ASPIRATION LUNG DISEASE IN CHILDREN WITH SEVERE NEURODISABILITY

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Abstract

Background: Children with severe neurodisability (ND) commonly suffer with respiratory disease and this is the leading cause of premature death. The nature of this respiratory disease is however, poorly understood. The underlying aetiology is often multifactorial, but aspiration (direct or reflux) is likely to play a key role. Unfortunately, diagnostic tests for reflux or direct aspiration are limited. There is a clinical need for high quality research on children with severe ND to define underlying mechanisms of respiratory disease and guide management.

Aims: In this study, the aims were to (i) characterise respiratory symptoms and their relationship with lower airway inflammation in children with severe ND, (ii) explore available bronchial lavage (BAL) biomarkers of reflux aspiration, assessing their validity and their relationship to clinical and airway inflammatory data, (iii) develop a novel assay for the accurate detection/quantification of pepsin in BAL, (iv) investigate the validity of an α-amylase assay in paediatric BAL and explore the relationship of α-amylase levels with lower airway inflammation and clinical symptoms, and (v) investigate the effects of pH alteration and pepsin exposure on airway epithelial cells (AEC).

Methods: Clinical data and BAL samples were collected from children with severe ND at times of stability and respiratory deterioration and also from healthy controls. BAL differential cell counts and cytokine measurements (ELISA) were performed. Lower airway microbial colonisation/infection was assessed. BAL pepsin measurement was attempted using a number of methods, and the feasibility of inhibitor affinity enrichment and LC-MRM-MS techniques to identify and quantify BAL pepsin was explored using SDS PAGE gel and mass spectrometry analysis. BAL alpha-amylase activity was measured using an ethylidene-pNP-G7 based assay. BEAS-2B cells were cultured in monolayer and the cytotoxic/inflammatory effects of pH alteration and pepsin exposure were explored through trypan blue staining and cytokine assays.

Results: Children with severe ND have burdensome chronic respiratory symptoms that impact on their quality of life and that of their families'. Symptoms may relate to lower airway inflammatory levels. A trend for greater airway

neutrophilia and respiratory symptoms was seen in those with lower airway microbial positivity. Available 'in house' pepsin ELISA assays were not robust but Western Blot results indicated a higher frequency of BAL pepsin positivity in ND patients with acute respiratory deterioration. Notably, positivity was also found in some healthy controls, highlighting the need for a quantitative assay. The use of an immobilized inhibitor (pepstatin agarose) to recover pepsin from paediatric BAL samples was shown to be feasible. Subsequent digestion of pepsin and detection by LC-MSMS with selective ion monitoring was demonstrated. Significantly increased BAL alpha-amylase levels were seen in Elective-ND patients compared to healthy controls and furthermore, significant correlations were observed with BAL lower airway inflammatory markers. BEAS-2B cells were sensitive to mild acidification, with up-regulation of TGFβ-1, IL-6 and IL-8 mRNA expression. A corresponding rise in protein secretion was not necessarily seen and in some cases, was significantly down-regulated. No significant cytotoxicity or decrease in cell viability was observed in any condition.

Conclusions: In children with severe ND, chronic respiratory symptoms may be directly related to lower airway inflammation and bacterial colonization. Measurement and quantification of pepsin in BAL (as a marker of reflux aspiration) is difficult. However, an inhibitor affinity enhanced mass spectrometry based technique has potential as a 'gold-standard' method for identification and quantification of pepsin in BAL. BAL alpha-amylase activity shows promise as a biomarker of direct aspiration. Mild alterations in airway pH may directly contribute to the pathophysiology of inflammatory airway disease and specifically, reflux-aspiration related lung disease in this group. This requires further study as a potential novel therapeutic target.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is the result of my own work. Specifically, Tina Samuels and Ass. Prof. Nikki Johnston (University of Wisconsin) performed Western Blot analyses for human pepsin on BAL samples, as described in Section 2.5.2.2. Also, Professor Lesley Turnbull (Consultant Cytopathologist) reported selected lipid laden macrophage samples, as described in Section 2.3.2.4. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree of qualification.

This research was carried out at the Institute of Child Health, Alder Hey Children's Hospital and the School of Biological Sciences, University of Liverpool.

Signature…….

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Abbreviations and Notations

Ab	antibody
AEC	airway epithelial cell
AmBic	ammonium bicarbonate
BAL	non-bronchoscopic broncho-alveolar lavage
BEGM	Bronchial Epithelial Cell Growth Media
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CF	cystic fibrosis
CO_2	carbon dioxide
CP	cerebral palsy
CT	cycle threshold
Cu	copper
CV	coefficient of variation
dH_20	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ELF	epithelial lining fluid
ELISA	Enzyme-linked immunosorbent assay
ERS	European Respiratory Society
ESI	electrospray ionisation
EtOH	ethanol
eV	electronvolt
fmol	femtomole
g	grams
Glu-fib	glu-1-fibrinopeptide B
GMFCS	Gross Motor Function Classification System
GORD	gastro-oesophageal reflux disease
HCl	hydrochloric acid
HDU	High Dependancy Unit
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IAN	iodoacetamide
IL	Interleukin

IL-6	Interleukin 6
IL-8	Interleukin 8
IQR	inter-quartile range
kg	kilograms
L	litres
LC-MSMS	liquid chromatography-tandem mass spectrometry
LDH	lactate dehydrogenase
LLM	lipid laden macrophages
LLMI	lipid laden macrophage index
LPS	lipopolysaccharide
LRSQ	Liverpool Respiratory Symptom Questionnaire
LRSQ-Neuro	Liverpool Respiratory Symptom Questionnaire (Neuro version)
m/z	mass to charge ratio
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight
mins	minutes
ml	millilitres
mm	millimoles
mmHg	millimetres of mercury
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSMS	tandem mass spectrometry
NaOH	sodium hydroxide
ND	neurodisability
ng	nanograms
nm	nanometres
nM	nanomoles
ºC	degrees centigrade
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline and tween 20
pg	picograms
pH-MII	combined pH and impedance testing
PICU	Paediatric Intensive Care Unit
pmol	picomole
qPCR	quantitative polymerase chain reaction

rDNA	ribosomal deoxyribonucleic acid
REM	rapid eye movement
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
secs	seconds
TBS	tris buffered saline
TBS-T	tris buffered saline and tween 20
TCA	trichloroacetic acid
TGFβ-1	Transforming Growth Factor Beta-1
U	units
UPLC	ultra performance liquid chromatography
URTI	upper respiratory tract infection
V	volts
VFS	videofluoroscopy
WB	Western blot
µg	micrograms
µl	microlitres
µM	micromoles

Chapter 1 – Introduction

1.1 Respiratory disease is a major cause of morbidity and mortality in neurodisabled children

1.1.1 Definition, classification and incidence of neurodisability

'Neurodisability' is a term used to describe conditions where there is functional compromise as a result of central or peripheral nervous system impairment. There are hundreds of causes of paediatric neurodisability (ND) including structural, genetic and metabolic defects (Nelson Textbook of Pediatrics, 2011). Cerebral palsy (CP) is the commonest cause of paediatric ND, affecting 2-2.5/1000 live births in the United Kingdom (UK), with 20-43% of this group having quadriplegic CP (Odding, Roebroeck, & Stam, 2006). There are various causes of CP, including intra-cerebral haemorrhage, infection and intra-partum asphyxia (Gupta & Appleton, 2001). CP is essentially an 'umbrella term' used to describe the resulting clinical presentation. It is described by Rosenbaum *et al* as 'a group of permanent disorders of the development of movement and posture, causing activity limitations that are attributed to non-progressive disturbances that occurred in the developing foetal or infant brain. The motor disorders of cerebral palsy are often accompanied by disturbances of sensation, perception, cognition, communication and behaviour, by epilepsy and by secondary musculoskeletal problems' (P. Rosenbaum et al., 2007).

There are a number of different classifications of CP (depending on the type, area and extent of brain injury and resulting clinical signs) and it is important to be aware that there is considerable variability in the extent of disability and accompanying co-morbidities. Consequently, there is a degree of unavoidable inter-subject heterogeneity which must be considered when studying children with CP, and even more so when studying a wider group of children with 'paediatric ND'. Therefore, this thesis focuses only on children with central causes of ND (not disorders of the peripheral nervous system) and severe physical impairment (not independently mobile).

1.1.2 Gross Motor Function Classification and correlation of degree of impairment with morbidity and mortality

A commonly used method of classifying physical impairment in children with CP is the Gross Motor Function Classification system (GMFCS). This internationally recognised system was devised by Palisano and colleagues in 1997 and has been

widely used within the literature (R. Palisano et al., 1997; R. J. Palisano, Rosenbaum, Bartlett, & Livingston, 2008). Similar systems are required for use in other areas of ND (non-CP). However, these are not available to date, likely due to the inherent challenges of developing such systems in children with ND, as discussed by Rosenbaum *et al*. (P. L. Rosenbaum, Palisano, Bartlett, Galuppi, & Russell, 2008).

Studies have shown that GMFCS level is often highly correlated with other aspects of disability (such as fine motor function, learning disability and epilepsy) (Beckung & Hagberg, 2002). Severity of motor impairment is also closely linked to co-morbidities such as gastro-intestinal problems, feeding difficulties and respiratory complications (Erkin, Culha, Ozel, & Kirbiyik, 2010; Liptak et al., 2001). It is perhaps unsurprising therefore, that degree of motor impairment is a strong predictor of overall survival (Reid, Carlin, & Reddihough, 2012; Westbom, Bergstrand, Wagner, & Nordmark, 2011). In their study of outcomes in CP, Hutton and colleagues considered four distinct areas of disability; ambulation, manual dexterity, cognitive ability and visual disability (Jane L. Hutton, 2008). They used internationally agreed definitions for severe impairment in all four areas. **Figure 1.1** shows survival statistics in a UK cohort of 596 people with CP, presented according to severity of impairment in each of the four areas of disability (Jane L. Hutton, 2008; J. L. Hutton, Colver, Mackie, & Rosenbloom, 2000). Within each of these disability groups, mortality was increased in those with severe impairment. Respiratory conditions were the commonest cause of premature mortality in those who died under the age of forty. This is in agreement with other studies, an Australian population study specifically identifying aspiration pneumonia as a significant cause of death (Jane L. Hutton, 2008; Reid et al., 2012). Therefore, the severely impaired are a particularly pertinent group to study when investigating aspiration lung disease in children with ND.

Figure 1.1 Survival of a UK cohort of 596 people with CP.

Graphs show that survival is reduced in those with severe disability in all four disability categories studied. Hutton JL *et al*. *Outcome in cerebral palsy: life-expectancy. Paediatrics and Child Health Volume 18, Issue 9 2008 419 – 422*

1.1.3 Burden of respiratory disease in children with ND

The exact burden of respiratory disease in children with ND has been poorly documented, with the majority of published literature being based on hospitalisation and mortality data rather than daily burden of symptoms and the associated effects on quality of life.

Children with ND make up a significant proportion of those children seen in the emergency department (Massin, Montesanti, Gérard, & Lepage, 2006). Reviews of admission data have concluded that respiratory tract infection and increased seizure activity are the commonest reasons for hospital admission in this group, who are at high risk of prolonged admissions and high readmission rates (Mahon & Kibirige, 2004; Young et al., 2011). Berry *et al* conducted a retrospective cohort analysis of >300,000 patients and documented a high incidence of ND in the sub-group of patients who required four or more admissions per year (Berry Jg & et al., 2011).

A local retrospective audit at Alder Hey Children's Hospital (UK) included data on 132 patients with severe ND treated at the hospital over a thirteen year period (courtesy of Dr C. Halfhide and Dr A. Curran, unpublished data). The audit indicated an increased frequency of admission compared to the general population (40 times), markedly increased mean length of stay (0.9 vs. 22 days) and also high PICU and HDU admission rates. These data also indicated that admission for respiratory deterioration seemed to increase with age (**Figure 1.2**), suggesting deterioration in respiratory status over time. National PICU audit data obtained from PICANET suggests that following admission to PICU with a respiratory deterioration, the number of ventilated days and mortality rate are approximately double that of other admission groups. These data imply that prophylactic and early community based acute interventions may be central to improving quality of life for patients and families and saving healthcare resources in this group.

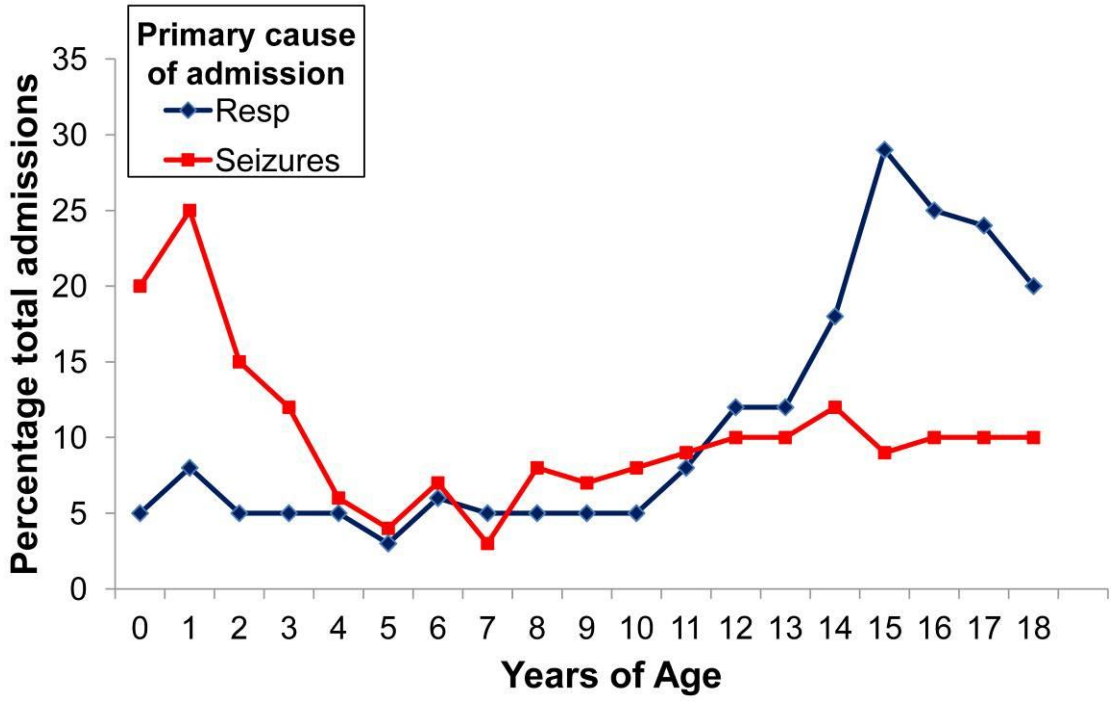

Figure 1.2 The percentage of total admissions which are due to primary respiratory causes in children with ND increase with age

Line graph to show admission data from 132 patients with severe ND treated at Alder Hey Children's Hospital over a thirteen year period. This indicates that the proportion of total admissions that are due to primary respiratory causes increase with age. Graph provided by Dr Clare Halfhide (Consultant Respiratory Paediatrician, Alder Hey).

Authors have highlighted the importance of child and family quality of life as the priority outcome in both clinical management and research in this area (Marks, 2008) (Baxter, 2007). It is surprising therefore, that there are no high quality data available on the frequency and type of respiratory symptoms in this patient group and the effects on the child and family. Also, the limited interventional studies that have been conducted often do not prioritise quality of life and daily symptomatology as an outcome measure, more commonly focusing on objective medical measures, which may hold less importance to the family and child (Mahant, Pastor, DeOliveira, Nicholas, & Langer, 2011; Samson-Fang, Butler, & O'Donnell, 2003). This may partly be due to the challenges of objective assessment in this group and the lack of validated outcome measures.

1.1.4 Respiratory care of children with ND has considerable healthcare cost implications

The impact of recurrent and prolonged hospital admissions for children with ND on health service expenditure is significant. A review of US paediatric hospital admissions in 2006 documented that 29% of US children's hospital expenditure was on inpatient care of children with ND and at least 10% of these admissions were for respiratory tract infection (Berry et al., 2012). The cost of recurrent hospital admission should not be underestimated; the afore mentioned (Section 1.1.3) 2011 retrospective cohort study documenting that those with recurrent admissions (>4 times/year) accounted for 23% of the total inpatient charges for the study follow up period (39% of this group being children with neurological disorders) (Berry Jg & et al., 2011).

It has been suggested that early, community co-ordinated care may reduce admission rates and consequent expenditure in children with ND (Berry Jg & et al., 2011; Berry et al., 2012). A recent pilot study at Alder Hey Children's Hospital (UK) demonstrated substantial financial benefits through employment and formalisation of this approach, using early, co-ordinated community physiotherapy in children with complex ND (Jackson S, 2012). Positive effects of adjuvant therapy (such as nebulised hypertonic saline and antibiotics) on symptoms were discussed within this study. However, these therapies have not been formally assessed in randomised controlled trials for this group of patients. Again, this highlights the need for improved evidence based care, ultimately

leading to standardisation of respiratory management for children with severe ND.

1.1.5 Mortality

Various international groups have explored survival rates in children with either non-specific neurological diagnoses or specifically CP, following the establishment of several population based databases. The life expectancy of children with severe disability is undoubtedly shorter than the general population (Thomas & Barnes, 2010). Estimates of survival at twenty years of age in children with severe CP range from 40-60% and studies agree that severity of impairment is the strongest predictor of survival, those with mild impairment having a 99% chance of survival at 20 years (J. L. Hutton, 2006; Jane L. Hutton, 2008; Westbom et al., 2011). Both CP specific studies and studies of children with non-specific neurological diagnoses are in agreement that level of immobility and the need for feeding assistance are strong predictors of survival and that generally, the more functional disability a child has, the higher the mortality risk (A. Cohen, Asor, & Tirosh, 2008; Eyman, Grossman, Chaney, & Call, 1990; Jane L. Hutton, 2008; Katz, 2009; Strauss, Eyman, & Grossman, 1996; Strauss, Kastner, Ashwal, & White, 1997; Thomas & Barnes, 2010). Clearly, functional disability itself places the child at increased risk of complications such as respiratory compromise secondary to immobility, but importantly, also reflects the level of neurological disability. Finally, all these studies are in agreement that respiratory causes are the leading cause of death in this group.

Conclusions differ regarding change in survival statistics over recent decades. Reid *et al* cited no improvement since the 1970's in their Australian study which spanned 1970-2004 (Reid et al., 2012). However, Strauss and colleagues found a 3.4% fall in mortality rate per year in severely disabled children in a Californian population study (1983-2002) (Strauss, Shavelle, Reynolds, Rosenbloom, & Day, 2007). The latter group highlight methodological weaknesses in the Australian paper (Strauss, Rosenbloom, Shavelle, & Brooks, 2012). They advocate a 'person-year' approach, taking account of motor function, factors such as gastrostomy usage and age when calculating any change in the probability of death in a given calendar year over the study period. This approach was not taken by Reid *et al* and was a fundamental weakness in their study; increases in

survival of preterm infants and accompanying influences on the severity of impairment in this population may well have influenced survival statistics.

The limitations of studies in this area have been extensively discussed in the literature (J. L. Hutton, 2006; Jane L. Hutton, 2008; Thomas & Barnes, 2010). Multiple factors potentially influence the findings of epidemiological studies and the complex task of predicting life expectancy. These include clarity of disorder definition, use of crude classification of functionality, length of follow up period, numbers of subjects included, appropriate range of subject disability and age at which life expectancy is calculated in the developing child. Thomas and colleagues concluded that no prospective, large cohort studies to date can be relied on to predict life expectancy and that a pragmatic clinical approach should be undertaken (Thomas & Barnes, 2010).

1.1.6 Aetiology and patho-physiology of respiratory disease in ND

Children with ND pose a unique set of challenges to respiratory healthcare professionals. They are complex to assess and manage clinically due to the multi-factorial aetiology of their respiratory disease which includes poor cough, muscle weakness, scoliosis, poor nutrition, sleep apnoea, gastro-oesophageal reflux and direct (salivary) or indirect (reflux) aspiration, all of which can ultimately lead to chronic lower airway inflammation and bacterial colonisation **(Figure 1.3)** (Seddon & Khan, 2003).

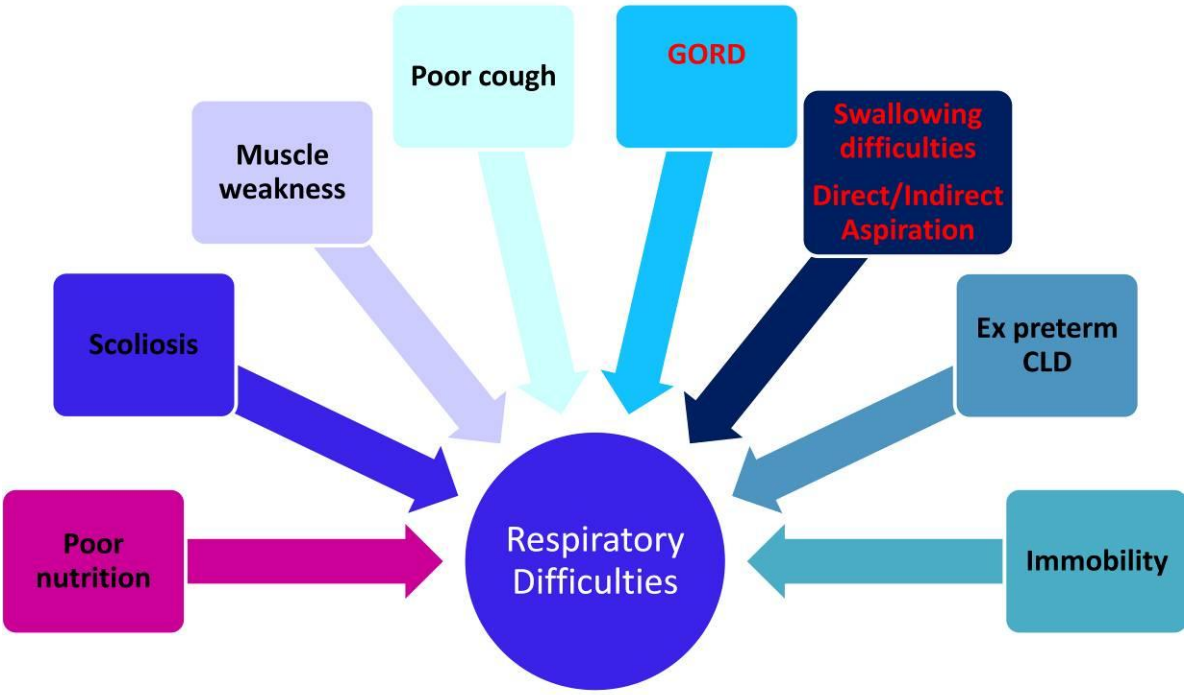

Figure 1.3 Aetiology of respiratory disease in children with ND

Diagram to highlight the complex multifactorial aetiology of respiratory disease in children with ND (created using Microsoft Office Powerpoint)

Children with ND commonly present to hospital with respiratory symptoms and a recent systematic review highlighted the severe consequences of lower respiratory tract infection in this group. The authors discussed the impact of prolonged recovery on a child's quality of life and the increased risk of further hospital acquired infections with recurrent, prolonged admissions (Kirk, 2008).
It is difficult to define the relative contribution of the various co-morbid factors to respiratory outcomes in this complex group of patients. Consequently, outcomes of respiratory interventions are difficult to assess and the evidence base for clinical management is extremely limited. Berry *et al* refer to 'crisis driven care' when discussing respiratory management and they highlight that a reactionary approach to care potentially contributes to high admission rates, long hospital stays and a high re-admission rate (Berry et al., 2012). The paucity of high quality clinical studies can partly be explained by the difficulty in undertaking an objective respiratory assessment of children with ND and the consequent challenges in designing study outcome measures (Rajendu Srivastava et al., 2009). However, the potential to reduce the burden of respiratory disease, thus improving patient and family quality of life and the saving of healthcare resources, makes investment in this field a priority (R. Srivastava & Stone, 2011).

Much of the literature on respiratory disease in children with ND is based on clinical experience rather than scientific knowledge. Over the following sub-sections, some important areas for consideration in the respiratory management of this patient group are discussed. Gastro-oesophageal reflux disease (GORD), swallowing dysfunction and pulmonary aspiration are all major considerations in the pathogenesis of respiratory disease in this group and are the main focus of this thesis. They are discussed in increased detail in Section 1.2.

Further understanding of the relative contribution of each of these aetiological factors to respiratory symptomatology would aid our understanding of the patho-physiology of respiratory disease and guide the best approach to clinical management. A multi-disciplinary approach to clinical care has been shown to be effective, but is currently variable by region (Waespi, Seddon, & Khan, 2000).

1.1.6.1 Poor cough and muscle weakness

Cough efficiency is often impaired in children with ND (Seddon & Khan, 2003). An effective cough is important for airway protection against aspiration and clearance of airway secretions, both in health and importantly, at times of airway infection. The physiological mechanism of cough generation is complex. It requires adequate coordination and function of inspiratory, expiratory and bulbar muscles in order to produce a forceful contraction and propel airway secretions to the top of the airway (Healy & Panitch, 2010; Seddon & Khan, 2003). Muscular control and co-ordination may be particularly impaired in children with central ND. Additionally, immobility and chest wall deformity (secondary, for example, to scoliosis) will ultimately contribute to muscle weakness.

Afferent airway receptor sensitivity is central to the initiation of cough. There are many potential reasons for this to be reduced in children with severe ND, including the high incidence of gastro-oesophageal reflux leading to laryngo-pharyngeal sensory deficits (Aviv, Liu, Parides, Kaplan, & Close, 2000). This has been observed in videofluoroscopy studies, which have demonstrated a high incidence of 'silent' aspiration in this patient group (K. A. Weir, McMahon, Taylor, & Chang, 2011).

Ultimately, poor cough and muscle weakness lead to pooling of airway secretions, recurrent infection and likely chronic inflammation. Chest physiotherapy and airway clearance devices are used in the management of these common problems, although with a limited evidence base (Marks, 2008).

1.1.6.2 Kyphoscoliosis

Kyphoscoliosis affects many children with ND (Healy & Panitch, 2010; Persson-Bunke, Hägglund, Lauge-Pedersen, Ma, & Westbom, 2012). It develops primarily as a result of unequal muscle tone and gravity in children with central ND. From a respiratory perspective, this results in decreased tidal volumes (with subsequent effects on airway clearance and expansion) due to changes in chest wall compliance and decreased efficiency of respiratory muscle function. The effects on airway expansion can be asymmetrical and can lead to problems with ventilation perfusion mismatch (Tsiligiannis & Grivas, 2012). Ultimately, as

scoliosis progresses it leads to increased work of breathing and respiratory failure, as well as other undesirable effects such as further reduced mobility, gastro-intestinal problems and pain (McCarthy, D'Andrea, Betz, & Clements, 2006). Spinal braces and specialist seating are often unsuccessful in halting progression of scoliosis in this group (Koop, 2009). Scoliosis should be carefully monitored and in progressive cases, spinal fusion surgery may be considered (following careful multidisciplinary assessment) with the aim of stabilising posture and lung function.

1.1.6.3 Poor nutrition

Good nutrition is known to be important in the management of any chronic respiratory disease and there is evidence to support an influence on overall prognosis in some respiratory diseases, for example, cystic fibrosis (Nogay, 2012; Shepherd et al., 1986; Steinkamp & Wiedemann, 2002). Malnutrition directly influences the condition of respiratory muscles (through catabolism), leading directly to increased muscle weakness. It also has potential detrimental effects on various aspects of immune function, including cell-mediated immunity, phagocyte function, complement system function and antibody production (Chandra & Kumari, 1994). Finally, malnutrition has potential effects on other important aspects of health such as brain growth, developmental progress and bone mineral density (Andrew & Sullivan, 2010).

There are numerous potential causes of malnutrition in the neuro-disabled child including feeding difficulties (dysphagia or food aversion), poor positioning, effects of medication on feeding (such as constipation or sedation), decreased appetite, GORD and difficulties with communication (Andrew, Parr, & Sullivan, 2012; Seddon & Khan, 2003). Ideally, a multi-disciplinary approach to assessment and management should be undertaken. Peter Sullivan, a gastroenterologist with expertise in this area, stated that nutritional supplementation in this group should aim ‘to achieve the child's genetic growth potential within the limits of their neurological condition whilst maintaining good nutrition’ (Andrew & Sullivan, 2010).

1.1.6.4 Sleep apnoea

There is an increased incidence of sleep disordered breathing in children with ND compared to healthy children (Zucconi & Bruni, 2001). Sleep disordered breathing can be due to problems with central respiratory drive or due to obstructive sleep apnoea.

Obstructive sleep apnoea is common in children with CP (Shintani et al., 1998). Contributory factors can be enlarged tonsillar/adenoid tissue or poor pharyngeal muscle control (Shintani et al., 1998). This leads to increased upper airway resistance in rapid eye movement sleep, leading to hypoxaemia and hypercarbia which in turn can lead to increased seizure activity, daytime sleepiness, headaches, behavioural problems and ultimately, pulmonary hypertension (Marks, 2008; White, 2005). Over time, central chemoreceptors become more tolerant to hypercarbia, allowing longer periods of hypoxia during REM sleep, thereby increasing risk to the patient (White, 2005).

Alternatively, any neurological condition affecting central respiratory drive centres in the brainstem can lead to central sleep apnoea, particularly degenerative central neurological conditions such as chiari malformation and unrecognised hydrocephalus (Marks, 2008; White, 2005).

Management includes ear, nose and throat or neuro-surgical intervention, where indicated. The use of non-invasive ventilation (continuous positive airways pressure or biphasic support) is becoming more widespread and there is evidence that this can improve the quality of life (Hsiao & Nixon, 2008; Kitazumi, 1998). A final alternative for management of obstructive sleep apnoea, untreatable by other means, is tracheostomy placement.

1.1.6.5 Other factors

The potential for underlying primary respiratory disease, such as asthma or chronic lung disease of prematurity should always be considered in the respiratory management of the patient with central ND.

1.2 Recurrent aspiration is a likely key contributory factor in respiratory disease in children with ND

1.2.1 Gastro-oesophageal reflux disease is common in children with ND

Gastro-oesophageal reflux refers to the retrograde passage of stomach contents into the oesophagus. This physiological phenomenon can occur normally in healthy children and adults at any time, particularly after eating. In health, it lasts for short periods of time only and is asymptomatic (Vandenplas et al., 2009). This becomes a disease process when it causes recurrent symptoms or complications (Vandenplas et al., 2009).

The high incidence of GORD in children with ND is well recognised. Up to 75% of those with severe CP have GORD, with contributory factors including decreased lower oesophageal sphincter tone, delayed gastric emptying, impaired oesophageal motility, scoliosis, seizures, medications, and poor positioning (Gustafsson & Tibbling, 1994; Mazzoleni et al., 1991; Peter B. Sullivan, 2008). Somerville and colleagues found evidence of chronic oesophagitis on upper airway endoscopy in 57% of 182 children with severe developmental disability (Somerville et al., 2008). Respiratory symptoms and complications of GORD may include cough, wheeze, shortness of breath and recurrent pneumonia (Boesch et al., 2006). However, it is important to consider that although these sequelae may be a direct result of GORD through recurrent micro-aspiration or direct stimulation of the distal oesophagus, they may also be unrelated. It can be difficult to establish a causal relationship (Mansfield & Stein, 1978). The risk of micro-aspiration with GORD is potentially increased due to impaired laryngeal sensation, known to be associated with recurrent gastro-oesophageal reflux (Aviv et al., 2000). Equally, respiratory symptoms themselves can cause/increase GORD. This ‘chicken and egg’ scenario results in limitation of our understanding of the effects of GORD on the airway and has been discussed at length in the literature (Shields, Bateman, McCallion, van Wijk, & Wenzl, 2011).

GORD can be particularly difficult to diagnose in children with ND, due to difficulties in communication and interpretation of behaviour. Clinical investigations including upper gastro-intestinal contrast studies, upper gastrointestinal endoscopy, pH studies and newer modalities such as impedance

monitoring are all used by gastroenterologists to aid diagnosis (Vandenplas et al., 2009) (Peter B. Sullivan, 2008).

1.2.2 Swallowing dysfunction is common in children with neurodisability

Dysfunctional swallowing (from oral and pharyngeal motor impairment) is also common in children with ND (Andrew et al., 2012). This can present with a wide range of symptoms, from subtle feeding difficulties and nutritional deficiencies to overt symptoms of aspiration and recurrent lower respiratory tract infection. A 'safe swallow' requires a level of oral motor skill to ensure adequate oral food preparation, formation of a bolus and a co-ordination of oral, pharyngeal and oesophageal muscles, enabling safe passage of the bolus into the stomach with accompanying airway protection from aspiration. Estimations of swallowing dysfunction frequency (as assessed by videofluoroscopy) in children with severe ND can be up to 70%, depending on the population studied (Andrew et al., 2012; Arvedson, Rogers, Buck, Smart, & Msall, 1994; Mirrett, Riski, Glascott, & Johnson, 1994; Somerville et al., 2008; K. Weir, McMahon, Barry, Masters, & Chang, 2009; K. A. Weir et al., 2011). An important observation from videofluoroscopy studies is the high frequency of silent, symptomless aspiration in this group, leading many authors to conclude that any child with neurological dysfunction who presents with feeding difficulties should undergo a videofluoroscopic assessment (Andrew et al., 2012; K. A. Weir et al., 2011).

1.2.3 Differentiation of direct and reflux aspiration

Pulmonary aspiration is the abnormal passage of the contents of the upper airway into the lower airway. The clinical consequences of recurrent aspiration can range from recurrent cough and wheeze and recurrent lower respiratory tract infection to acute life threatening events and catastrophic pulmonary injury and death in cases of large volume aspiration (Boesch et al., 2006). The commoner clinical scenario of repeated small-volume aspiration can potentially lead to chronic pulmonary disease, as discussed in Section 1.5.

The high incidence of GORD and swallowing dysfunction in children with severe ND places them at higher risk of recurrent aspiration than the general population. Aspiration can be direct (aspiration of oral contents such as food or

saliva into the lungs), reflux-aspiration (aspiration of refluxed stomach contents into the lungs) or a combination of both. It is important to be able to differentiate direct and indirect aspiration as the clinical management is distinct, as detailed in Sections 1.2.4 and 1.2.5. In a research setting, we are unable to fully assess the outcomes of intervention without being able to assess the relative contributions of the different types of aspiration to clinical symptoms.

1.2.4 Clinical management of direct aspiration

Direct aspiration can include aspiration of solid food, liquid or saliva. Diagnosis of direct aspiration is discussed in Section 1.3. Following diagnosis, interventions such as alteration of food consistency and optimal positioning of the child for feeding can be useful. Ideally, feeding interventions should be undertaken following multi-disciplinary assessment, as discussed in Section 1.1.6.3.

In patients where oral feeding cannot be maintained (due to risk of direct aspiration or inadequate nutritional intake) percutaneous gastrostomy feeding may be considered. This intervention potentially eliminates the risk of direct aspiration of food. However, it does not alter the frequency of salivary aspiration. There is little documented about the frequency of salivary aspiration and its direct clinical consequences in the literature, due to limitations in diagnostic abilities, as discussed in Section 1.3. However, salivary aspiration is potentially an important contributory factor in aspiration lung disease in children with ND, particularly due to the potential for instillation of pathogenic bacteria from the oral cavity into the lungs in a group who may have poor oral and dental hygiene, increasing the incidence of anaerobic pneumonia (Wallis & Ryan, 2012).

There are various medical and surgical options for management of salivary aspiration. Anti-cholinergic medications (such as glycopyrrolate), which interact with peripheral muscarinic receptors, are commonly used in the management of sialorrhoea (Eiland, 2012). The side effect profile of these medications, which includes anti-cholinergic symptoms and mucous plugging, and the limited evidence base for their use are discussed in Chapter 3. Most of the literature concerning their use has focussed on control of sialorrhoea rather than improving

respiratory outcomes, perhaps again because of the difficulties in diagnosing salivary aspiration and limited data regarding the role of salivary aspiration in causality of respiratory symptoms (Eiland, 2012; Zeller, Davidson, Lee, & Cavanaugh, 2012; Zeller, Lee, Cavanaugh, & Davidson, 2012).

Surgical management options include sub-mandibular and parotid gland botulinum toxin or removal of sub-mandibular glands and ligation of parotid glands (Ellies et al., 2002; Walshe, Smith, & Pennington, 2012; Wilken, Aslami, & Backes, 2008). The evidence base for these interventions is similarly limited and they are used with variable success, particularly from a respiratory symptom perspective (as discussed in Section 3.5 of this thesis).

1.2.5 Clinical management of reflux aspiration

The aim of reflux aspiration management is ideally to stop the reflux of gastric contents into the oesophagus. However, a secondary aim may be to minimise symptoms caused by the refluxate by neutralising the acidity of the stomach contents. Management options include lifestyle, pharmacological and surgical approaches. Simple measures aimed at reducing the frequency of reflux events include changing the feed volume and frequency, altering the child's position for feeding, and thickening feeds (Vandenplas et al., 2009).

Where these simple measures fail, there are various pharmacological options. Pro-kinetic agents, such as domperidone and erythromycin, are commonly used with the aim of encouraging prompt gastric emptying. However, the evidence for their efficacy in children is limited (as discussed in Section 3.5) (Pritchard, Baber, & Stephenson, 2005; Vandenplas et al., 2009). Proton pump inhibitors are often used for reflux symptom control and treatment of oesophagitis, limiting the acidity of the refluxate by blocking the proton pump (H+/K+ ATPase) of the gastric parietal cells. There is some evidence that these are effective when used for the purposes of gastroenterological reflux symptom control (Hassall, 2005). However, their role in the management of reflux aspiration is questionable because in the context of chronic, small-volume reflux-aspiration the refluxate is likely to be mildly acidic or neutral pH on reaching the lower airway (as discussed in Chapter 7). Indeed, some studies suggest that there may be an increased risk of lower respiratory tract infection in those on proton pump

inhibitors, possibly due to gastric bacterial overgrowth (Fohl & Regal, 2011; Theisen et al., 2000).

Where medical management fails, patients are referred for surgical assessment for anti-reflux procedures. Fundoplication is the commonest anti-reflux procedure. In the United States, children with ND represent approximately 40% of the paediatric population undergoing fundoplication (Lasser, Liao, & Burd, 2006). This technique involves wrapping the upper part of the stomach (the fundus) around the lower end of the oesophagus, creating a muscular tunnel, aiding the lower oesophageal sphincter in preventing reflux of stomach contents into the oesophagus (Shields et al., 2011). However, surgery associated morbidity and mortality rates are higher in children with ND and primary failure and 're-do' fundoplication rates are increased (Hassall, 2005; Lasser et al., 2006; Pearl et al., 1990; C. D. Smith et al., 1992; Subramaniam & Dickson, 2000; Wockenforth, Gillespie, & Jaffray, 2011). Results from studies on respiratory outcomes, gastrointestinal outcomes, and quality of life after fundoplication have been contradictory and some surgical centres are now advocating increased scrutiny in selecting children who may benefit from fundoplication (Fonkalsrud et al., 1998; Goldin, Sawin, Seidel, & Flum, 2006; Martinez, Ginnpease, Caniano, Vinocur, & Golladay, 1992; Shields et al., 2011; C. D. Smith et al., 1992; Rajendu Srivastava et al., 2009). Other options used in the surgical management of GORD include jejunal feeding and gastro-oesophageal dissociation (Verey & Cusick, 2008; Wales et al., 2002; Wockenforth et al., 2011). High quality prospective trials are needed to assess the outcomes of these interventions in children with ND, with careful attention being paid to patient group specific outcome measures (Vernon-Roberts & Sullivan, 2007).

1.3 Existing diagnostic tests for aspiration are limited

1.3.1 Clinical assessment

Clinical assessment of feeding, swallowing and risk of aspiration is made by a speech and language therapist, with multi-disciplinary input into any subsequent management. Clinical assessment is known to have limited sensitivity in identifying aspiration in many patient groups, but particularly in those with ND, due to the high incidence of 'silent' aspiration. Therefore, most authors

recommend a videofluoroscopy assessment in any child with ND who presents with feeding difficulties (Andrew et al., 2012; K. A. Weir et al., 2011).

1.3.2 Videofluoroscopy

Videofluoroscopy has long been considered the 'gold standard' for assessment of swallowing. This radiographic technique, in which real-time fluoroscopic images of a swallow of a radio-opaque food or liquid bolus are obtained, provides dynamic information on all phases of swallowing, from oral management of a food bolus to the pharyngeal and oesophageal phases. In paediatric practice, it is undertaken following a thorough clinical assessment by a speech and language therapist; it is used as a complementary tool to bedside assessment (Hiorns & Ryan, 2006). Bedside assessment alone has repeatedly been shown to have a lower sensitivity in aspiration diagnosis compared to videofluoroscopy, particularly with aspiration of solids and at times when 'silent' aspiration occurs (a common scenario in the neuro-disabled children)(K. Weir et al., 2009; K. A. Weir et al., 2011). A speech and language therapist should be present at the time of the procedure, during which recommendations about food consistency and positioning of the child for feeding can be made.

There are several important limitations to videofluoroscopy and consequently some authors have questioned whether it should be regarded as a true 'gold standard'. Studies comparing videofluoroscopy with direct endoscopic visualisation of the airway (FEES) in adults have found discrepancies between the two techniques (Chih-Hsiu, Tzu-Yu, Jiann-Chyuan, Yeun-Chung, & Shiann-Yann, 1997). There have also been concerns about the interpretation of videofluoroscopy in cases where frank aspiration was not observed; inter and intra- assessor variability has been demonstrated in reporting on adequacy of swallow (Kuhlemeier, Yates, & Palmer, 1998; Ott, 1998; Stoeckli, Huisman, Seifert, & Martin-Harris, 2003). In view of aspiration being an intermittent event and videofluoroscopy providing a 'one off' examination of a swallow, aspiration cannot be totally ruled out within the limits of this test. Furthermore, micro-aspiration may be below the level of sensitivity of the investigation. Radiation exposure precludes the possibility of multiple repeat examinations and therefore, fatiguability cannot be tested using this technique. Finally, videofluoroscopy requires the child to be able to feed by mouth and if the child

only takes small quantities, the evaluation is limited (Boesch et al., 2006). Therefore, a videofluoroscopy examination would generally not be conducted on days when children with ND may be at most at risk of aspiration for example, when they have received supplementary anti-seizure medication. Finally, and importantly in the context of this thesis, videofluoroscopy does not directly differentiate direct and reflux aspiration.

