils to ingest and kill pathogens, which these assays test, are key to neutrophil function (52, 53). Failure to perform these functions are likely to result in an inability to clear pathogenic organisms. Furthermore, neutrophil presence in the airway mucosa has been implicated in the generation of inflammation, through degranulation and other mechanisms (63, 224-226). Therefore the presence of dysfunctional neutrophils within the subglottis of long-term intubated patients may actually have detrimental effects in driving harmful inflammation and further mucin secretion.

An important finding was the impairment of neutrophil chemotaxis at high concentrations of mucin, further supporting the findings of Matsui *et al* (76). The ability of neutrophils to migrate towards an attractant is crucial to clearance of pathogens (52, 53). It has been extensively demonstrated that mucin has an impact on the rheological properties of mucus in the airway and that hypersecretion increases viscosity (79). It would therefore follow that high viscosity mucin may inhibit the migration of cells. However, I was unable to demonstrate that this was a purely rheological dysfunction in chemotaxis. I attempted to utilise a non-mucin agent, with similar rheological properties, to investigate this, however all agents tested were toxic to the neutrophils. The reversibility of the neutrophil dysfunction with DTT further supports the hypothesis that this is a rheological effect however. The finding that neutrophil chemotaxis increased at 5 mg/mL compared to the control (no mucin) concentration is intriguing and requires further investigation. I would hypothesise that this mucin concentration recapitulates a normal physiological concentrations of mucus (and viscosity), unlike the 0 mg/mL control, therefore is a preferential environment for neutrophil chemotaxis.

The preservation of neutrophil viability after incubation with higher concentrations of mucins is an intriguing finding. This indicates that the loss of neutrophil function is due to a functional deficiency and not loss of viability. The assay used was also able to detect even early signs of death, such as apoptotic cells. There was however a slight, non-significant increases in the proportion of late apoptotic and necrotic cells at the highest mucin concentrations. A further vitality marker was included in experiments such as the pHrodo® Bioparticles® phagocytosis experiments to negate this potential bias. Necrosis is a form of

non-programmed cell death that allows for the release of inflammatory agents, previously contained within the cell, into the surrounding environment (227). It is often associated with precipitating airway inflammation in respiratory conditions, such as asthma, CF and COPD, unlike apoptosis which is considered non-inflammatory (227). Neutrophils are however predominantly pro-inflammatory cells when viable too (52, 53, 63, 189). Neutrophils release a multitude of pro-inflammatory cytokines and proteases through degranulation and in the formation of neutrophil extracellular traps (NET) (52, 53). NETs are produced by neutrophils as an additional method of pathogen killing, where intracellular contents, such as human neutrophil elastase and histones, are released into the extracellular environment (228, 229). It could be hypothesised that in response to impaired chemotaxis or phagocytosis in the high mucin environment, neutrophils may be stimulated into "netosis". Production of NETs has been implicated in many respiratory and autoimmune diseases as precipitating inflammation (228, 229). I attempted to perform assessment of NET formation *in vitro* with mucin, however NETs are extremely fragile and the assay was impractical with viscous mucin.

Bacterial-epithelial co-culture experiments demonstrated that high mucin concentrations, found in the subglottic mucus of long-term intubated ICU patients, induce increased bacterial growth, compared to lower mucin concentration (Figure 6.5). The mucus layer is intended to act as a chemical barrier and to clear pathogens via the mucociliary escalator (78, 79, 84, 86). Therefore mucin may be acting as a deterrent, protecting the epithelial membrane by providing an alternative location for bacterial growth (230). However the finding that there is also increased bacterial growth in the epithelial homogenate suggests a seriously detrimental effect of higher mucin levels. Once bacteria are able to adhere to the epithelial membrane they are more likely to invade, increasing their growth and potentially resulting in disseminated infection (231, 232). Furthermore in long-term intubation it is thought that aspiration of infected mucus from around the cuff that is responsible for VAP development; hence increase growth in the mucus layer may be more harmful (14, 233). Further to this, the protective effects of bacteria accumulating in the mucus layer (as apposed to in the epithelium) relies on the airways ability to clear these pathogens through cough or the mucociliary escalator (84). There is evidence that long-term intubation, through cuff pressure and inflammation, prevents cough and induces ciliary dysfunction, preventing clearance of infected mucus from the airway (1, 14).

A key consideration when interpreting the findings in this chapter is the use of purified mucin. I used well-established methods to purify mucin from the patient mucus samples

(136-139), however it is possible that these methods may have altered the properties of the mucin molecules through the extensive processing (136-139). Steps were undertaken to ensure purity of the sample, including assessment of protein and DNA content (136-139). Furthermore it was not possible to purify all the samples of mucin together or individually, therefore I pooled batches of mucin into three purification runs, and this does introduce the potential for batch effects between these samples. Nevertheless non-purified mucin contains a multitude of substances, such as proteins, DNA and bacteria (81, 84), which would affect the assays described and not allow for reproducible *in vitro* investigation.

The findings in this chapter will of course need further validation. However they do suggest potentially interesting translational aspects to be investigated. The reversibility of the mucin-induced neutrophil dysfunction with a mucolytic agent would suggest the potential to trial mucolytic agents in the subglottis of long-term intubated ICU patients, as a method of potential VAP prevention. I utilised the mucolytic agent DTT in my experiments as it is a well established laboratory agent for neutrophil extraction from mucus samples (216). DTT is however a toxic agent and not used in humans (234). Nevertheless various other mucolytic agents have been effectively used in patients with cystic fibrosis, where mucin concentration is also thought to be a factor in disease development (235) (236). These agents include simple substances with milder and relatively acceptable side effect profiles, such as hypertonic saline (used successfully in cystic fibrosis (36)), which could be topically applied to the subglottic region, given the available access to this area in long-term intubated ICU patients.

5.5 Conclusions

Mucin at concentrations found in the subglottis of long-term intubated ICU patients, but not newly intubated control patients, had a significant impact on neutrophil function. The neutrophil functions measured included several crucial to pathogen clearance in the airway (52). Furthermore, in an *in vitro* subglottic epithelial model, mucin at concentrations found in the subglottis of long-term intubated ICU patients was associated with increased bacterial growth, in both the mucus layer and epithelial membrane.

These findings demonstrate, for the first time, that concentrations of mucin found in the subglottis of long-term intubated ICU patients may prevent neutrophil clearance of pathogenic bacteria, as well as contribute to bacterial growth. Neutrophil function and bacterial growth was unaffected at the concentrations of mucin found in newly intubated control patients. This may explain the microbiological differences between these two cohorts, as demonstrated in chapter 3 of this thesis. The data also demonstrated that mucin-induced neutrophil dysfunction is reversible with the application of a mucolytic agent, suggesting potential therapeutic strategies to restore host defences in this region and possibly prevent VAP development.

5.6 Future work

The work in this chapter was limited by the yield of purified mucin from mucus available from patients in the ICU. Further validation of these findings with larger numbers of repeats would be vital. Another important consideration would be time course analysis, with extension of the mucin incubation time used. Finally the addition of higher concentrations of mucin would give greater understanding of the impact of mucin on neutrophil function and bacterial growth.

Further understanding of the mucin-induced neutrophil dysfunction could be ascertained from analysis of other key neutrophil functions related to pathogen clearance (221). These would include: (a) measurement of neutrophil oxidative burst, a key element of the neutrophil's killing machinery; (b) investigation of actin polymerisation, crucial to neutrophil phagocytosis and chemotaxis; and (c) measurement of neutrophil degranulation, which may give valuable insights into neutrophils' functional abilities in mucin beyond just phagocytosis (52, 53).

The finding that neutrophils have reduced chemotaxis in higher concentrations of mucins raises the question of whether this is a physical, viscosity-related dysfunction or whether it is a reversible physiological effect of the mucins on neutrophils. The use of an agent with a similar viscosity to the mucin concentrations used would allow investigation of this. I attempted some preliminary investigation into this but found all compounds available to be toxic to the neutrophils. Furthermore it would be interesting to expand on the impact of mucin on neutrophil chemotaxis over serial time points (rather than just at one time point) to investigate neutrophil chemotaxis speeds.

While the lab strain of *P. aeruginosa,* PA01, used in the experiments described is appropriate, it would be useful to investigate clinical strains of *P. aeruginosa* and other organisms, such as *S. aureus* (9). This would be especially relevant in the case of the bacterial-epithelial co-cultures, where different organisms may interact differently with mucins. The bacterial-epithelial co-cultures would also benefit from the addition of immune cells, particularly neutrophils, to increase comparability of the model to that of the *in vivo* environment (237).

Chapter 6: Development of subglottic epithelial cell cultures

6.1 Introduction

The subglottis is an anatomical region of the upper airway that forms the inferior portion of the larynx and is continuous inferiorly with the trachea (238, 239). Its boundaries are defined superiorly by the inferior arcuate line of the vocal cord and inferiorly by the lower margin of the cricoid cartilage (238). In ventilated patients, after introduction of a tracheal tube (endotracheal or tracheostomy), the cuff of the tube is inflated in the trachea, directly below the subglottic region (14). This close association suggests that the subglottic region may have a particular importance in the pathogenesis of VAP (14, 15). This significance is demonstrated by the universal incorporation of subglottic drainage into international guidelines as an effective method for VAP prevention (12, 25, 27, 240, 241). Despite this, little is known about the subglottic environment in long-term ventilated patients at high risk of developing VAP (242, 243)

It follows that valid experimental models are required to further elucidate the pathogenesis of subglottic disease in ventilated patients prior to VAP development and to evaluate novel, potentially therapeutic compounds. A number of animal models of tracheal tube placement, ventilation and VAP development have been described, including mice, pig, primate, dog and cat (98-101). These models, while useful, have several limitations, including inter-species differences in submucosal gland distribution, innate immunity, epithelial cell composition and ion channel expression (108-111).

Human primary cell cultures and cell lines have previously been established from other respiratory epithelial sub-sites, including the trachea and small airways of the lung (118-120). These are, however, unlikely to reflect the subglottic region. The subglottis is an anatomically distinct region of the airway, differentiated from the trachea due to its circumferential binding to the cricoid cartilage, giving it unique physical properties (26, 238). These unique properties include a large number of seromucous glands present in the submucosa of the subglottis (238). In addition, subglottic blood vessels include a dense sub-epithelial capillary plexus with numerous anastomoses. This hypothetically results in stronger functional blood flow with resultant warming of air (238). During inflammation this is likely to lead to rapid widening of vessels with subsequent oedema and recruitment of defence cells into the region (238). Therefore *in vitro* culture of primary subglottic epithelial cells (PSECs) from newly intubated theatre attenders is likely to produce a superior cellular model to those produced by primary or immortalised cell lines from other respiratory sub-sites, with

characteristics that replicate more accurately those found *in vivo*. Furthermore, well differentiated PSEC cultures maintained at air-liquid interface (ALI) may therefore be a useful model of airway epithelial function and have the potential to allow for developments in our current knowledge of the pathophysiology of VAP and subglottic disease (121, 244, 245).

To the best of my knowledge, prior to the commencement of this work, PSECs had not been cultured. PSECs therefore represent a novel and potentially highly valuable resource for upper airway and VAP research.

6.2 Chapter aims

In this chapter I wished to answer the following questions:

1. Is it possible to collect and culture epithelial cells from the subglottic region?
2. Can these cells be cultured at an air-liquid interface?
3. What are the characteristics of subglottic epithelial cells subjected to *in vitro* culture and do they approximate to the *in vivo* environment?

6.3 Results

6.3.1 Development of a method to culture primary subglottic epithelial cells (PSECs)

The detailed methods that were used to collect and culture subglottic epithelial cells are described in the methods section (chapter 2). The culture techniques published by Forrest *et al* (118) and Fulcher *et al* (121) were used as an initial template, and were subsequently optimised through experience and supervisor input.

The process of obtaining subglottic samples for culture from patients attending for a laryngeal examination under general anaesthesia required not only technical skills of sampling and culture, but also excellent working relationships between a multidisciplinary team within the otolaryngology unit. The most successful recruitment method was through the theatre waiting list team identifying suitable patients for recruitment. Clinic letters were then reviewed and potential study participants sent information packs. Furthermore the helpful co-operation of the operating surgeon, theatre scrub team and other team members was also vital to successful recruitment.

6.3.2 Outcomes of primary subglottic epithelial cell culture

Subglottic brushings were collected between August 2014 and May 2016. In total, 33 patients were screened for recruitment, of these 26 had subglottic brushings taken. Reasons for not sampling were mainly related to theatre scheduling issues, such as patients not attending or being cancelled. There were 4 refusals to participate in the study. Feedback, at one-week post procedure, was received from 6 of the initial study participants. Patients reported some initial throat discomfort after the procedure as would be expected with intubation and laryngeal examination. There were no reported adverse events or complaints about the study process or sampling.

The yield of ciliated respiratory epithelial cells was ascertained from the subglottic mucosal brushings by differential cell count on a haemocytometer. The median number of ciliated epithelial cells per brush was 940,000, of which 15% (median 145,000 cells) were alive and 84% (median 790,000 cells) were dead (Figure 6.1). A number of other cells were visible on the haemocytometer and cytospin preparations. These included neutrophils, macrophages and squamous cells, which were not included in cell counts (Figure 6.2).

Submerged culture was successful in 17/26 (65%) patients from whom samples were taken (Figure 6.3). When unsuccessful this was initially due to failed growth as the sampling and culture techniques were optimised. There was a steep learning curve for the optimal brushing technique, which is key to obtaining the appropriate number of cells for successful culture. Later failure was mainly due to fungal infection in the first three days of culture. Overall 6 (23%) of all the samples taken had microbiologically proven *Candida albicans* infection despite the potent anti-fungal agent Amphotericin B being included in all the medium used for culture.

In those samples passaged to ALI culture 13/17 (77%) were successfully cultured to full differentiation (presence of cilia and mucus production apically on visual inspection with a light microscope). Of those that were unsuccessful the failure was usually within the first 2 weeks after the apical medium was removed. The cells became fibroblastic and lost epithelial resistance. Overall half of the total samples taken made it to full differentiation without incident.

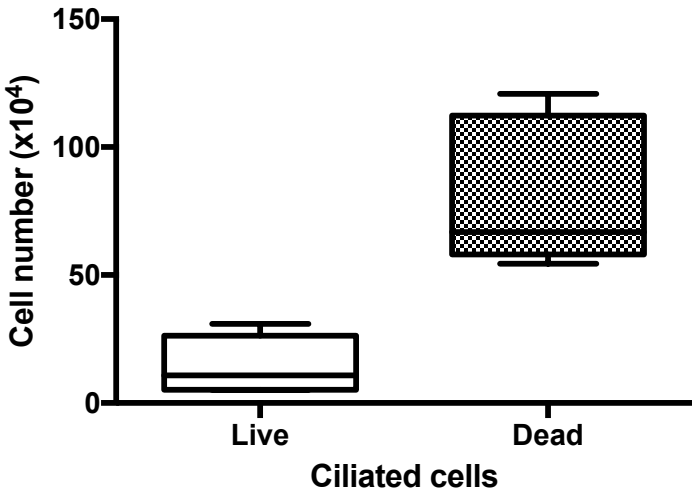

Figure 6.1 - Ciliated epithelial cells extracted from subglottic mucosal brushings.