1.3.3 Lipid laden macrophages

Lipid laden macrophages (LLMs) (alveolar macrophages with ingested lipid within their cytoplasm) can be seen after Oil-Red-O staining on microscopic examination of BAL cells (Reid-Nicholson, Kulkarni, Adeagbo, Looney, & Crosby, 2010). Their detection has been reported to be of value in diagnosing aspiration for decades (Pinkerton, 1928). In 1985, the LLM index (LLMI), which grades the amount of lipid in the cytoplasm of 100 macrophages (grade 0-4, giving an index of 0-400), was first described (Corwin & Irwin, 1985). Two years later, Colombo and Hallberg assessed this technique in BAL samples from children with and without suspected aspiration (Colombo, Hallberg, & Sammut, 1992). They concluded that LLMs were a non-specific finding, but a quantitative index with a diagnostic 'cut-off' might be useful. Animal studies have shown rapid macrophage uptake of exogenous lipid after intra-tracheal milk instillation, which lasts for 2 to 4 days after a single instillation and longer with recurrent instillations (Colombo & Hallberg, 2000).

Subsequently, many studies have reported LLMI as a useful clinical marker of gastric aspiration in children (Ahrens et al., 1999; Collins et al., 1995; Yang, Steele, Anbar, Sinacori, & Powers, 2001). More recently, Borrelli *et al* showed correlation of proximal non-acid reflux episodes (detected with the newer modality of pH and multichannel intraluminal impedance monitoring, or pH-MII) with LLMI, although interestingly this contrasted with the findings of Rosen et al (Borrelli et al., 2010; Rosen, Fritz, Nurkoa, Simon, & Nurko, 2008).

The role of LLMI as a criterion standard for diagnosing reflux aspiration is widely disputed. Studies have reported significant variation in the sensitivity and specificity of the LLMI (57%-100% and 57%-89%, respectively) (Ahrens et al., 1999; Colombo & Hallberg, 1987; Corwin & Irwin, 1985; Ding et al., 2002; Yang

et al., 2001). Ahrens *et al* examined BAL from children in 3 groups: with chest disease and GORD, with recurrent pneumonia but no evidence of GORD, and healthy controls. They found LLMs to be present in all samples, raising questions about whether the source of the lipid was always exogenous (Ahrens et al., 1999). Known alternative endogenous sources of lipid include parenteral nutrition, tissue/cell membrane breakdown, and airway surfactant phospholipids. These last two sources of lipid alter in respiratory infection and inflammation (Ahrens et al., 1999; Colombo & Hallberg, 1987; Knauer-Fischer & Ratjen, 1999; Ratjen, Rehn, & Costabel, 1996). Thus, LLMI is elevated independent of reflux aspiration in other pulmonary diseases such as cystic fibrosis, allergic alveolitis, and bronchiolitis obliterans (Knauer-Fischer & Ratjen, 1999).

The idea of establishing an LLMI cut-off value or range has been proposed; this would take into account time since the last aspiration event and the potential for subclinical aspiration in otherwise healthy children. However, the range suggested is particularly broad (Ahrens et al., 1999; Colombo & Hallberg, 1987). Also, previous studies have reported poor inter-rater reliability in LLMI scoring and, worryingly, intra-rater reliability between blinded, experienced cytopathologists (Camoretti-Mercado & Solway, 2005; Reid-Nicholson et al., 2010; Yang et al., 2001). These discrepancies would directly preclude the possibility of having a reliable LLMI 'cut-off' range.

In summary, it would seem that LLM lack the required specificity to be used as a diagnostic test for reflux-aspiration. Their current role may be in providing supportive evidence (in patients with highly positive results) for a diagnosis of aspiration but they should be interpreted with caution and within the context of the clinical assessment and other investigation results.

1.3.4 Other tests

Multiple other tests for reflux and direct aspiration have been used in clinical practice and are discussed within the literature. These include gastric scintigraphy, radionucleotide salivograms and dye studies in patients with a tracheostomy. However, none of these tests have been shown to have the required sensitivity to be used as a diagnostic test for aspiration in routine

clinical practice (Boesch et al., 2006; Peruzzi, Logemann, Currie, & Moen, 2001). Finally, Boesch *et al*, in their review of chronic pulmonary aspiration in children, discuss 'difficult cases' where all investigations are negative but clinical suspicion of aspiration is high. In some cases, a trial of stopping oral feeding has improved respiratory symptoms (Boesch et al., 2006). These clinical observations reinforce the limitations of the currently available diagnostic tests for aspiration.

1.4 Novel tests for aspiration

Several novel bronchial lavage (BAL) or sputum sample based tests of direct and reflux aspiration have been discussed within the research literature but interestingly, these have not previously been investigated in children with ND, a group at particularly high risk of aspiration. The available evidence regarding these novel tests is discussed below.

1.4.1 Pepsin

Pepsin (an acidic protease) is the primary proteolytic enzyme of the digestive system. It is produced by the chief cells of the stomach lining as pepsinogen and cleaved to pepsin in an acidic environment. Pepsin is not known to be produced by extra-oesophageal tissues and is therefore, first, a measure of reflux if found in the upper airway, and second, reflux aspiration if found in the lungs. Importantly, it is potentially a causative agent in extra-oesophageal injury. Animal studies have shown that BAL pepsin is a highly sensitive and specific marker of aspiration after forced gastric juice aspiration, with raised pepsin levels being detected up to 48 hours post aspiration event (Badellino et al., 1996; N. A. Metheny et al., 2004; H. B. Zhang, Van Hook, Fan, Mallory, & Elidemir, 2005). Various methods of pepsin measurement are discussed within the literature.

1.4.1.1 Enzymatic pepsin assays

The enzymatic activity of human pepsin 3b is pH dependent, having maximum activity at pH 2 becoming inactive at pH 6.5. It remains stable up to pH 7 but becomes irreversibly damaged at pH 8 (Nikki Johnston, Dettmar, Bishwokarma, Lively, & Koufman, 2007). Enzymatic assays include a sample re-acidification step (to re-activate any pH deactivated pepsin) followed by a quantification of

substrate digestion measured by protein digestion, spectroscopy, or other methods. Sample pepsin concentration is determined relative to a standard curve of substrate digestion activity for known pepsin concentrations, and then normalized to total sample protein. In samples up to pH 7, pepsin retains approximately 79% of its enzymatic activity on re-acidification (Nikki Johnston et al., 2007). Previous studies have shown correlative relationships between airway sample pepsin measurements and other clinical parameters of reflux or disease using the enzymatic assay (Sabeena Farhath et al., 2006; S. Farhath et al., 2008; U. Krishnan, Mitchell, Messina, Day, & Bohane, 2002; Potluri et al., 2003).

However, this type of assay has a number of limitations. In addition to the stomach, pepsinogen is also produced in the lung (pepsinogen C) and is found in serum (pepsinogen A and C) (Foster, Aktar, Kopf, Zhang, & Guttentag, 2004). Thus, activation of pepsin by acidification could also potentially cleave pepsinogen to pepsin, leading to false positive results. Other potential problems include interference from other proteases (such as cathepsins), freeze/thawing of samples affecting activity and highly proteinaceous samples inhibiting pepsin activity (Badellino et al., 1996; He et al., 2007; U. Krishnan et al., 2002)). Most importantly, if the sample pH is above pH 7, enzymatic activity may be reduced to a variable degree between samples upon reactivation, leading to potential false-negative results and difficulties in quantitative comparisons between samples (Nikki Johnston et al., 2007).

1.4.1.2 Immunologic pepsin assays

Enzyme Linked Immunosorbent Assays (ELISAs) and Western blotting techniques can be used to assess pepsin concentrations, but both are dependent on the quality and specificity of the pepsin antibody, many of which are known to cross-react with pepsinogen. More specific antibodies to human pepsin have been developed, and these have been shown to have a high specificity through gel electrophoresis and mass spectrometry studies (Crapko, Kerschner, Syring, & Johnston, 2007; Knight, Lively, Johnston, Dettmar, & Koufman, 2005). Both sandwich and indirect pepsin ELISA methods have been published. These techniques are cheap, more sensitive (0.2 ng/ml) than published enzymatic methods, and they allow multiple sample analysis. Sandwich ELISAs are probably less liable to non-specific antibody capture as initial antigen “capture” with a

primary antibody increases the ratio of antigen to non-specific protein bound to the platform. There is only one commercially available assay (USCN Life Science). However, assay documentation suggests that it is not fully validated across multiple respiratory sample types, and on inquiry, the antibody used has significant cross-reactivity with pepsinogen. Much pepsin research is therefore still dependent on 'in-house' kits.

Western blot analysis (WB) provides the advantage of being able to differentiate positive signal due to pepsin, pepsinogen, or other interfering proteins because proteins are separated by molecular weight. A further advantage is that, unlike enzymatic assays, it measures protein levels, and thus clinical samples above pH 7 should not test 'false negative' by WB analysis. WBs for pepsin analysis have been reported, including in the analysis of laryngeal and sub-glottic biopsies (Kim, Lee, Yeo, Kim, & Cho, 2008; Knight et al., 2005; N. A. Metheny et al., 2004). The Wisconsin group have now validated this method for analysis of BAL, with a pepsin lower limit of sensitivity of 0.05 ng (Johnston *et al*, unpublished data). The main disadvantage of WB is that it is semi-quantitative at best and in bronchial lavage samples, there is no reliable and validated protein which can be used for normalisation.

1.4.1.3 Human studies

Pepsin has been proposed as a useful biomarker of reflux aspiration in adults and in paediatric studies. Krishnan et al. detected pepsin enzymatic activity in 31 of 37 tracheal aspirates from children with GORD compared with 0 of 26 healthy controls (A. Krishnan et al., 2007). Significantly raised BAL pepsin levels have also been found in children with chronic cough and proximal reflux (Farrell, McMaster, Gibson, Shields, & McCallion, 2006). Similarly, Starosta *et al*. showed raised BAL pepsin levels in 96 children with chronic lung disease, with and without GORD (Starosta et al., 2007). However, specificity was low, and there was extensive group overlap. Pepsin has also been used as a marker of aspiration in children ventilated on intensive care. However, results vary widely with reported pepsin positivity in tracheal aspirate ranging from 13.5% to 70% (Gopalareddy et al., 2008; Kathleen L. Meert, Daphtary, & Metheny, 2002), possibly due to differences in assay sensitivity. Interestingly, recent studies using very sensitive assays have found low pepsin levels in healthy paediatric control

patients (McNally et al., 2011). The authors acknowledge that reflux of gastric contents into the pharynx occurs frequently in normal, asymptomatic children, and healthy adults have been shown to aspirate nasopharyngeal secretions during sleep. Therefore, this finding is perhaps unsurprising (Gleeson, Eggli, & Maxwell, 1997; Ramaiah, Stevenson, & McCallion, 2005).

Other groups have correlated pepsin levels with respiratory pathology. pH-MII studies in lung transplant recipients have shown that acid and alkaline reflux is common (Blondeau, Dupont, et al., 2008). Pepsin levels in BAL from lung transplant patients correlate with acute rejection, implying a contribution of pepsin to allograft injury (Stovold et al., 2007; Ward et al., 2005). Farhath *et al* found 92% pepsin positivity on analysis of tracheal aspirates from preterm ventilated neonates, and correlated pepsin levels with development of bronchopulmonary dysplasia (Sabeena Farhath et al., 2006; S. Farhath et al., 2008). Finally, correlations between BAL pepsin levels and airway neutrophils or inflammatory cytokines have been reported in GORD, cystic fibrosis, and post lung transplant (Blondeau, Merters, et al., 2008; McNally et al., 2011; Starosta et al., 2007).

Pepsin levels in more readily available samples such as sputum, saliva, and exhaled breath condensate have also been examined (A. Krishnan et al., 2007). Analysis of induced sputum from healthy children found pepsin was detectable in saliva from 17 of 19 children pre-sputum induction, and 19 of 19, post-sputum induction (median 14.7 ng/ml, range 8.3-25.6 ng/ml) (Ervine, McMaster, McCallion, & Shields, 2009). There is no evidence that pepsin is produced by the salivary glands; thus, these results are again consistent with the concept of normal physiological reflux in children (Ramaiah et al., 2005). In the adult literature, a variety of assays have been used to detect pepsin within sputum and saliva in patients with GERD and respiratory symptoms (Kim et al., 2008; Knight et al., 2005; Li et al., 2008; Potluri et al., 2003; Strugala, Dettmar, & Morice, 2009; Wang et al., 2010)). The variation in the frequency of pepsin detection in control subjects and the levels of pepsin reported in salivary/sputum samples is striking. In addition, pepsin levels detected in BAL studies appear to conflict with some of the sputum literature. Similar or lower levels have been reported in sputum/salivary studies compared with those reported in BAL studies, where samples are subject to significant dilution during

collection. When clinical specimens are analyzed by ELISA, antibody interference is always possible and particularly so when dealing with thick and proteinaceous samples, such as sputum. Therefore, rigorous assay validation is essential. Authors have called for assay standardization as a priority, so that normative values can be established for paediatric and adult age groups (Davis et al., 2010).

1.4.2 Bile acids

Bile acids are present in gastric juice as a result of duodeno-gastric reflux. They are not present consistently and are therefore not as potentially useful as a biomarker of reflux-aspiration. However, it is important to consider them in investigating aspiration lung injury, as they have been implicated in extra-oesophageal injury in the oesophagus, larynx, ear, and lung (Blondeau, Dupont, et al., 2008; Blondeau, Merters, et al., 2008; D'Ovidio et al., 2005; Woodland & Sifrim, 2010; Wu et al., 2009). Pearson and Parikh measured bile acid levels in fasting gastric juice in adults and found median concentrations of 0 μmol/L (range 0-150 μmol/L) in normal subjects and 100 μmol/L (range 0-410 μmol/L) in patients with oesophagitis (Pearson & Parikh, 2011). Although gastric juice and airway bile acid concentrations have not been investigated in children with ND, one would hypothesise that they may be increased, given the high incidence of bowel dysmotility in this group (Peter B. Sullivan, 2008). Under normal circumstances, the majority of bile acids within gastric juice are conjugated (Vertzoni, Archontaki, & Reppas, 2008). However, many patients with ND take proton pump inhibitors, and this can result in bacterial overgrowth, causing de-conjugation of bile acids (Theisen et al., 2000). Secondary bile acids are potentially more damaging to extraesophageal tissues due to increased lipid solubility (Pearson & Parikh, 2011). Therefore, bronchial lavage bile acid measurement, although limited in potential for use as a biomarker of reflux-aspiration, is potentially useful in research of the pathogenesis of aspiration lung disease.

1.4.3 Alpha-amylase

Alpha-amylase is an enzyme which has various isoforms and is predominantly found in pancreatic juice or in saliva in humans (Ferey-Roux et al., 1998; Hirtz et al., 2005). It's function in saliva is to start the digestive process through

hydrolysis of alpha bonds in large polysaccharides (such as starch and glycogen) (Ragunath, Manuel, Kasinathan, & Ramasubbu, 2008).

When studying amylase as a biomarker of direct aspiration, it is important to acknowledge that a low level of amylase can be detectable in the normal lung, as demonstrated in Nandapalan's study of tracheal aspirate amylase levels on laryngectomised patients (Nandapalan, McIlwain, & England, 1995). Similarly, the increased pulmonary production of amylase in certain pulmonary conditions, including malignancy and TB, should also be considered and this is further discussed in Section 5.5 of this thesis (Joseph et al., 1992; G. D. Lee et al., 2003; Villena, Echave-Sustaeta, Lopez-Encuentra, & Navarro, 1995; Villena et al., 2002). However, amylase has the potential to be a useful biomarker of direct aspiration if clear differentiation can be demonstrated between those with clinical evidence of aspiration and healthy or disease controls. In a small study of 9 patients with clinical or radiological evidence of aspiration and 19 controls undergoing diagnostic bronchoscopy or general anaesthetic, Smith *et al* concluded that there was a clear separation in amylase levels between the groups (D. D. Smith & McNamara, 1976). However, since that time, there have been few further studies of amylase as a biomarker of direct aspiration (Clarke, Bain, Davies, Levin, & Lambert, 1981; Nandapalan, McIlwain, & Hamilton, 1995). Interestingly, a clinical study by Nandapalan and colleagues suggested that high tracheo-bronchial amylase levels were predictive of subsequent respiratory deterioration in patients with tracheostomies in the Intensive Care setting (Nandapalan, McIlwain, & Hamilton, 1995). However, no other studies have subsequently made comparisons of lower airway alpha-amylase levels with lower airway inflammatory markers and respiratory symptoms.

Consequently, the literature would suggest that alpha-amylase has shown promise as a biomarker of direct aspiration but has been inadequately investigated in the clinical research setting to date.

1.4.4 Milk laden macrophages

Some groups have attempted to improve the specificity of the lipid laden macrophage test by looking specifically for human and bovine milk proteins within the alveolar macrophages (α-lactalbumin and ß-lactoglobulin,

respectively). This would aid differentiation of exogenous and endogenous lipid, a significant limitation of the lipid laden macrophage index (as discussed in Section 1.3.3). An initial study of single and repeated forced aspiration in a murine model suggested that testing for alveolar milk proteins in BAL was both specific and offered improved sensitivity when compared with lipid laden macrophage staining alone (Elidemir, Fan, & Colasurdo, 2000). Two further studies investigated alveolar macrophage milk protein in infants and young children, either ventilated on Intensive Care or undergoing bronchoscopy for respiratory symptoms. The Intensive Care based study by Miller *et al*, showed positivity in 12 patients who were being fed and had clinical or radiological evidence of aspiration (Miller et al., 2002). A later study found some correlation between positivity and incidence of gastro-oesophageal reflux and laryngo-tracheal anatomical abnormality (De Baets et al., 2010). However, there were concerns about specificity of staining in both studies, some positivity being demonstrated in control groups.

The proposed measurement of a more specific lipid in alveolar macrophages perhaps, still deserves further investigation. However, it could be argued that the measurement of milk proteins is limited because a) it would only be useful in infants, young children or potentially the gastrostomy fed with chronic disease, who take milk as a regular part of their diet throughout the day, b) it would not only be influenced by the frequency of aspiration events but also the frequency of feeding and c) it could potentially indicate reflux or direct aspiration in the orally fed child and would be unhelpful in differentiating the two.

1.5 Clinical outcomes of recurrent aspiration

Available clinical studies examining the pulmonary patho-physiological outcomes of recurrent direct and reflux aspiration are based solely on association rather than evidence of causation, due to the limitations of diagnostic testing, as discussed above. However, it is thought that recurrent aspiration can be associated with recurrent lower respiratory tract infections, potentially leading to chronic inflammatory changes, which subsequently can result in longer term airway damage, such as bronchiectasis or lung parenchymal damage (Seddon & Khan, 2003). Animal studies of forced aspiration provide supportive evidence of this disease process, although the physiological relevance of forced instillation

of hydrochloric acid directly into the lungs (as often performed in a number of these studies) is questionable (Amigoni et al., 2008; Chang et al., 2012; Ishizuka et al., 2001; Maniatis et al., 2012; Westphalen, Monma, Islam, & Bhattacharya, 2012). Indeed, animal studies of forced instillation of both acidic and pH neutral gastric juice have suggested that other components of the gastric juice (such as enzymatic or microbial elements) may also be damaging to the airway epithelium, independent of pH (Mertens et al., 2010; Tang et al., 2012). The airway epithelium is the first to encounter inhaled substances following aspiration and therefore, the direct immunological response of airway epithelial cells to elements of the refluxate is relevant in furthering our patho-physiological understanding of aspiration lung disease. The evidence regarding airway epithelial cell response following exposure to potentially injurious elements of the aspirate is discussed further in Chapter 7 of this thesis.

Two recent clinical studies have added further interesting evidence to this proposed patho-physiological process in children with severe ND. Somerville *et al* studied 294 paediatric patients with severe developmental disability in the clinic setting. Within this group, 92% had symptoms of dysphagia, 67% had symptoms (or had previously had symptoms) of GORD and 65% had recurrent lower respiratory tract infections (Somerville et al., 2008). On investigation 53% had evidence of GORD related damage on upper airway endoscopy (182 patients examined), 41% showed evidence of aspiration on videofluoroscopy (174 patients examined) and 68% showed evidence of bronchiectasis on CT scan of the thorax (62 patients examined). A more recent retrospective study of 100 paediatric patients who had established videofluoroscopy or airway endoscopy diagnoses of aspiration and had undergone CT scan of the thorax, found that 66% had evidence of bronchiectasis, alarmingly including 51% of those less than two years of age (Piccione, McPhail, Fenchel, Brody, & Boesch, 2012). They found that severe neurological impairment and history of GORD were significant predictors of bronchiectasis, but notably clinical history/examination and respiratory symptoms were not. Sixteen patients underwent repeat CT scan following instigation of clinical management including feed modification, airway clearance therapy, fundoplication and salivary secretion management. In 81% the changes were stable or improving (44%). The authors did not specify whether these children had ND but the results would imply that pulmonary outcomes associated

with direct or reflux aspiration may be modifiable with appropriate management.

Both of these studies are significantly limited as they are solely based on clinical associations, with no evidence of direct causation. The children with ND included in these studies were likely to have had other factors contributing to their respiratory disease, independent of aspiration. More detailed prospective observational and interventional studies are needed in order to tease out the relative contributions of these factors.

1.6 Closing remarks and aims and objectives of this thesis

Children with severe ND often suffer with burdensome respiratory disease and respiratory complications are the leading cause of premature death in this group. Respiratory complications often result in repeated, prolonged hospital admissions which are costly and are likely to impinge on child and family quality of life. The aetiology of this respiratory disease is undoubtedly multi-factorial. However, aspiration (whether reflux-associated or direct) is likely to play a significant role. Due to the challenges of research in this area and limitations in diagnostic testing, the evidence base for clinical management and outcomes of interventions is limited. With improved understanding of the burden of respiratory disease and relative contribution of reflux and direct aspiration in this group, there is potential to work towards optimisation and standardisation of clinical care.

The original objectives of this study were firstly, to characterise the burden of respiratory symptoms in children with ND and explore the relationship of respiratory symptoms with co-morbidities and lower airway inflammatory markers. Secondly, to explore both established and novel bronchial lavage biomarkers of reflux aspiration in this group, assessing their validity and the relationship of any results generated to clinical and inflammatory data. Finally, to conduct an investigation into the effects of mild pH alteration and pepsin on the airway epithelial cell.

As the study progressed, further objectives were generated. Development of a novel test for accurate detection and quantification of pepsin in bronchial lavage fluid became a key study objective. Also, investigation of the validity of alpha-amylase as a biomarker of direct aspiration and the relationship of alpha-amylase levels to lower airway inflammatory markers and clinical symptoms was explored.

The study hypotheses were that 'children with severe neurodisability have a high burden of chronic respiratory symptoms and concomitant high levels of lower airway inflammation. Both direct and reflux aspiration contribute to respiratory symptoms and lower airway inflammation through direct airway epithelial injury by elements of the aspirate.'

In order to address these hypotheses, a prospective observational cohort study was performed. Clinical data and non-bronchoscopic bronchial lavage samples were collected from children with ND both at a time of stability and a time of respiratory deterioration and also from a group of healthy control children. Using the clinical data, BAL samples and an airway epithelial cell culture model, it was possible to explore;

1) Respiratory symptoms (as assessed by a respiratory symptom questionnaire) and their relationship to clinical characteristics and co-morbidities in children with ND.

2) Lower airway inflammatory profile in children with ND both at a time of stability and respiratory deterioration compared to healthy controls, and the relationship to respiratory symptoms.

3) Biomarkers of direct and reflux aspiration in BAL fluid, including validity of available laboratory techniques.

4) Development of a novel assay for measurement of pepsin in BAL.

5) The effects of pepsin and mild pH alteration on airway epithelial cells in a monolayer airway epithelial cell culture model.

A chapter on each of these areas is included within this thesis.

Chapter 2 Materials and Methods

2.1 Patient recruitment, consent, data collection and ethics

To realise the aims of this study, three groups of patients were recruited during their admission to Alder Hey Children's Hospital, Liverpool. The first group consisted of children with ND, admitted to the paediatric intensive care unit (PICU) with a respiratory deterioration. It was anticipated that a number of these children may have experienced an acute aspiration event shortly prior to admission and therefore, BAL biomarkers of aspiration may be increased. A second group of children with ND was included, admitted electively for non-urgent surgical procedures. Theoretically, this group should be stable from a respiratory perspective. They were an important group to include, in order to assess the influence of chronic aspiration and any relationship between chronic aspiration and background respiratory symptoms and lower airway inflammation. Finally, a group of healthy control children, admitted for elective surgical procedures were involved as a comparison group.

It would have been ideal to include a non-ND PICU patient group however, because of competing studies on PICU, recruitment to such a group was not possible. It was also not possible to recruit every eligible patient during the study period due to researcher availability. Consequently, a convenience sequential approach was taken to recruitment.

Unfortunately, there were no previous data on which to base a power calculation for study group sample sizes. Therefore, a pragmatic sample size target of thirty patients per study group was set.

2.1.1 Paediatric intensive care neurodisability group (PICU-ND)

Children were eligible for inclusion in the study if they were over two years of age, had a central ND (not neuromuscular disease), were non-ambulant (GMFCS IV-V) and were ventilated on the PICU for a respiratory deterioration (R. Palisano et al., 1997). Patients who had been ventilated for more than 48 hours were not eligible. Demographic details, past medical history, medication history, current clinical management (including time since intubation), previous respiratory management and respiratory sample microbiology results were recorded on a bespoke, standardised data collection form (**Appendix 1**). Results of

investigations for suspected GORD and videofluoroscopy assessments were recorded, as well as information about surgical feeding interventions or anti-reflux procedures.

2.1.2 Elective neurodisability group (Elective-ND)

Children were eligible for inclusion in the study if they were over two years of age, had a central neurodisability (not neuromuscular disease), were non-ambulant (GMFCS IV-V) and were being admitted for elective surgery, which would require a general anaesthetic and endo-tracheal intubation (as opposed to a laryngeal mask airway, which is commonly used for minor surgical procedures). Clinical information was collected and collated from all participants on a standardised data collection form (**Appendix 1**).

2.1.3 Healthy control group

Children over the age of two years were recruited at the time of admission for routine elective minor surgical procedures for which they would require a general anaesthetic and endo-tracheal intubation. Children were not eligible for inclusion if they had a chronic illness or were taking any regular medication. Following a period of recruitment, children who were admitted for grommet insertion or tonsillectomy were excluded from the study due to the possible associations of otitis media and adeno-tonsillar hypertrophy with GORD (Crapko et al., 2007; Stapleton & Brodsky, 2008; Tasker et al., 2002b). Demographic details, past medical history, medication history and reason for admission were recorded on a standardised data collection form (**Appendix 1**).

2.1.4 Ethics and Consent

This study was approved by the local research ethics committee (REC reference 09/H1002/58). All patients and parents received written information about the study (**Appendix 2** - patient/parent information leaflets). Written informed consent (and assent where appropriate) was taken from all parents (and patients) prior to collection of data or clinical samples using standard Alder Hey Trust consent forms.

2.2 Assessment of clinical symptoms

2.2.1 Respiratory symptom questionnaire

No validated respiratory symptom score exists within the literature for children with ND. In this study, parents of all recruits completed a modified version of the Liverpool Respiratory Symptom Questionnaire (LRSQ). Previously, the LRSQ has been partially validated in preschool children with wheeze and in children with cystic fibrosis (Powell, McNamara, Solis, & Shaw, 2002; Trinick, Southern, & McNamara, 2012). The questionnaire enquires about respiratory symptoms over the previous three months and is divided into eight domains; daytime, nocturnal, URTI, interval, activity, other respiratory symptoms and the effect of these symptoms on both child and family. For the purposes of this study, we removed the 'activity symptoms' domain and a question about effects of respiratory symptoms on physical activity from the 'Effects of symptoms on the child' domain as by definition, the ND study group were not independently mobile. Thus, we created a modified version of the questionnaire (**Appendix 3** - LRSQ-Neuro). This modification negates validation data gathered from previous studies using the LRSQ (Powell, McNamara, Solis, & Shaw, 2002; Trinick, Southern, & McNamara, 2012). However, the questionnaire scores would not have been comparable between control and ND groups without these changes and no alternative validated respiratory questionnaire exists for this patient group. There were 27 questions in total, spread across seven domains, with a maximum total score of 108. Parents/patients completed the questionnaire independently following recruitment to the study.

2.2.2 GORD and pulmonary aspiration symptom assessment

No prospectively validated gastro-oesophageal reflux symptom score exists within the literature for use in children over two years of age. A commonly used score, which has been partially validated in the paediatric population, is the Reflux Symptom Index (Belafsky, Postma, & Koufman, 2002; Simons et al., 2008). It was anticipated that children recruited to the ND study groups may be fed via gastrostomy and may not communicate verbally, thus potentially limiting parental assessment of difficulty in swallowing and change in voice. The questions from the Reflux Symptom Index were adapted to create a set of five questions which were appropriate for use across the study population. The

questions enquired about cough, pain, choking, shortness of breath and vomiting with, or shortly following, feeds (whether they were oral, nasogastric or via gastrostomy). Questions were answered on a Likert scale from 'not at all' (score 0) to 'every day' (score 4). Some of the questions within the LRSQ-Neuro and GORD symptom questionnaires also address commonly encountered symptoms of pulmonary aspiration. The GORD symptom questionnaire is included in **Appendix 4**.

2.3 Broncho-alveolar lavage samples

2.3.1 Sampling, equipment and technique

Non-bronchoscopic bronchoalveolar lavage (BAL) samples were collected either following induction of anaesthetic for elective surgery in the anaesthetic room or when the child was muscle relaxed on PICU. The European Respiratory Society (ERS) guidelines on collection of BAL in children were followed (de Blic et al., 2000).The equipment required for BAL sampling included a sterile 10G suction catheter (Pennine Healthcare), a three-way tap (Codan Steritex), a tracheal suction set (Unomedical), two 10ml sterile syringes, sterile 0.9% saline solution and a suction machine **(Figure 2.1)**. A suction catheter was passed down the endo-tracheal tube until resistance was felt. Two 1 ml/kg aliquots of normal saline were instilled separately (maximum aliquot volume 20ml). BAL fluid was recovered with constant suction pressure of <100mmHg and collected into a tracheal suction set (mucus trap). Oximetry monitoring was performed throughout the procedure.

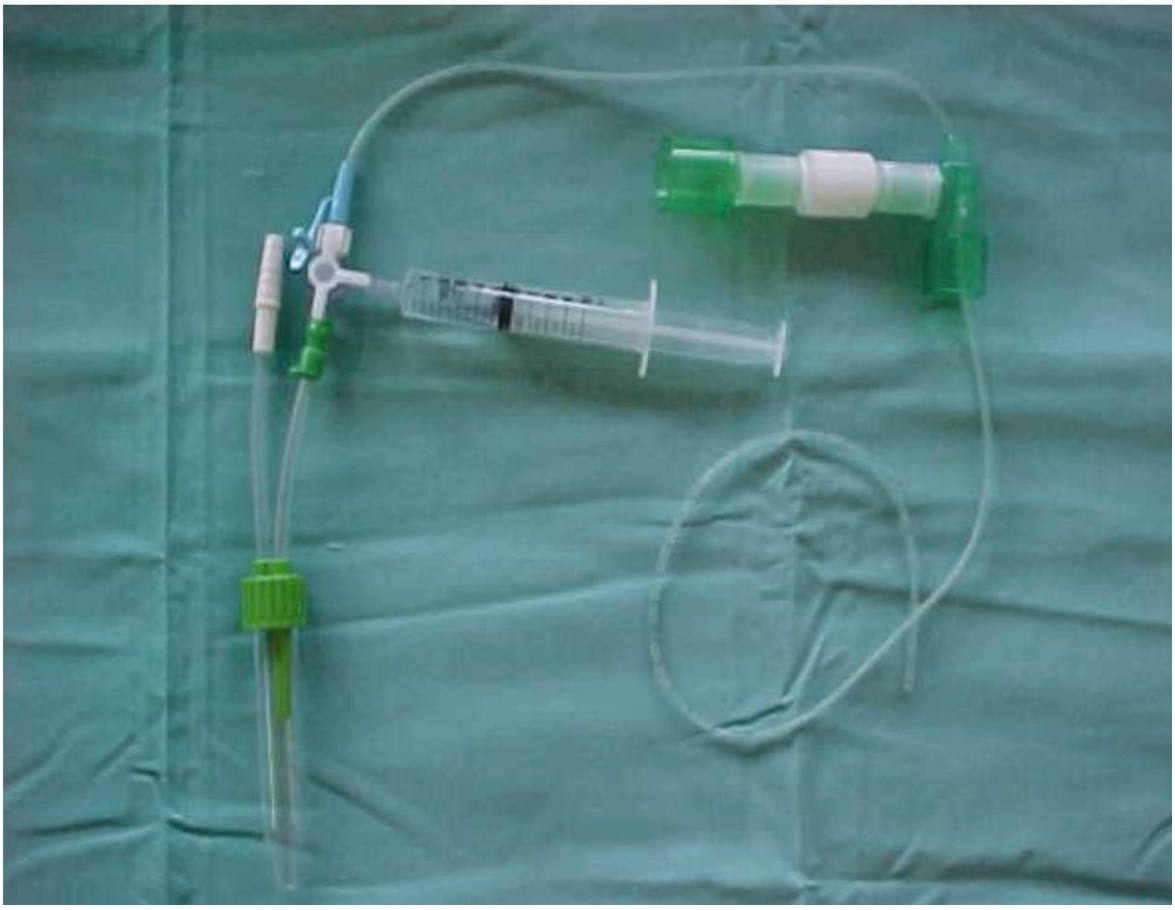

Figure 2.1 Equipment required for non-bronchoscopic broncho-alveolar lavage sample collection

The equipment required for non-bronchoscopic broncho-alveolar lavage sample collection includes a sterile 10G suction catheter (Pennine Healthcare), a three-way tap (Codan Steritex), a tracheal suction set (Unomedical) and a 10ml syringe with sterile 0.9% saline.

2.3.2 BAL sample processing

Following collection, BAL samples were processed immediately in the laboratory. The ERS guidelines on the processing of BAL in children were followed (de Blic et al., 2000).

2.3.2.1 Cell counting

Total volume of BAL sample returned was recorded and percentage recovery was calculated. The sample was gently mixed using a vortex, following which three 10µl aliquots were removed for cell counting. An equal volume (10µl) of trypan blue stain (Sigma Aldrich, Dorset, UK, diluted 1:2) was added to each aliquot. Following mixing, 10µl was pipetted under the positioned cover-slip of a Neubauer haemocytometer. Using a light microscope (Vickers 20/0.5 objective lens), the cell number in four 1x1mm squares (each holding 100 nanolitres) was counted. To calculate the number of cells per millilitre (ml), the following equation was used; (count x 2)/4 x 10,000). This figure was multiplied by the sample volume recovered (ml) to give the total cell count. Non-viable cells (which take up trypan blue stain differentially) were not included in the cell count. Bronchial epithelial cells (the number of which was low in adequate samples) were also excluded from the count. Samples with a cell count of less than 500,000 were immediately excluded from further analysis. Sample adequacy was assessed, based on volume returned and numbers of bronchial epithelial cells. Total counts were performed on three separate aliquots and the mean value was recorded.

2.3.2.2 Processing and storage

BAL pH measurement was made within fifteen minutes (mins) of collection using a pH meter (Philips PW 9418), which was calibrated and cleaned prior to each reading. An aliquot of whole BAL, sufficient (from the total cell count) to provide adequate cell numbers to produce four cytospin slides, was set aside (50,000 cells/slide). The remaining sample was centrifuged at 2656g for 5 mins. The BAL supernatant was aliquoted into 1.5ml Eppendorf tubes and stored at minus 80 ºC until further analysis, with a minimum of 200µl in each tube. The cell pellet was divided for protein (2 million cells) and/or mRNA (1 million cells)

analysis. Protein pellets were stored dry. Cell pellets for mRNA analysis were stored in 350µl RLT lysis buffer (1ml RLT buffer (Qiagen Ltd, Crawley, UK) and 10µl Beta-mercaptoethanol (Sigma Aldrich, UK))

2.3.2.3 Cytospin slide preparation, modified Wright-Giemsa staining (Diff-quick) and differential cell counting

Cytospin slides were prepared from a volume of whole BAL, calculated (from the total cell count) to contain approximately 50,000 cells. In samples of high cellularity, the total sample volume was made up to 100µl with sterile phosphate buffered saline (PBS). The samples were applied to cyto-chambers which were secured in slide carriers containing Xtra-adhesive slides (Surgipath® Consumables) and centrifuged at 500 xg for 5 mins in a Rotofix 32A.

'Diff-quick' stain is a modified Wright-Giemsa stain. Following brief air drying, slides were fixed with Diff Quick fixative (100% methanol, Reastain CE) by dipping five times and allowing excess to drip off the slide. Slides were then dipped five times in the eosinophilic staining 'Solution 1' followed by five dips in the basic 'Solution 2' (eosin Y and azur blue respectively, Reastain CE). Slides were rinsed in dH_20 before being allowed to air-dry. They were then briefly dipped in xylene before a cover slip was applied, using DPX mounting media.

A differential cell count was made using a light microscope with an oil emersion lens (**Figure 2.2**). Three hundred cells were counted and any bronchial epithelial cells or unidentifiable cells were not included in the count. Cell types were expressed as a percentage of the total cell count. A mean value was calculated from three cell counts.

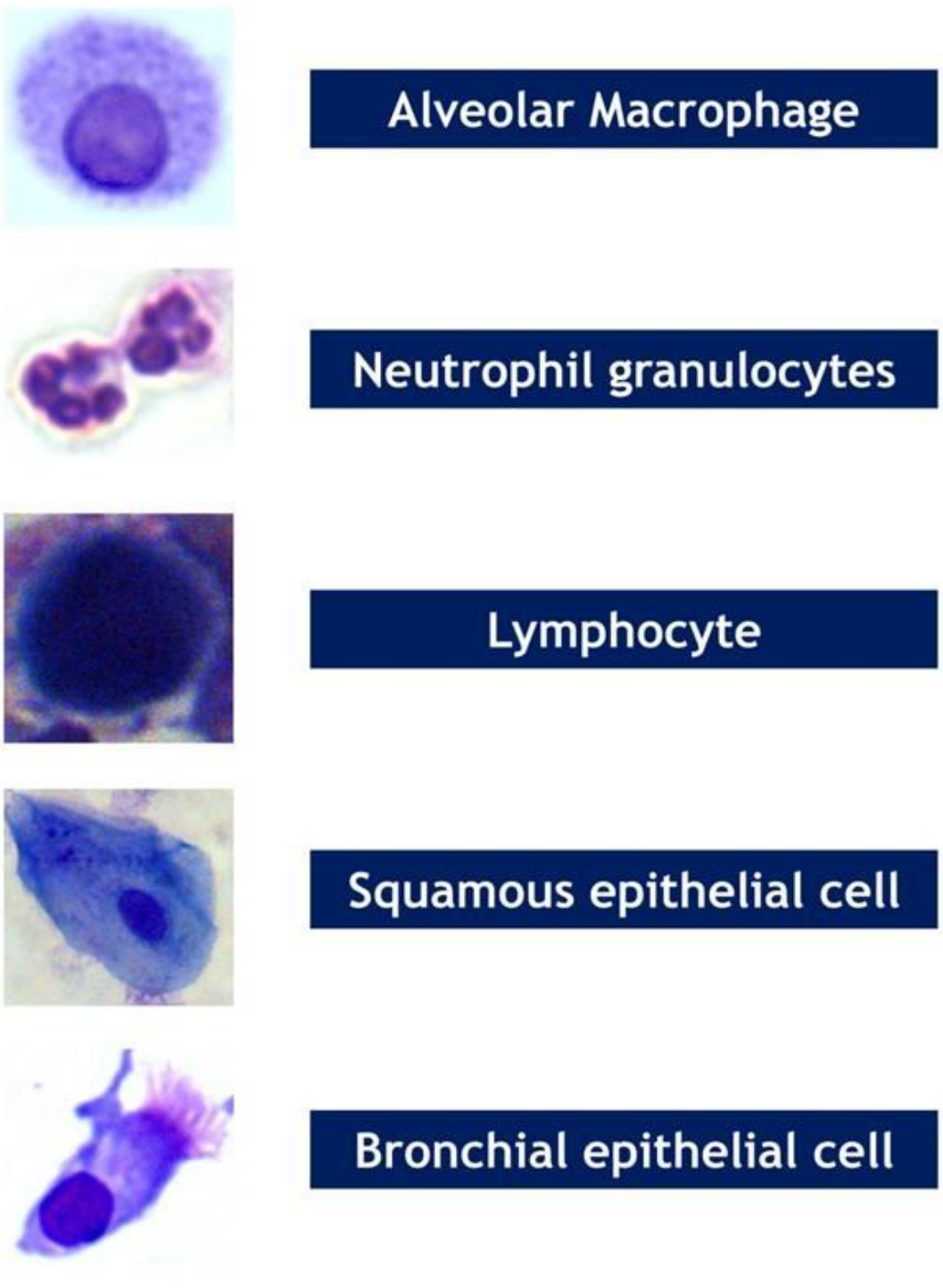

Figure 2.2 Examples of cell types observed in BAL fluid from study recruits

Diff-Quick staining (Reastain CE) was performed on BAL cytospin slides. Cells were visualised using a light microscope with an oil emersion lens**.**

2.3.2.4 Oil Red-O staining and lipid laden macrophage assessment

Stock Oil Red-O stain was prepared by adding 1 gram (g) of Oil Red-O (Sigma Aldrich, UK) to 100ml of 60% triethyl phosphate (Sigma Aldrich, UK). This was incubated at 60ºC for 2-3 hours, stirring occasionally. The mixture was filtered through fluted filter paper whilst still hot.

Cytospin slides were prepared as previously described. Following centrifugation, the slides were rapidly air dried using low flow cold air, followed by rinsing in 60% triethyl phosphate for 30 seconds (secs). Oil Red O stain was applied for 15 mins. Differentiation was achieved by washing off excess dye with 60% triethyl phosphate. Slides were then rinsed in dH_20 for 15 secs. Counterstaining was performed using haematoxylin (Gill III Surgipath®, Leica Microsystems) for 15 secs followed by a further dH_20 rinse for 15 secs. Scott's tap water substitute was used to blue the haematoxylin for 15 secs. Following further rinsing in dH_20, a cover-slip was applied using glycerol gelatine aqueous mountant (Sigma Aldrich, UK).

Slides were assessed for lipid staining of the alveolar macrophages using a light microscope with an oil emersion lens. The limitations of lipid laden macrophage index reporting are discussed in Chapter 1. In cases where evidence of alveolar macrophage lipid staining was seen, slides were forwarded to the local cytopathologist for interpretation according to local guidelines (**Figure 2.3**). All slides were reported by the same cytopathologist (Professor Lesley Turnbull). Lipid laden macrophage index scores were not reported due to concerns regarding misinterpretation (as discussed in section 1.3.3). Any lipid laden macrophage positivity was assessed within the context of the inflammatory background of the sample and a positive or negative report was issued. It was agreed that any cases of a positive report would be discussed with the medical team in charge of the child's care and that an appropriate course of clinical action would be discussed with the family.

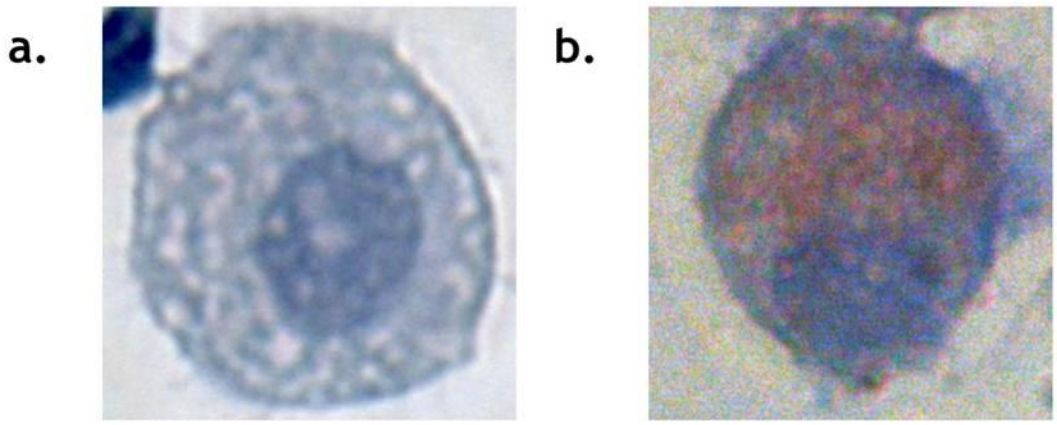

Figure 2.3 Examples of a. negative and b. positive BAL alveolar macrophages for cytoplasmic lipid, tested using Oil Red O staining.

Oil Red O staining was performed on cytospin slides of BAL samples and alveolar macrophages were visualised using a light microscope with an oil emersion lens. All samples with any positive staining were interpreted by a cytopathologist according to local policies.

2.3.2.5 Quantipro Bicinchoninic Acid Assay

Measurement of BAL total protein content was performed using a Quantipro (Bicinchoninic acid) assay kit (Sigma Aldrich, UK). The Bicinchoninic acid assay is based on the principle of Cu^{2+}-protein complex formation and reduction of Cu^{2+} to Cu^{1+} under alkaline conditions. This reduction is proportional to the amount of protein present in a linear relationship between 0.5-30 µg/ml.

A bovine serum albumin (BSA) stock solution of 900 µg/ml was prepared to use as a standard. All samples and standards were processed in duplicate. PBS (150µl) was added to all standard wells, with the exception of the two top standard wells, on a 96 well plate (Corning, Costar). For each top standard well, 10µl of the BSA stock solution was added to 290µl of PBS, giving a concentration of 30µg/ml. Serial 1:2 dilutions were then made to give a seven point standard curve. BAL supernatants were pre-diluted, as detailed below, and 150µl volume was added to sample wells, each sample being assayed in duplicate. The Quantipro working solution is a mixture of 25 parts 'QA' (sodium carbonate, sodium tartate, and sodium bicarbonate in 0.2 M NaOH, pH 11.25) with 25 parts 'QB' (4% (w/v) bicinchoninic acid solution, pH 8.5). and 1 part 'QC' (4% (w/v) copper(II) sulfate, pentahydrate solution). The working solution was mixed with samples 1:1. A cover was applied and the plate was incubated on a plate agitator at 60ºC for 60 mins. The absorbance was measured at 560 nm using a microplate reader (Bio-tek) and results were recorded using KC Junior software (Bio-tek). The sample total protein concentration was calculated from the standard curve.

For sample dilution optimisation, six samples (a range of elective and PICU samples) were run at dilutions (with PBS) ranging from 1:20 to 1:160. Optimal sample dilution was found to be 1:20 for healthy controls and 1:80-1:160 for PICU samples, allowing all samples to fall within the range of the standard curve. Linearity of dilution was checked and was found to be acceptable (**Figure 2.4**). Mean intra-assay and inter-assay coefficients of variation were found to be 4.04% (n=20) and 14.5% (n=4), respectively.

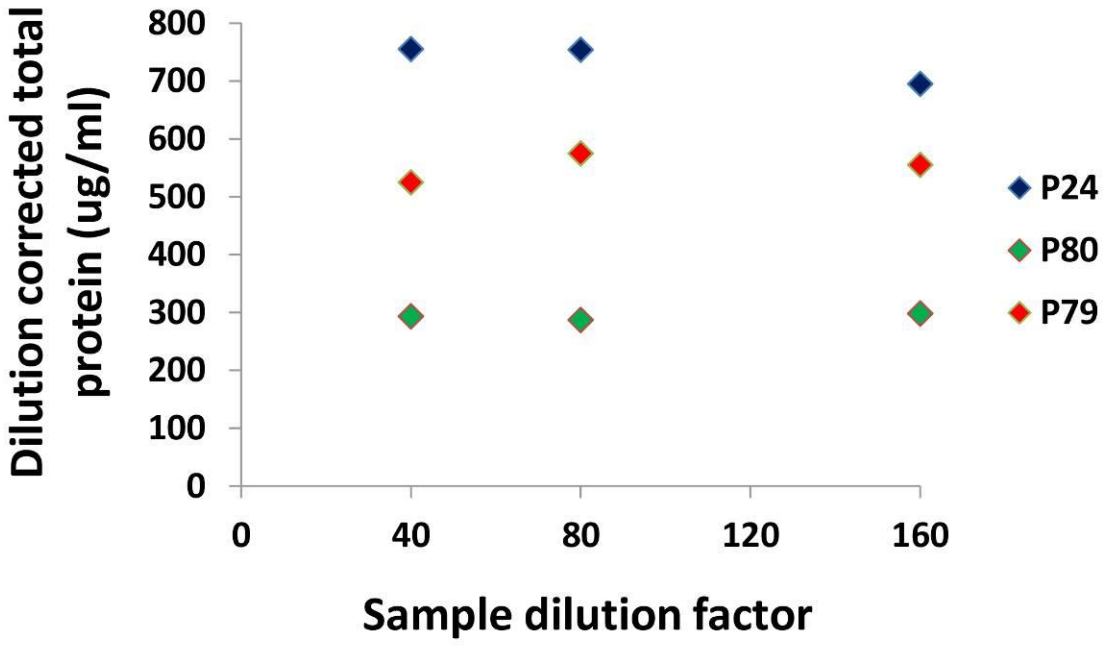

Figure 2.4 Good linearity of dilution was observed when assessing three BAL supernatant samples run at three dilutions using the Quantipro total protein assay (Sigma, UK)

Scatter graph to show dilution corrected total protein measurements for three BAL supernatant samples, run at three dilutions**.** Good linearity of dilution was seen and therefore, there was little variation in total protein measurement by sample dilution. This provided validation for use of varying, optimised sample dilutions according to patient group.

2.3.2.6 Correction for epithelial lining fluid dilution

BAL fluid collection provides a useful method of assessing the lower airway in both research and clinical settings. However, a widely recognised limitation of this technique is the variable dilution of the airway epithelial lining fluid within the returned sample. The possibility of using a dilutional marker to correct for this has been debated within the literature and various internal markers, such as albumin and urea, have been considered. However, all have potential significant limitations (Shields & Riedler, 2000; Ward, Fenwick, Booth, & Walters, 1997; Ward, Thien, Secombe, Gollant, & Walters, 2000). An ERS taskforce (2000) made recommendations that BAL data should not be corrected for dilution until a reliable method had been established. There has been no change to this recommendation to date (de Blic et al., 2000).

2.4 Enzyme linked immunosorbent assays (ELISA) and enzyme activity assays

R&D systems Duo-Set ELISA Development kits were used for Interleukin-8 (IL-8), Interleukin-6 (IL-6) and Transforming Growth Factor-Beta1 (TGFβ-1) protein measurement in BAL samples and/or cell culture supernatants. Antibody (Ab) and protein standard concentrations used for the ELISAs are listed in **Table 2.1**. The manufacturer of these ELISAs had not validated their use in BAL supernatants. Standard steps of assay validation include calculation of intra- and inter-assay coefficients of variation and spike recovery experiments (Food and Drug Administration, 2001). These validation steps were performed using BAL at the dilutions stated in the individual IL-8 and TGFβ-1 ELISA methods (Sections 1.4.2 and 1.4.4, respectively) and the results are documented in **Table 2.1**. Inter- and intra-plate coefficients of variation were calculated as detailed in Section 2.9. Spike recovery experiments were performed by spiking a known concentration of the protein of the interest into a BAL supernatant sample (also processing the same BAL supernatant alone with an equivalent volume dH_20 spike, in order to measure the baseline level of the protein of interest in the sample and thus, calculate the true spike retrieval). An identical protein spike was also performed in reagent diluent, on the same plate. Percentage spike retrieval was calculated by dividing the amount retrieved in sample matrix by the amount retrieved in reagent diluent and multiplying by 100. Acceptable spike recovery is considered to be 80-120% (Administration).

Table 2-1 Table of commercial ELISA Ab concentrations and validation data for use of IL-8 and TGFβ-1 duoset ELISA (R&D systems, UK) with BAL

ELISA	**IL-8**	**IL-6**	**TGF**β-1
Capture Ab (µg/ml)	4	2	2
Detection.Ab (ng/ml)	20	50	300
High standard (pg/ml)	2000	600	2000
Low standard (pg/ml)	31.25	9.3	31.25
Mean intra-assay CV (% n>20)	2.06	n/a	7.51
Mean inter-assay CV (% n=3)	5.86	n/a	5.31
Spike retrieval (% n=4)	94.5	n/a	101.5

2.4.1 Optimisation of sample dilution

Optimal sample dilution was ascertained for each ELISA by running a range of samples (healthy control and PICU-ND) several times at various dilutions and identifying the dilution factor which would allow the majority of samples to fall within the linear portion of the standard curve and all samples to fall below the top standard. Corrections for dilution were made at the end of the analysis.

2.4.2 Measurement of interleukin-8 in BAL and cell culture supernatants

An aliquot of capture antibody (calculated to give the desired Ab concentration on dilution in 10ml volume) was diluted in 10ml PBS. All wells of a 96 well immuno-plate (Maxisorp^{TM}, Nunc Brand) were coated with 100µl of capture antibody solution. The plate was sealed and incubated at room temperature (RT) overnight. Following aspiration of the capture antibody, an automatic plate washer was used to aspirate and fill the wells with 400µl wash buffer (0.05% Tween 20 in PBS, pH7.2-7.4) three times. The plate was then inverted and blotted. All wells were blocked with 300µl of block buffer (1% BSA in PBS) and the plate was incubated at RT for one hour. The three aspiration/wash cycles were then repeated. BAL samples were diluted 1:10 in reagent diluent (0.1% BSA, 0.05% Tween® 20 in Tris-buffered saline, pH7.2-7.4) and cell culture supernatants were processed neat. Samples (100µl/well) were loaded in duplicate. A seven point standard curve was run in duplicate, using two fold dilutions (in reagent diluent) from a high standard of 2000pg/ml. The plate was resealed and incubated at 37ºC for one hour. The aspiration/wash step was repeated. The detection antibody was diluted in 10ml reagent diluent, giving the required Ab concentration, and 100µl was added to each well. The plate was resealed and incubated at 37ºC for one hour. Following the aspiration/wash step, 100µl of Streptavidin-HRP (diluted 1:200 in reagent diluent) was added to each well. The plate was resealed and incubated at RT out of direct light for 20 mins. The aspiration/wash step was repeated, then 100µl 3,3',5,5'-Tetramethylbenzidine (Sigma, UK) was added to each well. The plate was incubated for up to 20 mins in the dark until the lowest standard had developed colour, following which 50µl of 'stop' solution (2N H_2SO_4) was added to each well. The plate was read using a microplate reader (Bio-tek) at 450nm and the

standard curve was generated using KC Junior software (Bio-tek), from which sample IL-8 concentrations were calculated.

2.4.3 Measurement of Interleukin–6 in cell culture supernatants

The methods for measurement of IL-6 using the IL-6 Duoset ELISA (R&D systems, UK) were identical to the methods described above, except the reagent diluent used was 1% BSA in PBS (pH 7.2-7.4) and the standard curve high standard was 600pg/ml. Cell culture supernatants were processed neat or at 1:2 dilution, depending on sample volumes available.

2.4.4 Measurement of TGFβ-1 in BAL and cell culture supernatants

Latent TGFβ-1 required activation before measurement by ELISA, using acid activation and neutralisation. For cell culture supernatants, 30µl 1N HCL was added to 150µl of sample, mixed and incubated at RT for 10 mins. Neutralisation was with 30µl 1.2N NaOH/0.5M HEPES. Following mixing, the sample was assayed in duplicate with no further dilution (100µl/well). For BAL, 50µl 1N HCL was added to 100µl sample, mixed and incubated at RT for 10 mins. Neutralisation was with 50 µl 1.2N NaOH/0.5M HEPES. Following mixing, the sample was assayed with no further dilution (total 100µl/well).

The TGFβ-1 assay protocol was identical to the IL-8 protocol except the block buffer was 5% Tween® 20 in PBS and the reagent diluent was 1.4% Reagent Diluent Concentrate 1 (R&D systems) and 0.05% Tween® 20 in PBS. The high standard was 2000pg/ml.

2.4.5 Measurement of pepsin in BAL

2.4.5.1 ELISA 1

This is the protocol of an 'in house' assay developed at the University of Newcastle (Tasker et al., 2002a). Primary and secondary Ab details are listed in **Table 2.2**.

For the standard curve, ten standards of 0-10 ng/ml porcine pepsin A solution (100µl volume) were constructed in duplicate across the top of a 96 well immuno-plate (Maxisorp™, Nunc Brand), using lyophilised porcine pepsin A

(Sigma Aldrich, UK) and reagent diluent (0.1% BSA, 0.05% Tween 20 in PBS). The rest of the plate was divided into two: the first half was for analysis of samples using the primary and secondary Abs and the second half was for analysis of samples using the secondary Ab only (to check and correct for any non-specific binding of the secondary Ab). BAL samples were loaded at a 1:5 dilution and analysed in duplicate on both halves of the plate. The plate was covered and incubated at RT overnight. All wells were then aspirated and washed three times with wash buffer (0.05% Tween 20 in PBS (pH7.2-7.4)). Following this, all wells were blocked with 300µl block buffer (1% BSA in PBS) and incubated for 1 hour at RT. The aspiration/wash step was then repeated. The primary Ab was then added to all wells (100µl, 5ug/ml). The plate was covered and incubated at RT for 1.5 hours. Following the aspiration/wash step, the secondary Ab was added to all wells (100µl, 1:10,000 dilution following reconstitution according to the manufacturer's instructions). The plate was re-covered and incubated for a further 1.5 hours. Following the aspiration/wash step, 100µl 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sµlfonic acid) liquid substrate system (Sigma, UK) was added to each well (pre-warmed to RT). The plate was incubated in the dark for 45 mins (optimised based on rate of standard curve colour development). To stop the reaction, 100µl 1% SDS was added to each well. The plate was read using a microplate reader (Bio-tek) at 405nm and the standard curve was generated using KC Junior software (Bio-tek). Any fluorescence emitted from samples due to non-specific binding of the secondary Ab was subtracted from the corresponding readings with the primary and secondary Ab. Pepsin levels were calculated from the standard curve and multiplied by two (the team who developed the ELISA have previously established that the porcine pepsin primary antibody is cross reactive with human pepsin with half the level of affinity for porcine pepsin).

2.4.5.2 ELISA 2

This is the protocol of an 'in house' assay developed at Queens University Belfast (Farrell et al., 2006). Primary and secondary Abs used in this method are listed in **Table 2.2**.

All wells of a 96 well immuno-plate (Maxisorp^{TM}, Nunc Brand) were coated with 100µl of primary Ab (10ug/ml 0.05M carbonate-bicarbonate buffer, pH 9.6). The

plate was sealed and incubated overnight at RT. All wells were aspirated and washed five times with 300µl wash buffer (0.05% Tween 20 / PBS wash buffer) following which the plate was blotted dry. All wells were blocked with 300µl block buffer (3% filtered Ovalbumin (Sigma, UK) in PBS) and the plate was incubated for two hours at RT. The 3% Ovalbumin / PBS was filtered through 50µm nylon gauze. The aspiration/wash step was repeated. Porcine pepsin standards (lyophilised porcine pepsin A, Sigma Aldrich, UK) were prepared in 0.1% BSA/PBS at concentrations of 1, 5, 7.5, 10, 50, 75, 100ng/ml and assayed in duplicate (100µl volume). BAL samples were assayed neat (100µl) in duplicate and 100µl of 0.1% BSA/PBS was added to blank wells. The plate was sealed and incubated at RT for 2 hours, following which the aspiration/wash step was repeated. Following blotting, all wells were coated with secondary Ab (2ug/ml in 3% filtered Ovalbumin/PBS) and the plate was incubated for two hours at RT. Following a final aspiration/wash step, 100µl of 3,3',5,5'-Tetramethylbenzidine substrate was added to all wells and the plate was incubated for 30 mins under subdued light. The reaction was stopped with 100µl 2M Sulphuric acid. The plate was read using a microplate reader (Bio-tek) at 450nm (with a wavelength correction at 540nm) and a four parameter logistic standard curve fit was generated using KC Junior software from which sample pepsin values were calculated.

Table 2-2 Table of 'in house' pepsin ELISA Abs, suppliers and working concentrations

Assay	***Ab***	***Working concentration***	***Host***	***Supplier***
ELISA 1 primary	Purified polyclonal anti-porcine pepsin A	5ug/ml	Goat	Biodesign, US (W59117G)
ELISA 1 secondary	Monoclonal anti goat/sheep IgG-peroxidase	1:10000 dilution	Mouse	Sigma Aldrich, UK (A9452)
ELISA 2 primary	Purified polyclonal anti-porcine pepsin A	10ug/ml	Goat	US Biological, US (P3270-06)
ELISA 2 secondary	Purified polyclonal anti-porcine pepsin A-peroxidase	2ug/ml	Goat	Biodesign (W59117P)

2.4.6 Lactate dehydrogenase activity assay

Cell lysis and death was assessed using a colorimetric cytotoxicity detection kit, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant (Roche Applied Science, UK). LDH activity is determined by the LDH catalysed conversion of lactate to pyruvate, which in turn reduces NAD^+ to $NADH/H^+$. The catalyst (diaphorase) then transfers H/H^+ from $NADH/H^+$ to tetrazolium salt INT, which is then reduced to formazan.

Cell culture supernatants were tested neat and assayed in duplicate (100µl/well) on a 96 well flat-bottomed plate (Corning, Costar). A 14-point standard curve was constructed from a high standard of 2U/ml, which was also run in duplicate (LDH, hog muscle, Roche, UK). A background control (assay medium alone) and low control (100µl cell culture media) was run on each plate. For 100 tests, 250µl of 'bottle 1' was combined with 11.25µl of 'bottle 2' and mixed. This was added 1:2 (100µl) to each well and the plate was incubated with agitation at 20 ºC for up to 30 mins. Absorbance was measured using a microplate reader (Bio-tek) at 490nm and the standard curve constructed using KC Junior software. LDH activity was calculated from the standard curve.

2.4.7 Alpha amylase activity assay

A commercially available alpha amylase activity kit (Abcam) was used for measurement of alpha amylase activity in BAL supernatants. The assay is based on specific cleavage of ethylidene-pNP-G7 by alpha amylase to smaller fragments, which can be acted upon by alpha-glucosidase, leading to release of a chromophore which can be measured at an absorbance of 405nm.

BAL supernatants (20µl volume) were assayed in duplicate. The samples were made up to 50µl volume with dH_20. A six point nitrophenol standard curve of 20, 16, 12, 8, 4 and 0 nM/well was also constructed in duplicate, all wells being made up to a final volume of 50µl. An alpha amylase positive control was reconstituted in 50µl assay buffer and run on each plate. A negative control of PBS was also run on each plate. Assay buffer and substrate mix were combined 1:1 and 100µl was added to each well. The plate was analysed immediately in a microplate reader (Bio-tek) at 405nm for 'T0' values. The plate was then

incubated at 25 ºC with agitation and reanalysed by the plate reader every two mins for a total of ten mins. Reaction kinetics were observed and the optimal time for 'T1'-'T0' to fall in the linear portion of the curve (for the majority of samples), was found to be 4 mins. Alpha amylase activity was calculated by applying the change in OD ('T1'-'T0') to the standard curve to calculate a value of 'B' nM of nitrophenol generated between T1 and T0. Amylase activity (U/L) was then calculated by multiplying 'B' by the sample dilution factor and then dividing this by time between T1 and T0, multiplied by the pre-treated sample volume.

This assay was not validated by the manufacturer for use with BAL supernatant samples. Therefore, previously described validation steps were performed. The intra-assay coefficient of variability was found to be 6.49% (n=20), the inter-assay coefficient of variability was 7.04% (n=3) and the mean spike recovery was 94.3% (n=3).

2.5 1D SDS-PAGE Gel Analysis and Western Blotting

2.5.1 1D SDS-PAGE (Polyacrylamide Gel Electrophoresis)

The spacer and short plates were secured in a casting frame and a 1mg/ml ammonium persulphate solution was prepared, prior to making the resolving gel solution. The resolving gel solution (enough for two gels) consisted of 4ml 30% acrylamide/bisacrylamide, 2.5ml 1.5M Tris (pH8.8), 50µl 10% Sodium Doecyl sµlphate (SDS) and 3.4ml ultrapure H20. When ready to pour, 75µl ammonium persulphate solution and 7.5µl TEMED was added. The solution was briefly mixed and then pipetted between the plates, filling the cavity to 1 cm from the top. A small volume of water-saturated butan-2-ol was added across the width of the gel before covering with cling-film and leaving for one hour to polymerise. Following this, excess butan-2-ol was poured off and 25µl ammonium persulphate (1mg/ml) and 2.5µl TEMED were added to the stacking gel buffer (0.65ml 30% acrylamide/bisacrylamide, 1.25ml 0.5M Tris (pH6.8), 50µl 10% SDS and 3.025ml ultrapure water). The solution was mixed prior to adding to the gels (filled to the top of the plates). The well comb was positioned and the casting frame was covered with cling-film for a further hour.

SDS sample buffer (1.2ml 0.5M Tris (pH6.8), 5.3ml ultrapure water, 1ml glycerol, 2ml 10% (w/v) SDS, 0.5ml 0.1% Bromophenol blue and 150mg dithiothreitol) was aliquoted and stored at -20 ºC until use. A broad range molecular weight marker (Bio-Rad) was pre-prepared by dilution 1:20 with sample buffer, heating to 95 ºC for 5 mins and storing in aliquots at -20 ºC. 5µl of marker was loaded in the first well, followed by the samples. Samples were prepared by mixing 1:1 with SDS sample buffer and heating to 95 ºC for 5 mins.

A twenty-five millimolar (mM) electrophoresis buffer (10x solution–30.3g tris base, 144g glycine, 10g SDS, 1 litre ultrapure H20) was used and the gel was run at 200V for 30-45 mins, until the samples reached the bottom. The gel was stained using Coomassie R-250 and destained using a 10% methanol/10% acetic acid solution. Following adequate destaining, gels were imaged using an Epson Expression 1680 Pro scanner and Corel Photoshop Pro (v3). Densitometry analysis was performed on grey-scale images using TotalLab Quant software (TotalLab Ltd, Newcastle, UK).

2.5.2 Western blotting

All Abs used in Western blotting analysis are listed in **Table 2.3**.

2.5.2.1Porcine Pepsin Western Blot

12% mini-PROTEAN® TGX™ precast gels and a 4-gel mini-PROTEAN® tetra cell were used (Bio-Rad, UK). Samples were diluted 1:2 with sample buffer (62.5mM Tris pH6.8, 10% glycerol, 2% SDS, 100mM DTT, bromophenol blue) and heated to 95 ºC for 5 mins. A Precision Plus Protein® Kaleidoscope Standard (Bio-Rad) was loaded into the first well (10µl) and samples were then loaded into the remaining wells. Following cell loading with 1x electrophoresis running buffer (10x buffer = 6.06g Tris, 28.8g glycine, 2g SDS in 200ml dH20), the gel was run at 150 volts until the samples reached the bottom of the gel.

Transblot® Turbo™ MIDI PVDF transfer packs and the Transblot® Turbo™ transfer system were used for gel blotting (Bio-Rad, UK). The gel was blotted on the seven minute turbo setting, following which the membrane was transferred into a tray in which it was blocked with 20ml 5% BSA in TBS-T for 1 hour. The membrane was then washed 7-8 times with TBS-T for 30 mins. The primary Ab incubation was for 1.5 hours at 1:5000 dilution (2ug/ml concentration) in 20ml 3% TBS-T. The wash step was repeated for 30 mins. The secondary Ab incubation was for 1.5 hours at 1:10,000 dilution in 3% TBS-T, following which the wash step was repeated. AGFA fixative and developer solutions were used at 1:4 and 1:6 dilutions respectively. Two ml of GE Healthcare ECL plus solution (25µl solution B:1ml solution A) were added to cover the membrane, laid flat on the bench. This was incubated for two mins, following which excess ECL was allowed to drip off and the membrane was wrapped in cling-film before being positioned in the developing cassette. In a completely dark room (using a red light head lamp), the blot was exposed to Amersham ECL Hyperfilm for 30 secs (optimised using a range of time-points). The film was then placed in the developer solution and agitated until bands were seen. Following a dH_2O rinse, the film was placed in the fixing solution for 1 min and then rinsed. The film was imaged using a Syngene Ingenius gel documentation system and Genesnap image acquisition software (Syngene, UK). The lower limit of detection was found to be 10ng.

2.5.2.2 Human Pepsin Western Blot

Human pepsin western blots were performed at the Otolaryngology research lab, University of Wisconsin (courtesy of Dr Tina Samuels and Dr Nikki Johnston). Sample buffer (4x concentration) was pre-prepared and stored at -20 ºC (4% SDS, 20% glycerol, 500mM Tris-HCL (pH6.8), 200mM DTT and 0.001% bromophenol blue). 6.25µl sample buffer was added to 18.25µl sample (unless this would result in loading >30µg protein, in which case a smaller sample volume was used) and heated to 95ºC for 5 mins. 10% precast Tris-HCl gels (Bio-Rad) were used. Kaleidoscope Precision Protein Plus® standard was loaded into the first well (8µl, Bio-Rad) followed by a 4ng human pepsin positive control (isolated from human gastric juice, by ion exchange chromatography) and a 40ng pepsinogen 1 negative control (Sigma, UK), followed by the samples. The gel was run at 120V for one hour, or until the samples reached the bottom of the gel. The PVDF transfer membrane (Bio-Rad) was pre-wetted in transfer buffer (10x buffer, 145g glycine, 29g tris base in 1L dH_20) and a transfer sandwich was set up. This was placed in apparatus filled with transfer buffer and run at 30V. The membrane was removed and blocked with 20ml block buffer (5% non-fat dry milk in PBS) for 1 hour. Three ten-minute washes were performed with PBS-T. All Abs were diluted in 5% non-fat dry milk/PBS-T. The blot was trimmed and 1.5ml primary Ab was pipetted to cover the membrane which was lying on parafilm on the bench top. Following this, the wash steps were repeated. Secondary Ab incubation was for 1 hour, followed by a final wash step. A Kodak fixer and developer was used in a darkroom (Kodak D-19). Equal volumes of ECL reagent 1 and 2 were combined and 1.5ml was used to cover the membrane (Santa Cruz Western Blot Luminol Reagent). The membrane was incubated for 1 minute. Excess reagent was removed and the blot was exposed to Amersham ECL Hyperfilm. Exposures were usually for 15 secs and developing for 45 secs, until bands appeared on the film. The film was then washed in dH_20 and fixed for 45 secs until the film was clear. Membranes were also probed with a mouse anti-beta-actin Ab (CP01, EMD Chemicals, USA) and an anti-mouse IgG-HRP secondary Ab (Dako, USA) as a loading control. This was purely for the purposes of demonstrating sample loading (assuming low numbers of host cells would still be present in supernatants) and was not for normalisation. The lower limit of detection of pepsin in sample matrix was 0.5ng.

Table 2-3 Antibodies used for porcine pepsin and human pepsin Western blot assays

Ab	Dilution	Host	Supplier
Anti porcine pepsin (polyclonal)	5000	Goat	Biodesign (USA)
Anti goat/sheep IgG-HRP (monoclonal)	10000	Mouse	Sigma (UK)
Anti human pepsin (polyclonal)	350	Rabbit	Johnston, N (Wisconsin)
Anti-mouse IgG-HRP (polyclonal)	5000	Goat	Dako (UK)

2.5.3 Trichloroacetic acid sample precipitation

Various experimental samples (Chapter 6) required a precipitation step in order to reduce the sample loading volume for subsequent 1D SDS-PAGE gel or Western Blot analysis. Trichloroacetic acid (TCA) precipitation is a widely used method (Jiang, He, & Fountoulakis, 2004).

A stock solution of 60% TCA was prepared. A volume of this stock solution was added to the sample to result in a 5-15% (final v/v) TCA concentration. The sample was gently vortexed and then incubated at 4ºC for 15 mins. Following centrifugation (14,000rpm for 10 mins), the supernatant was removed from the protein pellet and discarded. The protein pellet was washed twice in diethyl ether. Following the second ether wash, the tube was placed in a heating cabinet at 37ºC to evaporate any remaining ether. The protein pellet was then ready for the addition of sample buffer and processing for gel loading, as detailed in the relevant sections.

2.6 Polymerase Chain Reaction

2.6.1 RNA Extraction

Lysate samples were thawed on ice and the 350µl sample volume was applied to a QIAshredder and spin column sitting in a 2ml collection tube (Qiagen Ltd, Crawley, UK). This was centrifuged at 14,000 rpm for 2 mins. An equal volume of ethanol was added to the resulting lysate and mixed. The 700µl sample was then added to an RNeasy mini column, placed in a 2ml collection tube (Qiagen). This was centrifuged at 10,000rpm for 15 secs. The flow through was discarded. Buffer RW1 (Qiagen) was added (350µl) and the centrifugation step was repeated, followed by discarding the flow through. DNase 1 (80µl, prepared by adding 10µl resuspended DNase 1 to 70µl buffer RDD (Qiagen)) was pipetted directly onto the column membrane and incubated at RT for 15 mins. A further wash with 350µl Buffer RW1 was performed followed by centrifugation as previously and replacement of the collection tube. The membrane was then washed twice with 500µl Buffer RPE (Qiagen). Following the second wash, the centrifugation time was increased to 2 mins at 10,000rpm. The flow through was discarded and then the sample was re-centrifuged at 14000rpm for 1 minute to ensure complete buffer removal. The column was transferred to a 1.5ml collection tube and 50µl RNase-free water was pipetted directly onto the membrane, followed by centrifugation at 10,000rpm for 1 minute to elute the RNA. A NanoDrop ND-1000 spectrophotometer was used for RNA quantitation using 1µl sample at 260nm. The A260/A280 ratio was assessed as an indicator of RNA purity (1.8-2.1 being acceptable). The RNA was then stored at -80°C.

2.6.2 Reverse Transcription

Following thawing on ice, 0.2 µg total RNA was used for first strand cDNA synthesis. The oligonucleotide primer was annealed to the RNA by adding 1 µl random primers (0.5 µg/µl Promega, Madison, WI, USA) and nuclease-free H_2O to the volume of RNA to make a total volume of 15 µl in a micro-centrifuge tube. The mixture was incubated at 70°C for 5 mins and then put on ice. For the reverse transcriptase reaction, a master-mix of 1 µl M-MLV reverse transcriptase (Promega), 5 µl 5x M-MLV reverse transcriptase buffer (Promega), 0.5 µl ribonuclease inhibitor (Promega), 1.25 µl nucleotides (10 mM ATP, CTP, GTP, UTP) (Promega) and 2.25 µl nuclease-free H_2O were added to the RNA and

oligonucleotide mixture and incubated at 42°C for 1 hour. The cDNA was then diluted 1:4 (final volume 100µl) with nuclease-free H_2O and stored at -80 ºC until use. No further dilution was needed for the quantitative PCR (qPCR) experiments which followed.

2.6.3 Quantitative Polymerase Chain Reaction

Taqman® gene expression assays (Applied Biosystems, UK) were used for all qPCR experiments. All assays used are detailed in t**able 2.4**. For all reactions, 10µl 2x universal master mix, without UNG (Taqman®), 4µl H_20, 5µl cDNA and 1µl gene expression assay (Taqman®) were used and reactions were run in a 96 well PCR plate. For each assay, a positive control and blank were always included in duplicate. The positive control for IL-8 and IL-6 mRNA expression was BEAS-2B cells exposed to 5µg/ml lipopolysaccharide for 24 hours and harvested for mRNA analysis (a gift from Dr Angela Fonceca, University of Liverpool) (Schulz et al., 2002). The positive control for TGFβ-1 mRNA expression was human lymphocyte cDNA (a gift from Dr Angela Midgely, University of Liverpool) (Kohut et al., 2009). For each sample, the assay of interest and a housekeeping gene assay appropriate to the BEAS-2B cell line (ribosomal protein L32) was run, in order to assess and correct for differences in cDNA loading (Elizur et al., 2007). All mRNA target gene expression was normalised to this internal standard.

Table 2-4 Details of gene expression assays used for qPCR analysis (Applied Biosystems, UK)

Assay	*Assay Identifier*	*Amplicon length*	*Assay Design*
Interleukin-8	Hs00174103_m1	101	Probe spans exons
Interleukin-6	Hs00985639_m1	66	Probe spans exons
TGFβ-1	Hs00998133_m1	57	Probe spans exons
Mitochondrial ribosomal protein L32	H200388310_m1	81	Probe spans exons

A MX3000P® Multiplex Quantitative PCR System (Agilent Technologies) was used and cycling conditions were - hold at 95 ºC for 10 mins followed 40 cycles of 95 ºC for 15 secs and 60 ºC for 60 secs. MxPro software (Agilent) was used for

programming, data collection and analysis. Following viewing of amplification plots and setting of baseline and threshold values, the comparative CT method was used for data analysis. This compares the CT value of the target gene to another reference gene, using the formula $2^{\Delta\Delta CT}$ (Schmittgen & Livak, 2008). This method gives a fold change in mRNA expression rather than absolute quantification. For accuracy, the comparative CT method is reliant on 100% amplification efficiency, which can be assumed for Taqman gene expression assays. (Applied Biosystems, 2012)

2.6.4 Bacterial DNA Extraction and OprL PCR for Pseudomonas aeruginosa

PCR was performed for the detection of *Pseudomonas aeruginosa (P.Aeruginosa)* using a previously validated, specific PCR protocol based on an outer membrane lipoprotein gene (OprL) (DeVos et al., 1997). Analysis was performed on BAL supernatant that had been centrifuged for 5 mins at 6000 rpm and stored at -80 ºC. Bacterial DNA was extracted from 0.2mls of BAL using the DNeasy Blood and Tissue Kit following the Gram Negative Bacterial DNA Extraction Protocol (Qiagen). In order to harvest the bacterial cells, the sample was spun at 14,000 rpm for 10 mins in microfuge tubes. The supernatant was then discarded. Proteinase-K (Qiagen) was added to each sample (20µl) followed by thorough mixing and incubation at 56 ºC overnight. Following 15 secs of mixing using a vortex, 200µl Buffer AL (Qiagen) was added to each sample followed by 200µl 100% ethanol (with mixing in between and after both steps). The mixture was then pipetted into the DNeasy Mini-Spin Column (Qiagen) which was placed in a 2ml collection tube. The sample was centrifuged at 8000rpm for 1 minute, following which the flow through was discarded and the collection tube replaced. Buffer AW1 was added (500µl) and the sample was re-centrifuged at 8000rpm for 1 minute. Following this, 500µl Buffer AW2 was added and the sample was centrifuged at 14000rpm for 3 mins, until dry. The collection tube was carefully removed, ensuring no ethanol carryover. The column was placed in a 1.5ml microcentrifuge tube and 50µl (optimised volume) of Buffer AE was pipetted directly onto the DNeasy membrane. This was incubated at RT for 1 minute and then centrifuged at 6000g for 1 minute for DNA elution. DNA purity was assessed at 260nm using a NanoDrop ND-1000 spectrophotometer using the 260/280 ratio.

The primer sequences were PAL1, 59-ATGGAAATGCTGAAATTCGGC and PAL2, 59-CTTCTTCAGCTCGACGCGACG-39. Primers were custom ordered from Sigma Aldrich (UK), desalted, with a scale of synthesis of 0.025. They were used at 10μM concentration. The PCR mix consisted of 5μl of GoTaq Flexi Buffer (Promega), 0.5μl 10mM dNTPs (Bioline), 0.75μl PAL1/18S forward, 0.75μl PAL2/18S reverse, 2.5μl MgCl2 (Promega), 0.25μl GoTaq Flexi DNA polymerase (Promega), 14.25μl H20 and 1μl DNA per reaction, as optimised by the Medical Microbiology Department, University of Liverpool. All experiments included a positive control of *P. aeruginosa* DNA taken from bacterial colonies (a gift from the Medical Microbiology Department), a known *P. aeruginosa* culture positive CF BAL cDNA sample (stored and processed by identical methods to the study samples), a negative control without cDNA to check for contamination and a negative bacterial control of *Escherichia Coli* DNA taken from bacterial colonies (a gift from the Medical Microbiology Department, Liverpool). All samples were run in duplicate. Previous literature documents a lower limit of detection of 2-3 bacterial cells (DeVos et al., 1997).

In order to confirm adequacy of DNA extraction, PCR for 18S ribosomal RNA (eukaryotic cell) was also undertaken. This assumed that host airway cells were contained in the BAL and hence DNA extracted from these. Since BAL may have been aseptic and hence not containing prokaryotic bacterial DNA, 16S PCR was not performed for these purposes. Primer sequences for 18S PCR were 18S forward TCCGATAACGAACGAGACTC and 18S reverse CAGGGACTTAATCAACGCAA (Sigma UK) and primers were used at a concentration of 10μM.

The thermocycler (Techne) conditions used for both PCRs were 95ºC for 5 mins followed by 30 cycles of 95ºC for 40 secs and 57ºC for 1 minute (annealing) then 72ºc for 2 mins (extension). This is followed by a final 10 mins at 72ºC. The samples were then removed and placed on ice. 10μl of the PCR solution was loaded into a 2.5% agarose electrophoresis gel in a Thermo EC Midicell Primo EC330, in 1 x TBE buffer (10x TBE buffer Tris 108g, boric acid 55g, EDTA disodium salt 9.3g). A 1 kilobase plus DNA ladder (Invitrogen, UK) was used. The gel was run at 90 volts for 45 mins and analysed using an InGenius gel documentation system (Syngene UK, Cambridge, UK) with the accompanying GeneSnap software

2.6.5 Molzym

Personnel in the Molzym laboratories (Bremen, Germany) performed the Molzym SepsiTest™ analysis. Briefly, BAL was processed using the UMD™-Liquid test (Molzym, Bremen, Germany), a variant of the SepsiTest™ kit designed for extraction of DNA and PCR analysis of body liquids other than whole blood. BAL samples (0.2ml) were made up to 1ml with dilution buffer, according to the manufacturer's instructions. The UMD™-Liquid test includes a sample pre-treatment step by which human and/or dead microbial extracellular DNA is degraded, thus leaving DNA from living microbes to be extracted and purified, with a lower detection limit of </= 10 genome equivalents per PCR reaction (Wellinghausen et al., 2009). The PCR analysis comprises of universal rRNA gene-based assay for bacteria. In the case of positive results, amplicons are purified and sequences analysed by Sanger sequencing techniques (Sanger, Nicklen, & Coulson, 1977)). Pathogen identification was made using the online SepsiTest™ BLAST tool. Genus identification was presumed in samples with sequence identities of ≥97% (family identification if <97%) and species identification was presumed in samples with ≥99% (Wellinghausen et al., 2009).

For the majority of our samples, BAL was stored in sterile microfuge tubes and not the recommended Molzym UMD™ sample storage tubes. To ensure that this did not adversely affect results, we prospectively analysed paired BAL samples stored in microfuge tubes and UMD tubes for six patients. We could show that the same microbes were detected, but processing and storing samples in microfuge tubes decreased assay sensitivity, with microbe detection occurring a mean of four PCR cycles later (**Appendix 5**). Subsequently, in this study all PCR analysis was undertaken on BAL samples stored in microfuge tubes.

2.7 Airway epithelial cell culture

BEAS-2B cells (Bronchial epithelial cell line - European cell culture collection) were grown and plated on 96 well plates (Corning Costar) for all airway epithelial cell experiments. Bronchial epithelial growth medium (Lonza, Wokingham, UK) was used and was supplemented with hormones at final concentrations as follows: 50ug/ml Bovine pituitary extract (Invitrogen UK), 0.5ug/ml hydrocortisone (dissolved in 95% EtOH (Sigma Aldrich, Dorset, UK)), 25ng/ml epidermal growth factor (Sigma), 0.5ug/ml epinephrine (dissolved in 10mM HCl (Sigma)), 5ug/ml insulin (dissolved in 4M HCl (Sigma)), 10ug/ml transferrin (dissolved in BEGM (Sigma)), 6.5ng/ml triiodothyronine (dissolved in 1N NaOH (Sigma)), 0.2ng/ml retinoic acid (dissolved in DMSO (Sigma), 50ug/ml gentamycin (Sigma) and 50ug/ml penicillin/streptomycin (Sigma).