Subglottic mucosal brushing samples (n = 6) were processed to extract cells, which were stained with Trypan Blue. Ciliated respiratory epithelial cells were counted under a light microscope using four quadrants on a haemocytometer. Live cells were identified through exclusion of Trypan Blue. Box and whisker plots are depicted which display median, quartiles and range.

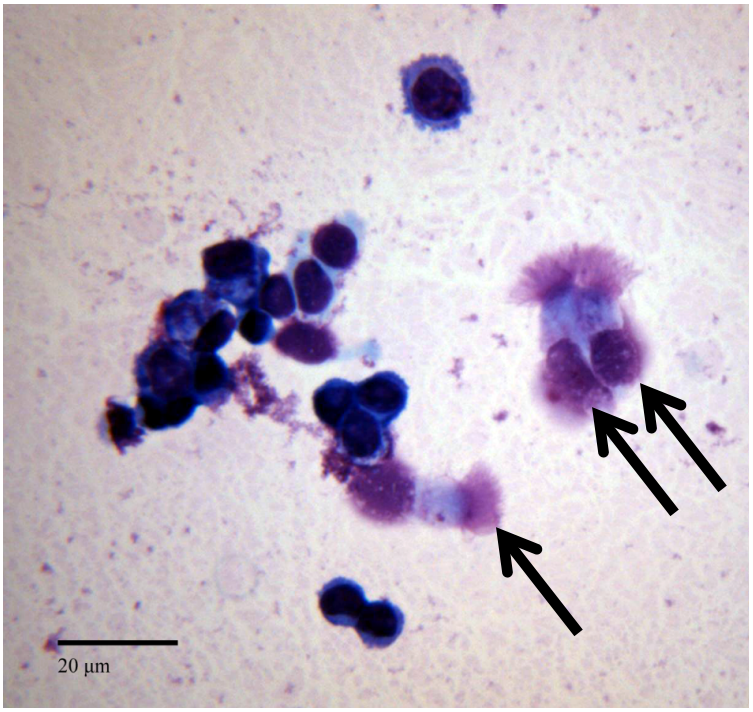

Figure 6.2 - Cytospin image of a Giemsa stained subglottic brushing sample.

Cytospins were prepared of the subglottic mucosal brushings to validate the morphology and appearance of the cell subsets identified by cell count. These demonstrated the presence of multiple cell types, including ciliated epithelial cells (arrows), red blood cells and immune cells. Cytospins were stained with Giemsa to visualise the cell nucleus.

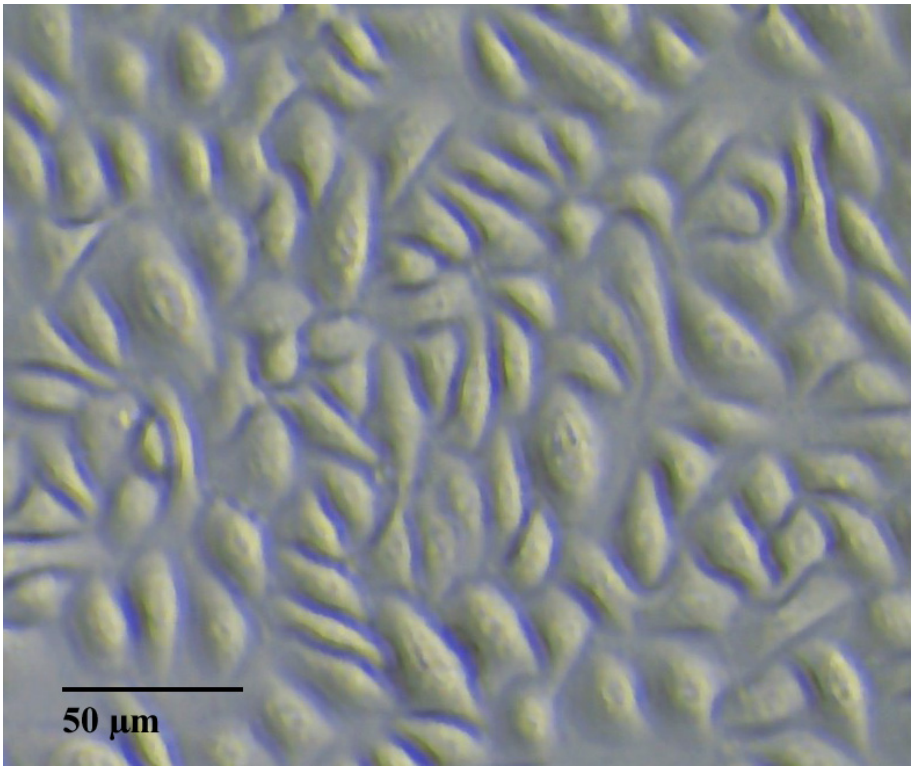

Figure 6.3 - Phase-contrast micrograph of primary subglottic epithelial cells cultured under submerged conditions.

Submerged cultures demonstrate typical ‘cobblestone’ appearance, typical of epithelial cells.

6.3.3 Characterisation of subglottic epithelial cells at air-liquid interface culture

The median time at which samples were passaged onto transwells from submerged culture was 10 (range 6 - 16) days. After removal of apical medium motile cilia were first visible at light microscopy at a median of 20 (range 16 - 34) days. In ALI culture phase-contrast microscopy demonstrated, on repeated occasions, characteristic epithelial morphology and growth, including tight epithelial junctions, cilia coverage and beating and mucus production.

I characterised these cultures using a variety of techniques. Firstly, subglottic epithelial cells grown on collagen-coated transwell membranes, and differentiated at ALI, were stained with an epithelial marker recognising pan-cytokeratin. The subglottic epithelial cells stained positively for cytokeratin (Figure 6.4) in a similar pattern to other respiratory epithelial sub-site brushings (excluding fibroblast or other cell contamination) (118). Scanning electron microscopy (SEM) was used to confirm cilia coverage apically (Figure 6.5). The transmission electron microscopy (TEM) further demonstrated the structural appearance of an epithelial membrane (Figure 6.6).

Paraffin-embedded sections of cultured cells were prepared. Haematoxylin and eosin (H and E) staining was used to highlight epithelial architecture within these sections. This allowed examination of the histological appearance of the *in vitro* epithelial culture and demonstrated a pseudostratified (rather than monolayer) epithelial culture, consistent with cellular differentiation (121) (Figure 6.7). Diastase resistant periodic acid-Schiff (DPAS) staining was used for recognition of mucin glycoproteins in paraffin-embedded epithelial sections. This demonstrated relevant glycoprotein coverage through purple staining on the apical surface. This is a classical marker for epithelial *in vitro* differentiation as it demonstrates mucus production (Figure 6.8) (121). DPAS is a standard marker of cell differentiation but is non-specific, as it stains for all glycoproteins. Therefore antibodies against MUC5B and MUC5AC were used to test for the individual mucins within paraffin-embedded sections of epithelial membranes. This suggested a predominance of MUC5B within the epithelium over MUC5AC (Figure 6.9).

A characteristic of normal airway epithelial cells is the ability to form a tight epithelial junction and specific ion channel activity across the apical membrane (246). In order to investigate whether the differentiated subglottic epithelial cells retained electrophysiological phenotypes found in other respiratory epithelial sub-sites, Ussing chamber electrophysiological studies were performed on differentiated subglottic epithelial cells

grown on Snapwells at ALI over a period of days of exposure to air apically (247). At just 8 days a high level of epithelial resistance was measured (>1000 $\Omega.cm^2$) (Figure 6.10). This continued to increase when measured at day 15. There was still an epithelial resistance of over 400 Ω.cm2 up to 79 days. The cells also retained the characteristic electrophysiological channel profile expected of a normal airway, such as apical epithelial sodium channel (ENaC) and cystic fibrosis transmembrane conductance regulator (CFTR) expression (Figure 6.10). Tight epithelial junctions (>800 Ω.cm2) were further confirmed in the transwell culture systems at day 20 – 30 of apical air exposure using chopstick electrodes to measure trans-epithelial electrical resistance (TEER) (Figure 6.11).

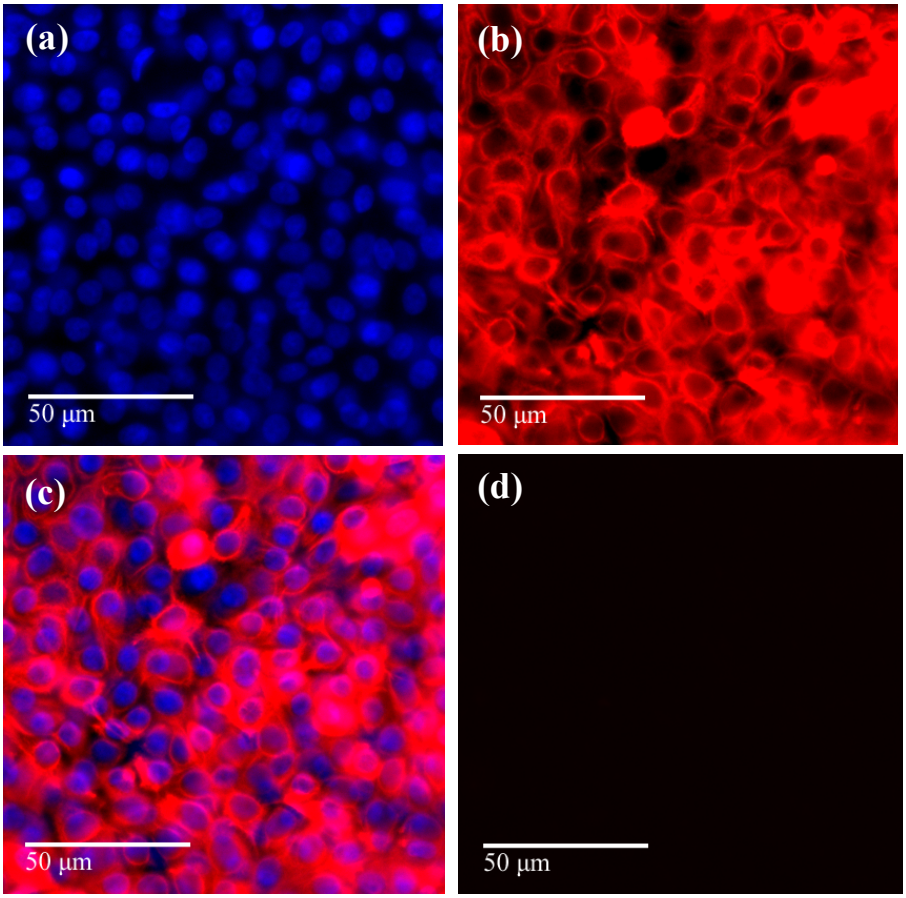

Figure 6.4 - Pan-cytokeratin staining of primary subglottic epithelial cells.

Fully differentiated epithelial cells, fixed and permeabilised, were stained with anti-cytokeratin (pan reactive) antibody, conjugated with Alexa Fluor® 647. The cells were then mounted in medium containing DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride). A confocal fluorescent camera (Zeiss Axio Imager II, Germany) was used to image the cells. (a) DAPI nuclear stain (blue), (b) anti-cytokeratin (pan reactive) antibody, conjugated with Alexa Fluor® 647 (red), (c) combined image of DAPI and cytokeratin, (d) unstained control to which the other images were referenced (no isotype control used).

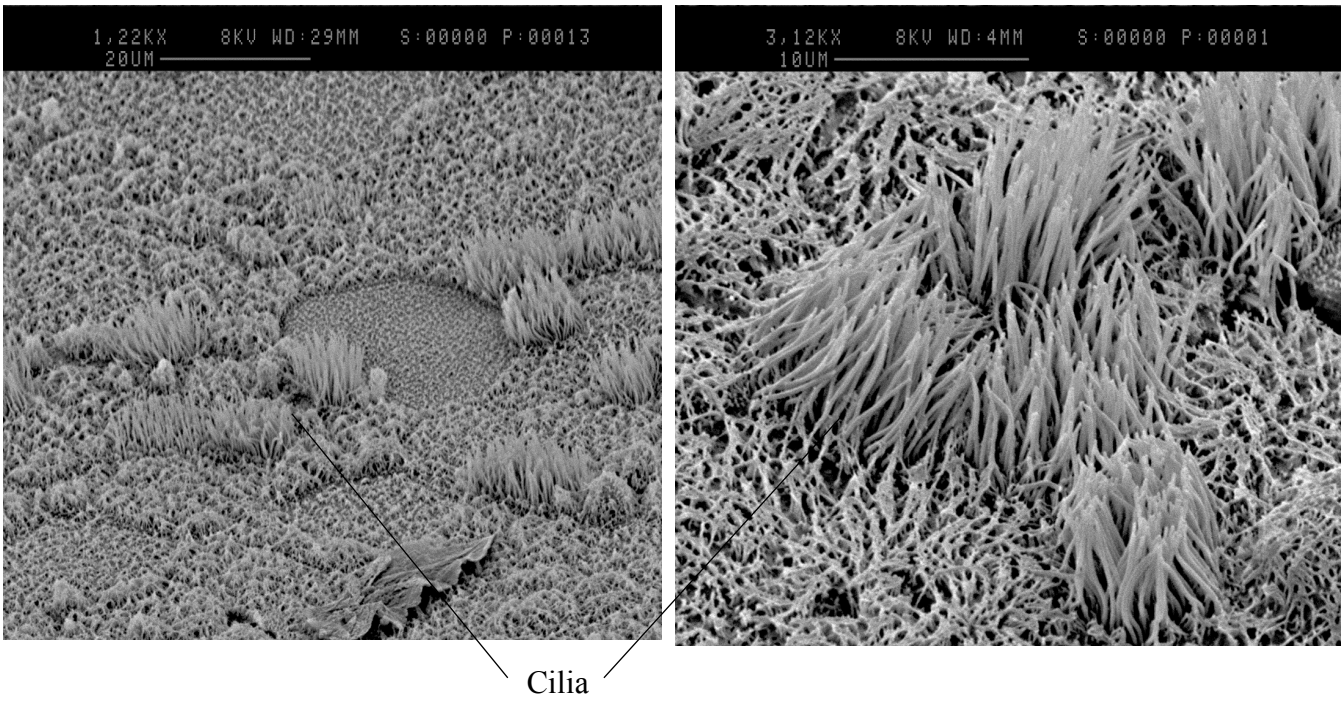

Figure 6.5 - Scanning electron microscopy of a differentiated epithelial culture.

Images taken by scanning electron microscopy (SEM) of the apical aspect of the epithelial cells cultured in air-liquid interface conditions.