Cells were plated in T25 flasks coated with 0.01 mg/ml fibronectin (Sigma UK), 0.03 mg/ml collagen (BD Biosciences Oxford, UK) and 0.01 mg/ml BSA (Sigma). They were seeded at a concentration of 30,000 cells per cm^2 (cell counting as described in Section 2.3.2.1) and incubated in 15ml supplemented BEGM at 37 ºC with 5% CO_2. The media was changed every 48 hours, following visual inspection for normal cell morphology and growth and any signs of contamination by phase-contrast microscopy (**Figure 2.4**)

At 80% confluence, the cells were re-plated. The flask was washed with 2 aliquots of pre-warmed 20ml sterile PBS. Pre-warmed trypsin (3ml 25g/L Sigma) was then added to the flask and it was incubated at 37ºC for three mins. Following this, the trypsin was inactivated with 5ml BEGM. The 8ml mixed solution was removed from the flask using a sterile graduated pipette and put in a 10ml sterile tube for centrifugation at 1300rpm for 5 mins in order to pellet the cells. The supernatant was poured off and the cell pellet was resuspended in 5ml pre-warmed supplemented BEGM. A trypan blue cell count was made (as detailed previously) and the cells were either re-plated onto a pre-coated T25 flask at 30,000 cells/cm^2 or in pre-coated 96 well plates for experimentation purposes.

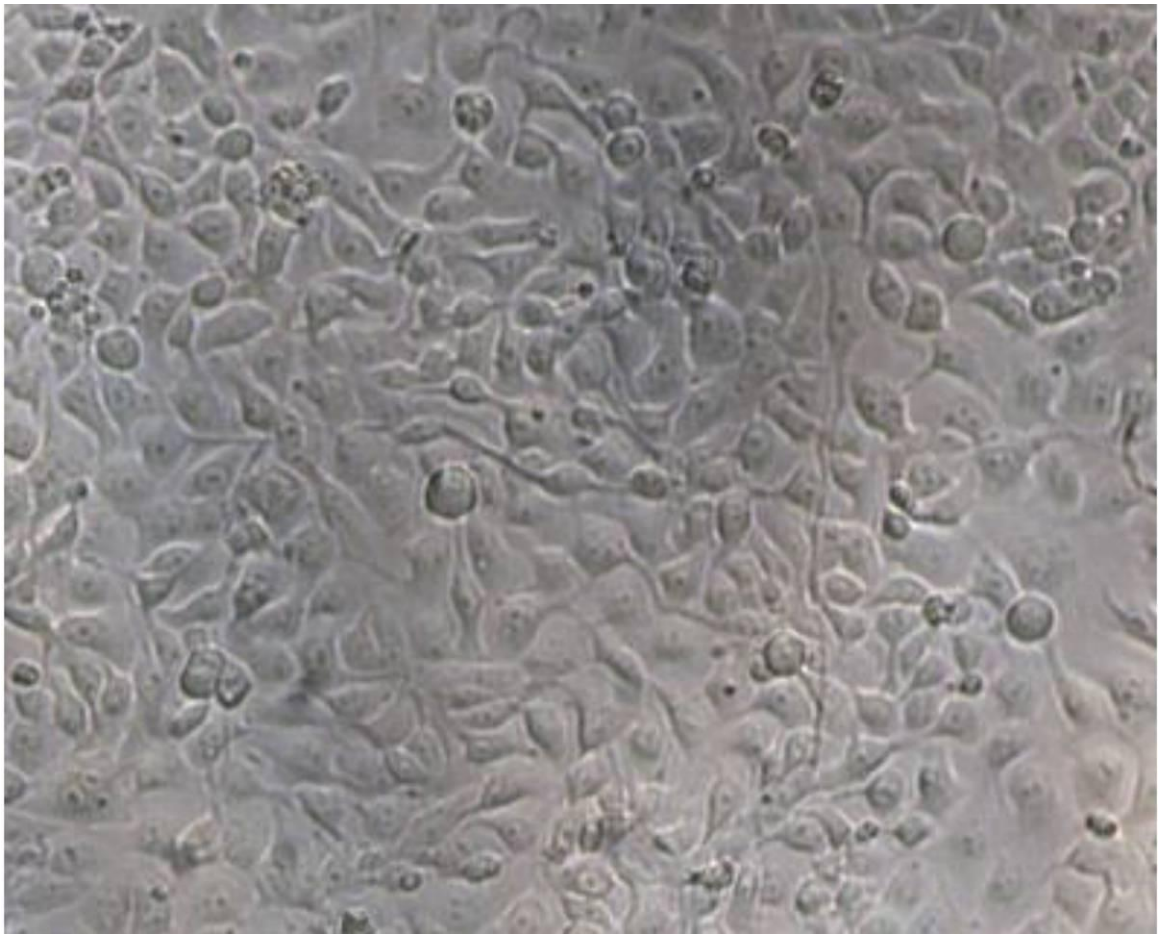

Figure 2.5 Normal morphology of bronchial epithelial cell line (BEAS2B) cells in culture

BEAS2B cells (purchased from the European Cell Culture Collection) were grown in collagen/fibronectin coated T25 flasks in supplemented BEGM media (Lonza)**.** The cells were visualised by phase contrast microscopy.

2.8 Mass spectrometry analysis

2.8.1 In Solution Tryptic Proteolysis

The following protocol formed the basis for in solution digests detailed in this thesis and was adapted according to the specific experiment, as detailed in later chapters.

Up to 100µg of protein was suspended in 25mM ammonium bicarbonate buffer containing a proprietary surfactant (0.05% Rapigest™, Waters, UK) to enhance proteolysis. This was incubated at 80ºC for 10 mins, with a brief mix after 5 mins. Sample reduction was with 3mM dithiothreitol for 10 mins at 60ºC. Alkylation was with 9mM iodoacetamide for 30 mins at RT in the dark. Sequencing grade trypsin (Sigma, UK) was added at a concentration of 0.01µg/µl and samples were digested for a minimum of 18 hours at 37ºC. The reaction was stopped and the surfactant inactivated and precipitated by addition of trifluoroacetic acid to a final volume of 0.5% (v/v), incubated for 30-45 mins at 37ºC. The samples were then centrifuged at 14,000rpm for 20 mins at a temperature of 4ºC to remove any residual precipitate.

2.8.2 Peptide mapping

The theoretical masses of peptides generated by trypsin cleavage of proteins (cleavage at C terminal side of lysine (K) or arginine (R), with exceptions) were calculated using PeptideMass (ExPASy Bioinformatics Resource Portal). Fixed carbamidomethyl modification for cysteine and variable oxidation modification for methionine were specified where applicable.

2.8.3 MALDI-TOF analysis

The matrix solution prepared for all MALDI-TOF analyses was 10mg/ml solution of alpha-hydroxy cinnaminic acid in 50% acetonitrile/0.1% tri-fluoroacetic acid (v/v). On addition of the alpha-hydroxy cinnaminic acid to the solvent, the mixture was vortexed well for 30 secs. It was then centrifuged at 14,000rpm for 10 mins in order to pellet any un-dissolved solid. 1µl trypsin digested sample was applied to a ground steel target plate (Bruker, US), followed immediately by 1µl of matrix solution over the top. This was air dried at RT for ten mins. A peptide

calibration mixture (detailed in **Appendix 6**) was loaded in the same way as a sample.

MALDI-TOF analysis was performed using the Bruker ultrafleXtreme MALDI TOF/TOF mass spectrometer. The calibration spot was analysed first with an initial laser energy set to 30%. The laser energy was subsequently adjusted as required to achieve optimum peak shape and baseline. Samples were then analysed in turn (MS), using a complete sample raster and 500 shots per spectrum. Spectra were analysed using Flex Analysis software (Bruker Daltonics, US). Spectra were searched manually if a specific m/z peak was being sought or the results could be exported and analysed using a MASCOT database search (Matrix Science).

2.8.4 High Resolution Liquid Chromatography

The digest solution was separated using a nanoAcquity UPLC™ system (Waters MS Technologies, Manchester, UK). The sample (of calculated volume for the experiment) was loaded onto the trapping column (Waters MS Technologies, C18, 180 µm X 20 mm), using partial loop injection, for three mins at a flow rate of 5 µl/min with 0.1% (v/v) tri-fluoroacetic acid. The sample was resolved on an analytical column (nanoACQUITY UPLC™ HSS T3 C18 75 µm x 150 mm 1.7 µm column) using a gradient of 97% A (0.1% formic acid) 3% B (99.9% ACN 0.1% formic acid) to 60% A 40% B at a flow rate of 300 nL/minute over 120 mins.

2.8.5 Synapt G1 and G2 mass spectrometry analysis

The nanoAcquity UPLC™ was coupled to either Synapt G1 or G2 mass spectrometers (Waters MS Technologies, Manchester, UK). These mass spectrometers were used (according to laboratory availability) to acquire high definition MS data as described in further detail Chapter 6. They acquired data using a data independent program with 1 second scan times and a collision energy ramp of 15 to 40eV for elevated energy scans. The mass spectrometer was calibrated before use against the fragment ions of glufibrinopeptide and throughout the analytical run at 1 minute intervals using the NanoLockSpray™ source with glufibrinopeptide. The data were processed and database searched using ProteinLynx Global Server v2.5 (Waters MS Technologies, Manchester, UK). The data were processed using a low energy threshold of 100 and an elevated

energy threshold of 20 and the processed spectra were searched against the appropriate database using MASCOT (Matrix Science). The automatic settings in PLGS for the precursor ion and fragment ion mass tolerance were used. The search thresholds used were: minimum fragment ion matches per peptide 3; minimum fragment ion matches per protein 7; minimum peptides per protein 1; and a false positive value of 4. The threshold score/expectation value for accepting individual spectra was the default value in the program, such that the false detection rate value was less than 4.

2.8.6 Xevo™ mass spectrometry analysis (triple quadripole)

The nanoAcquity UPLC™ was coupled to a Xevo™ TQ triple quadrupolar mass spectrometer (Waters), operated in scheduled Multiple Reaction Monitoring (MRM) mode with Q1 and Q3 operating at unit resolution. This analysis method was used for the development of an MRM assay for porcine and human pepsin detection (detailed in Chapter 6). MRM methods were built (including generation of fragment peptide lists and collision energies) using Skyline (MacCoss Lab Software). The program was set to acquire 15 data-points over a 15 sec chromatographic peak and minimum dwell time of 50ms was used. Specific details regarding fragment ions and collision energies used for fragmentation are detailed in Chapter 6**.** Data analysis was with Skyline software (MacCoss Lab Software, US).

2.8.7 LTQ Orbitrap Velos (discovery run MSMS analysis)

For discovery proteomics, the LTQ-Orbitrap Velos system (Thermo Scientific, UK) was used. For each digested sample, 100 ng of protein was injected onto a 75-µm x 150-mm BEH C18 column, and the peptides were resolved over a 120 minute linear organic gradient of 3–40% buffer B (0.1% formic acid in acetonitrile). Data acquisition was data-dependent, with the top twenty most intense peptides in each MS scan selected for fragmentation. The raw data files were processed initially using default parameters (s/n threshold =3, minimum peak count =6) in Proteome Discoverer (Thermo (1.3)) and searched against the Unihuman database in MASCOT (Matrix Science), as described below.

2.8.8 MSMS Database Searches

Using the appropriate software for the analytical system, MSMS data files were submitted to MASCOT (Matrix Science), searching within the required database (for example, Unihuman). The basic search parameters were - fixed carbamidomethyl modification for cysteine and variable oxidation modification for methionine, one trypsin miscleavage allowed, 1.2Da peptide tolerance, 0.6Da MSMS tolerance, 1^+-3^+ charge states and appropriate instrument specified. A minimum ion score was set (as suggested by MASCOT) along with an identity significance value of $p<0.05$.

2.8.9 Progenesis LC-MS Nonlinear Dynamics software

Progenesis LC-MS Nonlinear Dynamics software was used for discovery run proteomic analysis where label free relative protein quantification was required. This data analysis program enables identification and relative quantification of unlabelled proteins across samples. Raw MSMS data files for each sample were loaded into the program. Ion intensity maps were then created from each data file using peak models that retain all quantification and positional information. In order to perform comparative expression analysis, the results for each sample were then aligned by overlaying individual sample ion intensity maps on a chosen reference sample ion intensity map. This enabled correction for run variance in liquid chromatography separation. The aggregate data (containing information from all samples included in the analysis) was then combined and subsequently applied to all samples, allowing 100% peak matching. A complex peak picking algorithm is used and a list of peptide ions detected in the aggregate file was then generated. The top five MSMS peaks for each peptide ion were exported to MASCOT (Matrix Science) and searched in Unihuman as described in Section 2.8.8. The identity significance value was set to $p<0.05$, minimum ion score was set to 30 and a search of a decoy database was used to monitor the false detection rate (aiming for under 4%). The data was then imported back into the Progenesis software. Survey scan ion abundance data were used for quantification, using only unique peptides ions to the protein of interest. Analysis of variance (ANOVA) and q values (method of correction for multiple comparisons) were used for statistical analysis of differences between samples in experimental groups. The q value is a measure of significance according to the

false discovery rate rather than the false positive rate (Storey & Tibshirani, 2003).

2.9 Statistical analysis

All statistical analysis was performed using SPSS for windows version 19 (IBM®).

2.9.1 Clinical Data

Due to the relatively small study numbers, all patient sample data were treated as non-parametric (Altman & Bland, 2009). Standard hypothesis tests were used to determine any demographic differences between the groups, using the Mann Whitney U test for continuous data and the Chi Squared test for categorical data. Data were expressed as median [IQR]. A Mann Whitney U test was used for comparison of continuous data between two groups. Correlation of two sets of continuous data was assessed using Spearman's rank correlation. The Kruskal Wallis test was used for continuous data comparisons across more than two groups.

2.9.2 Cell culture experiment data

All cell line experimental data were assumed to be normally distributed. Data are expressed as mean [SEM]. The student t-test was used for comparison of continuous data between two groups. Within the SPSS analysis, Levene's test of equality of variances was used to assess whether equal variance could be assumed and the appropriate p value was subsequently referenced. A one way analysis of variance (ANOVA) was used where continuous data was analysed across more than two groups, with post hoc analysis to clarify where any differences between groups lay.

2.9.3 The Coefficient of variation

The coefficient of variation (% CV) is a measurement of the variability of quantitative assays. It is a relative standard deviation, which is calculated by dividing the standard deviation of the data points by the mean and multiplying by 100. This can be calculated for sample replicates within the same assay (intra-assay %CV) and also for replicates analysed on different plates (inter-assay %CV). Inter and intra-assay coefficients of variation should be <15% (Food and Drug Administration, 2001).

2.9.4 Graphical presentation of data

Data are presented as bar, line, scatter, vertical scatter or box and whisker plots, created using SPSS for windows version 19 (IBM®) or Graphpad Prism® version 5.

In box and whisker plots, the box represents the inter-quartile range values and the median is marked as a thick line. The whiskers represent the highest and lowest values that are not extreme values. Outliers (values that are between 1.5 and 3 times the inter-quartile range) and extreme values (values that are more than 3 times the inter-quartile range) are represented by circles or asterisks, respectively, beyond the whiskers.

On bar charts, the standard error of the mean is displayed by the error bars. On vertical scatter plots the median or mean value is marked as a horizontal line, as indicated in the legend.

Chapter 3 – Clinical Characteristics

3.1 Introduction

Respiratory disease is a major cause of morbidity and mortality in children with ND, accounting for over 35,000 hospital admissions in the US in 2006 (Berry et al., 2012). Previous studies providing information about respiratory disease in children with ND have largely focussed on incidence of acute deterioration or mortality (Mahon & Kibirige, 2004;Morton, 1999;Reid, 2012;Westbom, 2011;Sullivan, 2006). However, it is likely that the daily burden of respiratory symptoms and the impact of these symptoms on quality of life are equally important issues for children with complex needs (and their carers), yet there doesn't appear to have been a study which has attempted to quantify and characterise these (Marks, 2008). This may be partly explained by the challenges of objective respiratory assessment in these children. A reliable, non-invasive measure of patient/parent reported respiratory symptomatology, such as a respiratory symptom score, could potentially be an extremely useful clinical and research tool for use in children with ND. No validated respiratory score for assessment of symptoms currently exists for this group.

Clinical management of respiratory symptoms in these children is challenging because it is complex to differentiate which factors cause their symptoms. Thus, it is important to consider respiratory symptoms alongside other potentially contributory co-morbidities. Level of mobility, swallowing dysfunction, GORD, chronic lung disease of prematurity and obstructive sleep apnoea all potentially impact on respiratory health in this group (J. L. Hutton et al., 2000; Marks, 2008; Morton, Wheatley, & Minford, 1999; Seddon & Khan, 2003; K. Weir et al., 2009). In view of this complexity, multi-disciplinary respiratory assessment would be the ideal, aiding early identification of modifiable contributory factors and thus maximising respiratory health (Waespi et al., 2000, Piccione, 2012). However, the evidence base supporting this approach is limited and indeed, much of respiratory management in children with ND is anecdotal. This can in part be explained by the lack of suitable outcome measures for high quality clinical trials (Cella et al., 2007; Rajendu Srivastava et al., 2009). In some centres, patients have access to multi-disciplinary care in specialist neuro-respiratory clinics (Waespi et al., 2000). However, regional service provision and clinical management are variable and there is evidence that this may influence outcome (Westbom et al., 2011).

This chapter describes the clinical characteristics of children with severe ND admitted to a large tertiary children's hospital both at a time of respiratory deterioration (on admission to PICU) and when otherwise well (on admission for elective surgical procedures). Respiratory symptoms over the preceding three months were assessed using a respiratory symptom score (LRSQ), previously validated in other paediatric groups and modified for use in this study (Section 2.2.1). Symptoms of gastro-oesophageal reflux disease (GORD) were assessed through use of a set of five questions, modified from the Reflux Symptom Index, as described in Section 2.2.2. Clinical data on previous interventions and current management were recorded for all study recruits.

3.2 Aims

In order to assess the burden of respiratory symptoms, incidence of other co-morbidities and current management, the aims of this chapter were to;

1) Assess respiratory symptoms in children with severe ND at a time of stability and at a time of respiratory deterioration (on admission to PICU) compared to healthy controls, using a modified respiratory symptom questionnaire (LRSQ-Neuro).

2) Document clinical information about swallowing dysfunction, salivary management, GORD, symptoms of aspiration, surgical interventions and where possible, assess the relationship of these to respiratory symptom scores.

3) Document any previous specialist respiratory assessment and intervention in ND patients.

3.3 Specific methods

The data collection proforma used to document clinical information is shown in **Appendix 1**. Respiratory symptoms were assessed using a respiratory symptom score, which had previously been validated in other paediatric groups (Powell et al., 2002; Trinick, Southern, et al., 2012). No specific respiratory symptom score exists for children with ND and therefore, the LRSQ was modified for the purposes of this study, as described in Section 2.2.1. The questionnaire is included in **Appendix 3** and a summary of the symptoms assessed is provided in **Table 3.1**. Symptoms of GORD were assessed using modified questions taken from the Respiratory Symptom Index, as detailed in Section 2.2.3 (Belafsky et al., 2002). The questions are included in **Appendix 4**. Statistical analysis was performed as described in Section 2.9. All data were treated as non parametric due to sample size.

Table 3-1 Summary of symptoms assessed by the LRSQ-Neuro

Questionnaire Domain	Symptoms Assessed
Daytime symptoms, Symptoms with colds, Interval symptoms (between colds)	Cough, wheeze, shortness of breath, 'rattly chest'
Night-time symptoms	Cough, wheeze, shortness of breath, 'rattly chest', snoring
Other symptoms	Noisy breathing not from chest, noisy breathing from throat, fast breathing
Effect on child	Feeding disturbance, sleep disturbance, fatigue
Effect on family	Family activities, adjustment to family life, sleep disturbance, worry/anxiety

3.4 Results

3.4.1 Clinical characteristics

Nineteen children with ND were recruited at a time of respiratory deterioration on PICU (PICU-ND) and 16 children with ND were recruited when otherwise well, on admission for routine elective surgery (Elective-ND). Eleven healthy control children were also recruited. There were no significant differences in median age or gender between the groups (**Table 3.2**). In the PICU-ND and Elective-ND groups, 42% and 56% of children had cerebral palsy, respectively. The remaining children had other central neurological diagnoses. All children with ND were in Gross Motor Function Classification Group V. Half of the children in the Elective-ND group had a history of prematurity vs. 26% in the PICU-ND group. The median [IQR] time to recruitment and sample collection following PICU admission was 15 [12-22] hours.

Table 3-2 Study Participant Demographics

No significant differences in age (p=0.175) or gender (p=0.383) were found between the groups included in the analysis

	Healthy Controls	***Elective-ND***	***PICU-ND***
N number	11	16	19
Age (years)	5 [2-9]	10 [2.25-14.5]	7 [5-11]
Gender (% male)	50	50	46
GMFC IV (%)		0	0
GMFC V (%)		100	100
Cerebral palsy (%)		56	42
Other 'central' cause of ND (%)		44	58
Ex-premature (%)		50	26

3.4.2 Incidence of surgical feeding or anti-reflux interventions

Previous surgical feeding or anti-reflux interventions were recorded for all ND patients. A total of 12 patients had no prior intervention, 14 patients had undergone gastrostomy alone, 8 patients had a gastrostomy and fundoplication and 1 patient had a gastro-oesophageal dissociation (included from this point in the fundoplication group). Overall, more PICU-ND patients (18/19) had undergone a surgical intervention than Elective-ND patients (5/16) ($p<0.001$ **Figure 3.1a**).

3.4.3 Gastro-oesophageal reflux disease

Assessment of the impact of GORD in study recruits was made firstly, through documentation of current and previous anti-reflux interventions. Secondly, symptoms of GORD over the previous three months were recorded through enquiry about five symptoms, derived from the Reflux-Symptom Index (Belafsky et al., 2002).

Twenty out of thirty-five children with ND were currently receiving anti-reflux medication and overall 24/35 had received a surgical and/or medical intervention for treatment of GORD. 5/9 children who had undergone a surgical anti-reflux procedure continued to take anti-reflux medication **(Figure 3.1b)**. Anti-reflux medication prescribed included Domperidone (13/20), Omeprazole (13/20), Gaviscon (2/20), Ranitidine (5/20) and feed thickeners (1/20).

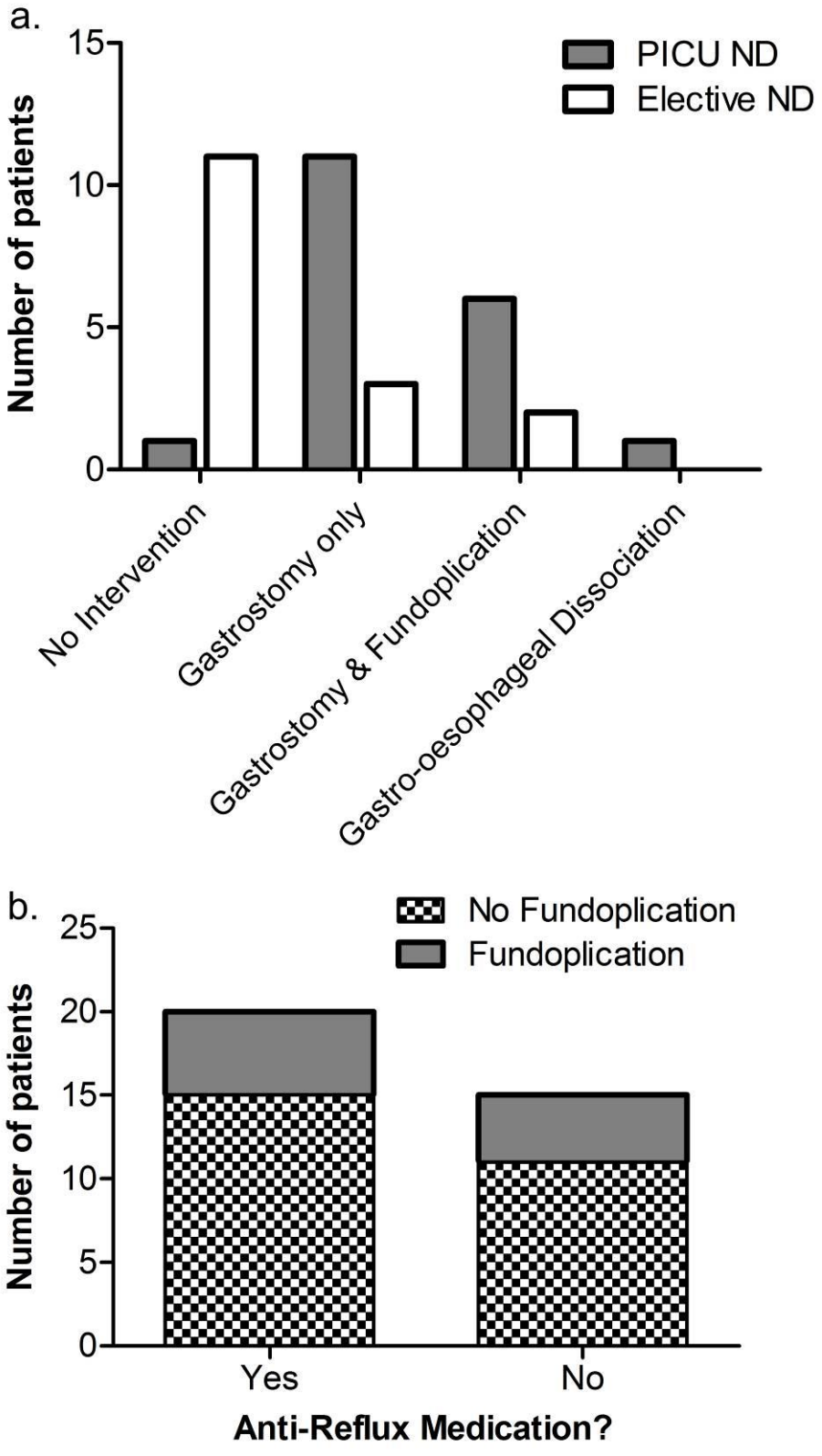

Figure 3.1 Histograms to show frequency of (a) surgical and (b) medical anti-reflux interventions in ND patients

Current medication and previous surgical intervention were recorded. Graph (a) shows frequency of surgical feeding and/or anti-reflux intervention between ND groups and graph (b) shows frequency of medical anti-reflux treatment in all children with ND. The bar division highlights the frequency of fundoplication within the two medical treatment groups.

Parents answered five questions about GORD symptoms on a Likert scale, scored from zero (not at all) to four (symptoms every day). Therefore, the maximum score possible was twenty. Median PICU-ND (5[2-8]) and Elective-ND scores (7[3-10]) were significantly higher than median healthy control scores (0[0-0], $p<0.001$, **Figure 3.2**). 'Cough with or following feeds', 'choking with or following feeds' and 'vomiting with or following feeds' were the most commonly reported symptoms (25/35, 19/35 and 19/35 patients, respectively). **Table 3.3** contains a summary of individual question scores for the ND groups.

Table 3-3 Median [range] scores for questions about GORD symptoms in ND patient groups

Symptom	PICU-ND	Elective-ND
Cough	1 [0-4]	2 [0-4]
Shortness of breath	0 [0-3]	0 [0-3]
Choking	0 [0-3]	2 [0-4]
Vomiting	1 [0-4]	0.5 [0-4]
Pain	0 [0-4]	0 [0-4]

No significant differences were observed in total score between PICU-ND vs. Elective-ND patients ($p=0.464$), ND patients who had a gastrostomy and fundoplication ($n=9$) vs. gastrostomy alone ($n=14$, $p=0.326$), ND patients who were orally fed ($n=14$) vs. non-orally fed ($n=21$, $p=0.245$), and ND patients on anti-reflux medication ($n=20$) vs. no anti-reflux medication ($n=15$, $p=0.441$).

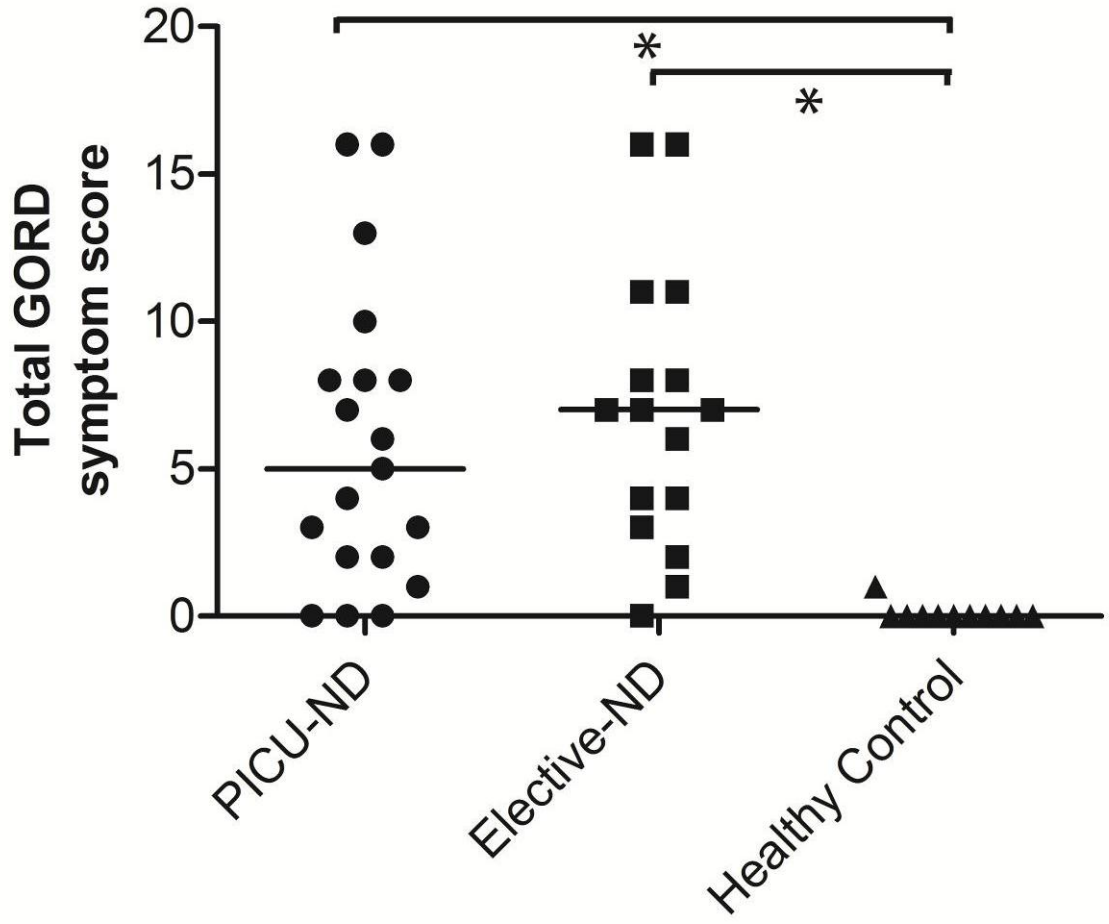

Figure 3.2 Vertical scatter graph to show GORD symptom scores for all study groups

Recruits were asked to answer questions about five symptoms of GORD on a likert scale from 'not at all' (score zero) to 'symptoms every day' (score four). Total scores are displayed for study groups. The median score is indicated by the horizontal line. * $p<0.001$

3.4.4 Swallowing assessment, oral feeding and medical management of salivary pooling

Previous VFS results were recorded for all ND patients. Overall, 13/19 PICU-ND patients and 8/16 Elective-ND patients had undergone VFS assessment. Of those who underwent VFS examination, 10/13 PICU-ND patients and 6/8 Elective-ND patients were positive to some degree (clear aspiration or transient laryngeal penetration). Few patients in the PICU-ND or Elective-ND group were prescribed glycopyronnium bromide or hyoscine hydrobromide for control of salivary pooling (5/19 (26%) and 3/16 (19%), respectively).

A higher proportion of PICU patients were nil by mouth and this was not necessarily guided by VFS results; 3 children with negative VFS results were exclusively gastrostomy fed ($P<0.01$, **Figure 3.3**). Possible explanations include clinical symptomatology or feed aversive behaviour. The limitations of VFS assessment are discussed in Chapter 1. Six out of eighteen children were orally fed despite a degree of aspiration being identified on VFS (**Figure 3.3**).

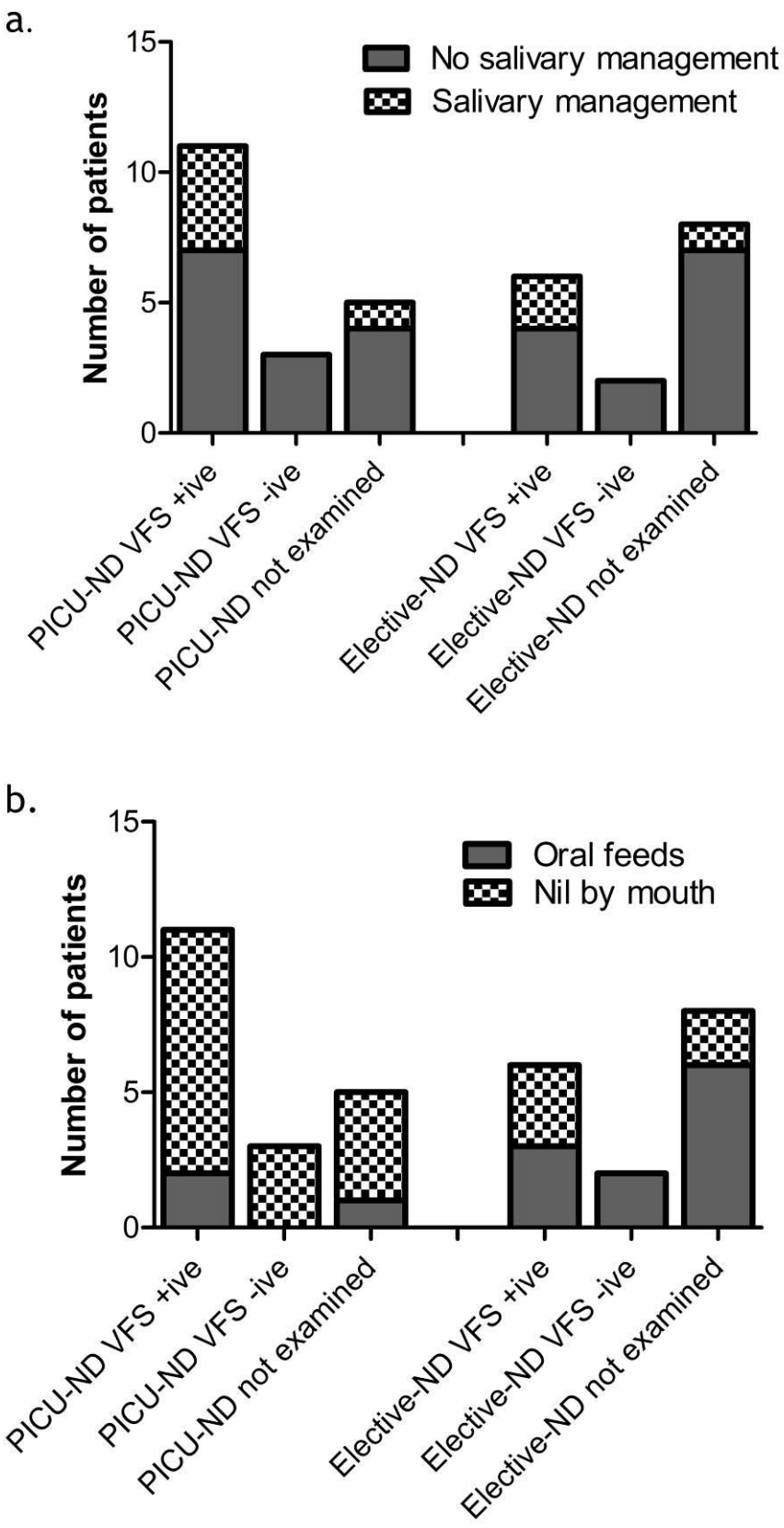

Figure 3.3 Histograms showing VFS results and related clinical interventions for ND patients

Clinical data were recorded and where possible, results of clinical investigation were confirmed in the clinical notes. Graph (a) shows the frequency of VFS assessment and outcome between ND groups. The bar division highlights frequency of salivary medical management within these groups Graph (b) shows the frequency of VFS assessment and outcome between ND patients. The bar division highlights the frequency of oral feeding.

3.4.5 Respiratory symptom scores

As expected, the PICU-ND group reported most respiratory symptoms over the three months prior to admission to hospital, with median [IQR] LRSQ-Neuro scores of 55[38-64]. However, high levels of respiratory symptoms were also reported in the Elective-ND group compared to healthy controls (Elective-ND, 26[7-45] vs. Control, 4[0-7], $p<0.01$). Each questionnaire domain contained between three and five questions which were scored on a Likert scale from 'not at all' (score zero) to 'every day' (score four). Individual domain scores in Elective-ND patients were significantly higher than healthy controls across all domains, except 'Symptoms with colds'. 'Effect of symptoms on the family' ($p=0.001$), 'Other symptoms' ($p=0.001$), and 'Night-time symptoms' ($p=0.004$) domain scores reached the highest levels of statistical significance (**Figure 3.4**)

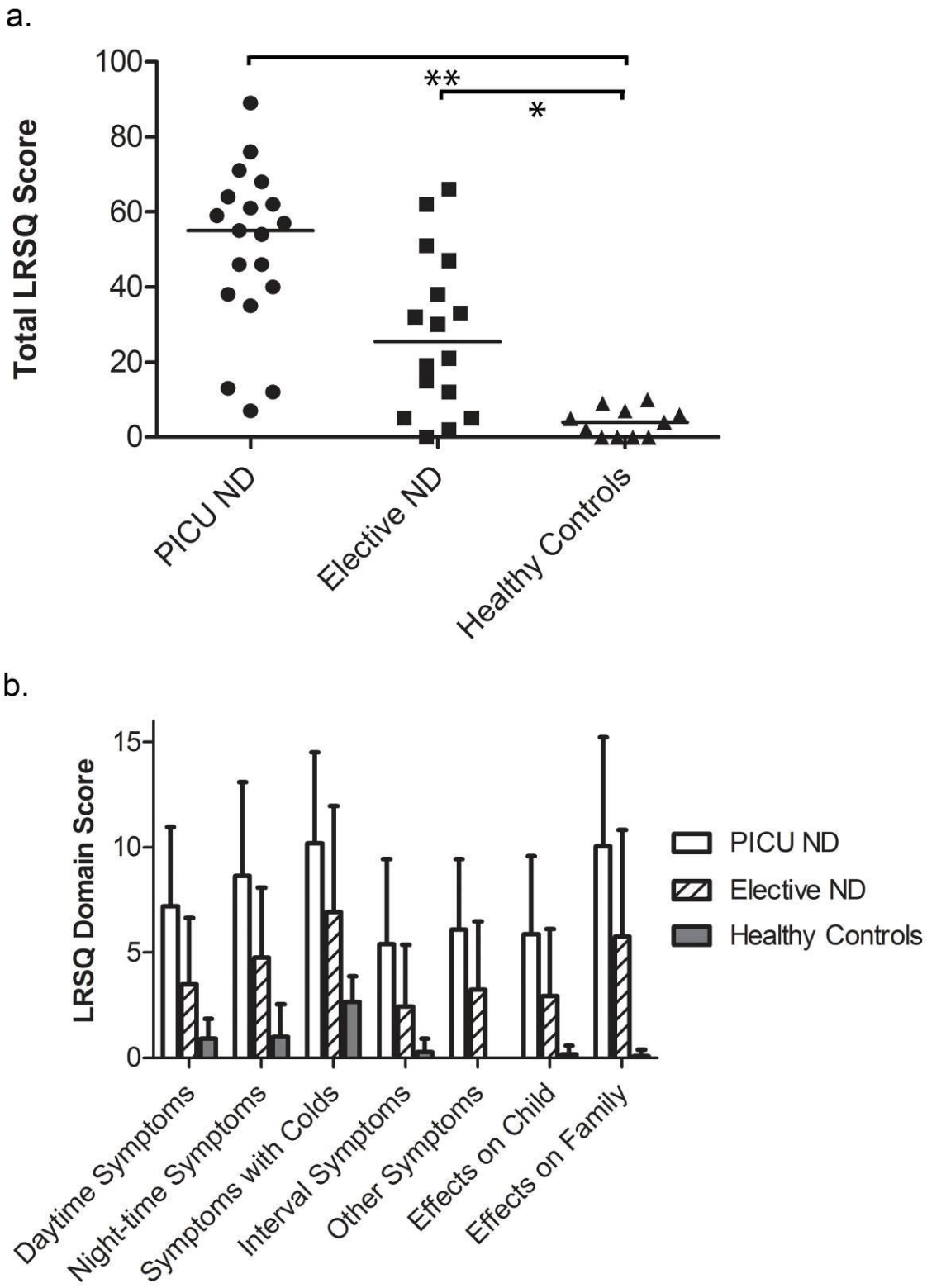

Figure 3.4 (a.) Vertical scatter graph to show total LRSQ-Neuro scores across study groups and (b.) bar chart to show individual LRSQ-Neuro domain scores between groups

All study participants completed a respiratory symptom score (LRSQ-Neuro) at the time of recruitment. Graph (a) shows total scores. The median total score is indicated by the horizontal line. Graph (b) shows individual domain scores. Mean and SEM are displayed for each group. Questionnaire domains contained between 3 and 5 questions, each question scored on a likert scale from 'not at all' (score zero) to 'every day' (score four).

3.4.6 Relationship of LRSQ-Neuro scores and surgical feeding and/or anti-reflux interventions

Significantly higher LRSQ-Neuro scores were observed in children who had undergone surgical feeding or anti-reflux interventions. Median total LRSQ-Neuro score was 17[5-32] in ND patients with no intervention vs. 37[13-55] in patients with gastrostomy only (p=0.001) and 40.5[7-62] in patients with gastrostomy & fundoplication (p=0.003, **figure 3.5**).

3.4.7 Relationship of LRSQ-Neuro scores and VFS results

Significantly higher total LRSQ-Neuro scores were recorded in Elective-ND children who had a positive VFS result (38[32-62], n=6) vs. those who had not been examined (13.5[5-20.5], n=8, p<0.05, **Figure 3.5**).

3.4.8 Other comparisons

A positive correlation was observed between GORD symptom score and total LRSQ-Neuro score in Elective-ND patients (r+0.66, P<0.01). Total LRSQ-Neuro scores were also significantly higher in Elective-ND patients who were taking anti-reflux medications (n=8) vs. those who were not (n=8, 35.5[13.75-59.25] vs. 18[2.75-31.5] respectively, p=0.028). No relationship was seen between history of prematurity and total LRSQ-Neuro score (p=0.959) or prescription of medications for control of salivary pooling and total LRSQ-Neuro score (p=0.269) in Elective-ND patients.

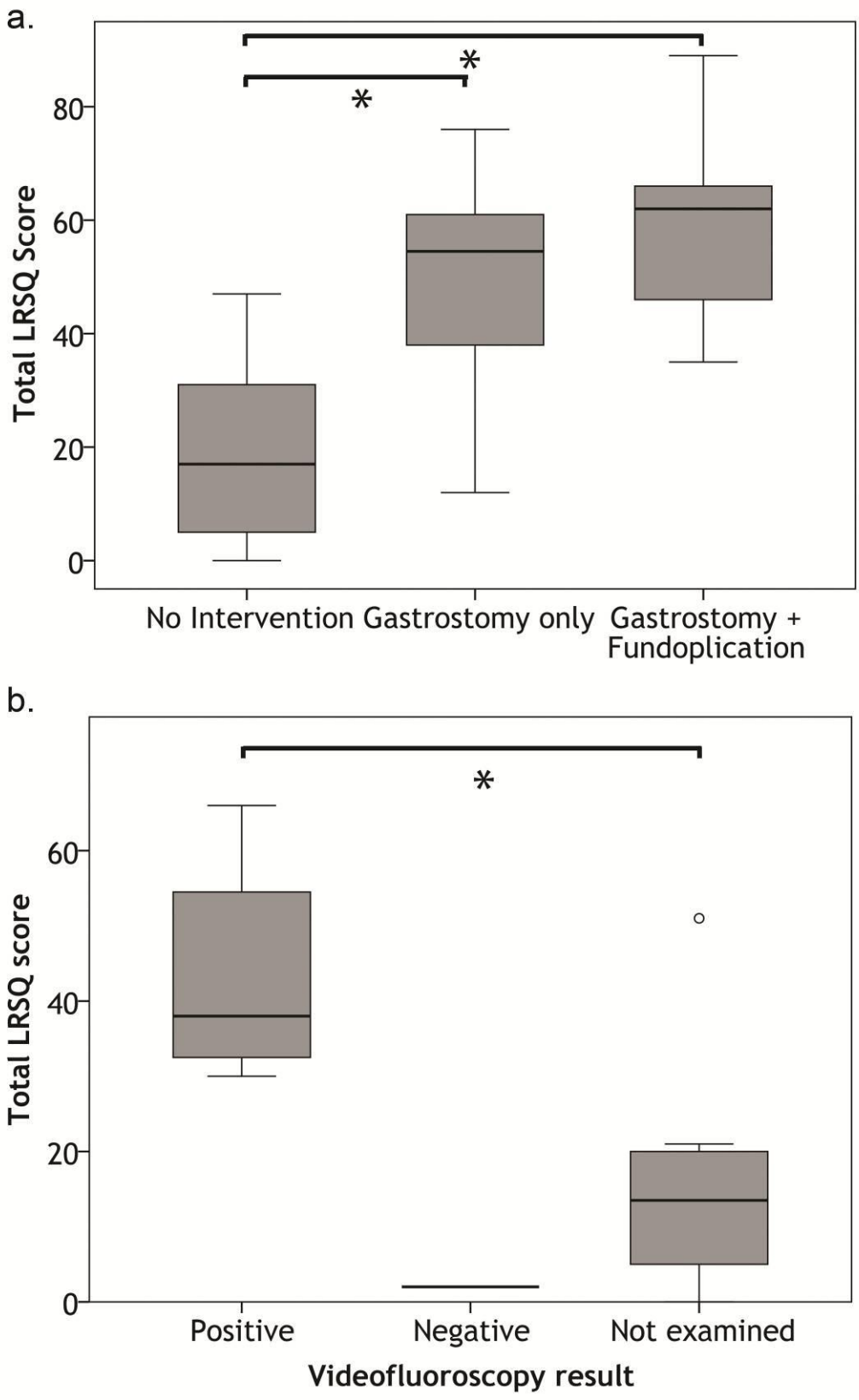

Figure 3.5 Box and whisker plots to show (a) total LRSQ-Neuro scores for all ND patients grouped according to surgical feeding anti-reflux interventions and (b) total LRSQ-Neuro scores for Elective-ND patients grouped according to VFS examination results

All study participants/families were asked to complete the LRSQ-Neuro on recruitment to the study. Clinical data regarding previous surgical interventions and VFS investigation were recorded on data collection proformas. Graph (a) * p=<0.01 Graph (b) * p<0.05

3.4.9 Previous respiratory input in children with ND

Table 3.4 shows some of the microbiological and clinical characteristics of the Elective-ND and PICU-ND patients. Most PICU-ND patients had previous and/or current admission airway microbiology results. 12/19 had cultured *P. aeruginosa* (some on multiple occasions) on sputum or tracheal aspirate samples. 5/19 cultured *P. aeruginosa* on tracheal aspirate on admission to PICU. Few patients were receiving anti-pseudomonal treatment. Most ND patients had never received a specialist respiratory paediatric opinion. Many of those being cared for by respiratory specialists were on home non-invasive ventilation or home oxygen. 10/19 PICU-ND patients were prescribed prophylactic antibiotics; treatment varied and included azithromycin (n=4), amoxicillin (n=2), co-amoxiclav (n=1), trimethoprim (n=2), cefaclor (n=1). Most PICU-ND patients had been on treatment doses of antibiotics for more than 24 hours prior to admission. Only 1/16 Elective-ND patients had previous airway microbiology results. This patient had previous *P. aeruginosa* detected on multiple sputum and tracheal aspirate samples. 2/16 Elective-ND patients were prescribed antibiotic prophylaxis (cotrimoxazole (n=1), trimethoprim (n=1)).

Table 3-4 Table to show airway sample *P.Aeruginosa* microbiology results and current respiratory management in ND patients

	Elective-ND	***PICU-ND***
Previous tracheal aspirate (ta) or sputum (s) samples M,C&S	1/16 (ta)	16/19 (13 ta, 3 s)
Previous *P.Aeruginosa* +ive	1/1	12/16
***P.Aeruginosa* +ive current admission**	N/A	5/16
Specialist respiratory paediatric care	1/16	7/19
Home oxygen	0/16	7/19
Home non-invasive ventilation	0/16	3/19
Prophylactic antibiotics	2/16	10/19
Anti-pseudomonal therapy	1/16	2/19
Treatment dose antibiotics at recruitment (>24 hours)	0/16	3/19

3.5 Discussion

This is the first detailed characterisation of respiratory symptoms in individuals with severe ND. Importantly, data presented within this chapter has shown that otherwise well children with ND have significant respiratory symptoms, particularly at night, that affect their families. The incidence of co-morbidity in this group is high and includes GORD, feeding difficulties and oral-motor discoordination. Respiratory symptom scores appear to correlate with some of these co-morbidities, although it is important to acknowledge that these are retrospective associations and may purely reflect severity of disability.

Although it is well known to paediatric healthcare professionals that children with severe ND often have chronic respiratory symptoms, this is the first time that they have been formally quantified and documented. It is perhaps no surprise that children admitted to PICU with respiratory deterioration were more symptomatic in the three months leading up to their hospitalisation. However, it is noteworthy that Elective-ND children, considered well enough by both parents and anaesthetists to undergo a general anaesthetic, also had significant respiratory symptoms. *Night-time* symptoms were especially prominent and predictably correlated with *Effect on Family* scores ($p<0.001$). Interestingly, *Other* domain scores, which covered extra-thoracic/back of throat symptoms (symptoms previously shown to be important in predicting aspiration), were also significantly different.

Recruitment targets were not achieved due to dependence on one researcher to recruit all patients, limiting recruitment opportunities. This may have reduced the power of the study to detect differences between study groups (increased type II errors), a significant limitation. All of the study recruits were in GMFC group V, indicating a high level of physical impairment across both study groups. However, the PICU-ND group had a higher incidence of surgical feeding and anti-reflux interventions, reflecting a higher level of co-morbidity in this group. This bias is difficult to avoid when selecting study recruits from PICU.

GORD is a well recognized phenomenon in children with ND and has previously been reported to affect up to 75% of those with severe cerebral palsy (Gustafsson & Tibbling, 1994; Mazzoleni et al., 1991). Data from this study were

in agreement, 69% of children with ND requiring GORD management. Interestingly, despite interventions, symptoms suggestive of reflux remained significantly higher in both ND groups when compared with healthy controls, highlighting the complexity of GORD management in children with ND. Initiation of any anti-reflux medication must be well considered in light of the limited evidence base and weighed against the possibility of side effects (Vandenplas et al., 2009). However, interpretation of the available clinical evidence is particularly difficult when treating children with ND, as this group have sometimes been actively excluded from studies of GORD treatment in order to limit the complexity of analysis (Peter B. Sullivan, 2008). In this study, the most commonly prescribed medications were proton pump inhibitors and the pro-motility agent, domperidone. Whilst there is some evidence in support of PPI use in gastroenterological symptom control, there is little robust evidence to support the use of domperidone (Hassall, 2005, Pritchard, 2005). Unfortunately, no conclusions can be drawn about the efficacy of medical reflux management from this study as data collection was retrospective. Although some GORD symptoms clearly persist in many, it is possible that patients were more symptomatic prior to commencing anti-reflux medications.

Children with ND are commonly referred for assessment for surgical anti-reflux procedures when GORD is considered to be pathological and symptoms persist despite medical management. Fundoplication remains the commonest surgical anti-reflux intervention (Lasser et al., 2006). In this study, 26% of ND recruits had undergone a surgical anti-reflux procedure. The complex relationship between GORD and respiratory symptoms makes assessment of the child with ND who predominantly has respiratory symptoms particularly challenging. In this study, analysis of respiratory symptoms in children with ND based on previous surgical intervention (nil, gastrostomy, gastrostomy & fundoplication) revealed a persistently high level of respiratory symptoms in those who have had both gastrostomy & fundoplication. Although these data cannot inform the debate regarding the efficacy of fundoplication for respiratory symptoms (due to recruitment bias), they do highlight the possibility that fundoplication may not always provide their complete resolution and many children continue to have a high level of respiratory symptoms despite gastrostomy & fundoplication.

Children may undergo gastrostomy placement either for feeding/nutritional difficulties or when there is concern about safety of swallow co-ordination. It is recommended that an anti-reflux procedure is considered at the time of this intervention if there are symptoms or signs of GORD. Forty percent of ND participants in this study had a gastrostomy without an anti-reflux procedure. Some literature has suggested that placement of gastrostomy alone is associated with an increase in GORD and respiratory morbidity (Jolley, Smith, & Tunell, 1985; Strauss et al., 1997). However, more recent prospective studies have found no such increase in respiratory symptoms (P. B. Sullivan et al., 2005; P. B. Sullivan et al., 2006). Although an increase in GORD is observed in a small proportion of children, this is not significant enough to recommend universal anti-reflux procedures (Burd, Price, & Whalen, 2002; Viswanath et al., 2010; Wilson, van der Zee, & Bax, 2006;Langer, 1988). The optimal management of GORD post gastrostomy placement is debated within the literature. A recent Cochrane review (2007) concluded that the evidence comparing the efficacy of fundoplication vs. post operative medical management for those patients with GORD undergoing gastrostomy insertion is not robust and that high quality studies are needed (Vernon-Roberts & Sullivan, 2007). In this study, no difference was found in GORD symptom reporting or total LRSQ-Neuro score between those who had a gastrostomy alone (n=15) vs. those who had a gastrostomy & fundoplication (n=9), although this data is limited as it is retrospective.

A significant correlation was observed between symptoms of GORD and LRSQ-Neuro scores in Elective-ND patients. As discussed in Chapter 1, respiratory symptoms and complications of GORD include cough, shortness of breath, wheeze, and recurrent lower respiratory tract infection (Boesch et al., 2006). However, it can be difficult to establish whether the respiratory symptoms are due to GORD (through direct stimulation of the lower oesophagus or recurrent micro-aspiration), whether they are unrelated or alternatively, whether the respiratory symptoms themselves are causing or increasing gastro-oesophageal reflux episodes. Therefore, although a relationship is frequently observed it can be difficult to draw any conclusions regarding causation. Finally, there is some overlap between respiratory symptoms and GORD symptoms and therefore, it is

perhaps unsurprising to observe a correlation between GORD and respiratory symptoms.

The high incidence of oral-motor dysfunction in children with ND is widely documented and swallowing assessment is advocated for children with ND who have feeding difficulties or symptoms of aspiration (Reilly, Skuse, & Poblete, 1996 Weir, 2009). In this study, it was notable that over 75% of children who had undergone VFS were found to have clear aspiration or transient laryngeal penetration in both the Elective-ND and PICU-ND groups. Interestingly, 5/16 children with positive VFS results had undergone VFS assessment on multiple occasions and had inconsistent negative and positive results. This highlights the limitation of VFS assessment in providing a limited 'snap shot' assessment of swallow co-ordination. Subsequently, there is potential to under estimate aspiration risk.

Respiratory symptoms were significantly higher in the Elective-ND group who were positive by VFS compared to those who had not been assessed. It seems likely that these children were selected for assessment on the basis of their respiratory symptoms or symptoms of aspiration. This highlights the necessity of careful history taking and structured symptom documentation in these complex patients. However, it is also important to recognise that many children with ND aspirate 'silently' (Mirrett et al., 1994; K. A. Weir et al., 2011). Within the VFS positive group in this study, 66% reported cough with feeds (33% most or every day), 61% reported choking with feeds (39% most or every day) and 78% reported noisy breathing coming from the back of the throat (44% most or every day), a symptom known to be sensitive for aspiration of thin fluids (K. A. Weir et al., 2011). Some children who had not been assessed by VFS also exhibited these symptoms whereas some children who were positive by VFS never or variably reported these symptoms. This may have been due to silent aspiration or management post VFS by gastrostomy feeding or thickened feeds, with subsequent improvement in symptoms. Information on feeding intervention directly following VFS was not consistently available and therefore, has not been included. It would be interesting to study respiratory symptom scores prospectively, pre and post any feeding intervention associated with VFS assessment, to assess the direct impact on respiratory symptoms.

Some parents chose to continue oral feeding despite positivity on VFS assessment. Interestingly these six children had significantly lower LRSQ-Neuro scores than those who were VFS positive but nil by mouth (29[4.5-58] vs. 54.5[36.7-62.5], $p<0.001$). Modifications to feeds can be made to minimise aspiration risk, for example, alteration of food consistency or addition of thickeners to fluids. The severity of respiratory symptoms may well influence parental choices, particularly if the child gains pleasure from oral feeding.

The evidence base for the management of salivary drooling and aspiration is limited and studies have generally focussed on drooling outcomes rather than respiratory outcomes (Walshe et al., 2012). The options for management include pharmacological (anti-cholinergic medications) and surgical interventions. In this study, very few patients were prescribed anti-cholinergic medications (8/35) and none had undergone surgical interventions. Three patients were prescribed Glycopyrrolate, four were prescribed hyoscine hydrobromide and one was prescribed both of these medications. The documented trials of these drugs have methodological limitations but generally conclude that statistically significant improvements are seen in drooling with glycopyrrolate or hyoscine hydrobromide treatment in ND patients. However, anticholinergic side effects including dry mouth, constipation, vomiting and behavioural changes were all reported and sometimes resulted in withdrawal of treatment (Eiland, 2012; Garnock-Jones, 2012; Mato et al., 2010; Walshe et al., 2012; Zeller, Davidson, et al., 2012). A recent survey of paediatricians would suggest that few refer for surgical interventions (such as submandibular botulinum toxin injection or parotid duct ligation) due to limited resources (Parr et al., 2012). The evidence base for such interventions was found to be limited by a recent Cochrane review (Walshe et al., 2012). It is likely that salivary pooling was a significant issue for many children with ND who participated in this study. It is possible that the low frequency of active management was due to the limited evidence base to support interventions and concerns about side effects (Fairhurst & Cockerill, 2011). Prospective comparative studies between pharmacological interventions and also between medical and surgical interventions are needed and it would be useful to include structured respiratory outcomes. The relative contribution of salivary aspiration to respiratory symptoms in children with ND is unclear however it was notable that those who were positive on VFS examination also

had significantly higher total LRSQ-Neuro scores. This association however, does not establish causality and respiratory symptoms may well have been the reason for selection for VFS assessment, thus introducing bias.

Contributory factors to respiratory morbidity are likely to be multi-factorial and therefore, it would be beneficial for children with persistent respiratory symptoms to be assessed by a multidisciplinary respiratory team. In this study, only 1/16 patients in the elective group and 7/19 PICU patients had received specialist respiratory input. Notably, the majority of those receiving input were either receiving home oxygen or non-invasive ventilation, suggesting that this group of patients are referred to respiratory paediatricians late in the disease process. Our data suggests that clinicians should be more active in identifying and assessing these patients. Future studies should focus on the outcomes of early respiratory intervention in terms of child and family quality of life.

This data is limited as it is retrospective and therefore, cause and effect cannot be established. However, it is useful in highlighting the complexity of this patient group, the need for structured multidisciplinary assessment and most importantly, in directing focuses for future prospective studies.

3.6 Conclusions

Children with severe ND often have high levels of chronic respiratory symptoms and complex co-morbidities. Early, tailored multi-disciplinary respiratory assessment may be useful in assessing and managing potentially modifiable contributory factors. Future studies need to prospectively assess the complex interactions of co-morbidities and respiratory symptoms with interventional studies using carefully selected, validated outcome measures.

Chapter 4 Lower airway pathology in children with severe ND

4.1 Introduction

Despite recognition of high levels of respiratory morbidity and mortality in children with ND, the underlying pathogenesis is poorly investigated within the literature (Reid et al., 2012; Westbom et al., 2011). The postulated cycle of recurrent aspiration, infection and chronic inflammation leading to airway damage is entirely based on clinical observation of a patient group who are inherently difficult to assess (Seddon & Khan, 2003). Aspiration and recurrent lower respiratory tract infections, although difficult to diagnose, are known to be common clinical problems in children with ND (Morton et al., 1999; Young et al., 2011). Piccione *et al* recently documented a high incidence of bronchiectasis (66%) in a large population of children with swallow study or airway endoscopy confirmed aspiration, occurring from an early age. Severe ND was identified as a significant risk factor for bronchiectasis on chest imaging (Piccione et al., 2012).

However, little is known about lower airway microbiology or levels of inflammation in children with ND, either acutely during a respiratory exacerbation or when otherwise well. No previous studies have sought to analyse lower airway samples in order to further define and characterise the relative roles of aspiration, infection and chronic inflammation and their potential relationship to respiratory symptoms and quality of life. This approach may give direction for new therapeutic targets and expand the limited evidence base for management of these patients who currently are often treated anecdotally.

In this chapter, several lower airway inflammatory markers are studied; bronchial lavage differential cell counts, Interleukin-8 (IL-8) and Transforming Growth Factor Beta-1 (TGFβ-1). Lower airway microbiology is studied through use of a 16S rRNA gene PCR assay (SepsiTest^{TM}, Molzym) and a specific PCR assay for *Pseudomonas aeruginosa (P. aeruginosa)*, based on the outer membrane lipoprotein gene, OprL (DeVos et al., 1997))(Handschur, Karlic, Hertel, Pfeilstöcker, & Haslberger, 2009).

4.1.1 Interleukin 8 (IL-8 or CXCL8)

IL-8, a potent neutrophil chemoattractant, is a key mediator of the pulmonary inflammatory response. A range of stimulants can induce production of this chemokine including bacteria, viruses, environmental factors and other pro-

inflammatory cytokines, such as IL-1 and tumour necrosis factor-alpha (Mukaida, 2003). Alveolar macrophages are the predominant cellular source of pulmonary IL-8 however, other sources include airway epithelial cells and fibroblasts (Kunkel, Standiford, Kasahara, & Strieter, 1991). Its key function is in enabling neutrophil transmigration and activation, which it performs through high affinity binding to CXCR1 and CXCR2 receptors on the neutrophil cell surface (Pease & Sabroe, 2002).

Neutrophil infiltration is characteristic of various paediatric airway diseases and more specifically, has been shown to be central to the pathogenesis of bronchiectasis, the end stage respiratory outcome often seen in children with severe ND (Angrill et al., 2001, Piccione, 2012). Raised pulmonary IL-8 and neutrophil levels have also been correlated with BAL pepsin and bile acid levels, potential biomarkers of reflux-aspiration (D'Ovidio et al., 2005; McNally et al., 2011; Starosta et al., 2007). Thus, measurement of pulmonary IL-8 is of interest in investigating the pathogenesis of the respiratory disease seen in children with ND, both in terms of potential contributory aetiological factors and pathological outcome.

4.1.2 Transforming Growth Factor Beta-1 (TGF β-1)

TGFβ-1 is a member of the transforming growth factor beta cytokine super-family. Three mammalian isoforms are recognised; TGFβ-1, TGFβ-2 and TGFβ-3. TGF β-1 plays a central role in the regulation of airway remodelling (N. Khalil & Greenberg, 1991).

Produced as a latent precursor complex by many cell types within the lung (including epithelial cells, endothelial cells, fibroblasts and various cells of the immune system), TGFβ-1 must be proteolytically or enzymatically activated (for example by proteases, reactive oxygen species or pH changes), in order to have physiological or pathological effects (Barcellos-Hoff & Dix, 1996; Camoretti-Mercado & Solway, 2005; Lyons, Keski-Oja, & Moses, 1988; Q. Yu & Stamenkovic, 2000). It functions through a specific heteromeric cell surface receptor complex (TGFβR type 1 and 2), present on most cell types (Camoretti-Mercado & Solway, 2005; LEASK & ABRAHAM, 2004). Key actions include control of cell growth,

proliferation, differentiation, and apoptosis (Dennler, Goumans, & ten Dijke, 2002).

TGFβ-1 expression has been demonstrated in epithelial cells and extracellular matrix from fibrotic lungs, localised to areas of repair and remodelling (N Khalil, O'Connor, Flanders, & Unruh, 1996). It is known to play roles in sub-epithelial fibrosis, airway smooth muscle changes and microvasculature changes as well as being a recognised driver of epithelial-mesenchymal transition (Camoretti-Mercado & Solway, 2005; Willis & Borok, 2007). Airway remodelling processes are implicated in the pathogenesis of a number of chronic inflammatory respiratory diseases and correlations have been shown with disease severity (Arkwright et al., 2000; Brown et al., 2012). Additionally, increased BAL TGFβ-1 has been noted in animal models of recurrent gastric juice aspiration and is induced in airway epithelial cell models on exposure to bile acids, a possible component of the refluxate (Li et al., 2008; Perng et al., 2007; Perng et al., 2008). These studies justify investigating levels of TGFβ-1 in BAL samples from children with ND.

4.1.3 16S PCR analysis

Our understanding of microbial-host relationships has increased over recent years with the development of novel molecular methods of airway pathogen identification (E. S. Charlson et al., 2011). 16S PCR techniques are based on the isolation of the 16S ribosomal RNA (rRNA) gene, a universal component of all prokaryotic ribosomes. However, whilst bacterial rDNA carries nucleotide sequences common to all species, it also carries variable regions that are genus or species specific. It is therefore possible to identify bacterium by referencing bacterial sequence libraries through nucelotide sequencing of PCR products (Harris & Hartley, 2003).

This technology removes the necessity (and subsequent bias) for 'growing' organisms on a culture plate and has highlighted the diversity and complexity of lower airway bacterial communities, both in health and disease, leading to rejection of the concept of a 'sterile' airway (E. S. Charlson et al., 2011; Emily S. Charlson et al., 2012; Huang, Lynch, & Wiener-Kronish, 2012). The traditional Sanger dideoxy sequencing methods have formed the basis of molecular

microbial identification for decades, leading to the identification of previously uncultured organisms and collection of large stores of phylogenetic data (Sanger et al., 1977). Newer pyrosequencing technologies are further advancing the field, allowing high throughput deep sequencing analysis and characterisation of complete microbial communities, rather than solely the predominant organisms (Petrosino, Highlander, Luna, Gibbs, & Versalovic, 2009). Broad microbial communities appear to differ in varying states of airway disease and with anti-microbial treatments, implying potential contribution to disease progression. However, the actual clinical implications of these differences are, to date, not fully understood (Hilty et al., 2010).

4.1.4 Pseudomonas aeruginosa

P. aeruginosa is a gram negative, aerobic, rod shaped bacterium commonly found in soil, water and on the surfaces of plants. In humans, it is an opportunistic pathogen with the ability to infect almost any organ if defences are compromised, the commonest site being the lung (Iglewski, 1996). Common predisposing conditions are cystic fibrosis, mechanical ventilation, immunodeficiency and other pre-existing respiratory diseases. The pathogenicity of *P. aeruginosa* infection is complex comprising advanced epithelial adhesion mechanisms, protease and cytotoxin secretion, cell-to-cell communication (quorum sensing) and alginate secretion (Kipnis, Sawa, & Wiener-Kronish, 2006; Pritt, O'Brien, & Winn, 2007). Individuals with cystic fibrosis become colonised, initially intermittently with pseudomonas from early childhood, and then chronically in adolescence. Aggressive treatment is warranted to delay the development of a mucoid phenotype, which is associated with increased morbidity and mortality (Banerjee & Stableforth, 2000; Pritt et al., 2007).

Children with ND often have abnormal, potentially pathogenic upper airway bacterial colonisation, particularly with *P. aeruginosa* (Thorburn et al., 2009). However, it's presence and role in lower airway pathology in this group of children is unclear. There are no clear management guidelines and consequently clinical management strategies are varied amongst clinicians (S. Smith & Puri, 2004).

4.2 Aims

To determine the relationship between lower airway inflammation/infection and clinical symptoms in children with severe ND, this study aimed to;

1. Assess lower airway inflammatory markers in BAL supernatant samples taken from children with severe ND at times of stability and during respiratory deteriorations on admission to PICU, in comparison to healthy controls.

2. Characterise lower airway microbial colonisation/infection at a time of stability and respiratory deterioration in comparison to healthy controls using a 16S rRNA gene PCR analysis and also a specific PCR for *P. aeruginosa*.

3. Assess the relationship of lower airway inflammation and microbial colonisation/infection with chronic respiratory symptoms (as characterised by total LRSQ-Neuro scores).

4.3 Specific Methods

Clinical data collection and bronchial lavage sampling were undertaken as described in Chapter 2. Differential cell counting was performed following Diff Quick staining (Reastain CE) of BAL cytospin slides, as detailed in Section 2.3.2.3. BAL IL-8 and TGFβ-1 measurements were made using commercial ELISA kits (R&D systems), as described in Section 2.4. The laboratory methods for the SepsiTest™ (Molzym, Bremen) and OprL PCR techniques are detailed in Sections 2.6.5 and 2.6.4, respectively. Further background information on these assays is detailed below.

4.3.1 SepsiTest™-16S/18S rDNA PCR with selective degradation of human DNA

It is generally acknowledged that when using 16S technology, the high human to pathogen DNA ratio in clinical samples can lead to problems with sensitivity and crucially to false identifications, due to homologous sequence motifs between host (eukaryotic) and bacterial (prokaryotic) genomes (Cridge et al., 2006; Handschur et al., 2009). However, by electively degrading human DNA, it is possible to enrich pathogen DNA and thus increase PCR sensitivity (Molzym Life Science, 2013).

SepsiTest™ differs from other commercially available 16S/18S PCR techniques in having a patented sample preparation step to remove human and extracellular bacterial DNA (MolYsis, Molzym, Bremen) (Horz, Scheer, Vianna, & Conrads, 2010). It also only yields information about pathogens with intact cell walls. This tackles another recognised limitation of 16S/18S studies; the inability to differentiate intact microbial DNA from free microbial DNA, which again potentially biases results (E. S. Charlson et al., 2011; Rogers & Bruce, 2010; Rogers et al., 2008).

Blood culture studies have demonstrated successful use of SepsiTest™ as a supplemental tool to accompany standard culture techniques in the diagnosis of septicaemia (Gebert, Siegel, & Wellinghausen, 2008; Wellinghausen et al., 2009). A high level of agreement with positive blood culture results was observed and benefits included increased speed of diagnosis and additional information about pathogenic organisms, previously challenging to identify by

standard culture techniques. The sequencing technique (based on Sanger methods) focuses on identifying the most predominant organisms within a sample, rather than community characterisation, as with pyrosequencing techniques (Molzym Life Science, 2013). Both bacterial (16S) and fungal (18S) rDNA can be identified using SepsiTest™. It has been used with other specimen types but has not been fully validated in BAL samples to date (Molzym Life Science, 2013). We are unaware of other 16S/18S rDNA PCR studies on BAL which have used a human DNA depletion step, although propidium monoazide has been used in a small number of studies to limit amplification of non-viable DNA (Rogers et al., 2008). Laboratory methods for this technique are documented in methods Section 2.6.5.

4.3.2 OprL PCR

This PCR technique is based on amplification of the OprL outer membrane lipoprotein gene (open reading frame 504bp). The OprL gene was previously validated as being specific to *P. aeruginosa* using various bacteria and twenty different fluorescent pseudomonad strains of both clinical sample and environmental origin (DeVos et al., 1997). The assay showed 100% sensitivity and 74% specificity in comparison to culture, when testing respiratory specimens. Laboratory methods are documented in methods Section 2.6.4.

4.4 Results

4.4.1 BAL characteristics

A total of 46 BAL samples were collected which met sample adequacy criteria, based on ERS recommendations (de Blic et al., 2000; Suen et al., 1999). Nineteen samples were from children in the PICU-ND group, 16 were from children in the Elective-ND group and 11 were healthy control patient samples. **Table 4.1** documents the BAL cellular component characteristics and BAL supernatant total protein levels.

Significant differences in BAL cell concentration (cells/ml) were seen between the PICU-ND and healthy control group ($p<0.001$). Significant differences in BAL total protein concentration (ug/ml) were seen between the PICU-ND and Elective-ND groups ($p=0.027$), Elective-ND and Healthy Control groups ($p=0.048$) and PICU-ND and healthy control groups ($P<0.01$).

Table 4-1 Table to show BAL cellular component characteristics and BAL supernatant total protein levels

	PICU-ND	***Elective-ND***	***Controls***
Number of samples	19	16	11
Median (IQR) total cell concentration (x 10^6 cells/ml)	3.9[1.3-7.4]	0.9[0.4-4.2]	0.6[0.3-0.9]
Median (IQR) % neutrophils	82[72-86.6]	48.8[29.1-71.2]	6.8[3-18.5]
Median (IQR) % alveolar macrophages	15.7[10.4-19.8]	47.5[24-69]	89.5[78.2-94.3]
Median (IQR) % lymphocytes	1.7[1.3-3.2]	2.0[1.3-3]	1.8[1.4-2.7]
Median (IQR) % eosinophils	0.3[0-0.6]	0.3[0-0.6]	0.3[0.1-0.6]
Median (IQR) total protein (ug/ml)	579[180-1412]	124[83-694]	73[49-160]

4.4.2 Children with severe ND have chronic lower airway inflammation, even at times of stability

BAL differential cell counts were made on 15/19 PICU-ND samples, 16/16 Elective-ND samples and 10/11 healthy control samples. As anticipated, a marked neutrophilia was found in PICU-ND BAL (82% [77-86]). However, a higher neutrophil percentage was also found in Elective-ND BALs compared to healthy control samples (Elective-ND, 49% [29-71]; Control, 6.8% [3-18.5]: $p<0.001$) (**Figure 4.1**).

IL-8 protein levels were measured in all BAL supernatant samples (46/46). BAL median [IQR] IL-8 concentrations were highest in PICU-ND BAL samples (5,641 [2,024-14,703] pg/ml) but were also significantly raised in Elective-ND samples compared to healthy controls (Elective-ND, 646 [0-3633] pg/ml; Control, 0 [0-222] pg/ml: $p<0.05$) (**Figure 4.2**).

TGFβ-1 levels were measured in all BAL supernatant samples (46/46). TGFβ-1 levels were also raised in PICU-ND BAL samples (157 [69-190] pg/ml; Control, 0 [0-0] pg/ml: $p<0.001$) and Elective-ND samples (Elective-ND, 0 [0-143] pg/ml; $p=0.051$) (**Figure 4.2**).

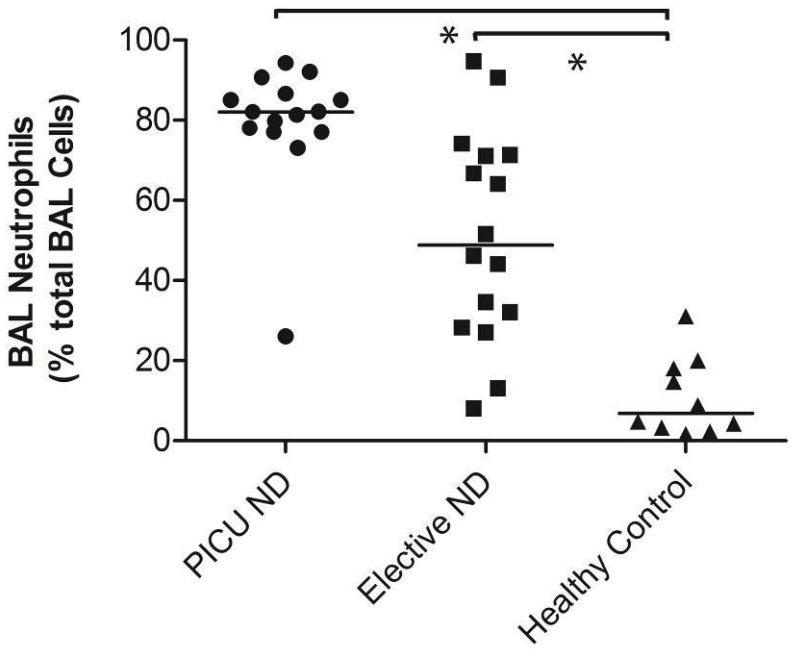

Figure 4.1 Vertical scatter graph showing BAL neutrophils (expressed as % total BAL cells) by study group

BAL differential cell counts were performed following cytospin slide preparation and Diff-Quick staining (Reastain CE). Neutrophil counts are expressed as % total BAL cells. * P <0.001. Horizontal line represents median value.

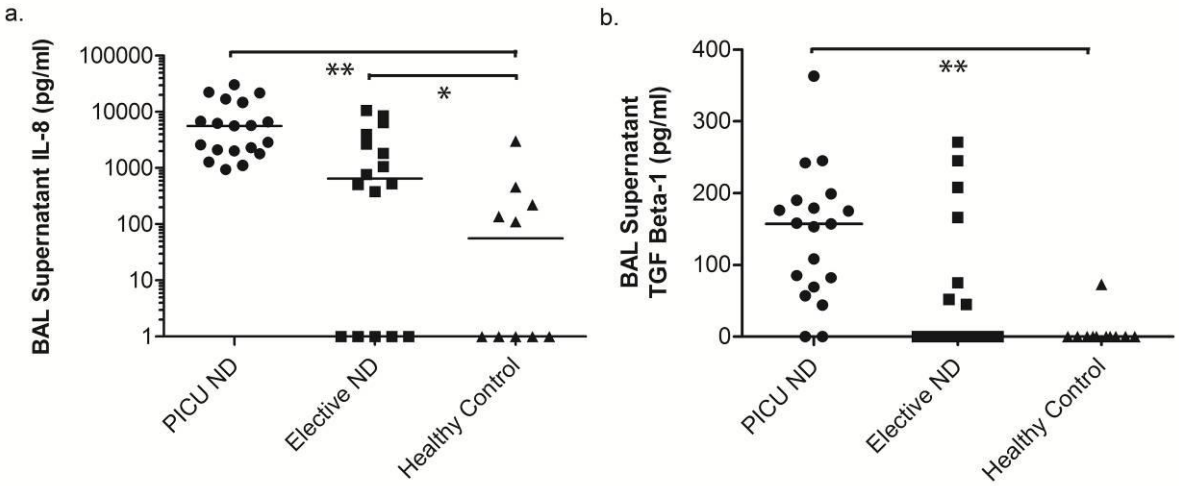

Figure 4.2 Vertical scatter graphs showing (a) BAL IL-8 levels (pg/ml) and (b) BAL TGFβ1 levels (pg/ml) by study group

BAL supernatant IL-8 and TGFβ1 levels were measured using commercially available ELISA sets (R&D systems). * P<0.05 ** P<0.001. Horizontal line represents median value

4.4.3 Lower airway inflammatory levels may relate to LRSQ-Neuro scores in Elective-ND patients

The relationships between total LRSQ-Neuro score and BAL neutrophil percentage, IL-8 and TGF-β1 were examined in the Elective-ND group.

No statistically significant relationships were found in this small patient cohort (n=16). However, it was notable that those patients with an LRSQ score >20 invariably had an accompanying BAL neutrophilia (**Figure 4.3**).

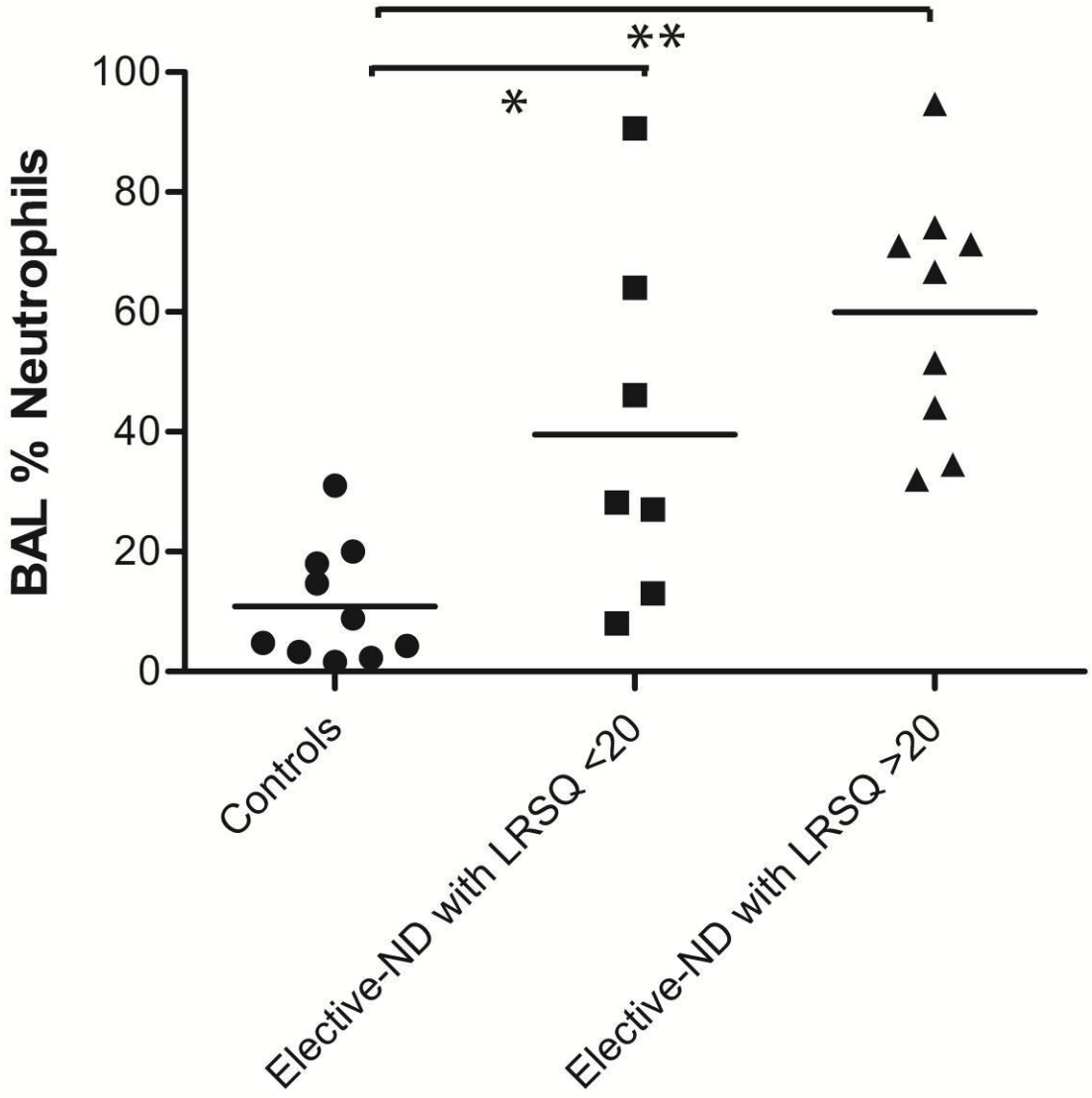

Figure 4.3 Vertical scatter graph illustrating observed patterns of BAL neutrophilia in Elective-ND patients divided according to LRSQ-Neuro score, compared to healthy controls.

BAL neutrophil counts were assessed following BAL cytospin slide preparation, Diff Quick staining and differential cell counting. All study recruits were asked to complete a respiratory symptom score (the LRSQ-Neuro) on admission to hospital. BAL neutrophils (% total BAL cells) were observed to be consistently raised in those Elective-ND patients with an LRSQ score >20. BAL neutrophil levels are plotted for Elective-ND patients (divided by LRSQ-Neuro score < or > 20) and healthy controls patients. Comparison of BAL neutrophil levels in Elective-ND patients with total LRSQ-Neuro score <20 vs. LRSQ-Neuro score >20, $p = 0.09$. * $p < 0.01$, ** $p < 0.0001$.

4.4.4 'Oral commensals' are often identified in the lower airway

Microbial rRNA gene 16S/18S (bacterial/fungal) analysis (SepsiTest™ Molzym, Bremen) was performed on 18/19 PICU-ND, 16/16 Elective-ND and 9/11 healthy control samples. The reason for not testing all samples was limitation of sample volumes.

Microbial DNA was detected in BAL samples from 66% PICU-ND patients, 81% Elective-ND patients and 55% healthy controls. In PICU-ND patients, microbial DNA was detected in 54% (7/13) of those who had been prescribed antibiotics for >24 hours prior to admission to hospital, and in 100% (5/5) patients who had not been prescribed antibiotics.

Various predominantly gram-positive bacteria were identified on sequencing (**Figure 4.4**). In both ND groups, microbial DNA from *Staphylococcus aureus*, *Streptococcus mitis* and *Rothia mucilaginosa* were most commonly detected. *Streptococcus mitis* and *Rothia mucilaginosa* were also identified in the two control patients in whom microbial DNA was detected <cycle 35. Despite *P. aeruginosa* being detected in airway secretions from multiple patients prior to this study, it was not detected in BAL samples from any patient using this assay. A total of 78% of the microbial identifications were with >97% sequence coverage and 22% were mixed sequences with the likely predominant species identified (71% *Rothia* species or *Streptococcus* species). In these cases, further detailed identification would require deep sequencing.