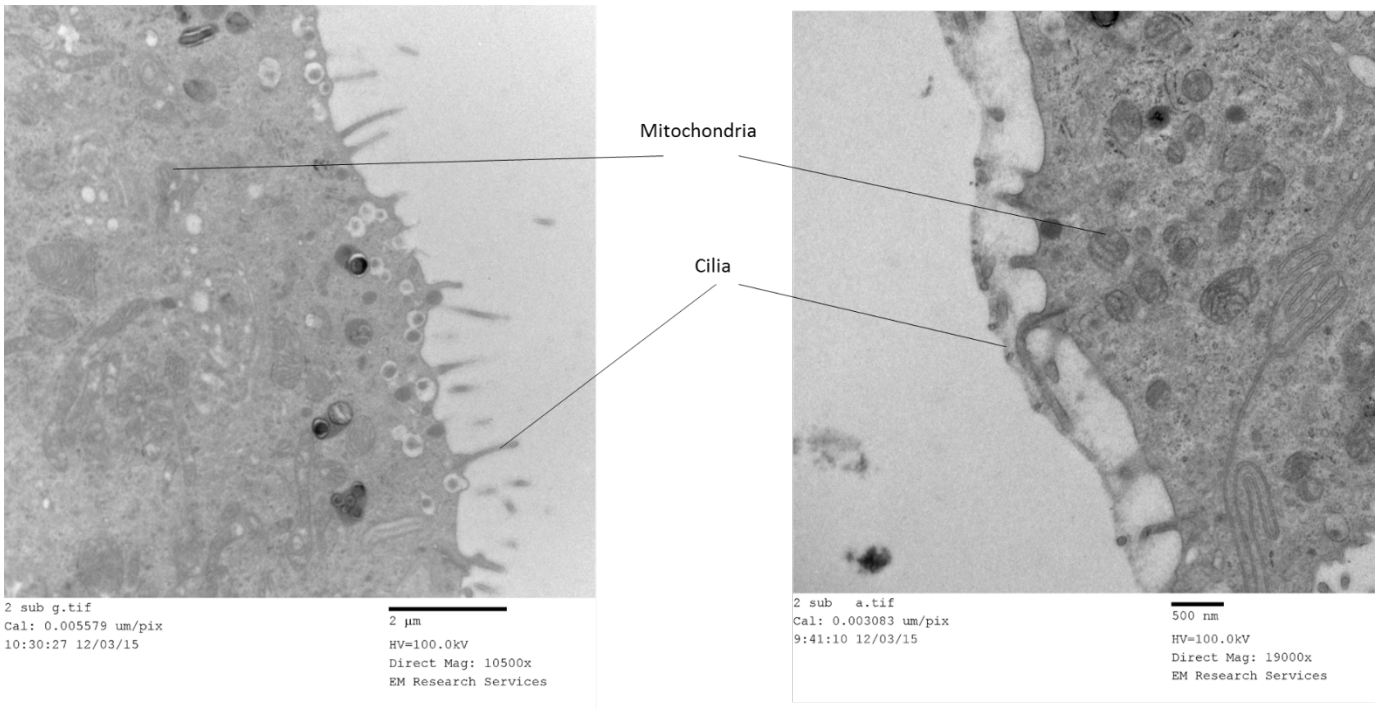

Figure 6.6 - Transmission electron microscopy of a differentiated epithelial culture.

Images taken by transmission electron microscopy (TEM) of the epithelial cells cultured at air-liquid interface conditions.

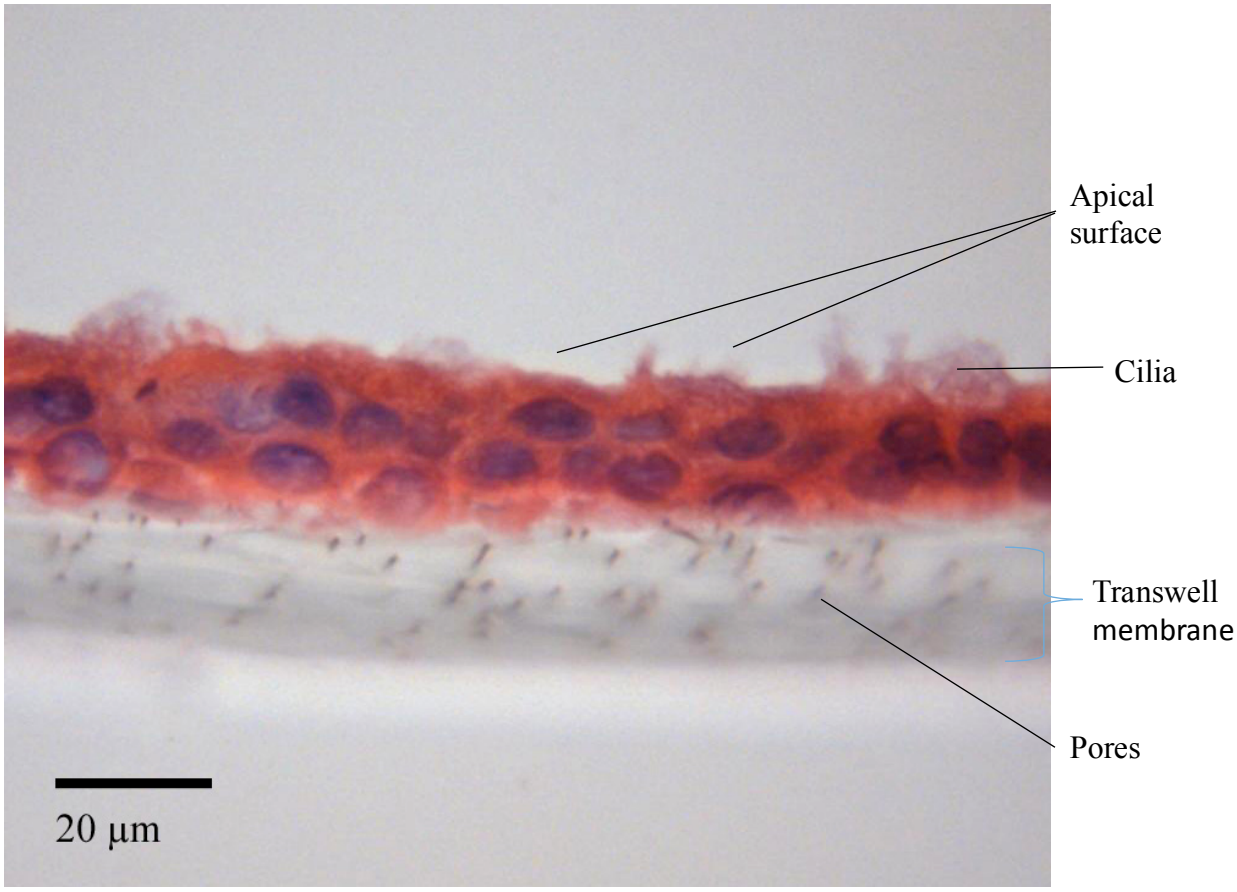

Figure 6.7 - Haematoxylin and eosin staining of a paraffin-embedded section of the differentiated epithelial culture.

Fixed cells were sectioned to 5 µm thickness and stained with Haematoxylin (nuclear/blue) and Eosin Y (cytosolic/red).

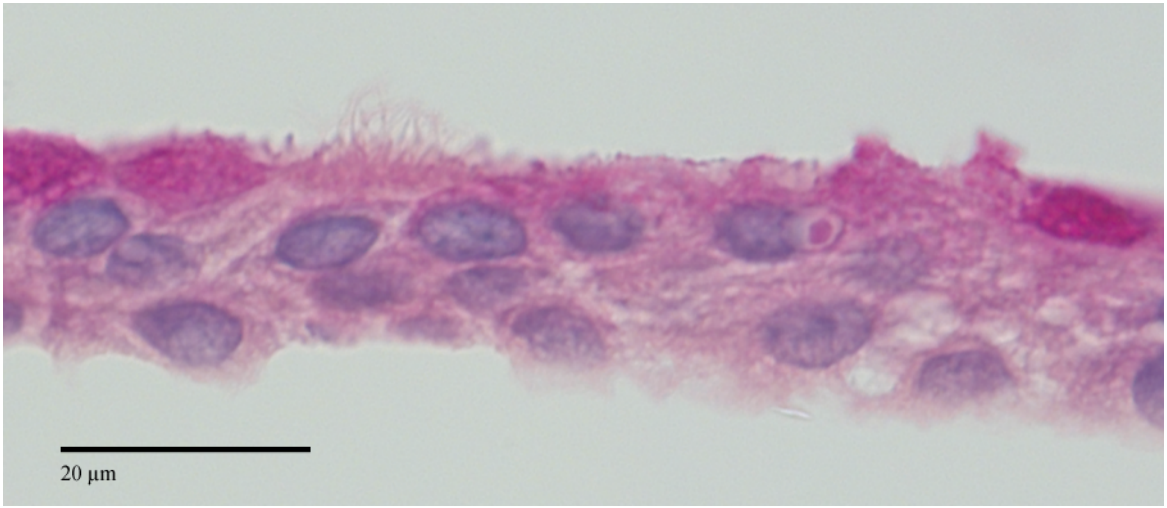

Figure 6.8 - Diastase resistant periodic acid-Schiff staining of a paraffin-embedded section of a differentiated epithelial culture.

DPAS was used as a stain for mucin glycoproteins (dark purple) and counter-stained with Haematoxylin (nuclear/blue) and Eosin Y (cytosolic/red).

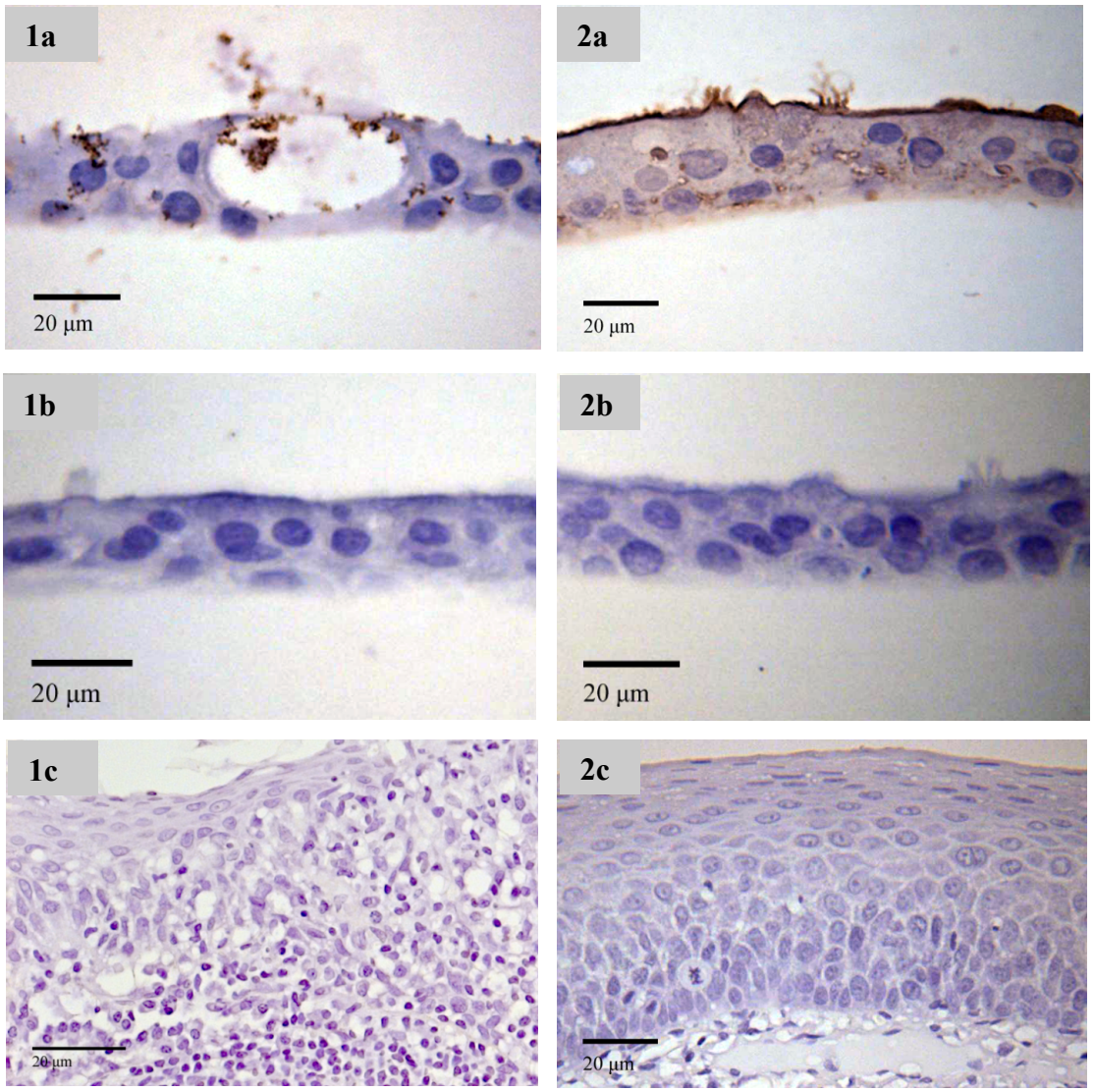

Figure 6.9 - Immunohistological staining for MUC5AC and MUC5B

Sections were incubated with primary antibodies to either MUC5B (Tepa II) or MUC5AC (45M1) and secondary horseradish peroxidase-conjugated antibody. The sections were counter-stained with haematoxylin. An isotype control (IgG) was also tested in place of the primary antibody in each experiment. Human tonsil tissue was used as a negative tissue control for the antibodies. MUC5AC and MUC5B (brown stain), counter stained with Haematoxylin (blue) - 1a and 2a respectively. 1b and 2b are isotype controls for MUC5AC and MUC5B antibodies respectively. 1c and 2c are tissue (tonsil) controls for MUC5AC and MUC5B antibodies respectively.

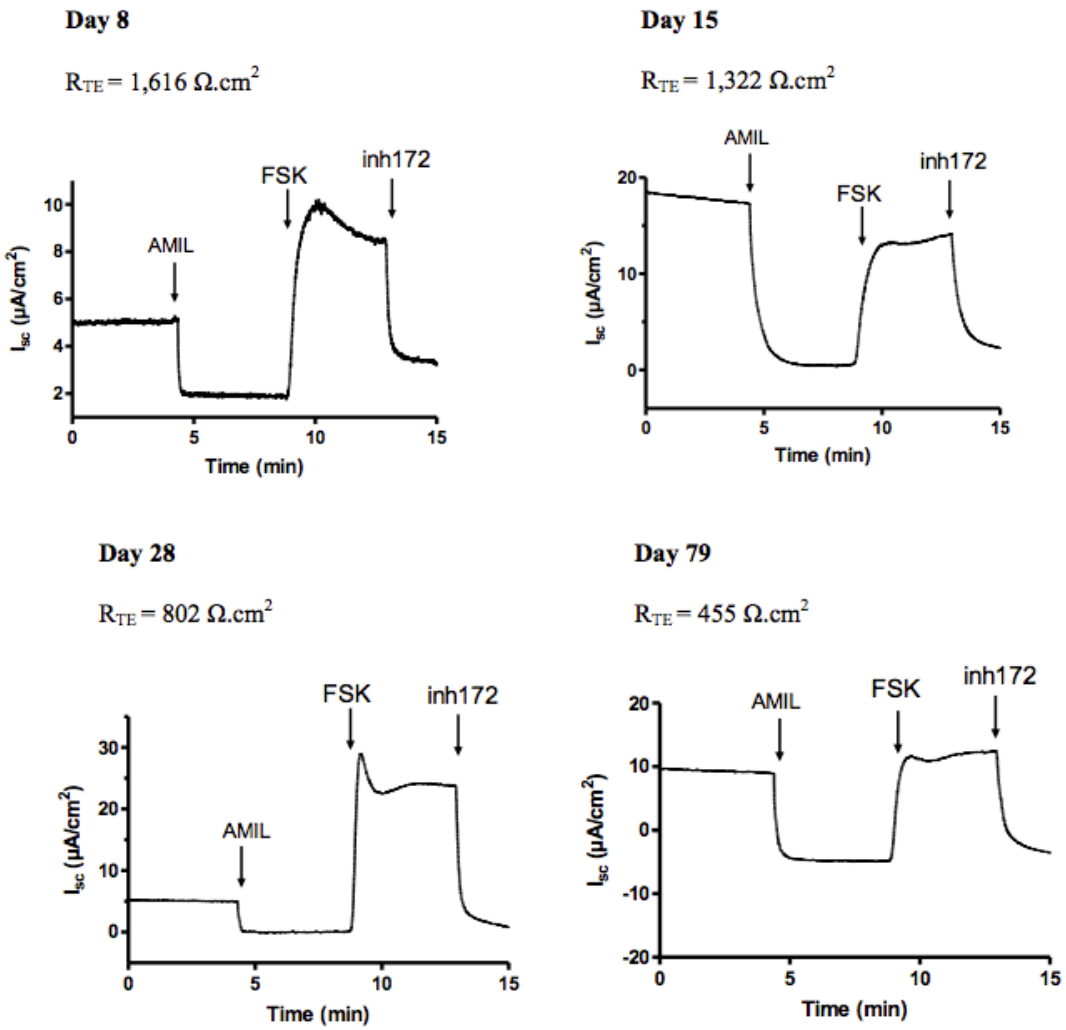

Figure 6.10 - Ussing chamber electrophysiological studies of the differentiated subglottic epithelial cell membranes over time.

Ussing chamber electrophysiological studies were performed on differentiated subglottic epithelial cells grown on Snapwells at air liquid interface over a period of exposure to air apically. Short-circuit current (Isc) values reflect anion (Cl^-) secretion and/or cation absorption (Na^+). Exposure of the epithelium to 10μM Amiloride (AMIL) blocked the apical epithelial sodium channels (ENaC) in the polarised epithelial cells. 10μM forskolin (FSK) was then added to activate cAMP cascades and caused a significant elevation in whole epithelial current. 20μM inh172, a specific CFTR inhibitor, reversed this elevated Isc current, which demonstrated that the vast proportion of the Forskolin response was accountable by CFTR. Resistance was monitored by applying 5mV voltage pulses at 30 second intervals and monitoring the change in Isc (not shown). This was multiplied by the surface area of the transwell to give an electrical resistance value in $\Omega.cm^2$.

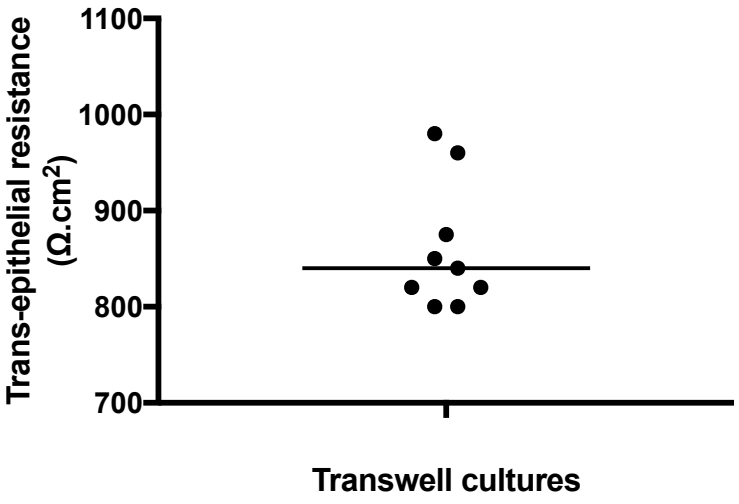

Figure 6.11 - Trans-epithelial electrical resistance measurements from differentiated epithelial membranes

Trans-epithelial electrical resistance (TEER) measurements were taken using chopstick electrode probes across differentiated epithelial cells in a transwell culture system (day 20 – 30 apical air exposure). Horizontal line represents median value, n = 9.

6.3.4 Characterisation of the mucus layer covering subglottic epithelial cells cultured at air-liquid interface

Differentiated PSECs at ALI culture produced an apical mucus layer. This *in vitro* mucus layer was sampled and quantification of gel forming mucins and cytokines was performed. The *in vitro* mucus layer contained the two main gel-forming mucins MUC5AC and MUC5B expected *in vivo* (Figure 6.12). The mucus layer also contained important cytokines relevant the *in vivo* airway (Figure 6.13). The concentrations of these agents were variable, as one would expect with the heterogeneity of the patients they were derived from.

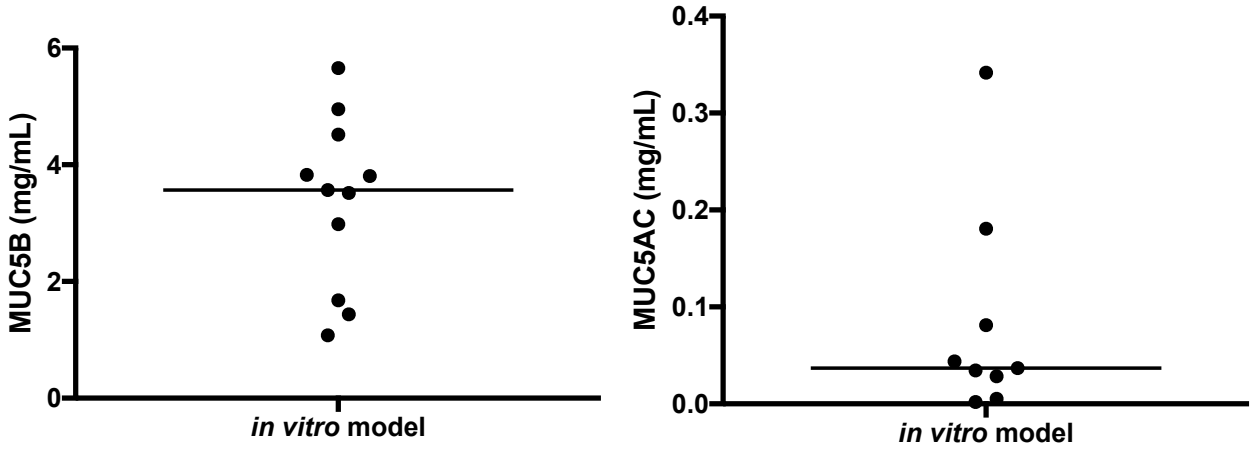

Figure 6.12 - Mucins MUC5B and MUC5AC concentration in mucus samples from *in vitro* air-liquid interface (ALI) subglottic epithelial cell cultures.

Mucin MUC5B and MUC5AC concentrations were quantified from aspirates of the apical mucus layer of *in vitro* PSEC cultures at ALI and measured via ELISA. Horizontal bars depict median values, n = 11 MUC5B, n = 9 MUC5AC

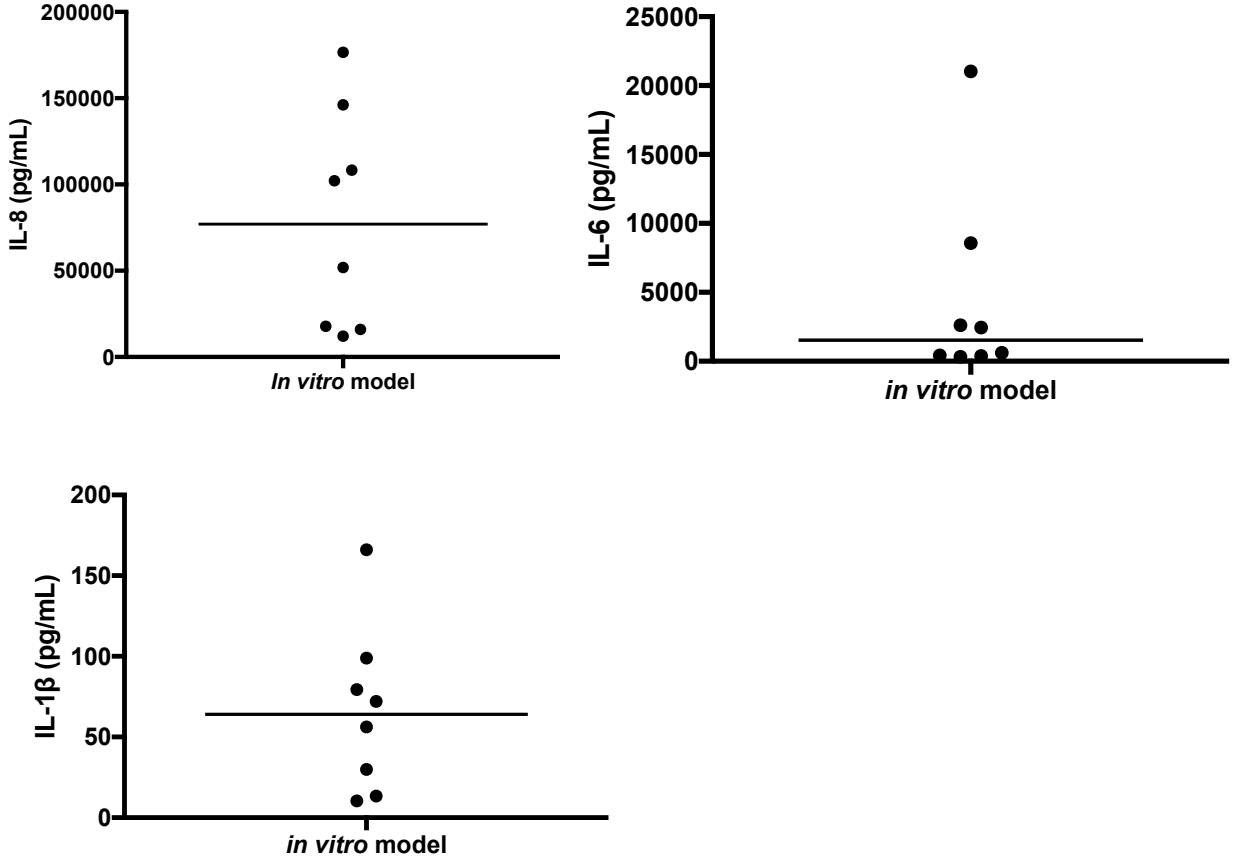

Figure 6.13 - Cytokine content in in mucus samples from in vitro air-liquid interface (ALI) subglottic epithelial cell cultures.

Cytokines were measured using MSD mutli-plex immunoassay. Horizontal bars depict median values, n = 8.

6.4 Discussion

Despite the challenges of new sampling methodologies and the presence of resistant microorganisms I have been successful in establishing a programme to culture PSECs from newly intubated theatre attenders. The PSECs were characterised in terms of their morphology (light microscopy, electron microscopy and haematoxylin and eosin staining) and immunohistochemistry (cytokeratin staining). In addition, it was possible to perform electrophysiological experiments to demonstrate epithelial tight junctions and appropriate ion channel expression (143). Under unstimulated ALI conditions the PSECs produced a mucus layer with relevant expression of mucins MUC5B and MUC5AC, in addition to a number of cytokines that would be expected *in vivo* (182).

A key consideration when using primary tissue in research is that the clinical care of patients is not compromised. The method described in this chapter allows PSECs to be collected from newly intubated theatre attenders without biopsy or significant airway trauma. This is important because the subglottic region is prone to stenosis after irritation or trauma, such as post-intubation (248).

The subglottis is an anatomically discrete region of the airway, distinct from the trachea due to the circumferential binding to the cricoid cartilage, giving it unique physical properties (26, 238). Furthermore there is increasing understanding of the importance of the laryngeal airway in immune signalling (249, 250). Therefore use of appropriate sub-site sampling is important to ensure that the *in vitro* model system accurately recapitulates the situation *in vivo.*

The culture of PSECs is demanding, both in terms of effort and expense, and is limited by their finite lifespan. However, immortalised cell lines have inevitably undergone significant mutations, thus limiting their applicability to the *in vivo* environment (26, 238, 251). Furthermore, well differentiated PSEC cultures maintained at ALI are an excellent model of airway epithelial function and have the potential to allow for developments in our current knowledge of the pathophysiology of VAP and upper airway disease (121).

While my aim was to develop a model of the subglottic region, it is clear from *in vivo* work (described in chapter 4 of this thesis) that the subglottic environment varies between individuals. The PSECs generally seem to reflect this inherent biological heterogeneity in terms of their function. The expression of mucins closely approximated the *in vivo* environment of newly intubated theatre attenders (from which population they are derived).

This recapitulation of the *in vivo* mucin environment is absent in many other epithelial models, which demonstrate significant up or down regulation of specific mucins *in vitro* (77, 252, 253). Interestingly the cytokine expression varied among common airway cytokines, some being up-regulated and some down-regulated. This is likely related to culture techniques and the lack of immune cells or other external stimuli in the *in vitro* culture (251).

It is often difficult to get large airway-derived primary airway samples from volunteers without respiratory co-morbidities (because there is no clinical indication for bronchoscopy). Therefore these cells, taken from theatre attenders without significant respiratory co-morbidities or acute disease may represent a useful 'control' tissue for respiratory researchers. Furthermore there are a number of subglottic diseases, including subglottic stenosis and malignancy that this model could be used to investigate (254-256).

While these theatre attenders were attending Ear, Nose and Throat (ENT) theatres for a laryngeal examination, and did not have significant respiratory co-morbidities or airway malignancy, these patients often had laryngeal symptoms. The origin of these symptoms was often not found but there may have been a multitude of undetected influences including laryngopharyngeal reflux (257). One must therefore be cautious in calling the participants 'healthy volunteers'. However given the difficulty of sampling this region via other methods, such as awake bronchoscopy (due to the close association with the vocal cords), this cohort represent an important resource.

Ideally I would have liked to develop a similar model taken from long-term ventilated patients on the intensive care unit (ICU). This was attempted in a small cohort of patients, however I found that it was not possible, due firstly to low yield of viable cells and secondly to fungal or bacterial infections, despite various antibiotic regimens trialled.

6.5 Conclusions

To my knowledge this is the first primary human epithelial model of the subglottic region to be described. I have characterised this model via a number of methodologies and have confirmed it to be highly representative of the subglottic environment of newly intubated theatre attenders. This unique model provides the potential to study subglottic diseases and potentially test therapeutic agents with a sub-site-specific *in vitro* model.