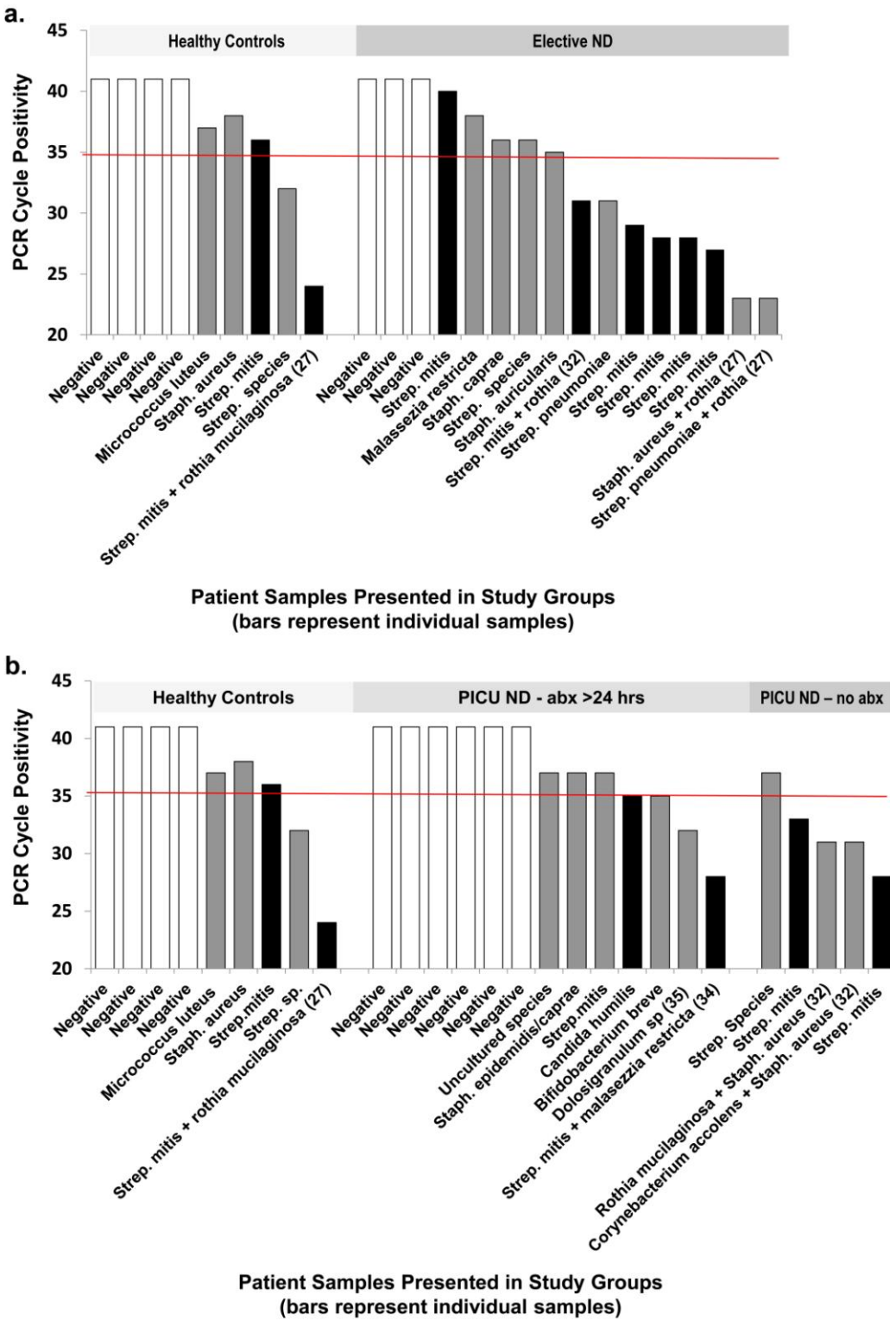

Figure 4.4 Bar charts showing predominant microbes with intact cell walls identified in BAL for (a) Elective-ND vs. healthy control patients and (b) PICU-ND vs. healthy control patients.

BAL samples were analysed using a novel 16S/18S rRNA gene PCR assay (SepsiTest$^{TM)}$ in the Molzym laboratories, Bremen, Germany. This assay incorporates a step to eliminate host and non-viable bacterial DNA. Microbial identity (post sequencing and referencing using the SepsiTest^{TM-} BLAST tool) for each patient sample analysed is displayed on the x axis and the PCR cycle at which they were identified is displayed on the y axis (each bar represents one sample). The bracketed numbers represent the PCR cycle at which the second indicated microbe was identified. The red line indicates the 'cut off' at which Molzym laboratories indicated '+/-' to microbe specific signal in the melt curve (i.e. the limits of confident detection).

4.4.5 Relationship between microbial DNA status, respiratory symptoms and lower airway inflammation in elective patients

The relationship between LRSQ-Neuro score, BAL neutrophilia and microbial DNA detection in Elective-ND and healthy control patients was examined.

Total LRSQ score was generally higher in patients who had samples that were positive for microbial DNA, although this did not reach statistical significance (microbial DNA +ve, 15[5-32]; microbial DNA -ve, 5[0-9]: p=0.097). Median [range] BAL neutrophil levels were also higher in samples that were microbial DNA positive (33%[18-70]) compared to microbial DNA -ve (8%[4-38]: p=0.065).

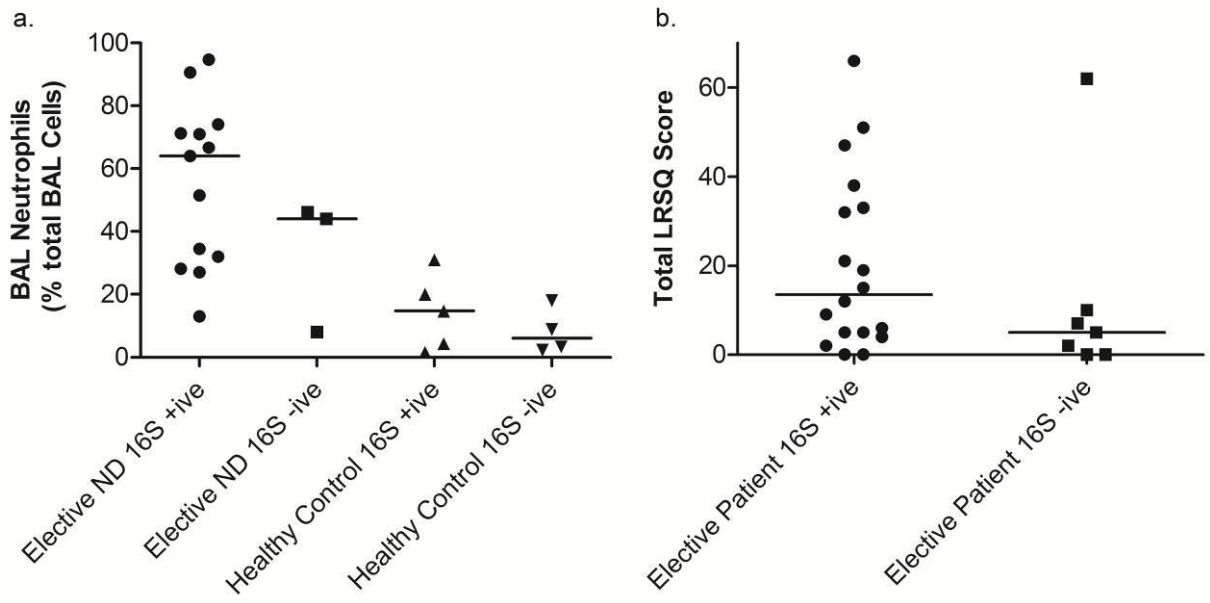

Figure 4.5 Vertical scatter graphs showing (a) relationship between BAL neutrophil levels and microbial DNA status within Elective-ND and healthy control study groups and (b) relationship between total LRSQ-Neuro score and DNA microbial status in both elective study groups combined.

BAL samples were analysed using a 16S/18S rRNA gene assay (SepsiTest^{TM}) in the Molzym laboratories, Bremen, Germany. This assay incorporates a step to eliminate host and non-viable bacterial DNA. BAL neutrophil counts were assessed following BAL cytospin slide preparation, Diff Quick staining and differential cell counting (expressed as % total BAL cells). All study recruits were asked to complete a respiratory symptom score (LRSQ-Neuro) on admission to hospital.

4.4.6 *P. aeruginosa* was not identified in lower airway samples of children with severe ND by OprL PCR

Given that 12/19 PICU-ND patients had cultured *P. aeruginosa* from their airways at some point prior to this admission and that 5/12 PICU-ND patients had cultured *P. aeruginosa* on tracheal aspirate during this admission, we sought to verify the SepsiTest™ data using OprL PCR. This would also investigate the possibility that *P. aeruginosa* had been rendered unviable during the freeze thawing process. *P. aeruginosa* was not detected in any PICU-ND, Elective-ND or healthy control BAL sample by this technique.

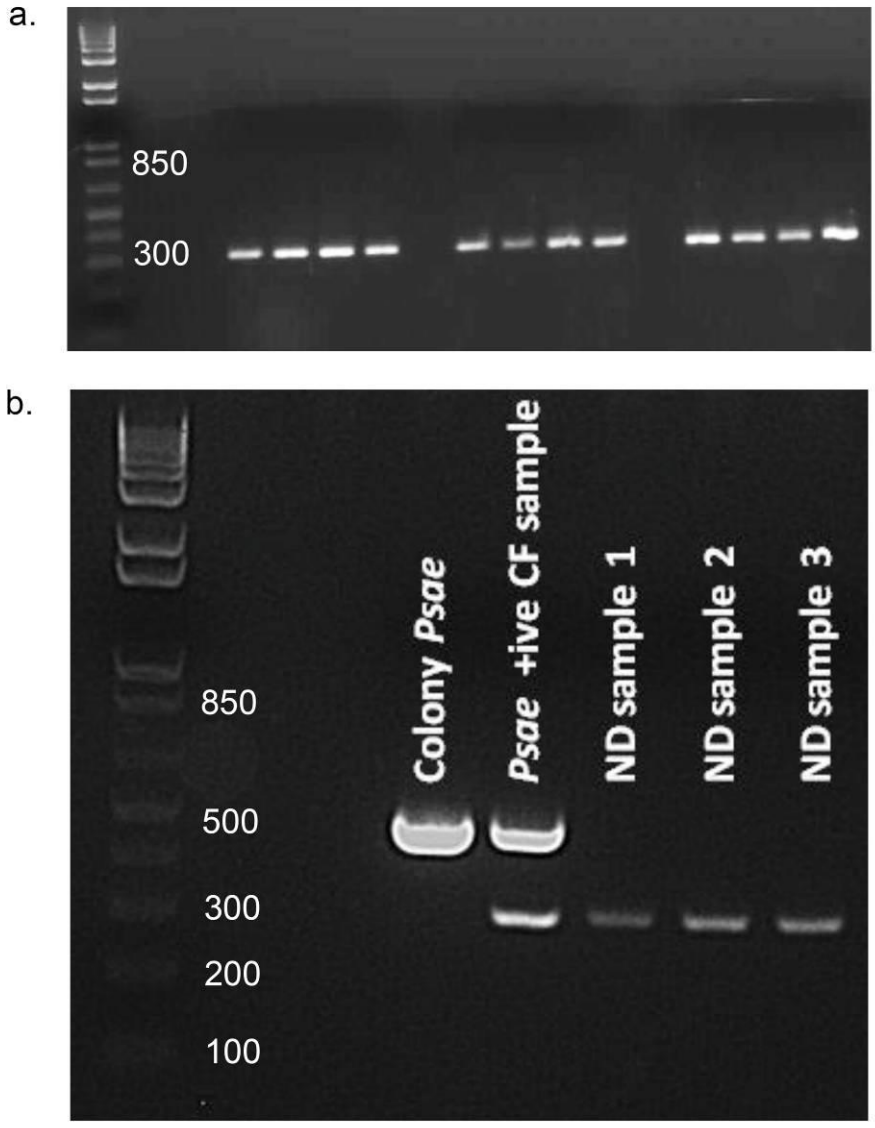

Figure 4.6 18S and OprL PCR Gel Images

Example PCR gel images showing (a) 18S PCR product at 315bp for 16 study samples (loading control) and (b) 18S and OprL PCR products at 315bp and 504bp respectively, for two positive control samples (Colony *P. aeruginosa* (Psae) and a culture positive cystic fibrosis (CF) sample) and three study samples.

4.5 Discussion

In Chapter three, chronic respiratory symptoms were documented in otherwise well children with severe ND, which impact on quality of life. In this chapter, we have identified chronic lower airway inflammation in these same children and there appears to be a relationship with respiratory symptoms in Elective-ND patients. Using novel 16S/18S PCR technology that only detects microbes with intact cell walls, we have identified the predominant organisms present within the airways of these children at the time of sampling. Of note, *Streptococcus mitis* was found in nearly half of those samples in which an organism was detected. In contrast, *P. aeruginosa* was not found in any lower airway sample despite being cultured from other, predominantly upper airway sample types in these patients (documented in chapter three). Lastly, a trend for greater airway neutrophilia and respiratory symptoms in those elective patients with lower airway microbial positivity was observed, suggesting that lower airway infection may play a significant role in the respiratory morbidity experienced by children with severe ND.

In this challenging group of patients, it is often difficult to elicit whether respiratory symptoms are coming from the lower or upper respiratory tract. The tendency for BAL neutrophil levels to be higher in those with more respiratory symptoms may indicate that at least some of these symptoms originate from the lower respiratory tract; in this study, patients with an LRSQ-Neuro score >20 invariably had lower airway neutrophilia. This observation certainly warrants further investigation since these types of relationship are not often found. In the CF literature for example, most studies show that respiratory symptoms correlate poorly with lower airway inflammation, although few have used a structured validated tool to assess symptomatology (Brennan et al.; Nixon et al., 2002; Stick et al.). It is important to acknowledge that any observations made within this study were significantly limited by study numbers. However, these preliminary results indicate that the LRSQ-Neuro may have potential as a non-invasive indicator of lower airway inflammation, and it is not inconceivable that this quick, simple test could be used to screen patients who may benefit from specialist respiratory assessment or even as an outcome measure in clinical trials. For this to happen, further psychometric validation is required, particularly with regard to sensitivity to change (US Department of Health and

Human Services Food and Drug Administration, 2006) . Future studies should include larger study numbers and children with ND with a range of disability levels rather than only those with GMFCS V.

The levels and type of inflammation recorded may provide some insight into why many of these children die early from respiratory complications in adolescence and early adulthood (Piccione et al., 2012). As well as finding significant airway neutrophilia and increased IL-8 levels, we also found elevated levels of TGFβ1, an important driver of airway remodelling and fibrosis, which is induced by neutrophil elastase (K.-Y. Lee et al., 2006). It is feasible that chronic inflammation and infection may be important in the development of airway damage seen in these patients in the end stages of their respiratory disease. However, in order to implicate TGFβ1 in the mechanism of chronic airway damage the data would need further consolidation. TGFβ1 ELISA kits usually measure 'active' TGFβ1 and therefore in order to measure total sample TGFβ1, an 'activation' step (usually by acidification followed by neutralisation) is required. The finding of raised TGFβ1 levels by ELISA quantification is interesting: future work would ideally investigate TGFβ1 activity in airway epithelial cells using, for example, immunohistochemical staining for pSMAD2/3 as a measure of TGFβ1 signalling (Lim et al., 2009).

The 16S/18S PCR results (SepsiTest^{TM}) provide a record of the predominant pathogens with intact cell walls, populating the airways of participants at the time of bronchoalveolar lavage. Interestingly, broadly the same microbes, often considered oral commensals, were found in the airways of children with and without severe ND. These groups did however differ in the timing of PCR cycle positivity. The microbes identified in healthy control samples generally came up after 35 PCR cycles whereas those within the ND samples came up before 35 cycles. The company classed any positivity above cycle 35 as +/- in terms of microbe specific signal in the melt curve and therefore, this 'cut off' line was drawn for comparison of samples between groups. Unfortunately, no firm conclusions can be drawn about relative microbial abundance from this observation. In any future study it would be interesting to formally assess this through use of standard curves.

Streptococcus mitis was the organism identified most commonly within the samples. This gram-positive organism is generally considered an oral commensal, and while thought to pose little threat to the immuno-competent, it is a significant cause of endocarditis and septicaemia in neutropenic and immuno-compromised individuals (Mitchell, 2011). *Streptococcus mitis* uses a variety of strategies including expression of adhesins, immunoglobulin A proteases and toxins, and modulating the host immune response, to colonise the human oropharynx. In neuro-disabled individuals lacking adequate airway protection, it is possible that these same strategies could also allow the colonisation of the lower respiratory tract. The pulmonary inflammatory potential of organisms which are regarded as oral flora requires further study, an issue also raised by Gangell *'et al'* in a traditional culture study of CF BAL specimens (Gangell et al., 2011).

A potential limitation of this work was that sheathed equipment was not used for BAL collection, increasing the risk of carryover of microbes from the upper to the lower respiratory tract (E. S. Charlson et al., 2011; Goddard et al., 2012). Nevertheless, the same collection technique was used for all patient groups. Therefore, the differences observed in timing of PCR cycle positivity are still valid, particularly as organisms (specifically *P. aeruginosa)* known to have been present in the upper airway of several PICU patients, do not appear to have been carried over to lower airway specimens. It would be useful to make direct comparisons of upper and lower airway sample microbiology in future studies, using sheathed equipment and fresh suction circuits to collect lower airway samples.

Microbial diversity is noticeably limited in results from this study compared to other lower airway 16S rRNA gene studies (E. S. Charlson et al., 2011; Hilty et al., 2010)). Sanger sequencing targets identification of the dominant species, rather than documentation of whole microbial communities. Newer pyrosequencing techniques offer superior depth of sequencing and enable monitoring of the broader lower airway microbiome, including information about low abundance species which may play important roles in disease pathogenesis (M. J. Cox et al., 2010). The use of deep sequencing would also allow clearer identification of organisms in cases where there is not a predominant species (as seen in 22% of microbial DNA identifications in this study). Other researchers

have found it useful to use these sequencing techniques in tandem (Flanagan et al., 2007).

Another potential explanation for differences in results between this study and other studies is the removal of host DNA and extracellular bacterial DNA (Rogers et al., 2008). Potential difficulties with molecular detection of both intact and extracellular bacterial DNA include increased signal to noise and increased rates of false microbial identification. Also, ability to judge the clinical relevance of species detected at the time of sampling may be influenced by the rate of DNA clearance from the lower airway (known to be delayed in conditions such as cystic fibrosis) (Stressmann et al., 2011). Similarly, effects of recently used antimicrobials may become difficult to assess (Rogers & Bruce, 2010; Sibley, Peirano, & Church, 2012). Of note, in this study, no microbes were detected in 6/13 PICU-ND samples from children who had received antibiotics in the preceding 24 hours, whereas they were identified in all 5 PICU patients who did not have antibiotics.

However, this strength in solely detecting organisms with intact cell walls may also be a limitation. Children often receive antibiotic treatment before sampling and this could decrease the ability of SepsiTest^{TM} to detect microbial causes of symptoms (Flanagan et al., 2007). The relative abundance of gram-positive organisms in our samples was surprising given the likely prevalence of reflux aspiration in our ND patients and theoretical potential for gram-negative infection (Morton et al., 1999). This may have been due to the purification step, in which human cells are lysed while viable bacterial cells ideally remain unaffected. A recognised limitation of this sort of process is that bacteria with less robust cell envelopes, such as gram-negative microbes, may also be lysed in this step, biasing the reported micro-flora (Horz, Scheer, Huenger, Vianna, & Conrads, 2008; Horz et al., 2010). Equally, this may have been influenced by how we stored our samples, as gram-negative bacteria are known to have lower survival following freezing (Miyamoto-Shinohara, Sukenobe, Imaizumi, & Nakahara, 2006). Future studies should analyse samples fresh, where possible. Finally, this may reflect a low relative abundance of gram-negative organisms in these patients, limiting detection via the sequencing technique used.

The advantages offered by molecular microbial detection over traditional culture are counter-balanced by potential confounders relating to the effects of sample processing, nucleic acid extraction efficiency, purification, amplification, depth of sequencing and analysis (Rogers & Bruce, 2010; Sibley et al., 2012). All this needs to be considered in interpretation of molecular studies and in research study planning. It would certainly be of interest to conduct BAL studies with culture and molecular techniques (including and excluding non-viable DNA) in tandem in the future. This was not performed in the current study as the original study aims did not include microbiological analysis and therefore, microbiological analysis was retrospective. This approach may also be useful in further validating the use of SepsiTest™ in BAL samples (previously validated in serum samples) (Gebert et al., 2008; Wellinghausen et al., 2009). However, validation in respiratory samples, which unlike serum samples would be expected to contain microbes under normal circumstances, would be more challenging. A deeper sequencing approach would need to be employed, as organisms grown on the culture plate may not be the 'predominant' species identified by Sanger sequencing.

Given the literature, and that so many of our ND cohort cultured *P. aeruginosa* on cough swab or tracheal aspirate (as recorded in chapter three), it was surprising that this organism was not detected on 16S analysis or PCR of the OprL gene (S. Smith & Puri, 2004; Thorburn et al., 2009). There are a number of possible explanations for this. It is well-recognised that microbial growth may differ between lobes of the lung and therefore, it is possible that in 'blindly' sampling the lower respiratory tract, we have missed *P. aeruginosa* infected lobes (Gilchrist et al., 2011). However, it is equally possible that our results accurately reflect lower airway microbiology and inferences about lower respiratory microbiology have been based on upper airways pseudomonal infection. Many of these children have regular upper airways suction and poorly coordinated swallows, a situation that provides the perfect opportunity and environment for *P. aeruginosa* to be introduced and then to colonise the oropharynx. Also, one would expect that tracheal aspirates would be more prone to contamination from the oropharynx than lower airway BAL samples. This potentially important clinical observation should be considered when treating respiratory exacerbations in these children, and certainly warrants further study

and investigation. Again, the same points regarding methodological bias apply and it would be useful to use culture and molecular techniques in tandem on paired tracheal aspirate and BAL samples to investigate this further in future studies (Deschaght, Van daele, De Baets, & Vaneechoutte, 2011). In particular, the sensitivity of the OprL PCR technique compared to culture should be revalidated within our laboratory.

The absence of BAL viral PCR results is a potential criticism of the data presented in this chapter. Viral infection may have triggered a respiratory deterioration and generated a lower airway inflammatory response in many of the PICU-ND group. However, the electively recruited patients were the main focus of conclusions drawn from this chapter. At the time of recruitment, all electively recruited patients were 'well' from a respiratory perspective. It was felt clinically unlikely that active viral infection was a relevant factor in generating airway inflammation and symptoms at this time and therefore, viral PCR testing was not pursued. However, it must be acknowledged that subclinical viral infection could potentially have contributed to the lower airway inflammation observed.

The trend for airway neutrophilia and symptoms to be greater in those electively recruited patients with lower airway microbial positivity points toward infection being the cause of respiratory symptoms and morbidity. This raises the clinical question as to whether antibiotic prophylaxis would be beneficial in this group of patients. There have been no high quality studies to date and little is known about antibiotic resistance patterns in neuro-disabled children. Within our region, there is an inconsistent approach (as documented in Chapter 3), with those attending joint neuro-respiratory clinics more likely to be prescribed antibiotic prophylaxis. Formal randomised controlled trials are needed to further assess this, possibly using the LRSQ-Neuro as an outcome measure: development of microbial resistance will be a key consideration in such studies.

4.6 Conclusions

Children with severe ND often have high levels of chronic respiratory symptoms, which possibly relate to levels of lower airway inflammation. This needs to be explored further in a larger patient cohort. Bacterial airway colonisation,

particularly with oral commensals, may play a role in both symptom generation and inflammation but requires further detailed investigation. Early tailored respiratory assessment of children with ND and chronic respiratory symptoms may be useful and further well-designed clinical studies are needed to determine the risks and benefits of prophylactic therapy in this group.

Chapter 5 – Biomarkers of Aspiration in Bronchial Lavage Fluid

5.1 Introduction

The aetiology of respiratory disease in children with severe ND is undoubtedly multi-factorial, however recurrent pulmonary aspiration is thought to play a significant role (Seddon & Khan, 2003). Gastro-oesophageal reflux disease and oral-motor discoordination are known to be common problems in ND patients (Mazzoleni et al., 1991; Morton et al., 1999; K. A. Weir et al., 2011). The high incidence of these co-morbidities means that children with severe ND represent a patient group at 'high risk' of recurrent direct and reflux pulmonary aspiration. Data presented in Chapter Three suggest that this is also true of recruits to this study.

Differentiating direct aspiration (of food and saliva) and gastric reflux aspiration is difficult. This is particularly the case when children present with predominantly respiratory symptoms, where there may be direct aspiration, reflux aspiration, neither, or both. This conundrum hinders clinical decision making, particularly concerning invasive procedures such as fundoplication (Shields et al., 2011). Clinical biomarkers to identify and quantify both reflux aspiration and direct aspiration would therefore be useful in clinical assessment, and may also be applicable as outcome measures for interventional trials. Longitudinal studies using validated biomarkers would further inform our understanding of the impact of aspiration on pulmonary health in children with severe ND and also clarify associations between other respiratory pathologies and GORD (Havemann, Henderson, & El-Serag, 2007; King, Iyer, Leidi, & Carby, 2009; McNally et al., 2011).

Useful biomarkers of direct or reflux pulmonary aspiration ideally need to be non-invasive, quantifiable, specific to the aspirate (gastric juice or saliva), not normally present within the lungs and detectable for sustained periods following aspiration (McNally et al., 2011). BAL fluid analysis, although invasive, provides a good starting point for the study of biomarkers of pulmonary aspiration. The significant limitations of the lipid laden macrophage index were discussed at length in Chapter One (Boesch et al., 2006). However, despite these limitations, this test continues to be used in clinical practice, as no other biomarker of reflux-aspiration currently exists (Reid-Nicholson et al., 2010). Various novel biomarkers of aspiration have been proposed in the literature. Two such markers

are BAL pepsin (reflux aspiration) and BAL alpha amylase activity (direct aspiration) (Abou Jaoude, Knight, Ohtake, & El-Solh, 2010; Clarke, Bain, & Davies, 1981). To date, clinical studies of BAL alpha amylase as a biomarker of direct aspiration have been limited; however, studies of pepsin as a biomarker of reflux aspiration have been much more extensive (reviewed in Chapter 1)(Clarke, Bain, & Davies, 1981; Nandapalan, McIlwain, & England, 1995; Nandapalan, McIlwain, & Hamilton, 1995). Interestingly, despite this, no firm conclusions have been drawn about the utility of BAL pepsin as a clinical test for a number of reasons. The first is that there is no diagnostic gold standard against which to compare results. Clinicians currently make the diagnosis of aspiration based on an accumulation of clinical evidence (Boesch et al., 2006). Therefore, it would be helpful to study BAL pepsin in a 'high risk' group by current diagnostic standards, such as children with severe ND. Secondly and as discussed in Chapter One, a number of different assay techniques have been used to measure BAL pepsin, potentially contributing to discrepancies in results between studies. Critical analysis of methods, to ensure high standards of protein identification and quantification across study samples, is needed and subsequent standardisation of methods is essential in order to conduct meaningful, longitudinal studies of these biomarkers.

The aims of this chapter were to study the relationships between BAL biomarkers of reflux and direct aspiration (lipid laden macrophages, pepsin and amylase) and respiratory symptoms, previous clinical investigation results and lower airway inflammatory markers, in children with severe ND compared to healthy controls. Specifically, the study took a critical approach to each analytical method used, utilising standard techniques (as described in Section 2.4) to assess assay validity across PICU and healthy control samples, and using a minimum of two analytical methods to assess each biomarker.

5.2 Aims

In order to assess BAL biomarkers of direct and indirect aspiration in children with severe ND, the chapter aims were to:

1) Assess the frequency of BAL LLM positivity in children with ND at a time of stability and at a time of respiratory deterioration (on admission to PICU) compared to healthy controls, also observing the relationship of positivity to (a) respiratory symptoms (b) symptoms suggestive of GORD (c) evidence of swallowing dysfunction (d) novel biomarkers of reflux-aspiration.

2) Critically assess previously published methods of BAL pepsin and amylase measurement in BAL across a range of clinical samples (PICU and healthy control), using (a) standard methods of assay validation and (b) a second method of measurement to corroborate results.

3) Assess the relationship of robust measurements of BAL alpha amylase activity and pepsin (as established in aim 2) to respiratory symptom scores, lower airway inflammatory markers and other clinical evidence of direct/reflux aspiration risk in children with ND (at a time of stability and at a time of deterioration), compared to healthy controls.

5.3 Specific methods

Clinical data collection and bronchial lavage sampling were undertaken as described in Chapter 2. Differential cell counting was performed following Diff Quick staining (Reastain CE) of BAL cytospin slides, as detailed in Section 2.3.2.3. BAL IL-8 and TGFβ-1 measurements were made using commercial ELISA kits (R&D systems, Section 2.4). All data relating to clinical samples was treated as non-parametric for statistical analysis due to small study group size (Section 2.9).

5.3.1 Lipid laden macrophage staining

The widely recognised limitations of the lipid laden macrophage index as a marker of reflux-aspiration were discussed in Chapter 1 (Boesch et al., 2006). Following concerns about inter and intra-rater reliability in reporting a lipid laden macrophage index and the limited overall specificity of the finding of lipid laden macrophages in BAL samples, it has been suggested that laboratories establish their own 'cut offs' for reporting lipid laden macrophage slide positivity (Ahrens et al., 1999; Colombo, Sammut, & Hallberg, 1986; Reid-Nicholson et al., 2010).

In this study, slides with any degree of alveolar macrophage lipid positivity by Oil Red O staining (Section 2.3.2.4) were forwarded to cytopathology for interpretation by a specified experienced cytopathologist, according to local guidelines. A positive or negative result was issued rather than a lipid laden macrophage index, which would require expert interpretation.

5.3.2 Identification and quantification of pepsin in BAL

Previously published laboratory techniques used to measure pepsin in BAL include ELISA, WB and enzymatic methods (Samuels & Johnston, 2010). All of these methods have strengths and weaknesses, as discussed in Chapter 1. The enzymatic method of analysis of pepsin A has various limitations, not least it's inability to differentiate pepsin activity from that of other aspartic proteases known to be produced locally in the lung and to be increased in pulmonary inflammatory conditions (Bewley et al., 2011; Kimura et al., 2005; Koslowski, Knoch, Kuhlisch, Seidel, & Kasper, 2003; Trinick, Johnston, Dalzell, &

McNamara, 2012). Immunological methods of pepsin analysis, such as ELISA and WB, offer potential for increased specificity. A variety of 'in house' assays have been used to measure pepsin in BAL with little published cross validation. Two widely used ELISA methods (one indirect and one sandwich ELISA using polyclonal antibodies to porcine pepsin A, shown to be cross reactive with human pepsin A) and one Western blot method (using a polyclonal antibody to human pepsin) were chosen for use in this thesis (Farrell et al., 2006; Rosen, Johnston, Hart, Khatwa, & Nurko, 2012; Tasker et al., 2002a).

5.3.2.1 ELISA methods for measurement of pepsin in BAL

The two published ELISA methods selected for analysis both used porcine pepsin antibodies (shown to be cross reactive with human pepsin A) to quantify human pepsin A in BAL. They followed different ELISA formats: ELISA 1 used an indirect ELISA technique whereas ELISA 2 used a sandwich ELISA technique.

Indirect ELISAS rely on the primary step of the antigen of interest being non-specifically adsorbed onto a microtiter plate. Blocking of residual binding sites on the plate is usually achieved by adding a formulation of protein that does not react with the antigen of interest or the antibody. A specific antibody for the antigen of interest is then added which binds to the antigen and following this, any residual antibody is washed off. An enzyme labelled secondary antibody is then added which, on addition of a substrate, gives off a colour signal which can be read using a spectrophotometer and is directly proportional to the amount of enzyme (and consequently antigen) on the plate. Non-specific binding of the secondary antibody was checked in pepsin ELISA 1 by running the samples in duplicate, with and without the addition of the capture antibody and subtracting any fluorescence resulting from non-specific binding of the secondary antibody from the results.

The most robust ELISA format (and hence, the common format of commercial assays) is the 'sandwich' ELISA. This utilises two primary antibodies to different epitopes of the antigen of interest, increasing the specificity of the assay. The first antibody is immobilised onto the plate and used to capture the antigen of interest. Following a wash step, to remove any unbound antigen, the second enzyme labelled antibody is added. The enzyme dependant colour change is

observed and can be measured by spectrophotometer on addition of the substrate solution (**Figure 5.1**).

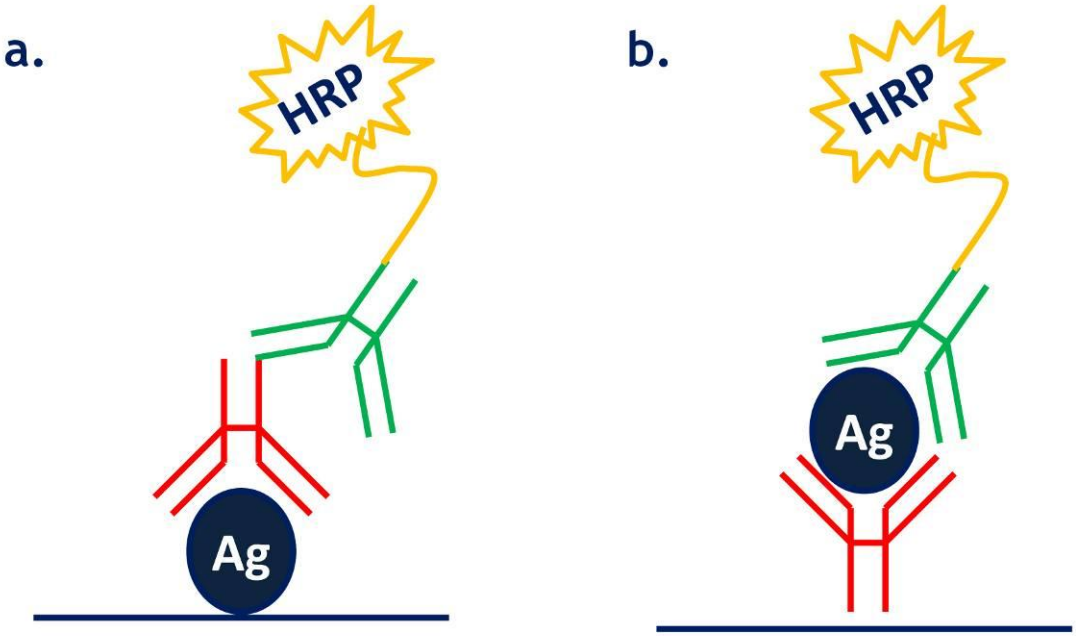

Figure 5.1 Schematic diagram to show the difference between an indirect and sandwich ELISA format

The antigen is labelled 'Ag'. The primary antibody is labelled red and the secondary antibody is labelled green. HRP is an abbreviation for horseradish peroxidase, the common enzyme used for detection in ELISA assays.

5.3.3 Western blotting

WB analysis provides the advantage of being able to differentiate positive signal due to pepsin, pepsinogen, or other interfering proteins because proteins are separated by molecular weight. Therefore, any difficulties with non-specific binding are clearly visible on analysis, an advantage over ELISA. However, the main disadvantage of WB is that it is only semi quantitative. The assay used for sample analysis in this chapter utilises a polyclonal anti-human pepsin A antibody. Johnston *et al* previously demonstrated that this antibody does not cross react with gastricsin or cathepsin D and has very low level cross-reactivity with pepsinogen A (N. Johnston, Knight, Dettmar, Lively, & Koufman, 2004). Any cross reactivity with pepsinogen A in samples would be clearly visible by differences in molecular weight on WB.

5.3.4 Alpha-amylase activity assay

Alpha-amylase is an enzyme, which has various isoforms and is predominantly found in pancreatic juice or in saliva in humans (Ferey-Roux et al., 1998; Hirtz et al., 2005). It's function in saliva is to start the digestive process through hydrolysis of alpha bonds in large polysaccharides (such as starch and glycogen) (Ragunath et al., 2008). In this study, a commercially available alpha amylase activity kit (Abcam) was used for measurement of alpha amylase activity in BAL supernatants. The assay is based on specific cleavage of ethylidene-pNP-G7 by alpha amylase to smaller fragments, which can be acted upon by alpha-glucosidase, leading to release of a chromophore. Chromogenic methods offer superior linearity, sensitivity and precision over previous saccharogenic (measuring the production of maltose or glucose) or amyloclastic (measuring the disappearance of a substrate) methods (Foo & Rosalki, 1986). Of the chromogenic methods available, specific cleavage of ethylidene-pNP-G7 and its subsequent reaction with alpha-glucosidase has been approved as the method of choice for unambiguous, robust measurement of alpha amylase (Lorentz, 2000).

5.4 Results

5.4.1 Lipid laden macrophages

1/16 PICU-ND, 6/12 Elective-ND and 2/10 healthy control BAL specimens showed occasional alveolar macrophage cytoplasm lipid staining. None of these slides reached the criteria for a positive report on review by the local cytopathologist.

5.4.2 Published ELISA methods for measurement of pepsin in BAL are not robust

Standard curves generated for pepsin ELISA 1 and 2 were found to be consistent and reproducible (**Figure 5.2 (a)**).

Spike and retrieval experiments were carried out as detailed in Section 2.4. Mean [SD] % spike recovery was found to be 16.4% [23.3] for ELISA 1 and 80.8% [46.9] for ELISA 2 on assessment in three samples (performed in at least duplicate). **Figure 5.2 (b)** shows that mean (SD) percentage spike retrieval improved with dilution of samples when analysis was performed using ELISA 1 and that recovery was worse with concentrated PICU samples. These results imply that BAL sample matrix interferes with antibody function and that this interference is reduced with sample dilution. Serial dilution (neat, 1:2, 1:5 and 1:10) of two PICU patient samples for analysis using ELISA 2 revealed poor linearity of dilution and subsequently **Figure 5.2 (c)** shows an apparent rising reported pepsin level with higher sample dilution (following correction for dilution).

The intra-assay coefficient of variation was calculated for 37-40 results (a range of PICU and healthy control samples), assayed in duplicate on the same plate and was found to be 3.94% for ELISA 1 and 9.05% for ELISA 2. The inter-assay coefficient of variation was calculated from repeated analysis of 3 samples on different days and was found to be 30.56% for ELISA 1 and 62.68% for ELISA 2.

Table 5-1 Table to show spike and recovery data and inter/intra-assay coefficients of variation for pepsin ELISA 1 and pepsin ELISA 2

	Pepsin ELISA 1	***Pepsin ELISA 2***
Inter-assay coefficient of variation (%CV (n=3/assay)	30.56	62.68
Intra-assay coefficient of variation (%CV (n=37-40/assay)	3.94	9.05
Spike retrieval (mean % (SD) (n=4-6/assay)	16.4[23.33]	80.8[46.9]

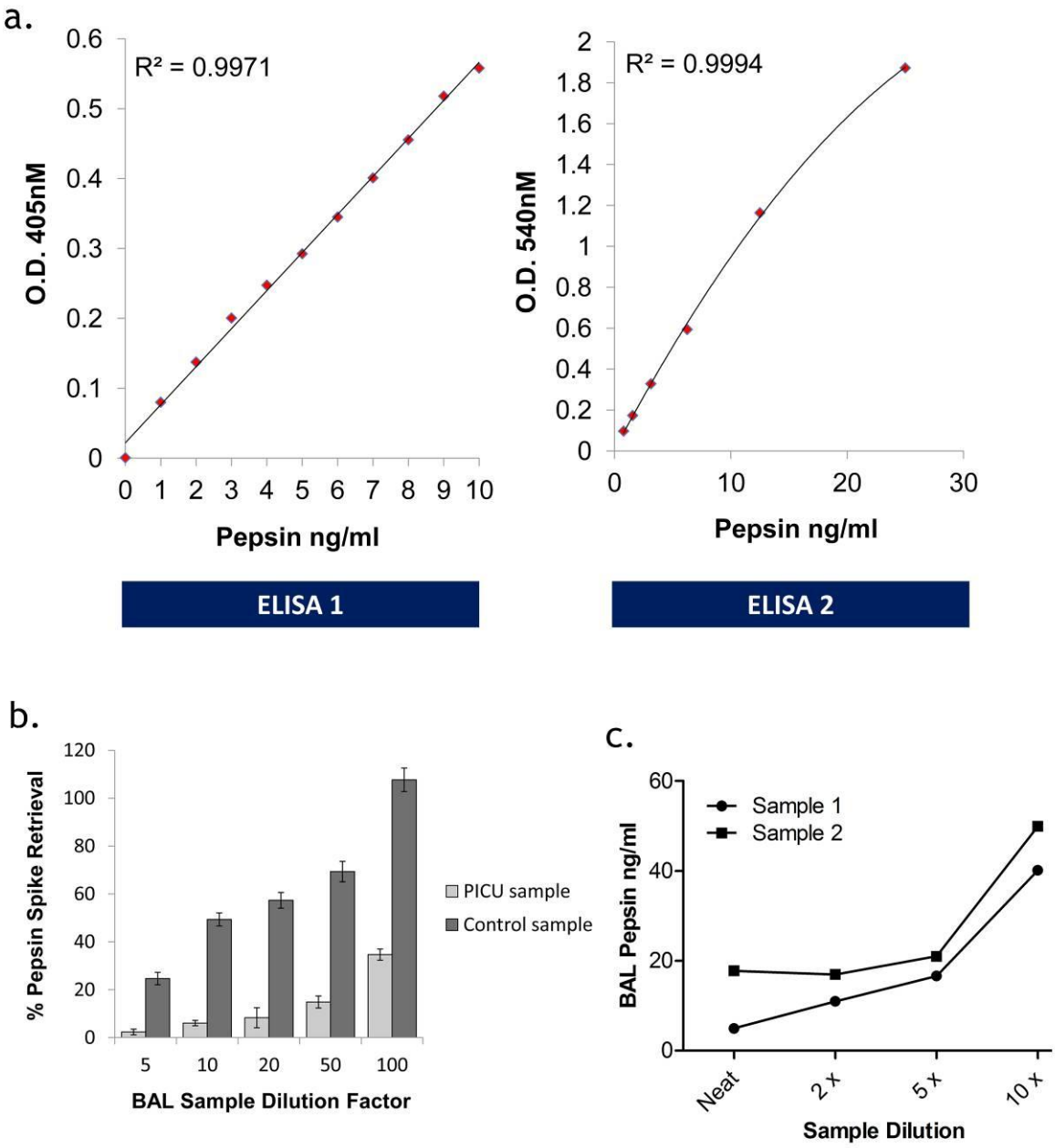

Figure 5.2 Published ELISA methods for measurement of pepsin are not robust when used with paediatric BAL

(a) Example standard curves for ELISA 1 and ELISA 2 (b) Bar chart to show mean (SD) pepsin spike retrieval following spiking two samples (a PICU sample and a healthy control sample) at a range of dilutions, compared to retrieval of an identical spike in reagent diluent using pepsin ELISA 1. Spike retrieval was worse in the PICU sample and improved in both the PICU and control samples with sample dilution. (c) Line graph to show pepsin measurements in two PICU samples, assayed in duplicate at various dilutions. Poor linearity of dilution was seen and therefore, on correction for dilution, rising reported pepsin levels were observed at higher dilutions.

Following concern about the validity of ELISA 1 and 2 for analysis of pepsin in paediatric BAL (specifically, poor linearity of dilution and spike retrieval and high inter-assay variability), samples were analysed by WB analysis using a polyclonal human pepsin antibody. Disagreement was observed on comparison of results for three samples analysed by the three pepsin assays (two ELISAS and one WB) both in reporting the presence or absence of pepsin and in the quantification of pepsin (**Figure 5.3 (a)**).

5.4.2.1 Anti-porcine pepsin antibodies used in published pepsin ELISAs are susceptible to non-specific binding to other BAL proteins

Ten microlitres of four different BAL samples were analysed by WB for pepsin A (porcine pepsin antibody WB protocol, Section 2.5.2.1) using primary (1:5000 dilution) and secondary antibodies from ELISA 1 and ELISA 2 and also secondary antibodies alone (to assess any non-specific binding of the secondary antibody). The primary antibody in ELISA 1 showed non-specific binding to a BAL protein with a higher molecular weight than porcine pepsin (**Figure 5.3 (b)**). Notably, the extent of non-specific binding was variable across samples. Both the primary and secondary antibodies in ELISA 2 showed variable non-specific binding to a BAL protein with a higher molecular weight than porcine pepsin (**Figure 5.3 (c)**).

Ten microlitres of three BAL samples spiked with 100ng porcine pepsin were also analysed by porcine pepsin WB (Section 2.5.2.1). The protein which the antibodies non-specifically bind to was clearly shown to be at a higher molecular weight than porcine pepsin (**Figure 5.3 (d)**).

One hundred nanograms of human pepsin and porcine pepsin were 'spiked' into (a) dH_20 and (b) BAL (P80) and analysed by porcine pepsin WB. Human pepsin was shown to run at a slightly lower molecular weight compared to porcine pepsin on WB. The unidentified protein was unlikely to be pepsinogen as the lower limit of detection of the porcine pepsin WB was 10ng. Therefore, levels of over 1000ng/ml would need to be present for detection on loading a 10ul sample.

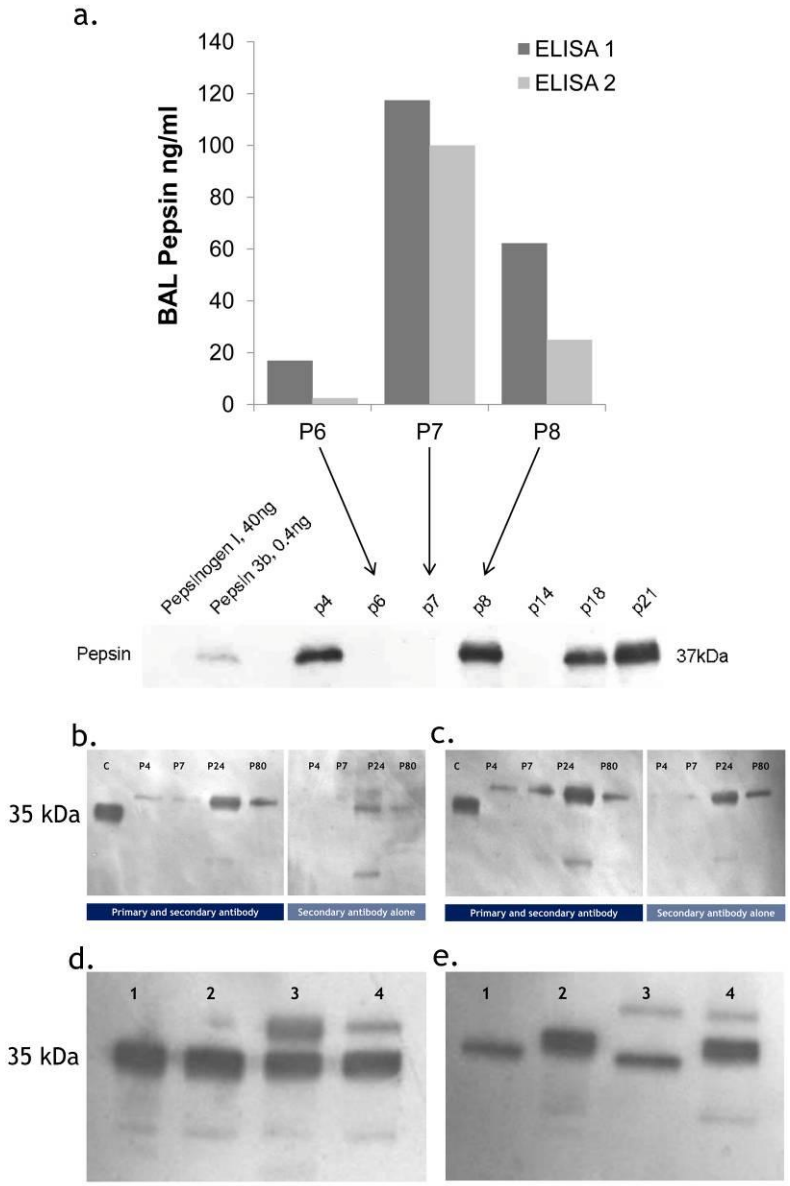

Figure 5.3 WB images and histogram to show (a) conflict between BAL pepsin results using ELISA and WB analysis (b-e) non specific binding of porcine pepsin antibodies used in published ELISA assays to other BAL proteins

(a) Histogram to show pepsin measurements in three BAL samples (two Elective-ND samples and one PICU-ND sample) by quantification using ELISA 1 and ELISA 2. Western blot image showing results of the same three samples analysed by WB, using a polyclonal antibody to human pepsin. Conflicting results are demonstrated in both identification of pepsin (between ELISA and WB methods) and quantification of pepsin (between ELISA methods 1 and 2). (b) (c) WB images to show analysis of four 10ul aliquots of different BAL samples (P4, P7, P24, P80) and a 100ng porcine pepsin control (C) by WB using (b) antibodies from pepsin ELISA 1 and (c) antibodies from pepsin ELISA 2. No pepsin was detected in the samples using this method (lower limit of detection optimised to 10ng porcine pepsin). However, variable cross reactivity was observed between porcine pepsin antibodies used in ELISA 1 and 2 and another BAL protein (shown by bands which appear to be at a higher molecular weight than pepsin). (d) WB image (porcine pepsin WB methods) to show analysis of 10ul of three BAL samples, spiked with 100ng porcine pepsin (2,3,4) compared to a porcine pepsin control in water (1). Variable non-specific binding to a protein at a higher molecular weight to pepsin is clearly shown. (e) WB image (porcine pepsin WB methods) showing identification of 100ng porcine pepsin (1) and 100ng human pepsin (2) at approximately 35kDa. Human pepsin is identified at a slightly lower molecular weight than porcine pepsin by this method and analysis of two BAL sample aliquots (10ul) spiked with 100ng porcine pepsin (3) or 100ng human pepsin (4) clearly demonstrates that the BAL protein which the porcine pepsin antibodies cross react with is not human pepsin.

5.4.3 Pepsin WB results

Forty four BAL samples were tested for the presence of pepsin using a WB technique with a polyclonal antibody for human pepsin. 6/19 PICU-ND samples (32%), 1/10 (10%) healthy control samples and 1/16 (6%) Elective-ND samples were positive. All samples which were positive had bands of greater density than a 5ng human pepsin loading control. PICU-ND patients who were positive had been ventilated for between 2 and 27 hours. 3/6 BAL pepsin positive PICU-ND patients had undergone a fundoplication procedure and 2/3 of these patients still had symptoms suggestive of GORD. 1/3 did not report symptoms suggestive of GORD but remained on anti-reflux medication. No significant differences were observed in BAL inflammatory markers, LRSQ-Neuro scores or GORD symptom scores between pepsin positive and negative PICU-ND patients. The BAL pepsin positive healthy control patient had no respiratory symptoms, no GORD symptoms and did not have raised lower airway inflammatory markers.

A spike-recovery experiment was conducted to test whether pepsin band density was equal for identical pepsin spikes in healthy control and PICU samples, in order to verify whether densitometry could be used to give an indication of the level of pepsin positivity in samples. 2ng and 1ng of pepsin were spiked into dH_20, a healthy control sample and a PICU sample (with high protein content). **Figure 5.4** demonstrates that although the spikes were visible in all samples, the band density appeared to be affected by sample protein content. Therefore, no inferences could be made about the level of pepsin in patient samples using densitometry and normalisation to a loading control. **Figure 5.4** also demonstrates that the non-specific binding experienced with the porcine pepsin antibodies in BAL did not occur with the human pepsin antibody.

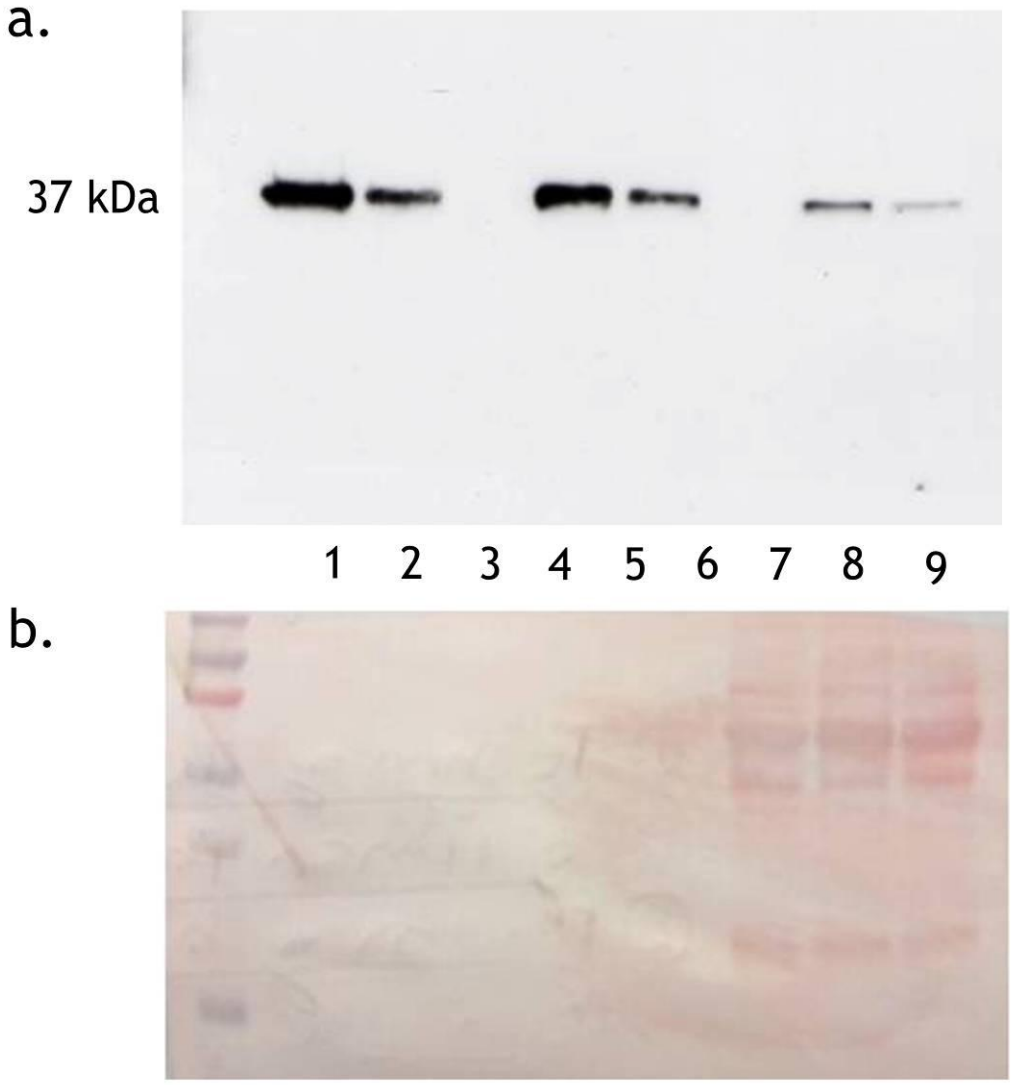

Figure 5.4 Western blot image (a) and corresponding ponceau stained PVDF membrane (b) to show effect of sample protein content on pepsin spike WB band density.

1 and 2ng purified human pepsin were spiked into water, a 10ul healthy control sample (1ug total protein in 10ul) and a 10ul PICU-ND sample (9ug total protein in 10ul). Image (a) shows WB analysis of 1. dH_20, 2. dH_20 + 1ng human pepsin, 3. dH_20 + 2ng human pepsin, 4. 10ul healthy control sample, 5. 10ul healthy control sample + 1ng human pepsin, 6. 10ul healthy control sample + 2ng human pepsin, 7. 10ul PICU-ND sample, 8. 10ul PICU-ND sample + 1ng human pepsin, 9. 10ul PICU-ND sample + 2ng human pepsin. The lower band density in the spiked PICU-ND samples implies sample interference with antibody binding. Image (b) shows the PVDF membrane post ponceau staining and confirms the increased protein content of the PICU-ND sample (lanes 7, 8 and 9).

5.4.4 Measurement of alpha amylase activity in BAL

Standard validation methods (as described in Section 2.4) were used to assess the function of the alpha amylase activity assay (Abcam UK, methods as detailed in Section 2.4.7) across a range of PICU and healthy control BAL samples. Good spike recovery and low inter and intra-assay variability were shown. Further validation of results by a second method of alpha amylase measurement is detailed in Section 5.4.4.1.

5.4.4.1 Significant correlation is observed between BAL alpha amylase activity levels and label free relative quantification of alpha amylase peptide ion intensity by LC-MSMS analysis

BAL alpha amylase activity results for eight preliminary samples were compared to results of discovery run proteomic analysis of the same eight samples using the LTQ Orbitrap Velos (as described in Section 2.8.7) followed by label free quantitative analysis of relative ion abundance using Progenesis LC-MS Nonlinear Dynamics software (as detailed in Section 2.8.9). Alpha amylase protein was identified in all samples by detection of five unique peptides to alpha amylase, post trypsin proteolysis. **Figure 5.5 (c)** shows a significant correlation between alpha amylase activity and combined relative ion intensity for the unique peptides used in the identification of alpha amylase by MSMS analysis (r +0.91, p=0.002).

5.4.4.2 Median BAL alpha amylase activity is significantly increased in Elective-ND patients compared to healthy controls

Alpha-amylase activity was measured in BAL supernatants from healthy control patients (n=10), Elective ND patients (n=16) and PICU ND patients (n=18). Alpha-amylase was detected in 97.8% samples. Median activity was significantly increased in Elective-ND patients compared to healthy controls (100.2[46.4-597] vs. 26.9[9-71.4] U/L, p=0.012). There was no statistically significant difference between PICU ND samples (54.2[14.8-254.4] U/L) and healthy controls (p=0.415).

5.4.4.3 Median BAL alpha-amylase activity is significantly lower in ND patients receiving anti-cholinergic medications

Median BAL alpha-amylase activity levels were compared between those children with ND who were taking anti-cholinergic medications (n=8) and those who were not on any medical salivary management (n=27). There was no significant difference in time since intubation between those PICU-ND children who were on salivary management (n=5) compared to those who were not (n=13, p=0.754). Therefore, the Elective ND and PICU ND groups were combined for this analysis without concern about differences in ventilation time (and subsequent relative airway protection) between groups. Median BAL alpha amylase activity levels were significantly lower in those who were on medical salivary management compared to those who were not (11.5[1.5-51.2] vs. 78.1[44.8-434.5] U/L respectively, p=0.010).

5.4.4.4 Relationship between time since intubation in PICU-ND patients and BAL alpha amylase activity

Time since intubation (hours) was noted on recruitment of PICU-ND patients. On plotting this against measured BAL amylase activity, no correlation was observed (r=-0.114 p=0.652).

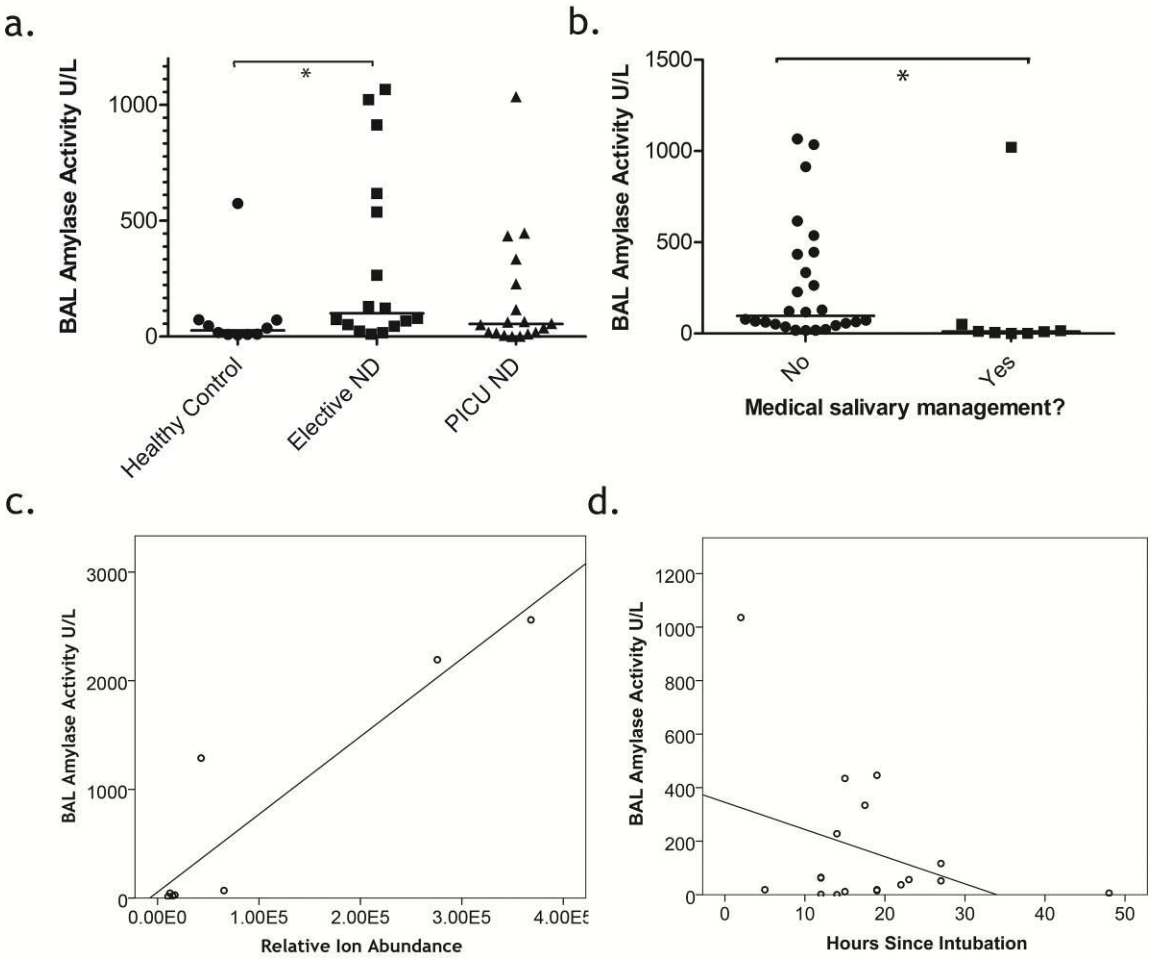

Figure 5.5 Results of alpha-amylase activity measurement in BAL

(a) Vertical scatter graph showing significantly increased median BAL amylase activity levels (measured using an ethylidene-pNP-G7 assay (Abcam, UK)) in Elective-ND patients compared to healthy control patients (100.2[46.4-597] vs. 26.9[9-71.4 U/L, p=0.012). (b) Vertical scatter graph showing significantly lower BAL amylase activity levels in those patients with ND who were prescribed anti-cholinergic medications compared to those on no medical salivary management (11.5[1.5-51.2] vs. 78.1[44.8-434.5] U/L, p=0.010). (c) Scatter graph showing a positive correlation between BAL alpha amylase activity and relative ion intensity for peptides used in the identification of alpha amylase by MSMS analysis of eight BAL samples (r +0.91, p=0.002). (d) Scatter graph to show a trend towards negative correlation between BAL alpha amylase activity levels and time since intubation in PICU-ND patients.

5.4.5 BAL inflammatory markers correlate with BAL alpha amylase activity levels in Elective-ND patients

BAL alpha-amylase activity levels positively correlated with BAL neutrophil levels in Elective-ND patients (n=16, r +0.81, p<0.001). BAL IL-8 and TGFβ-1 levels also positively correlated with BAL alpha-amylase activity levels in this group (n=16, r +0.77, p<0.001 and r+0.66, p=0.005, respectively) (**Figure 5.6 (a), (b) and (c)**).

5.4.6 Relationship between BAL alpha amylase activity levels and microbial DNA positivity in elective patients

Alpha-amylase activity levels were compared between BAL microbial rDNA positive elective patients (n=18) and BAL microbial rDNA negative elective patients (n=7). Microbial rDNA detection was assessed using SepsiTest™ (Molzym, Bremen), as detailed in Section 2.6.5. There was no statistically significant difference in median BAL alpha amylase activity level between the groups (70.7[15.7-546.3] vs. 51.2[16.9-72.6] U/L, p=0.495). However, the ranges were markedly different (7.06-929.2 U/L vs. 10.8-72.6 U/L) (**Figure 5.6 (d)**).

5.4.7 Relationship of BAL alpha amylase activity levels and VFS assessment results

There were insufficient patient numbers in the Elective-ND group who had undergone VFS assessment to make meaningful comparisons of BAL alpha amylase activity levels between VFS positive and negative patients.

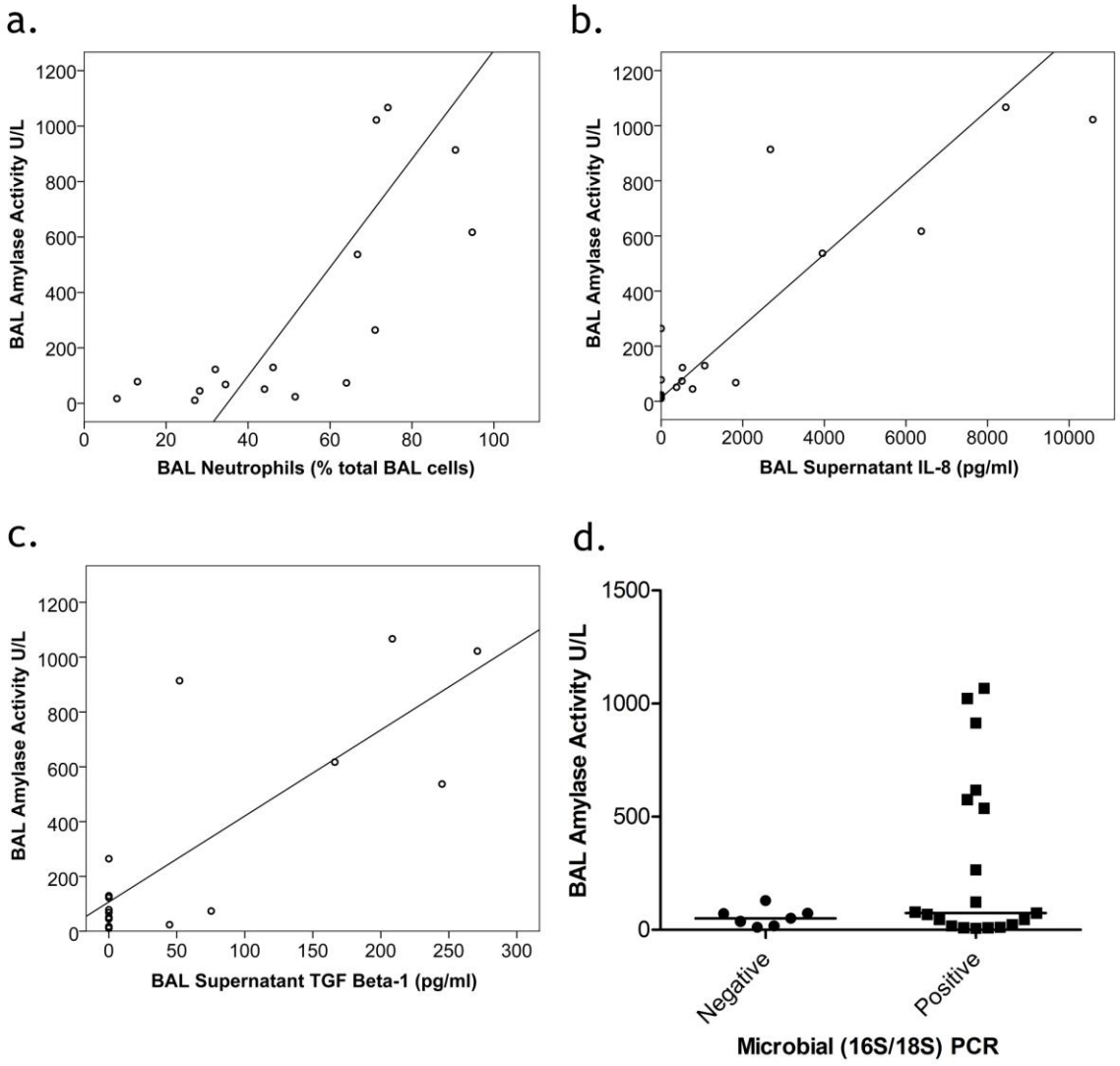

Figure 5.6 BAL inflammatory markers correlate with BAL alpha amylase activity levels in Elective-ND patients

Scatter graphs to show positive correlations between BAL alpha amylase activity and (a) BAL neutrophils (r+0.81, p<0.001), (b) BAL IL-8 (r+0.77, p<0.001) and (c) BAL TGFβ-1 in Elective-ND patients (r+0.66, p=0.005). (d) Vertical scatter graph to show the relationship of BAL microbial rDNA status with BAL alpha amylase activity in all elective patients (healthy control and Elective-ND groups).

5.5 Discussion

In this chapter, a thorough investigation of two novel BAL biomarkers of reflux and direct aspiration has been presented. Concerns were highlighted about the validity of two published pepsin ELISA assays when used with paediatric BAL samples and furthermore, disagreement between results from pepsin analysis by WB and ELISA techniques was documented. WB results indicated a higher frequency of BAL pepsin positivity in ND patients ventilated on PICU with a respiratory deterioration, suggesting pepsin may be useful as a biomarker of acute reflux aspiration. However, no firm conclusions can be drawn about the utility of this biomarker until an accurate method of BAL pepsin quantification is validated, due to occasional detection of pepsin in asymptomatic controls. Thus, the presence or absence of BAL pepsin becomes less relevant in the investigation of pepsin as a biomarker of pathological reflux aspiration and the question of whether there are significant differences in BAL pepsin load in those who are symptomatic becomes more important. Contrastingly, the alpha amylase activity assay (Abcam, UK) appears to be robust when used with paediatric BAL. We found significantly increased BAL alpha amylase levels in Elective-ND patients compared to healthy controls, suggesting this may be a useful biomarker of chronic direct aspiration. The correlation between BAL alpha amylase activity and lower airway inflammatory markers in Elective-ND patients suggests that the degree of chronic aspiration may be directly linked to lower airway pathology in these patients.

It was surprising not to find any BAL lipid laden macrophage positivity in this study, particularly considering the finding of likely aspiration in some study participants, as indicated by clinical assessments and other biomarkers. This could be explained by the need for conservative reporting of lipid laden macrophage positivity due to it's poor specificity as a test for aspiration, now widely acknowledged within the literature (Reid-Nicholson et al., 2010). Most authors support the view that lipid laden macrophage positivity can, at best, be viewed as supporting evidence of aspiration rather than diagnostic (Boesch et al., 2006). However, nine slides were reviewed by a local cytopathologist due to occasional staining of lipid laden macrophages (none of which ultimately fulfilled the criteria for a positive report); none of these samples were positive for pepsin by WB analysis and there was no correlation with BAL amylase. These findings

support previous researchers' conclusions about the poor specificity and sensitivity of BAL lipid laden macrophages as a biomarker of reflux aspiration (Colombo et al., 1986; Ding et al., 2002; Knauer-Fischer & Ratjen, 1999; Reid-Nicholson et al., 2010; Rosen et al., 2008).

When clinical specimens are analyzed by ELISA, antibody interference is always possible and particularly so when dealing with potentially thick and proteinaceous samples such as BAL or sputum. A standard method of assay validation involves 'spiking' samples with known amounts of the protein of interest and then measuring retrieval (Administration). One study, reporting the validation of a pepsin ELISA, showed reasonable retrieval of high concentration spikes, but inadequate and, importantly, variable recovery at lower concentrations (100 ng/mL 50%-93% recovery; 10 ng/mL 27%-82% recovery) (Knight et al., 2005). This highlights how clinical samples from different individuals can interact and interfere variably with assay efficiency. We conducted similar experiments using two published ELISAs on paediatric BAL samples. We found evidence of variable non-specific antibody binding and sample interference as well as high inter-assay variability. These findings are important, particularly considering the wide range of methods used for measuring pepsin within the literature and incongruity of study results, as discussed in Chapter 1.

The anti porcine pepsin antibodies used in ELISA 1 and 2 are known to be cross reactive with pepsinogen A. Although researchers have previously tested for cross reactivity with selected serum proteins (albumin, fibrinogen and gamma globulins), specific cross reactivity testing with other acidic proteases known to be present within the lung (such as pepsinogen C and cathepsin D) has not been documented (Tasker et al., 2002a). Recent BAL proteomic studies have highlighted the complexity of respiratory samples (Plymoth et al., 2006). Therefore, selective testing of antibody cross reactivity with predominant serum proteins would seem inadequate, particularly when utilising ELISA techniques, where non-specific antibody binding may not be easily recognised. We found variable non-specific binding of these antibodies to other unidentified proteins in BAL when used in WB analysis. These findings concur with those of He *et al* who documented non-specific binding of one of the porcine pepsin A antibodies used in both ELISA 1 and 2 to an unidentified serum protein at a slightly higher

molecular weight than pepsin (He et al., 2007). A commercial human pepsin ELISA (USCN life sciences) has recently become available and has been utilised in a small number of clinical studies. Lee *et al* recently documented a high overall incidence of BAL pepsin positivity in adults with idiopathic pulmonary fibrosis. However, no validation data was published for use of this ELISA with BAL (by either the manufacturer or the authors) and cross reactivity with pepsinogen is acknowledged by the manufacturers (J. S. Lee et al., 2012). 'Peptest' (RD Biomed, Hull, UK), a lateral flow device using two monoclonal human antibodies, has also recently become available for pepsin analysis. Again, no specific detailed BAL validation data was available on informal enquiry in 2010 and there has been no publication of specific data to support validation since this time.

WB analysis offers increased transparency where there are concerns about non-specific antibody binding, due to the separation of proteins by molecular weight. We undertook WB analysis using a polyclonal human pepsin antibody, previously shown not to cross-react with gastricsin or cathepsin D and to have very low cross reactivity with pepsinogen A (N. Johnston et al., 2004). **Figure 5.4** demonstrates that this antibody does not seem to be susceptible to non-specific binding to other BAL proteins on analysis by WB, despite high protein loads. This is in stark contrast to commercially available porcine pepsin antibodies, as highlighted in **Figures 5.3** and **6.9**. Unfortunately, WB analysis is semi quantitative at best and furthermore, **Figure 5.5** demonstrates that BAL sample protein load can influence the density of the band visualised by WB for identical pepsin spikes, even when using a highly specific antibody. Therefore, attempts to partially quantify pepsin by WB techniques using densitometry would potentially yield misleading results.

Our finding of 32% pepsin positivity in the PICU-ND group compared to a mean of 8% in the electively recruited groups suggests that pepsin may be a useful biomarker of acute reflux aspiration. PICU-ND patients had been ventilated for various time periods before sampling and therefore, it is possible that the frequency of pepsin positivity may have been higher at the time of intubation. However, pepsin was detectable in one study recruit 27 hours post intubation, suggesting it may remain in the lungs for some hours post aspiration. The possibility of ongoing gastric aspiration during ventilation on PICU also needs to be considered, as highlighted by a series of studies by Methany *et al* who

measured pepsin (by WB) in serial tracheal aspirates in ventilated patients and suggested a potential for ongoing gastric aspiration over the period of ventilation (Kathleen L. Meert et al., 2002; K. L. Meert, Daphtary, & Metheny, 2004; N. A. Metheny et al., 2006; Norma A. Metheny, Schallom, Oliver, & Clouse, 2008). These studies also highlighted that acute gastric aspiration may occur in other patient groups ventilated on PICU. Therefore, without a PICU control group, we are unable to draw conclusions about either the risk of gastric aspiration during ventilation on PICU in ND patients compared to other patient groups or whether gastric aspiration was the cause of deterioration in children with ND in this study (or alternatively, whether it was a result of clinical deterioration and mechanical ventilation). Unfortunately, we were unable to recruit a PICU control group due to competing studies and limited patient numbers. Interestingly, BAL amylase activity levels within the PICU-ND group did not correlate with pepsin positivity and median BAL amylase activity was not significantly higher in the PICU-ND group compared to healthy controls, in contrast to the Elective-ND patient group who had significantly raised BAL amylase activity level. This might imply that the airway is actually reasonably well protected from further aspiration during paediatric ventilation, as one would expect continued salivary aspiration to accompany any gastric aspiration.

It is noteworthy that the Elective-ND group had a low incidence of pepsin positivity (6%) by WB analysis. This could imply that regular micro aspiration of gastric contents is not common or alternatively that pepsin is not a good biomarker of chronic reflux aspiration. The latter seems more likely in view of the high incidence of GORD and salivary aspiration in this group. A potential limitation of pepsin as a biomarker of chronic reflux aspiration is that pepsin detection and quantification will be influenced by time since the last reflux-aspiration event. Knowledge of the rate of clearance of pepsin from the lungs after an aspiration event is limited to animal data (Badellino et al., 1996; N. A. Metheny et al., 2004). Therefore, although the theoretical specificity of pepsin A as a biomarker of reflux-aspiration is high, the sensitivity may be limited. Large studies using reliable quantitative assays are needed to draw conclusions about whether there are significant differences between the level of BAL pepsin detected in healthy, asymptomatic individuals and those with likely pathological reflux-aspiration. Subsequently, robust conclusions could potentially be drawn

about the utility of pepsin as a clinical biomarker of acute or chronic aspiration and it's clinical relevance could be studied through longitudinal studies.

Our validation data supports the suitability of Abcam's alpha amylase activity assay (based on amylase specific cleavage of ethylidene-pNP-G7) for use in BAL analysis. Previous studies of alpha amylase as a biomarker of direct aspiration have been limited (Clarke, Bain, Davies, et al., 1981; Nandapalan, McIlwain, & England, 1995; D. D. Smith & McNamara, 1976). Nandapalan *et al* clearly documented the presence of low levels of alpha amylase in the normal lung in their comparative study of undiluted tracheo-bronchial and salivary secretions in laryngectomized patients (Nandapalan, McIlwain, & England, 1995). In this study, salivary amylase activity measurements between 20-35,000 U/L were documented and the ratio of salivary to bronchial amylase in paired samples was 30-600:1. The levels recorded in undiluted tracheo-bronchial secretions were unlikely to have been of serum origin in the population studied (no pancreatic disease) and the authors concluded that amylase is produced locally in the lung. We have recorded (for the first time) alpha amylase activity control measurements in paediatric BAL, collected according to ERS guidelines. Alpha amylase activity was detectable in 98% of all samples and this is in keeping with the presence of low levels of amylase in the normal lung, as well as the potential for minor salivary contamination of the sample following intubation and for minor aspiration during sleep (Gleeson et al., 1997). Significantly higher levels of amylase activity were observed in Elective-ND patients in comparison to healthy controls. All patients in the two elective groups were intubated under controlled conditions, following which BAL was collected using standardised techniques. Therefore it seems reasonable to conclude that differences in BAL amylase activity are likely to represent increased salivary aspiration in the Elective-ND group rather than contamination at intubation. The high levels reported in some Elective-ND patients (1000 U/L) also support the salivary origin of the amylase, taking into account the BAL dilution factor. Interestingly, median amylase activity in PICU-ND samples was not significantly raised compared to healthy controls, raising the possibility that airway protection by intubation may have led to lower BAL amylase activity levels in this group of patients, who were matched by age and disability level to the Elective-ND group. A negative correlative trend between amylase activity and time since

intubation was observed, although this did not reach statistical significance. Clearly, levels of BAL amylase activity at intubation would be varied between patients (as displayed in the Elective-ND group) and therefore, it would be more useful to investigate this through serial sampling in a larger PICU population.

The correlation observed between BAL alpha amylase activity and inflammatory markers in Elective-ND patients is interesting and implies a direct relationship between degree of aspiration and lower airway inflammation. Fedrowitz *et al* documented anti-proliferative effects of alpha-amylase on rat mammary epithelial cells. However, there is no literature documenting the effects of alpha amylase on airway epithelial cells and further investigation of this is warranted (Fedrowitz, Hass, Bertram, & Löscher, 2011). It may be that alpha amylase is not directly damaging to airway epithelial cells and that other elements of the aspirate, such as bacterial and gastric contents are the cause of aspiration associated lower airway inflammation. Further study of the relationship of BAL alpha amylase activity and microbiology would be of interest and this is supported by the relationship between BAL amylase levels and microbial rDNA positivity observed in this study.

When assessing alpha amylase as a biomarker of direct aspiration it is essential to consider conditions in which pulmonary amylase levels are known to be raised. Pulmonary hyperamylasaemia has been shown (on direct testing of pulmonary effusions) in a variety of conditions including pancreatic disease, pulmonary malignancy, tuberculosis and parapnuemonic effusions (Joseph et al., 1992; G. D. Lee et al., 2003; Villena et al., 1995; Villena et al., 2002). However, the amylase activity levels recorded in effusions from patients with pancreatic or malignant disease were by far the highest in a series of 841 patients studied by Villena *et al; e*ffusion amylase activity levels in infective and cardiac causes being consistently lower (<500U/L). Considering that amylase activity levels of double this were measured in dilute BAL samples in certain patients in our study, it seems likely (on the basis of the available literature) that the BAL amylase activity measured was of salivary rather than pulmonary origin. Furthermore, the finding of no significant difference in BAL amylase activity between PICU-ND and healthy control patients also provides supportive evidence that local production of pulmonary amylase is not consistently triggered by lower airway inflammation/infection. However, in order to draw firm conclusions, further

study of BAL alpha amylase activity levels would need to be conducted in a large cohort of paediatric patients at high and low risk of aspiration and with a range of pulmonary conditions, using standardised BAL collection techniques. If clear, definable differences were demonstrable between 'normal' BAL alpha amylase levels and BAL alpha amylase levels in those at high risk of aspiration, then BAL alpha amylase may evolve into a useful clinical and research marker of direct aspiration.

Future work should focus on the development of robust quantitative techniques for measurement of pepsin in BAL. Researchers should subsequently aim for standardisation of measurement techniques across studies in order to draw firm conclusions about the utility of pepsin as a biomarker of acute or chronic gastric aspiration. Further research investigating alpha amylase as a potentially useful biomarker of direct aspiration should focus on development of paediatric and adult normative values. Subsequent longitudinal study of clinical outcomes in relation to BAL alpha amylase levels and their relationship to lower airway microbiology would potentially increase knowledge of the clinical consequences of aspiration in children with ND and also other patient groups, for example, in the study of ventilator associated pneumonia in intensive care patients.

Chapter 6 - Development of a novel assay for quantification of pepsin in BAL

6.1 Introduction

Designing an assay for accurate quantification of specific proteins in clinical samples is challenging, due to the effects of other substances within the sample matrix. We have highlighted how clinical samples from different individuals can interact and interfere variably with assay efficiency and accuracy, demonstrating contrasting results from three different immunological pepsin assays on repeated BAL sample analysis (Chapter 5).

Key properties of a high quality clinical assay are confident protein identification, accurate quantification and consistency across all samples. However, the previously published ELISA assays for measurement of pepsin in BAL showed susceptibility to variable non-specific antibody binding (limiting reliability of pepsin identification) and sample interference with antibody function (limiting accuracy of quantification) (Ervine et al., 2009; Stovold et al., 2007). Western blotting is potentially a more robust method of BAL protein analysis, although this technique is semi-quantitative at best (Rosen et al., 2012). In investigating pepsin as a biomarker of reflux aspiration, it is essential to establish an accurate quantitative assay technique because low levels of pepsin may be detected in healthy, asymptomatic individuals; there is a high incidence of physiological gastro-oesophageal reflux in children and the aspiration of nasopharyngeal secretions is common during sleep (Gleeson et al., 1997; Ramaiah et al., 2005). Following quantitative assay development, clear demonstrable differences in BAL pepsin level between those with physiological reflux-aspiration who are asymptomatic and those who have pathological reflux aspiration would need to be demonstrated in order for pepsin to be hailed a useful BAL biomarker of reflux aspiration. Authors have highlighted assay standardisation as a priority, so that normative values can be established for paediatric and adult age groups (Davis et al., 2010).

In view of the limitations of antibody-based assays for detection of pepsin in BAL, this chapter focuses on the development of an assay that uses mass spectrometry, the gold standard for protein identification (Pusch, Flocco, Leung, Thiele, & Kostrzewa, 2003). Mass spectrometers analyse the mass to charge ratio (m/z) of ionised molecules. Protein identification is by matching acquired peptide m/z to those in a database of theoretical protein digests; peptide

fingerprint mapping (Nielsen, Savitski, & Zubarev, 2005). Neubert *et al* recently developed a nanoflow liquid chromatography-tandem mass spectrometry (LC-MSMS) assay for measurement of pepsin A and its precursor, pepsinogen A, in saliva (Neubert, Gale, & Muirhead, 2010). They utilised a peptide immuno-affinity enrichment technique and multiple reaction monitoring (MRM) to increase assay sensitivity and specificity.