6.6 Future work

PSECs have been established in a number of newly intubated theatre attenders. Further sampling and establishment of cultures would of course be beneficial. An area for future work would be the development of PSECs from long-term intubated ICU patients. This would give a more relevant model of long-term ventilation from the specific patients who are most susceptible to VAP. However, it is not entirely clear whether these cells would retain their *in vivo* characteristics, such as mucin hypersecretion, when cultured *in vitro* (121). A further area for investigation would be the response of these cells at ALI culture to stimulations (such as LPS or cytokines) to investigate if the mucin and cytokine profile of ICU patients could be better recapitulated. There are a number of potential ways to improve the *in vitro* model through various advanced culture techniques. Firstly the co-culture of these epithelial cells with human immune cells, particularly neutrophils, as found in the airway (258, 259). Additionally, the use of a feeder lay (such as fibroblasts). These layers supply metabolites to their supporting epithelial cells and can better recapitulate inflammatory responses to challenges (260). These feeder layers can also be used in the context of three-dimensional culture systems (using supports such as collagen) which more recapitulate the *in vivo* environment (261). Another potential area for exploration is the development of immortal clones of these subglottic epithelial cells using commercially available kits (262). Viral agents can be introduced (by other viral transfection vectors), to provoke mutations in epithelial cells. These mutations can prevent or slow cellular senescence with division (262). Immortal subglottic cells would have the benefits of allowing rapid expansion of cell numbers and would be subsite specific. However these mutants would have the limitations of other cell lines, as discussed in section 6.4.

Chapter 7: General discussion

7.1 General discussion

7.1.1 Overview

VAP is associated with significant morbidity, mortality and costs (5, 263). Furthermore the excessive use of antimicrobial agents in VAP treatment and prevention is contributing to antibiotic resistance (150). The subglottic region appears key to VAP pathogenesis, however lack of understanding of the pathophysiology of VAP is limiting the development of further prevention strategies (1, 15, 24). I therefore chose to investigate the subglottic region in long-term intubated ICU patients, with a particular focus on mucins. I used a variety of techniques to address these questions and the four main strands of interest to emerge from this work are discussed below.

7.1.2 The microbiology of mucus derived from the subglottis

It is hypothesised that infected mucus pooled in the subglottis, immediately above the tracheal tube cuff, is aspirated into the lung and results in VAP. I therefore aimed to characterise the microbiology of subglottic mucus from patients on the ICU who were intubated for four or more days and compared these findings to newly intubated control patients' mucus. Utilising semi-quantitative microbiology cultures as well as bacterial/fungal gene sequencing I was able to demonstrate significant differences in the microbiology of the mucus from the two groups. Importantly there was significantly less diversity of organisms in the ICU mucus, compared to controls. Reduced microbial diversity has been associated with progression of several inflammatory respiratory conditions, particularly in CF (154-157, 264). However the importance of this change is debated in the literature, with some studies finding the converse (264). An important consideration is why the microbial diversity was lower in the ICU cohort compared to newly intubated controls. An obvious consideration would be the effect of antibiotics and immunosuppressive agents, received by 83% and 21% of the ICU cohort respectively. These rates were well within those expected from previously published studies (149, 150). It has however been suggested that lung microbiome changes during intubation and ventilation, are independent of antibiotic administration (151). Another consideration is whether my control population of newly intubated patients may have had increased microbial diversity due to the placement of the endotracheal tube, contaminating the subglottis with oropharyngeal commensal organisms (158, 159). Assessment of the microbiology of the subglottis in non-intubated patients would be needed to fully examine the

'normal' subglottic microbiome. However, all the ICU patients had also undergone endotracheal intubation (not tracheostomy), it could therefore be contended that the newly intubated group represented an appropriate control group. It has been extensively documented that oral health deteriorates with hospital admission, even without intubation, and this leads to subsequent microbiological changes (176, 265). Not all ICU patients were intubated at the time of hospital admission and this may be a confounding variable. If the oral microbiology had already changed with hospital admission or illness, and the patient was subsequently intubated, this could have potentially introduced different organisms into the subglottis, compared to a well patient, admitted for theatre (as in the control cohort). Subgroup analysis would be needed in a larger cohort to further investigate this.

I was also able to demonstrate different organisms in the two cohorts. Broadly the organisms identified in the ICU cohort were more pathogenic, in that they are more often associated with respiratory disease and antimicrobial resistance (genera such as *Pseudomonas*, *Escherichia* and *Klebsiella*) (266-270). However many organisms have the ability to be pathogenic, given the right circumstances. Furthermore, the microbiology of VAP is highly variable, depending on several factors, including the geographical location and patient group (8, 9, 11, 271). A large proportion of VAP cases, particularly early VAP (usually classified as up to day 3 after intubation) are associated with airway commensal organisms, including *Streptococcus* and *Actinomyces,* both found more frequently in the control cohort (8, 9, 11, 271). All ICU patients were intubated and mechanically ventilated for four or more days. Therefore based on previous VAP studies I would have expected the non-commensal type of organisms identified (8, 9, 11). The greater proportion of samples with fungal organisms in the ICU cohort, compared with controls, was an intriguing finding. The rates of fungus in VAP lavage samples are relatively low (8, 9, 11, 271). Further investigation of the potential synergistic relationship between fungal and bacterial organisms, in the upper airway, would be of interest.

When considering these results it is important to note that I sampled patients at four or more days after intubation. This was in order to understand the effect of prolonged intubation on the subglottic microbiology and host defences. One critique could be that most ICU patients are not in fact intubated for so long (median length of ICU stay is 1.4 days), and a shorter period would be more relevant (272). Further longitudinal sampling over the period of intubation would be beneficial. Another consideration is that these findings are not as translatable to the proportion of ICU patients who are tracheotomised. There is increasing

emphasis on early tracheostomies to improve lung mechanics, decrease the need for sedatives, enhance communication and prevent long-term complications, such as subglottic stenosis (273, 274). A tracheostomy provides a conduit between the skin and the trachea and it is likely that these patients would have different airway microbiology from the patients with endotracheal tubes (9).

Antimicrobial resistance is increasing and ICU patients are large contributors to antibiotic use and the propagation of resistance (275-277). It is obvious that prevention of VAP is preferable to treatment of the condition, in terms of reduced mortality and antibiotic usage (278). The subglottis is crucial to VAP development, therefore greater understanding of the reasons for these subglottic microbiological differences may lead to greater understanding of the pathophysiology of VAP, and inform novel, non-antimicrobial, preventive strategies (27). I therefore went on to investigate if the microbiological differences described could be associated with changes in local host defences.

7.1.3 Characterisation of subglottic mucosal host defences

Critical illness and subsequent intubation and mechanical ventilation are usually associated with an acute illness, possibly on the background of chronic disease (32). The most engaged host defences will therefore most likely be primarily from the innate immune system (30). This consists of structural defences, such as epithelial mucosal membranes, antimicrobial molecules, the complement system, as well as immune cells, such as neutrophils and macrophages (31). I utilised a number of techniques to quantify various aspects of host defences in the subglottic mucus and in the systemic circulation. Comparing long-term intubated ICU patients and newly intubated controls I was able to demonstrate a number of physical/constituent and immunological differences between the mucus samples (Figure 7.1).

Broadly the differences demonstrated were, within the ICU cohort, greater concentrations of MUC5B (but not MUC5AC), greater DNA content and lower water content, compared to controls. As would be expected the ICU mucus also exhibited increased viscosity. It is reasonable to hypothesise that this is related, in part at least, to the differences in the individual mucus components I demonstrated (81). Other mucus constituents, such as protein and lipids, can also contribute to viscosity, but to a lesser degree than elements such as mucins. In this sub-site MUC5B was found to be at far higher concentrations than MUC5AC. To my knowledge this has not been described previously, although the ratio of MUC5B and

MUC5AC (the two main secreted airway mucins) can vary according to the airway sub-site (77, 83, 84). Changes in the mucus constituents and viscosity can have significant impact on the function of mucin as a lubricant and selective barrier (81). Increased mucus viscosity is characteristic of several acute and chronic respiratory diseases, but is best characterised in CF, where this is thought to be crucial to disease pathogenesis and deterioration of respiratory health (77, 78, 81, 88, 203).

The immunological differences were broadly demonstrative of an inflamed airway in the ICU cohort, compared to controls (279, 280), with significantly greater concentrations of pro-inflammatory cytokines, higher neutrophil counts, and greater concentrations of neutrophil elastase and C5a. There was no significant difference in macrophage counts.

I went on to investigate the phagocytic function and activation markers expressed on neutrophils extracted from the mucus. No difference was observed in phagocytic function between neutrophils extracted from long-term intubated ICU patients and newly intubated controls. There was also no significant difference in activation markers CD62L or CD11b. I therefore proceeded to investigate these differences in ICU blood-derived neutrophils, and in blood-derived neutrophils from a cohort of healthy volunteers. Once again no differences were demonstrated. Neutrophils from critically ill patients have previously been shown to have an activated phenotype and significant phagocytic dysfunction (42, 72, 73). There are a number of potential explanations for these findings in both blood and mucus. A noteworthy consideration must be the ICU cohort I recruited. This was a heterogeneous cohort, not unified by any one severe illness, such as sepsis, as in other studies (42). Furthermore neutrophil dysfunction is typically only found in significant disease states, and even then only in a limited proportion of patients (42). The spread of results for phagocytic function was far greater in the ICU blood and mucus samples, compared to the corresponding controls. With the mucus samples in particular there is also the confounding variable of DTT used to extract neutrophils from mucus. This has been demonstrated to affect surface marker expression and possibly the functional ability of neutrophils (213-216). There is however another consideration; that the mucus-derived neutrophils are removed from the *in vivo* mucus environment, which they would normally function within.

Previous studies have demonstrated that even short-term (median time 3 hours) tracheal intubation can result in significant tracheal mucosal inflammation in humans, with a significant migration of neutrophils, rise in cytokines IL-6 and IL-1β, and a significant

increase in C5a concentrations in a longitudinal theatre patient cohort (50). My cohort of ICU patients had a median intubation time of 5 days, therefore it can be postulated that placement of the tracheal tube results in early inflammation and the increased neutrophil counts that persist. Neutrophils are predominantly pro-inflammatory cells (52, 53, 63, 189). Therefore are likely to be an important driver of further inflammation, in addition to the recruitment and retention of neutrophils through agents such as IL-8 IL-6, IL-1β, C5a and GM-CSF (188, 204). It has been previously established that tracheal intubation can reduce the function of the mucociliary escalator (45-48). This would likely lead to mucus stasis and accumulation of mucus constituents, such as DNA and MUC5B. Furthermore, IL-8, IL-6 and IL-1β have all been implicated in increased secretion of MUC5B by airway cells (183-186). A key consideration is why this inflammatory process is not attenuated by corresponded anti-inflammatory mechanisms. One possibility is because of the continued presence of the inflammatory stimulus (i.e. the tracheal tube) this is not possible. However another possibility is related to the microbiology differences I revealed between the two cohorts. The presence of bacterial agents would likely modulate local immune responses, through tissue damage and the presence of bacterial agents, such as LPS, leading to further inflammation (157). Nevertheless, given the larger number of neutrophils in the subglottic mucus of long-term ventilated ICU patients, it should be expected that pathogenic bacteria would be cleared by these highly phagocytic cells (52, 53). I therefore went on to investigate if the high concentration of mucin may have an effect on neutrophil function and bacterial growth.

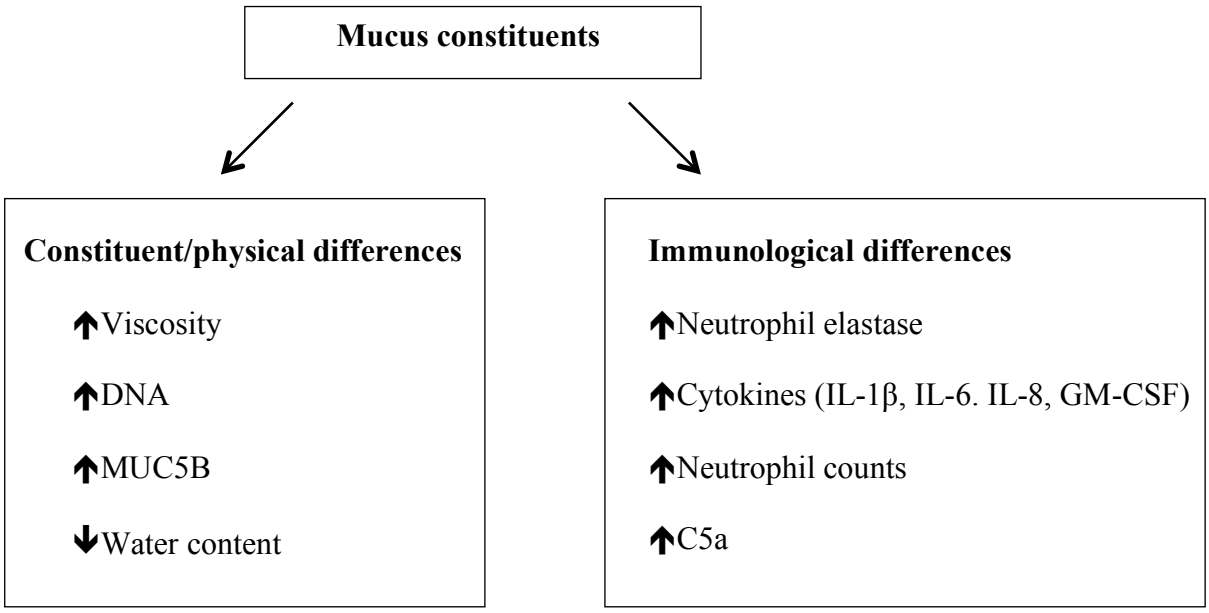

Figure 7.1 - Summary of findings related to the subglottic mucus constituents of long-term intubated ICU patients, compared with newly intubated controls.

7.1.4 The effect of mucin on neutrophil function and epithelial-pathogen interactions

VAP is typically a bacterial infection, and the neutrophil is the key innate immune cell involved in the clearance of bacterial pathogens (8, 9, 11, 52, 53, 271). I therefore investigated the relationship between purified mucin, extracted from the subglottic region of long-term intubated ICU patients, and neutrophils extracted from the whole blood of healthy volunteers. I established that 20 mg/mL mucin (representative of long-term intubated ICU patients), but not lower concentrations (representing newly intubated control patients), had a significant negative impact on neutrophil phagocytosis, bacterial killing and chemotaxis. The data also revealed that mucin-induced phagocytic dysfunction was reversible with the application of a mucolytic agent. It has previously been suggested in one *in vitro* CF model that high mucin concentrations (31.2 mg/mL total mucin) may adversely affect neutrophils' capacity for chemotaxis and bacterial killing (76). I extended these findings by using further assays and a lower concentration of mucin more representative of most of the ICU subglottic mucus.

In an *in vitro* subglottic epithelial model, I was also able to demonstrate that mucin concentrations found in long-term intubated ICU patients were associated with increased bacterial growth, in both the mucus layer and epithelial membrane, compared with concentrations found in control patients. The relationship between mucin and bacteria is contentious and poorly understood, particularly in the airway (77, 87). Mucins have no recorded anti-bacterial activity (281). However, they are able to offer binding sites similar to those of epithelial cells, preventing pathogen adhesion to the underlying epithelial cells, and clear pathogens via the mucociliary escalator (78, 79, 84, 86). This process is however subverted by pathogens in cases where the mucociliary escalator is dysfunctional, such as when there is particularly viscous mucus, or importantly during intubation (1, 14, 77). In these conditions mucins have been implicated in aiding bacterial propagation and biofilm formation, through acting as binding sites for pathogens (90-92). Another consideration is whether mucins may also act as a nutrient source for pathogens. This has been described mainly in the gastrointestinal tract, where a multitude of bacteria have been shown to be able to digest mucins and use them as an energy source (281-285). Increasingly there is evidence that similar processes may exist between airway pathogens and airway mucins (89, 93, 94, 286, 287). These studies are however limited by the nature of the mucus membrane. Mucus layers contain a multitude of constituents and understanding the relationship between any two components is challenging. For example, several other small molecules in the mucus layer could support the growth of pathogens, including sugars, fatty acids, phospholipids, and amino acids (84, 146).

I have summarised my hypothesised mechanism for the role of the subglottic region in VAP development in Figure 7.2. There are a multitude of potential interventions that could target various stages of the proposed pathogenic environment in the intubated subglottis, principally to restore the hypothesised neutrophil dysfunction and prevent bacterial proliferation. These could include agents to reduce inflammation, such as steroids or non-steroidal anti-inflammatory drugs (NSAIDs). However, many patients are already on systemic anti-inflammatory agents (149). Studies investigating the effect of these agents on infection are inconclusive (149, 288, 289). There would also be concerns about the impact of anti-inflammatory agents reducing the immune response too greatly, and precipitating bacterial growth (149). A far more targeted approach would be to change the mucus constituents, and in particular the concentration of mucin directly. This could be achieved through a variety of simple methods. Many of these interventions are already safely used in respiratory

conditions, for example nebulised saline, which increases mucus water content, thereby diluting mucins and other viscous agents (36). A host of other mucus-altering therapies are also in use, including anticholinergics, β_2-adrenoceptor agonists, mucolytics and macrolide antibiotics (290). There are also some interesting recent advances in targeted therapeutic agents, specifically focusing on mucin granule release. These include the myristoylated alanine-rich C-kinase substrate (MARCKS) protein (291). Several studies have demonstrated that diminished MARCKS expression reduces the mucin secretory response to stimulation (291-294). Conversely mucins cover all mucosal membranes and serve a multitude of purposes within the body (295). Their importance was elegantly exhibited in a murine knockout model of MUC5B, which revealed (amongst other harms) increased respiratory infection and earlier death compared to controls (86). The ease of access to the subglottis would however allow for the potential use of local, topical agents, reducing the likelihood of systemic complications.

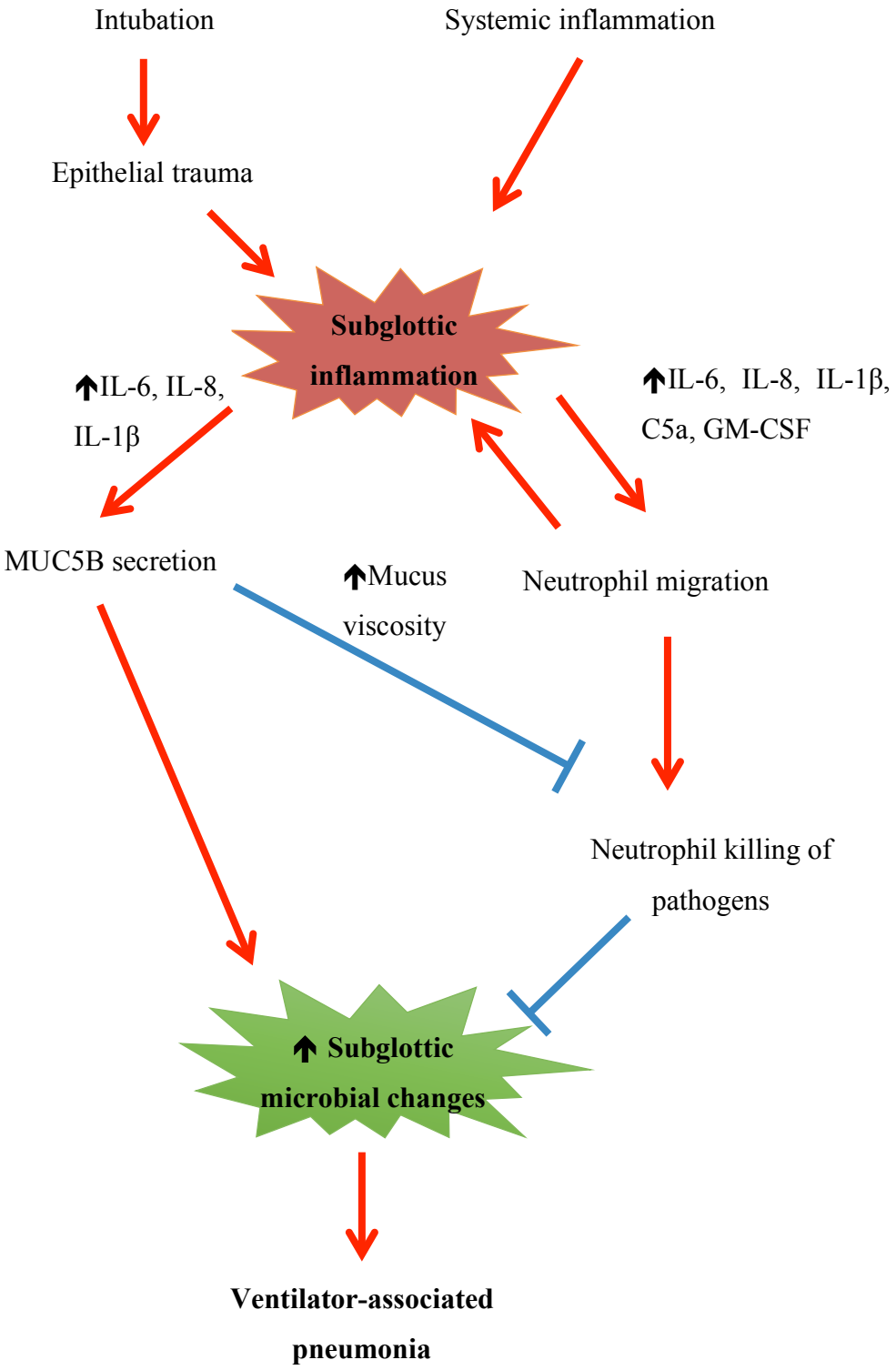

Figure 7.2 - Summary schematic regarding the proposed pathogenesis of a subglottic environment favouring development of ventilator-associated pneumonia (VAP).

7.1.5 Development of subglottic epithelial cell cultures

The development of human *in vitro* models is an important step in understanding disease pathophysiology and in allowing the testing of potential therapeutic agents. It is likely that preventive strategies for VAP are hampered by lack of good human cellular models of disease (100, 112). I therefore describe the establishment of human ALI primary subglottic epithelial cell (PSECs) cultures. A number of animal models of tracheal tube placement, ventilation and VAP development have been described (98-101). These models, while useful, have several limitations (108, 296). Firstly there are a large number of inter-species differences; particularly relevant to this work are differences in innate immunity and epithelial cell composition (108-111). There are also ethical considerations around the use of animals in laboratory research. The UK NC3Rs (National Centre for the Replacement, Refinement and Reduction of Animals in Research) have encouraged the development of advanced, human-derived, *in vitro* models (296). It is likely that animal testing would be needed at some stage in the future, particularly in the development of therapeutics, prior to human trials. However, the *in vitro* model allows for the reduction of animal use and may give superior early objective outcomes (108, 296). There are a number of *in vitro* models of respiratory epithelial sub-sites described, such as the small airways and trachea (118-120). However the subglottis is an anatomically discrete region, giving it unique physical properties (26, 238). Therefore a sub-site-specific model is likely to recapitulate the *in vivo* environment more faithfully. PSEC cultures at ALI were extensively characterised in terms of morphology, cytokeratin immunohistochemistry, electrophysiology and mucus constituents. Cultures demonstrated features typical of what would be expected of the *in vivo* subglottic region. A limitation of this model is that the samples originated from the control cohort of theatre attenders, not critically unwell ICU patients. The development of a model of the subglottic airway from ICU patients remains a future aim. This was not possible in the current work due to infections in the cultures, despite the use of appropriate antimicrobial agents. Greater understanding of the subglottic microbiology of ICU patients, as described in chapter 3 of this thesis, will hopefully allow more targeted antimicrobial use in the culture medium, and prevention of infections in these cultures.

I have therefore described, to my knowledge, the first primary human epithelial model of the subglottic region. This unique model will hopefully provide the potential to study subglottic diseases and test therapeutic agents.

7.2 Strengths and weaknesses

Strengths and weaknesses related to specific scientific approaches are summarised in Table 7.1. A global consideration is that this work comes under the umbrella term of translational research. This aims to define human disease and unmet clinical need through the characterisation of patients and disease mechanisms, thereby defining potential therapeutic targets, such as mucin (297). A limitation of early translational research is that it often uses *in vitro* methodology or small numbers of human samples. ICU patients are a heterogeneous group of patients and the sub-group who develop VAP are equally heterogeneous (148-150, 298-302). These patients are of varying ages, co-morbidities, and have differing reasons for attendance to the ICU, all of which may impact on their immune function and risk of VAP development (303, 304) (305). Furthermore there are a plethora of other potential genetic and environmental factors that may impact on these risk factors (306, 307). This variability is challenging and requires sufficiently sized cohorts to discover and validate observations. Another consideration is that even when associations are identified they cannot robustly link cause and effect. The work described in this thesis broadly fits into the remit of a proof of concept study (147). Many of the techniques describe here were novel and even aspects such as the acceptability of sampling in the ICU and control populations constitute methodological advances. Further work is needed to validate observations made here in larger cohorts. It is also likely that *in vivo* models would need to be utilised in order to study potential disease mechanisms or therapeutic targets. These models have intrinsic limitations when compared with human tissue, but also advantages, such as the whole body model of disease (98-101, 108-111). Ultimately human experimentation will be required to provide the most relevant insights into human disease.

Strengths	Weaknesses
• Long-term intubated ICU cohort • Newly intubated control cohort • Subglottic mucus examined from long-term intubated patients - unique • Matched ICU blood and mucus samples • Matched neutrophil functional assays from mucus and blood • Bacterial and fungal gene sequencing allowing microbiome assessment • Unique subglottic epithelial sampling and culture • Novel air-liquid interface cultures	• No longitudinal cohort data • Only one mucus sub-site sampled • No 'healthy' mucus controls • Unable to perform all assays in all subjects due to limited mucus yields • No matched blood and mucus samples from controls • Heterogeneous ICU cohort • No fully quantitative microbiology data • No subgroup analysis of clinical outcomes, such as VAP development • Inadequate volumes of purified mucin limited *in vitro* experimentation

Table 7.1 - Outline of specific strengths and weaknesses of the experimental design.

7.3 Future work

The findings discussed here are intriguing and suggest multiple avenues for further investigation. The first direction would be to increase the participant numbers and experimental repetitions in key experiments. This would help to validate the current findings described in this thesis. I would attempt to do this in two parallel strands. Firstly I would recruit an appropriately sized longitudinal cohort of ICU patients, performing microbiological and host defence measures, as describe in this thesis. This would allow for assessment of clinical outcomes and subgroup analyses of potentially important clinical factors or outcome measures. This may also lead to the identification of important biomarkers and further elucidate the pathogenesis of VAP. Secondly, I would perform further *in vitro* experimentation, investigating the interaction between ICU-derived mucin, neutrophils and bacterial organisms. There are several areas for further exploration, all of which could promote additional understanding of the mechanistic effect of mucins. These experiments would include use of agents other than purified mucin to investigate if it is specifically mucin, or the viscosity of the mucus, that inhibits neutrophil function. This would be a key consideration when contemplating potential therapeutic targets. Another key area for further research is further improvement of the subglottic epithelial model and utilising this to investigate the complex interaction between the epithelium, ICU-derived mucins, and bacterial species. These *in vitro* findings would be of interest to VAP and upper airway researchers, but also researchers in other fields where mucins are thought important, such as in CF, asthma, COPD, bronchiectasis and otitis media (202, 308-311).

Ultimately this research will hopefully lead to; a) a greater understanding of VAP pathogenesis, b) identification of potential biological markers for VAP risk, which are urgently needed, and c) trials of novel therapeutic/preventive targets (312, 313). There are a number of relatively low risk interventions available to target subglottic mucus, which have previously been used safely in other conditions, and could be trialled in VAP prevention studies. These include agents such as nebulised saline, which could be applied directly into the subglottic airway (235).

7.4 Final conclusions

In conclusion, the microbiology of subglottic mucus from long-term intubated ICU patients is distinctly different from newly intubated controls. I propose that these differences are due to changes in the composition and constituents of the subglottic mucus, particularly the greater concentration of the mucin MUC5B. These changes are likely caused by the inflammatory nature of long-term intubation, as illustrated by larger neutrophil counts in ICU subglottic mucus, as well as increased concentrations of inflammatory cytokines, proteases and other agents. I further propose that the high mucin concentrations, found in long-term intubation, prevent neutrophils from clearing pathogens from the subglottic mucus, and may further directly promote bacterial growth. I was unable to demonstrate any significant difference in the ability of subglottic mucus-derived neutrophils to perform phagocytosis, once removed from the mucus layer. However, blood-derived neutrophils from healthy volunteers became functionally deficient in representative ICU-derived mucin concentrations *in vitro,* and regained their function upon treatment with a mucolytic agent. I also describe a novel air-liquid interface culture of subglottic epithelial cells, which could be utilised for future translational research studies of disease mechanisms and testing of potential therapeutics. Given the morbidity, mortality and costs associated with VAP development, early prevention strategies are crucial. I suggest that reducing mucin concentrations in the subglottic region could reduce the infective burden of subglottic mucus prior to aspiration into the lungs, therefore potentially preventing VAP.

Overall I offer an intriguing preliminary insight into the microbiology and host defences of the subglottic region in long-term intubated ICU patients. These findings have implications for VAP disease pathogenesis, disease markers, as well as the generation of novel therapeutics. I believe that further research will allow optimisation of new VAP prevention strategies, such as the administration of mucolytic agents to the subglottic region in ICU patients.

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Appendix A: Additional data

Full results of semi-quantitative microbiology cultures

	Control mucus (n = 25)		ICU mucus (n = 23)		Fisher's exact test
	No of samples	**%**	**No of samples**	**%**	**P**
Streptococcus	23	92	9	39	**0.0001**
Streptococcus salivarius	17	68	0	0	**<0.0001**
Streptococcus vestibularis	2	8	0	0	0.4902
Streptococcus mitis	1	4	0	0	>0.9999
Staphylococcus epidermidis	0	0	1	4	0.4792
Streptococcus anginosus	0	0	2	9	0.2243
Other alpha haemolytic streptococci	22	88	7	30	**<0.0001**
Rothia	14	56	0	0	**<0.0001**
Rothia mucilaginosa	12	48	0	0	**<0.0001**
Rothia dentocariosa	1	4	0	0	>0.9999
Other rothia species	2	8	0	0	0.4902
Actinomyces	10	40	0	0	**0.0007**
Actinomyces odontolyticus	4	16	0	0	0.1105
Acinetomyces oris	2	8	0	0	0.4902
Actnomyces odontolyticus	1	4	0	0	>0.9999
Actinomyces graevenitzii	1	4	0	0	>0.9999
Other actinomyces species	1	4	0	0	>0.9999
Neisseria	7	28	2	9	0.1402
Neisseria flavescens	5	20	0	0	0.0507
Neisseria mucosa	1	4	1	4	>0.9999

Other Neisseria species	1	4	1	4	>0.9999
Haemophilus	6	24	2	9	0.2487
Haemophilus influenzae	3	12	1	4	0.6099
Haemphilus parainfluenzae	1	4	1	4	>0.9999
Haemophilus parahaemolyticus	2	8	0	0	0.4902
Haemophilus haemolyticus	0	0	1	4	0.4792
Staphylococcus	4	16	3	13	>0.9999
Staphylococcus aureus	2	8	1	4	>0.9999
Staphylococcus capitis	0	0	0	0	>0.9999
Staphylococcus epidermidis	2	8	1	4	>0.9999
Staphylococcus pasteuri	0	0	1	4	0.4792
Gemella	3	12	0	0	0.2354
Gemella haemolysans	3	12	0	0	0.2354
Veillonella	3	12	0	0	0.2354
Veillonella atypica	2	8	0	0	0.4902
Veilonella dispar	1	4	0	0	>0.9999
Candida	2	8	9	39	**0.0157**
Candida glabrata	1	4	2	9	0.6011
Candida tropicalis	0	0	1	4	0.4792
Other candida species	1	4	0	0	>0.9999
Candida guilliermondii	0	0	1	4	0.4792
Candida nivariensis	0	0	1	4	0.4792
Candida albicans	0	0	4	17	**0.0455**
Candida lusitaniae	0	0	1	4	0.4792

Candida parapsilosis	0	0	1	4	0.4792
Moraxella	1	4	0	0	>0.9999
Moraxella catarrhalis	1	4	0	0	>0.9999
Capnocytophaga	1	4	0	0	>0.9999
Capnocytophaga gingivalis	1	4	0	0	>0.9999
Lactobacillus	1	4	2	9	0.6011
Lactobacillus fermentum	1	4	0	0	>0.9999
Lactobacillus rhamnosus	0	0	1	4	0.4792
Lactobacillus gasseri	0	0	1	4	0.4792
Pseudomona	0	0	2	9	0.2243
Pseudomonas aeruginosa	0	0	2	9	0.2243
Enterococcus	0	0	4	17	**0.0455**
Enterococcus faecalis	0	0	4	17	**0.0455**
Achromobacter	1	4	2	9	0.6011
Achromobacter xylosoxidans	1	4	2	9	0.6011
Bifidobacterium	0	0	3	13	0.1024
Bifidobacterium breve	0	0	2	9	0.2243
Bifidobacterium dentium	0	0	1	4	0.4792
Atopobium vaginae	0	0	2	9	0.2243
Alloscardovia	0	0	2	9	0.2243
Alloscardovia omnicolens	0	0	2	9	0.2243
Enterobacteriaceae*	1	0	11	43	**0.0185**
Escherichia*	1	4	1	4	0.9520
Escherichia coli	1	4	1	4	0.9520

Raoultella*	0	0	1	4	0.4792
Raoultella ornithinolytica	0	0	1	4	0.4792
Prevotella*	0	0	1	4	0.4792
Prevotella melaninogenica	0	0	1	4	0.4792
Morganella*	0	0	1	4	0.4792
Morganella morganii	0	0	1	4	0.4792
Hafnia*	0	0	1	4	0.4792
Hafnia alvei	0	0	1	4	0.4792
Providencia*	0	0	1	9	0.2243
Providencia stuartii	0	0	1	4	0.4792
Enterobacter*	0	0	2	9	0.2243
Enterobacter cloacae	0	0	2	9	0.2243
Klebsiella*	0	0	1	4	0.4792
Klebsiella oxytoca	0	0	0	0	>0.9999
Klebsiella variicola	0	0	1	4	0.4792
Serratia*	0	0	2	9	0.2243
Serratia marcescens	0	0	2	9	0.2243

**Enterobacteriaceae* family

Appendix B: Ethical approval, consent forms and patient information sheets related to this thesis

Health Research Authority

West Midlands - Edgbaston Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Telephone: 0115 8839695

23 September 2015

Professor Janet Wilson
Prof of Otolaryngolgoy Head and Neck Surgery; Hon Consultant Otolaryngologist
Newcastle University
Department of Otolaryngolgoy Head and Neck Surgery
Freeman Hospital
Newcastle upon Tyne
NE7 7DN

Dear Professor Wilson

Study title:	**Modulation of the upper airway host defences to prevent pneumonia in ventilated patients - ENT volunteers**
REC reference:	**15/WM/0349**

Thank you for your letter of 22 September 2015. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 18 September 2015

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Participant information sheet (PIS)	2	22 September 2015
Response to Request for Further Information [Covering Letter]		22 September 2015

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Zurich Municipal insurance certificate]		30 July 2015
GP/consultant information sheets or letters [GP letter]	1	08 July 2015
IRAS Checklist XML [Checklist_07092015]		07 September 2015

NHS
Health Research Authority

North East - Newcastle & North Tyneside 2 Research Ethics Committee
Room 001
Jarrow Business Centre
Rolling Mill Road
Jarrow
NE32 3DT

Telephone: 0191 4283563

17 November 2015

Dr Stephen Wright
Consultant in Anaesthesia and Intensive Care
The Newcastle upon Tyne Hospitals NHS Foundation Trust
Perioperative and Critical Care
Freeman Hospital
Newcastle upon Tyne
NE7 7DN

Dear Dr Wright

Study title:	**Modulation of the upper airway host defences to prevent pneumonia in ventilated patients - ICU attenders**
REC reference:	**15/NE/0323**
IRAS project ID:	**186924**

Thank you for your letter of 9 November 2015, responding to the Committee's request for further information on the above research [and submitting revised documentation].

The further information has been considered on behalf of the Committee by myself as Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Mrs Helen Wilson, nrescommittee.northeast-newcastleandnorthtyneside2@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a **favourable ethical opinion** for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

Mental Capacity Act 2005

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

CONSULTEE CONSENT FORM

Title of Project: Subglottic study - ICU

Principal investigator: Dr Stephen Wright

Please initial

1. I (_____) have been consulted about (_____)'s participation in this research project. I confirm that I have read and understand the information sheet dated 8th November 2015 (Version 4) for the above study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily . [ ]

2. In my opinion he/she would have no objection to taking part in the above study. [ ]

3. I understand that I can request he/she is withdrawn from the study at any time, without giving any reason and without his/her care or legal rights being affected. [ ]

4. i) I understand that relevant sections of his/her medical notes and data collected during the study may be looked at by individuals from: the study team; the Sponsor (Newcastle upon Tyne Hospitals NHS Foundation Trust) or their representatives; and from regulatory authorities, where it is relevant to my taking part in this research. [ ]

5. I agree to their GP or other care professional being informed of their participation in the study. [ ]

_____	_____	_____
Name of consultee	Date	Signature

_____
Relationship to patient

_____	_____	_____
Researcher	Date	Signature

1 copy for consultee; 1 for research site file; 1 for case notes

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

PARTICIPANT CONSENT FORM

Title of Project: Subglottic study - ICU

Principal investigator: Dr Stephen Wright

Please initial

1. I confirm that I have read and understand the information sheet dated 8th November 2015 (Version 4) for the above study I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily . [ ]

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. [ ]

3. i)I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from: the study team; the Sponsor (Newcastle upon Tyne Hospitals NHS Foundation Trust) or their representatives; and from regulatory authorities, where it is relevant to my taking part in this research. [ ]

ii) I give permission for these individuals to have access to my records, even if I withdraw from the study, and I understand that my records will only be reviewed for information related to my participation in the study. [ ]

4. I agree that the research team may record information from my case records solely for the purpose of this study. [ ]

5. I agree that my throat samples may be used for this study. [ ]

6. I agree that my throat samples can be used in future studies

7. I agree that my blood samples may be used for this study. [ ]

8. I agree that my blood samples can be used in future studies [ ]

9. I consent to my GP being informed of my participation in this study. [ ]

10. I wish to receive a research summary after completion of the study. [ ]

Name of Patient	Date	Signature
Researcher	Date	Signature

1 copy for patient; 1 for research site file; 1 for case notes

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

INFORMATION SHEET FOR PATIENT (Personal consultee)

Subglottic study – ICU

Introduction

We feel your relative/friend is unable to decide for himself/herself whether to participate in this research study.

To help decide if he/she should join the study, we'd like to ask your opinion whether or not they would want to be involved. We'd ask you to consider what you know of their wishes and feelings, and to consider their interests. Please let us know of any advance decisions they may have made about participating in research. These should take precedence.

If you decide your relative/friend would have no objection to taking part we will ask you to read and sign the consultee declaration on the last page of this information leaflet. We'll then give you a copy to keep. We will keep you fully informed during the study so you can let us know if you have any concerns or you think your relative/friend should be withdrawn.

If you decide that your friend/relative would not wish to take part it will not affect the standard of care they receive in any way.

If you are unsure about taking the role of consultee you may seek independent advice.

We will understand if you do not want to take on this responsibility.

The following information is the same as would have been provided to your relative/friend:

You are being consulted about your opinion to take part in a research study. Before you decide whether or not to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

- Part 1 tells you the purpose of the study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Please ask if there is anything that is not clear or if you would like more information.

PART 1

What is the purpose of the study?

Patients in intensive care units (ICUs) often require help with their breathing. This involves placing a plastic tube between the vocal cords of the unconscious patient, and into the windpipe, after which the tube is attached to a ventilator machine. The tube is kept in place by inflating a balloon (or "cuff") at the end of the tube, to hold the tube against the windpipe, just below the vocal cords. In health, the vocal cords and a normal cough prevent germs entering the lung. Unfortunately, the combination of illness, reduced cough, and having a tube "bypassing" the vocal cords, leaves these patients extremely vulnerable to lung infection. This infection termed "ventilator-acquired pneumonia", causes more deaths than any other hospital-acquired infection. New ways to prevent it, preferably without using antibiotics, are urgently required.

A key starting point would be identifying how germs begin their journey into the lung. We know germs grow rapidly just above the cuff of the tube in the voice box (an area known as the "subglottis"), before passing into the lung. To understanding how this happens we need samples from people in ICU to use in the laboratory for experiments and to identify new treatments.

What will happen to you if you agree to take part in the study?

In addition to your normal care you will receive the following procedures–
1) An extra blood test.
2) Samples of mucus will be taken from your throat using a suction tube.
3) A sampled of the lining of your voice box will be taken using a small brush passed into your throat via a small telescope.

Rather than throw away any excess blood or throat samples that are not needed, with your permission, we will keep some of the samples to be used in any future research by our research group where ethical approval has been granted.

Why have you been chosen?

The research is specifically looking at the factors in the throat and blood that put patients in the intensive care unit (ICU) at risk of serious chest infection. The fact that you are on the ICU for several days means that you are eligible for entry into the study.

Do you have to allow your information and samples to be used for the study?

No, it is up to you to decide whether to take part in the research. If you decide to take part you will be free to change your mind at any time and without giving a reason. It is very important to understand that if you do not wish for your samples / clinical information to be used for the research then we shall fully understand and **this will not in any way alter your care now or at any stage in the future.**

If you decide you would rather not have taken part, the consent form that comes along with this document will give you 2 options. These are either to say

1. That you do not wish us to collect any further information about you for the study, but we may keep any information/samples collected up to now.
2. That you wish us to destroy all samples and information relating to you that have been collected as part of the study.

What are the possible disadvantages and risks of taking part in the study?

You should not experience any side effects from the drawing of blood. You should not experience any side effects from the sampling from your throat.

What are the potential benefits of you taking part in the study?

There are no benefits to you personally in taking part in the study. Our research in the lab, however, is aimed at helping prevent people, such as you, from getting serious infection while on the intensive care unit in the future.

Is there any reimbursement for taking part?

No

What if there is a problem?

Any complaint about the way you have been dealt with during the study will be addressed. The detailed information on this is found in Part 2.

Is the study confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making a decision.

PART 2

What happens if you change your mind?

You can change your mind at any point. If you subsequently decide that you do not want to take part in the study you can insist that all research data and samples be destroyed.

What if there is a problem?

If you wish to complain, or have any concerns about the way in which you have been approached or treated during the course of this study you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy, and with to complain formally, you can do so via the normal NHS complaints procedure.

What if something goes wrong?

In the event that something goes wrong, and you are harmed during the research, and this is due to someone's negligence, then you may have grounds for legal action for compensation against Newcastle Upon Tyne Hospitals Foundation Trust, but you may have to pay your legal costs. The normal NHS complaints service will still be available to you.

Will your taking part be kept confidential?

Yes, personal data will be regarded as strictly confidential. Any samples or data collected about you (such as your age and medical conditions) will be marked with a unique study number only, preventing you being recognised by anyone outside the study team. This study number will allow only an authorized member of the research team to identify you if absolutely necessary. Data will be stored in a secure location within your hospital and at the central study location at Newcastle University. Data will be retained at these sites for 5 years, following this time it will be disposed of as confidential waste.

Can you access the results of the research?

Yes. We aim to send all participant a summary of the study finding after the study is completed and analysis performed. This may take several years. Should you wish to know details before then please contact Dr Wright at the address shown at the end of this document.

Involvement of the GP

With permission we will inform your GP, by letter, that you have taken part in our study.

Will anyone else know I am involved in the study?

Only key member of the research team, your clinical team, and your GP, will know you are in the study.

Authorised people from the research team will look at anonymised data collected from the study whilst conducting the analysis. Auditors representing Newcastle Upon Tyne Hospitals Foundation Trust may have access to personalised data like consent forms as they are responsible for the research.

What will happen to the samples we obtained?

The samples will be stored in our laboratory and used in a series of experiments to investigate the interactions between airway cells, mucus, blood cells and germs. Samples may be retained for 5 years before being destroyed. They will be used by members of the study team and by our scientific collaborators.

Will any genetic tests be done?

No genetic tests will be performed as part of this study.

What will happen to the results of the research study?

We aim to publish the study results in high quality scientific/medical journals and present them at medical and scientific meetings. You will not be identified in any report/publication. We also propose to make results of the study available to patient groups (eg CritPal and ICUSteps – support groups for patients who have been on ITU and their relatives).

Who is funding this study?

The study is funded by the Wellcome Trust.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect the safety, rights, wellbeing and dignity of patients. This study has been reviewed and given a favourable opinion by such a Research Ethics Committee - Newcastle & North Tyneside 2 Research Ethics Committee. The scientific rigour and importance of the study was reviewed by several independent specialists and by a scientific panel chaired by the Wellcome Trust.

Contact for further information

If you would like further information now or at any stage in the future, please do not hesitate to contact

Dr Stephen Wright
Intensive Care Unit
Freeman Hospital
Freeman Road
Newcastle upon Tyne
NE7 7DN
0191 223 1059
Stephen.Wright@nuth.nhs.uk

If you would like to talk to an expert who is not involved in the project, we have an independent advisor for this specific purpose. This person is a fully qualified medical practitioner who is there to answer any questions or concerns you may have about the study. He is not in any way involved in the study, but understands all of the medical aspects of this particular project. The contact details are

Dr Bryan Yates
Consultant Intensive Care and Respiratory Medicine
North Tyneside General Hospital
Rake Lane
North Shields
Tyne and Wear
NE29 8NH
0191 2932582

You will be given a copy of this Information Sheet and a signed consent form to keep.
Thank you for taking time to read this sheet and for considering taking part.

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

INFORMATION SHEET FOR PATIENT (Participant)

Subglottic study - ICU

PART 1

You are being invited to take part in a research study. Before you decide whether or not to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish.

- Part 1 tells you the purpose of the study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Please ask if there is anything that is not clear or if you would like more information.
Thank you for reading this.

What is the purpose of the study?

Patients in intensive care units (ICUs) often require help with their breathing. This involves placing a plastic tube between the vocal cords of the unconscious patient, and into the windpipe, after which the tube is attached to a ventilator machine. The tube is kept in place by inflating a balloon (or "cuff") at the end of the tube, to hold the tube against the windpipe, just below the vocal cords. In health, the vocal cords and a normal cough prevent germs entering the lung. Unfortunately, the combination of illness, reduced cough, and having a tube "bypassing" the vocal cords, leaves these patients extremely vulnerable to lung infection. This infection termed "ventilator-acquired pneumonia", causes more deaths than any other hospital-acquired infection. New ways to prevent it, preferably without using antibiotics, are urgently required.

A key starting point would be identifying how germs begin their journey into the lung. We know germs grow rapidly just above the cuff of the tube in the voice box (an area known as the "subglottis"), before passing into the lung. To understanding how this happens we need samples from people in ICU to use in the laboratory for experiments and to identify new treatments.

What happened to you during the study?

You were unaware of our study at the time it was performed. This is because you were not well enough for the details of the study to be discussed with you.

We received permission (consent) from your relative/friend/carer (_____), who was not aware of you having any objection to helping with medical research.

In addition to your normal care you received the following procedures–
1) An extra blood test was performed.
2) We took samples of mucus from your throat.
3) We sampled some of the lining of your throat using a small brush passed into your throat via a small scope.

We also kept some of the samples we collected, this includes the liquid part of your blood (called plasma or serum) and some glass slides with some white blood cells on them. We also kept some of the samples from your throat. We now need to check with you whether you give us permission for these samples to be used in any future research by our research group, where ethical approval has been granted. We should emphasise that all samples and information collected up to now are marked with a unique study number only, preventing you being recognised by anyone outside the study team.

Why have you been chosen?

The research is specifically looking at the factors in the throat and blood that put patients in the intensive care unit at risk of serious chest infection. The fact that you are on the ICU for several days means that you are eligible for entry into the study.

Do you have to allow your information and samples to be used for the study?

No, it is up to you to decide whether to take part in the research. If you decide to take part you will be free to change your mind at any time and without giving a reason. It is very important to understand that if you do not wish for your samples / clinical information to be used for the research then we shall fully understand and **this will not in any way alter your care now or at any stage in the future.**

If you decide you would rather not have taken part, the consent form that comes along with this document will give you 2 options. These are either to say

1. That you do not wish us to collect any further information about you for the study, but we may keep any information/samples collected up to now.
2. That you wish us to destroy all samples and information relating to you that have been collected as part of the study.

What are the possible disadvantages and risks of taking part in the study?

You should not experience / have experienced any side effects from the drawing of blood. You should not experience / have experienced any side effects from the sampling from your throat.

What are the potential benefits of you taking part in the study?

There are no benefits to you personally in taking part in the study. Our research in the lab, however, is aimed at helping prevent people, such as you, from getting serious infection while on the intensive care unit in the future.

Is there any reimbursement for taking part?

No

What if there is a problem?

Any complaint about the way you have been dealt with during the study will be addressed. The detailed information on this is found in Part 2.

Is the study confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. The details are included in Part 2.

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making a decision.

PART 2

What happens if you change your mind?

You can change your mind at any point. If you subsequently decide that you do not want to take part in the study you can insist that all research data and samples be destroyed.

What if there is a problem?

If you wish to complain, or have any concerns about the way in which you have been approached or treated during the course of this study you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy, and wish to complain formally, you can do so via the normal NHS complaints procedure.

What if something goes wrong?

In the event that something goes wrong, and you are harmed during the research, and this is due to someone's negligence, then you may have grounds for legal action for compensation against Newcastle Upon Tyne Hospitals Foundation Trust, but you may have to pay your legal costs. The normal NHS complaints service will still be available to you.

Will your taking part be kept confidential?

Yes, personal data will be regarded as strictly confidential. Any samples or data collected about you (such as your age and medical conditions) will be marked with a unique study number only, preventing you being recognised by anyone outside the study team. This study number will allow only an authorized member of the research team to identify you if absolutely necessary. Data will be stored in a secure location within your hospital and at the central study location at Newcastle University. Data will be retained at these sites for 5 years, following this time it will be disposed of as confidential waste.

Can you access the results of the research?

Yes. We aim to send all participant a summary of the study finding after the study is completed and analysis performed. This may take several years. Should you wish to know details before then please contact Dr Wright at the address shown at the end of this document.

Involvement of the GP

We also asked your relative/friend/carer for permission to inform your GP, by letter, that you have taken part in our study.

Will anyone else know I am involved in the study?

Only key member of the research team, your clinical team, and your GP, will know you are in the study.

Authorised people from the research team will look at anonymised data collected from the study whilst conducting the analysis. Auditors representing Newcastle Upon Tyne Hospitals Foundation Trust may have access to personalised data like consent forms as they are responsible for the research.

What will happen to the samples we obtained?

The samples will be stored in our laboratory and used in a series of experiments to investigate the interactions between airway cells, mucus, blood cells and germs. Samples may be retained for 5 years before being destroyed. They will be used by members of the study team and by our scientific collaborators

Will any genetic tests be done?

No genetic tests will be performed as part of this study.

What will happen to the results of the research study?

We aim to publish the study results in high quality scientific/medical journals and present them at medical and scientific meetings. You will not be identified in any report/publication. We also propose to make results of the study available to patient groups (eg CritPal and ICUSteps – support groups for patients who have been on ITU and their relatives).

Who is funding this study?

The study is funded by the Wellcome Trust.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect the safety, rights, wellbeing and dignity of patients. This study has been reviewed and given a favourable opinion by such a Research Ethics Committee - Newcastle & North Tyneside 2 Research Ethics Committee. The scientific rigour and importance of the study was reviewed by several independent specialists and by a scientific panel chaired by the Wellcome Trust.

Contact for further information

If you would like further information now or at any stage in the future, please do not hesitate to contact

Dr Stephen Wright
Critical Care Unit (Ward 37)
Freeman Hospital
Freeman Road
Newcastle upon Tyne
NE7 7DN
0191 223 1059
Stephen.Wright@nuth.nhs.uk

If you would like to talk to an expert who is not involved in the project, we have an independent advisor for this specific purpose. This person is a fully qualified medical practitioner who is there to answer any questions or concerns you may have about the study. He is not in any way involved in the study, but understands all of the medical aspects of this particular project. The contact details are

Dr Bryan Yates
Consultant Intensive Care and Respiratory Medicine
North Tyneside General Hospital
Rake Lane
North Shields
Tyne and Wear
NE29 8NH
0191 2932582

You will be given a copy of this Information Sheet and a signed consent form to keep.
Thank you for taking time to read this sheet and for considering taking part.

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Attach patient label here

CONSENT FORM

Subject Number:

Title of Project: Modulation of the upper airway host defences to prevent pneumonia in ventilated patients – theatre attenders

Chief investigator: Prof Janet Wilson
Principal investigator at site: Mr Jason Powell

Please initial

1. I confirm that I have read and understand the information sheet dated 22nd September 2015 (Version 2) for the above study I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily. [ ]

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. [ ]

3. i)I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from: the study team or the Sponsor (Newcastle upon Tyne Hospitals NHS Foundation Trust), where it is relevant to my taking part in this research. [ ]

ii) I give permission for these individuals to have access to my records, even if I withdraw from the study, and I understand that my records will only be reviewed for information related to my participation in the study. [ ]

4. I agree that the research team may record information from my case records solely for the purpose of this study. [ ]

5. I agree that my throat samples may be used for this study. [ ]

6. I agree that my throat samples can be used in future studies on condition that my identity cannot be determined from the sample. [ ]

7. I consent to my GP being informed of my participation in this study. [ ]

Name of Patient	Date	Signature
Researcher	Date	Signature

1 copy for patient; 1 for researcher; 1 for case notes

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Patient Information Sheet

Modulation of the upper airway host defences to prevent pneumonia in ventilated patients – ENT volunteers

Invitation

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

What is the purpose of the study?

Critically ill patients in intensive care units (ICUs) are more prone to infections than other people. These patients are particularly vulnerable to a lung infection termed "ventilator-acquired pneumonia" (VAP). This infection causes more deaths than any other hospital-acquired infection. New ways to prevent it, preferably without using antibiotics, are urgently required. A key starting point would be identifying how germs begin their journey into the lung from the upper airway or throat. In order to understand why this happens we need samples of the throat from both patients on the ICU, and people who are not on ICU, to look at the differences, and to use these samples to devise new treatments.

Why have I been chosen?

You have been chosen because you are going to have an examination of the throat under a general anaesthetic - 'asleep'. This is something your doctor has planned but during this procedure is a good time to take these samples from your throat while you are asleep and don't feel anything.

Do I have to take part?

No, you do not have to take part in this study. If you do not agree, your care will not be affected in any way. You may also change you mind later and ask to be withdrawn

from the study. No reason need be given. If you do withdraw, your samples will be destroyed and your data will be removed from the study entirely, provided that the results of the study have not already been published in a scientific journal.

Will taking part affect my treatment?

Donating your tissue samples will not affect your treatment. We would like to perform brushings and collect mucus from your voice box. This would be done in agreement with your hospital doctor and it would not affect your treatment and should not cause you any further discomfort.

What will happen to me if I take part?

In theatre when your throat is being examined we will, with your hospital doctor's consent, take brushings, swabs and a sample of mucus from your voice box. You will be asleep during this and will not be aware of it. The samples taken by us will not be used for diagnostic purposes. The samples may be stored in our laboratory for future research studies. The samples will not be used for research that involves reproductive cloning, or be tested for inherited diseases. During the procedure, we may find we are unable to take the study samples for some reason. If this happens, we will discuss this with you afterwards. The findings of experiments using your tissue will only be presented as group results. No individual information about you will be shown or published. Only research staff will have access to your information and tissue samples.

What do I have to do?

You do not have to do anything right now. On the day of your procedure a member of the research team will contact you again and ask if you are happy to take part in the study. You will not have to come to the hospital again other than on the date planned by your doctor. After we take the samples during your procedure we will only contact you once more, by post, to let you know about the pooled results of the research study, if you wish us to. We will not contact you again at any other time. If you decide not to be involved with the study simply inform the researcher and we will not contact you further. If you have any further questions or are not sure, then please discuss your concerns with a member of the research team.

What are the possible risks and disadvantages of taking part?

You will be asleep while all these events happen and unaware so should not feel any discomfort at the time of sampling. We will be stroking the voice box with a miniature brush that is less than a centimetre wide, this takes a very small sample of superficial cells of the voice box lining. These cells will quickly grow back and be replaced. This will not affect your speaking voice. In order to sample your throat mucus we will suction this out with a small tube through your mouth. This should not cause any ill effect and your body will naturally replace this. Finally we will take a swab of the voice box to see what bacteria/fungus/viruses we might find. These procedures should not add on more than 5-10 minutes to the length of your procedure and time asleep under anaesthetic. This should not make the procedure or anaesthetic any more risky for you.

What are the possible benefits of taking part?

The results will not be of direct benefit to you, but will help towards better treatments for seriously unwell patients in hospital. If we find any unexpected incidental findings that relate to your sample we will contact your GP who will contact you to discuss this. This is highly unlikely. After the study has finished we will let you know about the findings of the research study in writing, for your interest. This will be related to general pooled findings, not personal results.

What if something goes wrong?

We do not envisage any complications but we really would like to hear about any possible side effects of taking part, please contact Prof Janet Wilson, Hon Consultant, ENT Department, Freeman Hospital, NE7 7DN, Tel: 01912231086, if you experience anything negative that you fell may be related to the study, however minor.

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the study, you can speak directly in confidence to any member of research team. The National Health Service complaints procedure is also available to you. Details can be found on our hospital web site www.newcastle-hospitals.org.uk or by contacting the Patient Relations Department on 0191 223 1382

Will my taking part in this study be kept confidential?

Yes. All patient data is treated as confidential and will be anonymised before any analysis. Only group data, not individual data, will be produced in this study. With your permission we will let your General Practitioner (GP) know that you have been in the study.

What happens when the research stops?

The pooled results of this project may be presented at scientific meeting or in scientific journals.

Who is organising and funding the research?

This study is being funded by the Wellcome Trust. None of the research team will receive payment for this study.

Who has reviewed the study?

The study has been reviewed by the West Midlands - Edgbaston Research Ethics Committee, and the Research and Development Department within the Newcastle upon Tyne Hospitals NHS Trust.

What happens if I change my mind?

You have the right to change your mind at any time before the procedure. If you change your mind after the samples have been taken we will withdraw your samples from the study as long as they have not been analysed. If you choose to do so, please contact the research team:

Contact Person- Prof Janet Wilson

Postal address- ENT Department, Freeman Hospital, Freeman Road, High Heaton, Newcastle upon Tyne, NE7 7DN

Telephone number: 01912231086

Who can I contact for further information?

For further information about the study:

Prof Janet Wilson, Hon Consultant, ENT Department, Freeman Hospital, NE7 7DN, Tel: 01912231086

Or for confidential advice about taking part: The Patient Advice and Liaison Service: 08000 320202, northoftynepals@nhct.nhs.uk

Appendix C: Presentations, prizes and publications pertaining to this thesis

Presentations

- **Powell J**, Garnett J, Verdon B, Wilson J, Pearson J, Ward C. A human in vitro model of the subglottic airway. **(Oral presentation)**
  29th September 2015, American Academy of Otolaryngology - Head and Neck Surgery (AAO-HNS) Annual Meeting, Dallas, USA.

- **Powell J**, Garnett J, Verdon B, Wilson J, Pearson J, Ward C. A primary in vitro model of the Subglottic Airway. **(Oral presentation)**
  9th October 2015, ORS Autumn Meeting, Liverpool.

- **Powell J**, Garnett J, Verdon B, Wilson J, Pearson J, Ward C. A human in vitro model of the subglottic airway. **(Poster presentation)**
  5th November 2015, British Laryngological Association Annual Conference, London

- **Powell J**, Garnett J, Verdon B, Wilson J, Pearson J, Ward C. A human in vitro model of the subglottic airway. **(Oral presentation)**
  9th November 2015, Faculty Development Day - Otolaryngology, Newcastle upon Tyne.

Prizes

- David Howard Prize, British Laryngological Association annual meeting, poster presentation prize, November 2015.
- Munro Black Prize, Northern Deanery Otolaryngology oral presentation prize, November 2015.

Publications

- **Powell J**, Garnett J, Verdon B, Wilson J, Pearson J, Ward C. A human in vitro model of the subglottic airway. *Otolaryngol Head Neck Surg. 2015; 153(1s):83*