The aim of this chapter was to develop a similar LC-MRM-MS assay but with an affinity enrichment step using the potent inhibitor of pepsin, pepstatin A. To our knowledge, inhibitor affinity enrichment has not been used for these purposes previously and it offers potential advantages over immuno-affinity methods, as discussed in Section 1.1.2. A number of preliminary experiments were required in order to confirm the feasibility of this proposed method prior to more detailed LC-MS assay development. The results of these experiments are presented in this chapter.

6.1.1 Pepsin

Pepsin, an aspartic protease, is the primary proteolytic enzyme of the digestive system. There are two broad categories of pepsin in human gastric juice; pepsin A (four different isoforms: pepsin 1, pepsin 3a, pepsin 3b and pepsin 3c) and pepsin C (gastricsin). Previous studies have established a high degree of homology between the four pepsin A isoforms (A. T. Jones & Roberts, 1992). Pepsins 3b and 3c differ only by one amino acid substitution and are the products of genes PGA5 and PGA3, respectively. Pepsin 3a differs by three amino acid residues and corresponds to gene PGA4. A much lower degree of homology is seen between gastricsin (gene PGC) and other human pepsins (A. T. Jones & Roberts, 1992). High performance ion exchange chromatography of human gastric juice has highlighted pepsin 3b as the most abundant gastric pepsin (66%), followed by gastricsin (20%) and lastly pepsins 3a, 3c and 1 (14%) (A. T. Jones, Balan, et al., 1993).

The chief cells within the stomach lining produce pepsin as an inactive precursor to prevent host tissue damage and self-autolysis. Pepsinogen A and pro-gastricsin are the precursors of pepsin A and C, respectively (A. T. Jones, Keen, & Roberts, 1993). These precursors, which are released on eating, have a 'pro-segment' at

their N terminus (47 residues in the case of pepsinogen A) that blocks the active site of the enzyme. Under acidic conditions (< pH 3), the pro-segment is autolytically removed, leaving a functional active site (Richter, Tanaka, & Yada, 1998). The pH dependence of the conversion of these zymogens to their active form is proposed to be due to the electrostatic interaction of basic residues in the pro-segment and acidic residues in the active enzyme (Richter et al., 1998).

The enzymatic activity of human pepsin A is pH dependent, having maximal activity at pH 2, becoming inactive at pH 6.5 and remaining structurally stable up to pH 8 (Nikki Johnston et al., 2007). Johnston *et al* found that human pepsin 3b retains approximately 79% of its enzymatic activity on re-acidification following exposure to pH 7 for twenty-four hours (Nikki Johnston et al., 2007).

Pepsin A is potentially a useful biomarker of gastric aspiration, as it is not known to be produced in extra-gastric tissues. However, pepsinogen C (the precursor of gastricsin) has been shown to be produced by type 2 alveolar cells where it has a role in the proteolytic processing of surfactant protein B (Elabiad & Zhang, 2011; Foster et al., 2004; Gerson et al., 2008). Cathepsin D, another aspartic protease sharing a high degree of homology with pepsin, is also known to be produced within the lungs and furthermore, to be increased in inflammatory and infective pulmonary conditions (Bewley et al., 2011; Plymoth et al., 2006; Shewale & Tang, 1984). It is essential therefore, that any assay used to investigate pepsin as a biomarker of gastric aspiration is capable of confidently differentiating progastricsin and cathepsin D from pepsinogen A and pepsin A, a little-recognised limitation of some previous enzymatic and immunological methods (Rosen et al., 2012; Samuels & Johnston, 2010).

Interestingly, there is a high degree of homology between human pepsin 3b and porcine pepsin A, indicating the potential use for porcine pepsin A, which is readily commercially available, as a surrogate for human pepsin 3b in assay development (**Figure 6.1**) (A. T. Jones & Roberts, 1992).

a.

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sp|P00791|PEPA_PIG        MKWLLLLSLVVLSECLV-KVPLVRKKSLRQNLIKNGKLKDFLKTHKHNPA 49
sp|P0DJD9|PEPA5_HUMAN     MKWLLLLGLVALSECIMYKVPLIRKKSLRRTLSERGLLKDFLKKHNLNPA 50
                          *******.**.****:: ****:******:.* :.* ******.*: ***

sp|P00791|PEPA_PIG        SKYFP--EAAALIGDEPLENYLDTEYFGTIGIGTPAQDFTVIFDTGSSNL 97
sp|P0DJD9|PEPA5_HUMAN     RKYFPQWEAPTLVDEQPLENYLDMEYFGTIGIGTPAQDFTVVFDTGSSNL 100
                           ****  **.:*:.::******* ****************:********

sp|P00791|PEPA_PIG        WVPSVYCSSLACSDHNQFNPDDSSTFEATSQELSITYGTGSMTGILGYDT 147
sp|P0DJD9|PEPA5_HUMAN     WVPSVYCSSLACTNHNRFNPEDSSTYQSTSETVSITYGTGSMTGILGYDT 150
                          ************::**:***:****:::**: :*****************

sp|P00791|PEPA_PIG        VQVGGISDTNQIFGLSETEPGSFLYYAPFDGILGLAYPSISASGATPVFD 197
sp|P0DJD9|PEPA5_HUMAN     VQVGGISDTNQIFGLSETEPGSFLYYAPFDGILGLAYPSISSSGATPVFD 200
                          ******************************************:********

sp|P00791|PEPA_PIG        NLWDQGLVSQDLFSVYLSSNDDSGSVVLLGGIDSSYYTGSLNWVPVSVEG 247
sp|P0DJD9|PEPA5_HUMAN     NIWNQGLVSQDLFSVYLSADDKSGSVVIFGGIDSSYYTGSLNWVPVTVEG 250
                          *:*:***************::*.*****::*****************:***

sp|P00791|PEPA_PIG        YWQITLDSITMDGETIACSGGCQAIVDTGTSLLTGPTSAIANIQSDIGAS 297
sp|P0DJD9|PEPA5_HUMAN     YWQITVDSITMNGETIACAEGCQAIVDTGTSLLTGPTSPIANIQSDIGAS 300
                          *****:*****:******: ******************.***********

sp|P00791|PEPA_PIG        ENSDGEMVISCSSIDSLPDIVFTINGVQYPLSPSAYILQDDDSCTSGFEG 347
sp|P0DJD9|PEPA5_HUMAN     ENSDGDMVVSCSAISSLPDIVFTINGVQYPVPPSAYILQSEGSCISGFQG 350
                          *****:**:***:*.***************:.*******.:.** ***:*

sp|P00791|PEPA_PIG        MDVPTSSGELWILGDVFIRQYYTVFDRANNKVGLAPVA 385
sp|P0DJD9|PEPA5_HUMAN     MNVPTESGELWILGDVFIRQYFTVFDRANNQVGLAPVA 388
                          *:***.***************:********:********
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b.

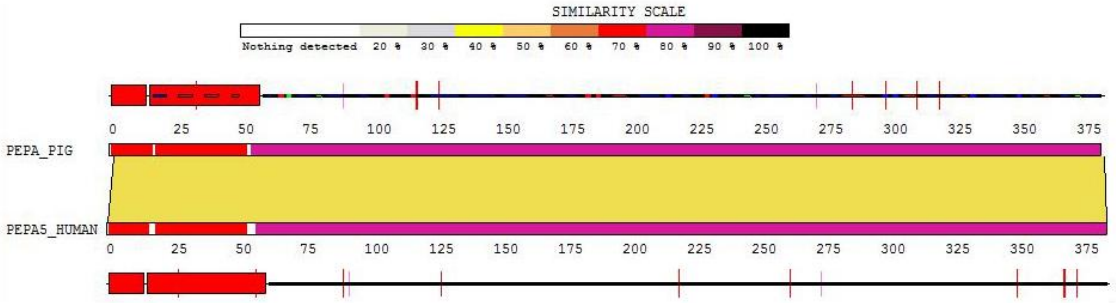

Figure 6.1 Amino acid sequence alignment for porcine pepsin and human pepsinogen A (PGA 5, corresponding to human pepsin 3b).

a. Amino acid sequence alignment (produced using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2)) of porcine pepsin (PEPA_PIG) and human pepsinogen A (PEPA5_HUMAN), following retrieval of protein sequences from the Universal Protein Resource (Uniprot (www.uniprot.org)). Key: (*) identical. (:) conserved substitution. (.) semi-conserved substitution. (red) small, hydrophobic, aromatic, not Y. (blue) acidic. (magenta) basic. (green) hydroxyl, amine, amide, basic.
b. Graph to show the degree of similarity between the amino acid sequences of porcine pepsin (PEPA_PIG) and human pepsinogen A (PEPA5_HUMAN), created using Expasy SIM (alignment tool for protein sequences, available at web.expasy.org/sim/) and Lalnview (a graphical program for visualising local alignments between two sequences, available at pbil.univ-lyon.fr/software/lalnview.html). A similarity score of 1706, identity of 83.9% and gap frequency of 0.8% was reported.

6.1.2 Affinity enrichment

Although there are concerns about antibody production and selectivity, ELISA methods continue to offer superior sensitivity over mass spectrometry (MS) techniques (Shi et al., 2012). Strategies employed to increase MS sensitivity and limit matrix effects of proteinaceous samples include multiple reaction monitoring (MRM, discussed in Section 6.3.3.3) and immuno-affinity techniques. Ackermann and colleagues describe the use of these techniques to enable quantitative MS analysis of sub-nanogram levels of low abundance proteins in clinical samples (Ackermann & Berna, 2007).

Affinity enrichment entails isolation of the protein or peptide of interest from the sample matrix, allowing presentation in a purified (reducing matrix effects) and concentrated (increasing sensitivity) format for MS analysis. Antibodies are commonly utilised for this purpose. Neubert *et al* (2010) achieved affinity enrichment and MS quantification of human pepsinogen A/pepsin A in salivary samples using endoproteinase AspN for sample digestion, then enrichment at the peptide level using an antibody which was directed against a human pepsinogen A and subsequently, pepsin A C-terminal sequence. However, the authors acknowledged that their technique relied upon the ability to consistently generate an anti-peptide antibody of high quality (Neubert et al., 2010).

It was hypothesised that pepstatin A, a potent inhibitor of pepsin, could similarly be used for affinity enrichment at the protein, rather than the peptide, level. This would theoretically allow both selective retrieval of pepsin with an active site and eliminate the need for high quality antibody generation.

6.1.3 Pepstatin A

Pepstatin A is a potent inhibitor of the aspartic proteases pepsin, cathepsin D, renin and chymosin. It non-covalently binds pepsin by a complex interaction at the active site of the protein. Pepsin subsequently undergoes a conformational change to enclose the inhibitor tightly (**Figure 6.2**) (Fujinaga, Chernaia, Tarasova, Mosimann, & James, 1995).

The potential use of bead immobilised pepstatin A for affinity enrichment offers selectivity for retrieval of pepsin with an active site. Critical assessment of novel

biomarkers of reflux-aspiration includes questioning the specificity (gastric origin) of the biomarker. Although pepsinogen A has not been shown to be produced by extra-gastric tissues, it is present at low levels in serum (Oishi et al., 2006). Therefore, previous researchers have gone to lengths to demonstrate no antibody cross reactivity with pepsinogen A when using antibody based methods for pepsin detection (Knight et al., 2005; Rosen et al., 2012). Pepstatin-A affinity enhancement offers selectivity for activated pepsin; it is unable to bind pepsinogen A unless it has encountered low pH values (<pH3) and been converted to active pepsin (Campos & Sancho, 2003). Production of pepsinogen A at a higher pH (extra-gastric) would lead to sustained active site blockage by the pro-peptide sequence. Therefore, it is reasonable to conclude that pepsin retrieved by pepstatin A is likely to originate from the stomach.

Should gastric pepsin be aspirated into the lungs, it will encounter pulmonary epithelial lining fluid (pELF). A review of available evidence suggests that pELF pH is likely to be mildly acidic (Fischer & Widdicombe, 2006). BAL samples collected from recruits to this study ranged from pH 5.5-7.4. Researchers have demonstrated (via spectroscopic structural characterisation studies) that despite a structural change in pepsin to an intermediate conformation between pH 4 and pH 6.5, the strength of subsequent pepsin-pepstatin binding remains unchanged (Campos *et al* 2003). However, their studies indicated that pepsin-pepstatin binding might not occur at pH 9, likely due to irreversible protein denaturation (Campos & Sancho, 2003).

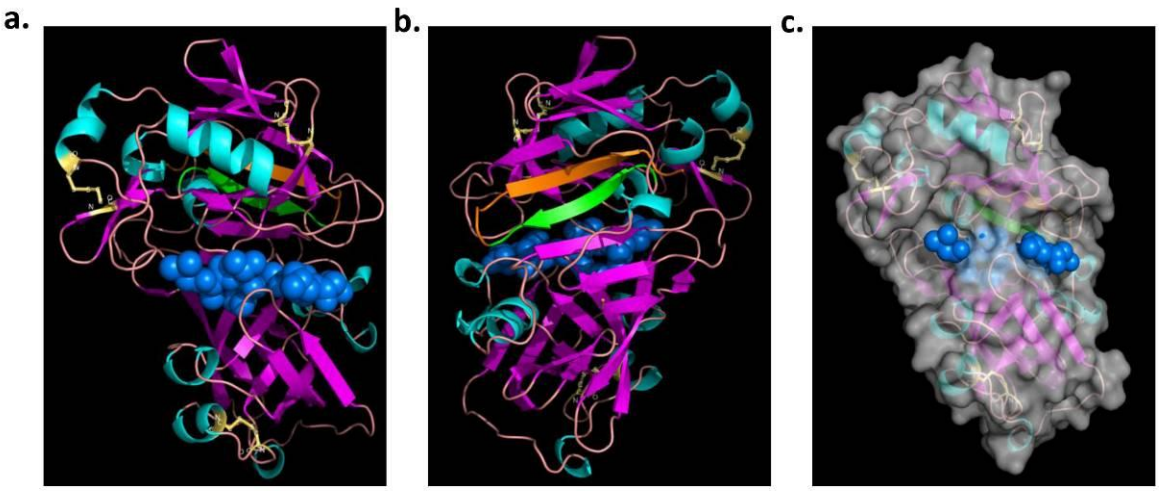

Figure 6.2 Schematic diagrams of human pepsin A structure and it's interaction with pepstatin

a.b. Human pepsin/pepsinogen A 'identification' peptides QYFTVFDR and ANNQVGLAPVA are highlighted in orange and green (explained in Sections 6.5.1 and 6.5.2). All atoms are labelled for the four disulphide bonds. Pepstatin is represented in blue spheres, showing it's position of interaction within the active site of pepsin.
c. Shows pepsin with a rendered surface, demonstrating how pepsin (grey) tightly encloses pepstatin (blue spheres) within the active site. All images generated using Pymol (http://pymol.org).

6.2 Aims

To establish the feasibility of using immobilised pepstatin A to extract active pepsin from paediatric BAL matrix for subsequent LC-MRM-MS analysis, we aimed to:

(i) Identify the predicted peptides generated by trypsin digestion of human and porcine pepsin A and their suitability for MS analysis, subsequently providing 'proof of concept' through preliminary experiments using porcine pepsin A, pepstatin A agarose beads and MALDI-TOF mass spectrometry analysis.

(ii) Optimise conditions for (a) binding porcine pepsin A to pepstatin A agarose beads in paediatric BAL matrix and (b) subsequent release of pepsin from beads for LC-MSMS analysis, using SDS-PAGE gel and Western blot (WB) analysis.

(iii) Establish an LC-MRM-MS assay for detection of porcine pepsin (for proof of concept) and subsequently human pepsin in BAL.

6.3 Specific methods

6.3.1 Peptide mapping

Trypsin is the most commonly used enzyme for proteolysis in mass spectrometry (Rodriguez, Gupta, Smith, & Pevzner, 2008). The theoretical masses of peptides generated by trypsin cleavage of proteins (cleavage at the C terminal side of lysine (K) or arginine (R), with exceptions) were calculated using PeptideMass (ExPASy Bioinformatics Resource Portal), as described in Section 2.8.2. MRM methods were built (including generation of fragment peptide lists and collision energies) using Skyline (Maccoss Lab Software https://sites.google.com/a/uw.edu/maccoss/) as detailed in Section 2.8.6.

6.3.2 Reversed phase chromatography

Liquid chromatography is an analytical chemistry technique used to separate compounds within a solution based on physical properties, using a 'stationary' phase and a 'mobile' phase (Dorsey, Cooper, Siles, Foley, & Barth, 1996). The type of chromatography described in this chapter is reversed phase chromatography, which separates molecules based on relative hydrophobicity (Amersham Biosciences, 1999). A non-polar stationary phase (silica column with immobilised hydrophobic alkyl chains: in this case, C18) binds the analytes, injected within an initial mobile phase, which is designed to favour hydrophobic binding (aqueous solution). The concentration of organic solvent (for example, acetonitrile or methanol) in the mobile phase solution is then gradually increased to encourage desorption of the analytes from the stationary phase (on the column), back into the mobile phase. This can occur at variable points in the gradient, giving a specific column 'retention time' for each molecule, depending on it's individual hydrophobicity (Biosciences, 1999). The mobile phase is also usually acidic to improve chromatography and subsequent MS performance. The retention times of the peptides of interest are used to schedule MRM assays (described in Section 2.2.3.3).

6.3.3 Mass spectrometry (MS)

The first step of MS analysis involves ionisation of the analyte: a liquid or solid sample is converted to gas-phase ions by the appropriate ionisation technique (commonly electron spray ionisation (ESI) in the case of liquid samples and

matrix assisted laser desorption/ionisation for solid samples) (Takáts, Wiseman, & Cooks, 2005). Compound ions are then separated in a mass analyser by electrostatic fields according to their m/z ratio, and then measured by a detector.

6.3.3.1 Tandem mass spectrometry

Tandem MS (MSMS), as the name suggests, consists of two stages of MS analysis. The mass selected ions (precursor ions) are isolated, undergo a dissociation step (for example, by high energy collision with an inert gas) and then the resulting fragment ions are subjected to a second MS analysis (**Figure 6.3**). This can occur 'in space' (where two mass spectrometers are used in tandem) or 'in time' (where there are discreet steps of selection, fragmentation and detection within the same machine) (Gross JH, 2011). This technique, coupled to liquid chromatography, offers excellent specificity and good sensitivity (Pitt, 2009). This has superseded older techniques such as liquid chromatography and radio-assays, and is used in both research and clinical settings (Carvalho, 2012).

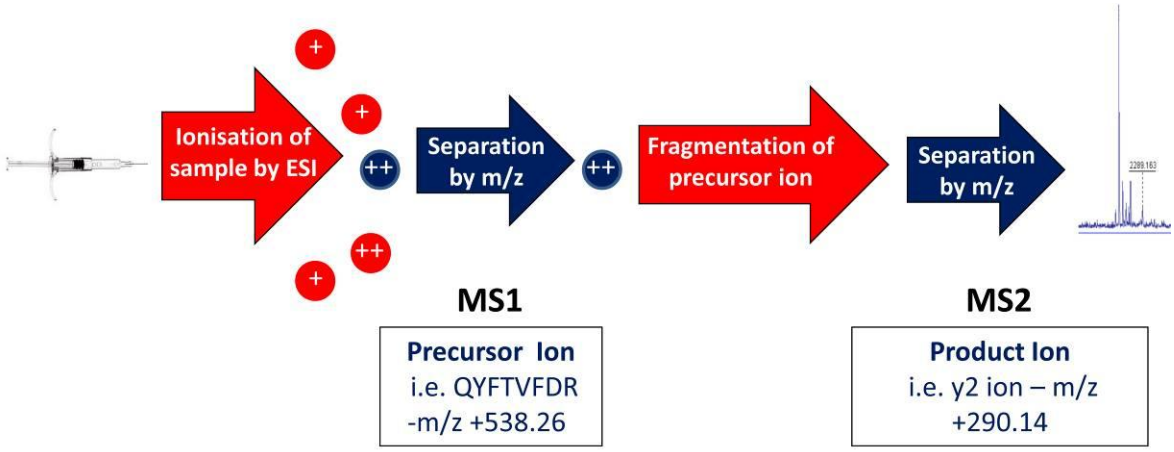

Figure 6.3 Pictorial representation of tandem mass spectrometry (MS-MS) process

This figure shows the process of sample entry into a mass spectrometer, followed by ionisation and subsequent separation by m/z ratio. When performing tandem mass spectrometry, a precursor ion is selected (e.g. in a human pepsin MSMS assay, ions with m/z 538.26 would be selected - this is the m/z ratio of the peptide QYFTVFDR, which is generated following trypsin digestion of human pepsin). This ion would be isolated and then further fragmented (by collision induced dissociation) to produce product fragment ions **(figure 6.13)**. The ions would be separated by m/z ratio and then measured by the detector. The tandem mass spectrometry technique offers high specificity as both the precursor ion and predicted product ions must be isolated for identification of the protein.

6.3.3.2 Matrix-assisted laser desorption/ionisation (MALDI)

MALDI is a laser-based ionisation method where the analyte is embedded in a chemical matrix. The choice of matrix is important and is selected on the basis of the type of sample and laser to be used; for example, alpha cyano-4-hydroxycinnamic acid is considered the 'gold standard' for use with a UV laser (Leszyk, 2010). The matrix crystals absorb the laser energy, transfer this energy to the analyte and subsequently, a small amount of the sample vaporises. Sample preparation and matrix crystal formation are key to maximise quality of resolution and subsequent sensitivity and reproducibility (S. L. Cohen & Chait, 1996). Various methods are discussed in the literature (S. L. Cohen & Chait, 1996). The dried droplet method was used in the work presented here (described in Section 2.8.3).

The ionised sample (mostly in the singly charged state) enters the mass analyser. Here, ions are often separated by 'time of flight' (the length of time it takes the ions to move down the flight tube to reach the detector, which is governed by m/z ratio).

6.3.3.3 Multiple reaction monitoring (MRM)

The complexity of clinical samples can limit the sensitivity of mass spectrometry analysis for low abundance proteins. Mass spectrometers can detect a limited number of peptides in a given time window and therefore, when 'discovery' mode is used with a complex sample the machine may detect dominant signals from major proteins, failing to trigger an MS/MS scan on low abundance ions. However, MRM experiments are designed to maximise the sensitivity for targeted compounds, using knowledge of their mass and structure to predict the precursor m/z ratio and subsequently, those of predicted fragment ions (D. M. Cox et al., 2005). In contrast to conventional MS-based analysis, trigger-dependent acquisitions are undertaken, only scanning each individual programmed transition with narrow m/z windows (D. M. Cox et al., 2005; Shi et al., 2012). In a triple quadrupole mass spectrometer, the precursor ion is selected in the first quadrupole (Q1). Fragmentation of this selected precursor ion then occurs by collision-induced dissociation in the second quadrupole (Q2) and finally, specified fragment ions are selected in the third quadrupole (Q3) (Shi et al., 2012). These are then detected and the protein can be identified.

This approach not only increases assay sensitivity (by limiting the signal to noise ratio) but also increases specificity, as compounds must be identified not only by HPLC retention time and precursor mass but also by identification of expected fragment ions. The use of this technique in low abundance biomarker research is well established (Kitteringham, Jenkins, Lane, Elliott, & Park, 2009; Shi et al., 2012).

6.4 Assay development

6.4.1 Assay concept

The proposed assay is based on the concept of using pepstatin A, immobilised on agarose beads, to bind all pepsin with an active site in a BAL sample; thus performing an affinity enhancement step. Having bound pepsin to the pepstatin A agarose beads, the rest of the sample would be discarded, leaving only the protein of interest (and possibly other aspartic proteases) attached to the washed beads. The protein would then be removed from the beads using an enzymatic digestion step (for example, with trypsin) and the resulting digest solution would be analysed using tandem mass spectrometry (specifically MRM) methods (LC-MRM-MS analysis). A quantitative assay could subsequently be built using a labelled peptide as an internal standard, the gold standard for LC-MRM-MS quantification (**Figure 6.4**) (H. Zhang et al., 2011). Porcine pepsin A was used for initial assay development as it shares a close homology with the predominant human gastric pepsin A isoform, 3b **(Figure 6.1, Table 6.1)**.

This assay concept would offer advantages over previous assays in being able to (a) selectively isolate aspartic proteases from the rest of the sample matrix using pepstatin A (b) provide reassurance that pepsin detected is of gastric origin, as the pro-peptide must have been cleaved in a low pH environment in order to bind to pepstatin and (c) clearly differentiate pepsinogen C and cathepsin D (both known to be produced locally in the lung) from pepsin A by LC-MSMS analysis. Affinity enrichment and MRM analysis techniques would also increase assay sensitivity, potentially to levels that could rival immunological assays (Ackermann & Berna, 2007).

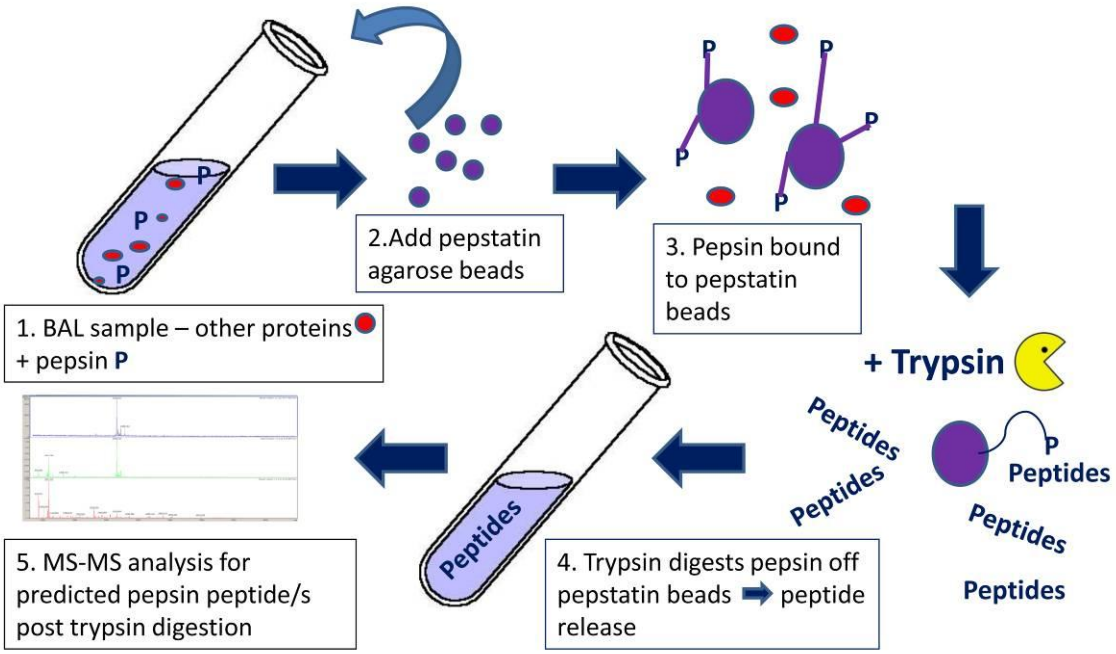

Figure 6.4 Pictorial representation of novel pepsin assay concept

This figure outlines the proposed novel method for the quantification of human pepsin A in BAL, using an inhibitor affinity enhanced LC-MSMS assay. Pepstatin A agarose beads (Sigma, UK) bind all aspartic proteases (with an accessible active site) in the BAL sample. The attached proteins are then digested off the beads using enzymatic proteolysis (for example, with trypsin). The resulting peptide solution is then analysed by LC-MSMS, looking specifically for precursor and fragment peptides produced by trypsin digestion and MSMS analysis of human pepsin. This method potentially offers excellent specificity and high sensitivity.

6.5 Identification of assay peptides

In order to confirm the feasibility of the proposed assay, information about protein amino acid sequences and theoretical masses of peptides produced by trypsin digestion were collected, as detailed below.

6.5.1 Porcine and human pepsin are theoretically identifiable, post trypsin digestion, by mass spectrometry

Peptide Mass (ExPASy Bioinformatics Resource Portal –http://web.espasy.org/peptide mass) was used to compute the theoretical masses of generated peptides following trypsin digestion of porcine pepsin A (PGA) and human pepsin 3b (PGA5) (with cysteine residues alkylated (carbamidomethylated) with iodoacetamide, methionine residues variably oxidised and allowing for no missed cleavages). Likely candidates for identification in a mass spectrometry analysis (based on m/z ratio) are highlighted in **Table 6.1**. The Swissprot database (www.uniprot.org) was searched using the online Basic Local Alignment Search Tool (BLAST http://blast.ncbi.nlm.nih.gov) to check that the two potential human pepsin 3b 'identification' peptides generated by trypsin digestion (QYFTVFDR and ANNQVGLAPVA) are unique to human pepsin A. Both peptides are specific to human pepsin A (PGA3, PGA4, PGA5; relating to isoforms 3c, 3a and 3b) or human pepsinogen A (as they are located at the C terminal end of the protein), with 100% query coverage and identity.

Table 6-1 Two peptides are highlighted as possible 'identification' peptide candidates in MS analysis for both (a) porcine pepsin A and (b) human pepsin A quantification.

a.

M	$[M+H]^+$	$[M+2H]^{2+}$	Peptide Amino Acid Sequence
32381.1	32382.11	16191.56	IGDEPLENYL DTEYFGTIGI GTPAQDFTVI FDTGSSNLWV PSVYCSSLAC SDHNQFNPDD SSTFEATSQE LSITYGTGSM TGILGYDTVQ VGGISDTNQI FGLSETEPGS FLYYAPFDGI LGLAYPSISA SGATPVFDNL WDQGLVSQDL FSVYLSSNDD SGSVVLLGGI DSSYYTGSLN WVPVSVEGYW QITLDSITMD GETIACSGGC QAIVDTGTSL LTGPTSAIAN IQSDIGASEN SDGEMVISCS SIDSLPDIVF TINGVQYPLS PSAYILQDDD SCTSGFEGMD VPTSSGELWI LGDVFIR
1090.508	1091.516	546.2614	QYYTVFDR
625.3799	626.387	313.6972	VGLAPVA
445.2285	446.2358	223.6215	ANNK

b.

M	$[M+H]^+$	$[M+2H]^{2+}$	Peptide Amino Acid Sequence
15300.33	15301.33	7651.169	SGSVVIFGGI DSSYYTGSLN WVPVTVEGYW QITVDSITMN GETIACAEGC QAIVDTGTSL LTGPTSPIAN IQSDIGASEN SDGDMVVSCS AISSLPDIVF TINGVQYPVP PSAYILQSEG SCISGFQGMN VPTESGELWI LGDVFIR
11167.26	11168.26	5584.637	FNPEDSSTYQ STSETVSITY GTGSMTGILG YDTVQVGGIS DTNQIFGLSE TEPGSFLYYA PFDGILGLAY PSISSSGATP VFDNIWNQGL VSQDLFSVYL SADDK
6099.79	6100.79	3050.903	VDEQPLENYL DMEYFGTIGI GTPAQDFTVV FDTGSSNLWV PSVYCSSLAC TNHNR
1074.513	1075.52	538.264	QYFTVFDR
1052.561	1053.56	527.288	ANNQVGLAPVA

6.5.2 Human pepsinogen A and pepsinogen C are differentiable by mass spectrometry

In order to confirm that the targeted 'identification' peptides for human pepsin A are not also predicted on trypsin digestion of pepsinogen C, the amino acid sequences of pepsinogen A (PGA5 gene (3b isoform)) and pepsinogen C (PGC gene) were checked using the Universal Protein Resource *(*www.uniprot.org). **Figure 6.5** shows that peptides QYFTVFDR and ANNQVGLAPVA would not be generated by trypsin digestion of pepsinogen C.

a.

Signal peptide

MKWLLLLGLV ALSEC

Pro-peptide

IMYKVPLIRK KSLRRTLSER GLLKDFLKKH NLNPARKYFP QWEAPTL

Chain

VDEQPLENYL DMEYFGTIGI GTPAQDFTVV FDTGSSNLWV PSVYCSSLAC
TNNRFNPEDS STYQSTSETV SITYGTGSMT GILGYDTVQV GGISDTNQIF
GLSETEGSFL YYAPFDGILG LAYPSISSSG ATPVFDNIWN QGLVSQDLFS
VYLSADDQSG VVIFGGIDSS YYTGSLNWVP VTVEGYWQIT VDSITMNGEA
IACAEGCQAI VDGTSLLTGP TSPIANIQSD IGASENSDGD MVVSCSAISS
LPDIVFTING VQYPVPPSAY ILQSEGSCIS GFQGMNLPTE SGELWILGDV
FIRQYFTVFD RANNQVGLAP VA

b.

Signal Peptide

MKWMVVVLVC LQLLEA

Pro-peptide

AVVKVPLKKF KSIRETMKEK GLLGEFLRTH KYDPAWKYRF GDL

Chain

SVTYEPMAYM DAAYFGEISI GTPPQNFLVL FDTGSSNLWV PSVYCQSQAC
TSHSRFNPSE SSTYSTNGQT FSLQYGSGSL TGFFGYDTLT VQSIQVPNQE
FGLSENEPGT NFVYAQFDGI MGLAYPALSV DEATTAMQGM VQEGALTSPV
FSVYLSNQQG SSGGAVVFGG VDSSLYTGQI YWAPVTQELY WQIGIEEFLI
GGQASGWCSE GCQAIVDTGT SLLTVPQQYM SALLQATGAQ EDEYGQFLVN
CNSIQNLPSL TFIINGVEFP LPPSSYILSN NGYCTVGVEP TYLSSQNGQP
LWILGDVFLR SYYSVYDLGN NRVGFATAA

Figure 6.5 Amino acid sequences for (a) human pepsinogen A and (b) human pepsinogen C, illustrating potential for differentiation by mass spectrometry analysis

a. The potential 'identification' peptides for pepsinogen A (and by inference human pepsin A (3b)) are highlighted in orange and green at the C-terminus of the protein. **b.** The corresponding peptides generated by trypsin digestion at the C-terminus of human pepsinogen C are also highlighted in orange and green.

6.6 Proof of assay concept

The aim of this experiment was to establish the feasibility of using immobilised pepstatin binding followed by LC-MS to detect pepsin in human BAL. The protocol below is for an artificial, *in vitro* experiment using relatively high, non-physiological quantities of pepsin. The volume of pepstatin agarose beads (Sigma Aldrich, UK) used in the experiment was calculated from the manufacturer's indication of bead binding capacity (20-40mg pepsin/ml beads, settled wet volume).

A 10μM solution of porcine pepsin A (Sigma Aldrich, UK) was made in 50mM acetic acid. A 50μl aliquot of pepstatin A agarose beads (Sigma Aldrich, UK) was centrifuged at 6000rpm for 3 minutes. The saline fraction was removed and the bead pellet was re-suspended in 50μl 50mM acetic acid. A 50μl aliquot of 10μM porcine pepsin A solution (500pmol) was mixed with 2μl of resuspended pepstatin A agarose beads (enough to bind >1000pmol pepsin based on the manufacturer's estimation of binding capacity) in an Eppendorf tube. This was incubated in an Eppendorf mixer (Thermomixer Comfort) for 1 hour at RT, 1200 rpm. Incubation time and temperature were optimised during preliminary experiments: temperature ranges of 21-40^{0}C and incubation times of 1-6 hours were assessed by SDS PAGE gel analysis for bead-pepsin binding (as described in Section 6.7.2).

Following incubation, the pepsin/pepstatin bead suspension was centrifuged at 6000rpm and the supernatant was discarded. The beads were washed three times in 200μl MilliQ H_20, and re-pelleted by centrifugation; the supernatant fraction was discarded. A 500μM solution of non-sequencing grade porcine trypsin (Sigma Aldrich, UK) was made in 25mM ammonium bicarbonate buffer, pH8.5 (AmBic) and 25000pmol (50μl) aliquot was subsequently added to the beads (protein:trypsin ratio 50:1). The solution was incubated in an Eppendorf mixer (Thermomixer Comfort) at 37^{0}C, 1200rpm overnight. Following centrifugation, the supernatant was removed from the beads and 0.5% (final v/v) trifluoroacetic acid (TFA) was added to stop the reaction. A positive control of 500pmol pepsin in 50μl 25mM AmBic in solution digest and a negative control of pepstatin A agarose bead binding and digestion but with no pepsin added was conducted. MALDI-TOF MS analysis of the resulting digest solutions was made

using a Bruker UltrafleXtreme MALDI TOF/TOF mass spectrometer, as detailed in Section 2.8.3. Theoretically, 10pmol pepsin A peptides (1µl digest solution) were loaded onto the MALDI target (for the experimental bead pepsin digest and positive control).

Figure 6.6 displays proof of the assay concept: identification of the m/z 1091 peak (relating to peptide QYYTVFDR, $[M+H]^+$) on analysis of the in-solution porcine pepsin A digest and the digest from pepstatin agarose beads post incubation with porcine pepsin A solution. This peak was not identified in the pepsin negative bead digest control. The other peak observed in all three samples (m/z 2163) was a porcine trypsin autolysis peak. The amount of trypsin used in subsequent experiments was reduced to 25:1 (protein:trypsin) and sequencing grade trypsin was used. The relative signal intensity of trypsin to pepsin was noted to differ between the 'pepsin digested off beads' and 'pepsin digested in solution' samples. This was possibly due to incomplete pepsin retrieval with 2ul pepstatin agarose beads and was further investigated in Section 6.7.2.

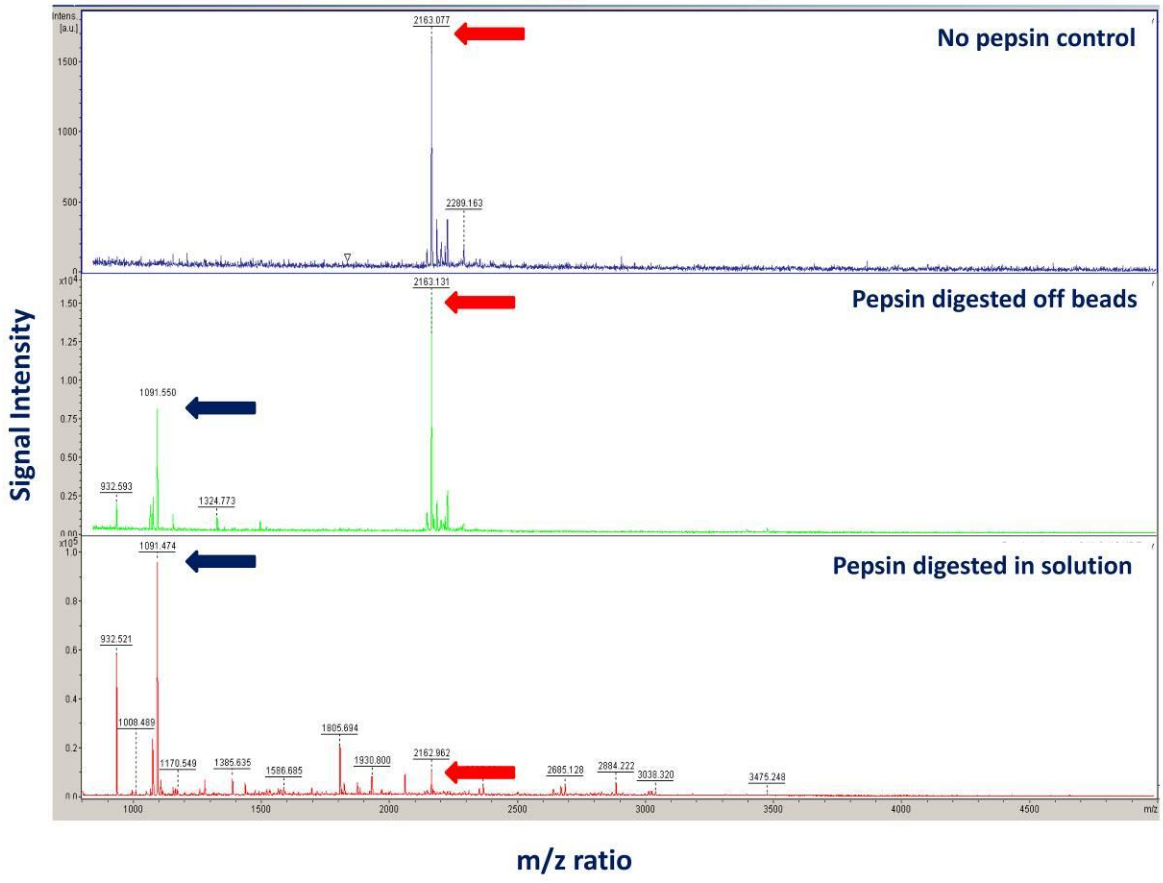

Figure 6.6 Proof of assay concept

MALDI chromatograms (generated using an UltrafleXtreme MALDI TOF/TOF mass spectrometer and Flex Analysis software, Bruker). Peak m/z 1091 (indicated by blue arrows) was identified in the porcine pepsin A in solution digest control and in the washed pepstatin agarose bead digest post incubation with porcine pepsin A. However, it was not seen in the pepsin negative bead digest control sample. This is the m/z of QYYTVFDR ($[M+H]^+$), predicted post porcine pepsin A digestion with trypsin (trypsin autolysis peak m/z 2163, indicated by red arrows).

6.7 Optimisation of pepstatin A agarose bead binding of porcine pepsin A

Following proof of concept, experimental conditions were explored in further detail to ensure maximum efficiency of porcine pepsin A binding to pepstatin A agarose beads in BAL sample matrix.

6.7.1 Pepstatin agarose beads are capable of binding porcine pepsin at an acidic or neutral pH

The aim of this experiment was to test whether pepstatin agarose bead binding of porcine pepsin A was affected by pH. This question is important because epithelial lining fluid may be mildly acidic or neutral and the pH of patient BAL samples ranged from pH 5.5-7.4 (Fischer & Widdicombe, 2006).

The experiment described in Section 6.6 was repeated, in duplicate, with two pH conditions: (a) porcine pepsin A dissolved in 50mM acetic acid (pH3.0) and (b) porcine pepsin A dissolved in 50mM HEPES (pH 7.4). The resulting bead digests were analysed in quadruplicate by MALDI-TOF analysis, using a raster pattern (as described in Section 2.8.3). The mean area under the m/z 1091 curve and standard deviation was calculated for each pH condition.

No significant difference was seen in the area under the m/z 1091 curve between acidic and neutral pH conditions (p=0.696, **Figure 6.7**). It is not possible to make a direct comparison between unrelated spots on a MALDI target however, despite this, we felt that this experiment provided convincing evidence that pepstatin agarose beads are able to bind porcine pepsin A at a neutral pH. From this point, all experiments were performed at pH 7.4, as this was the highest pH measured in recruit BAL fluid samples.

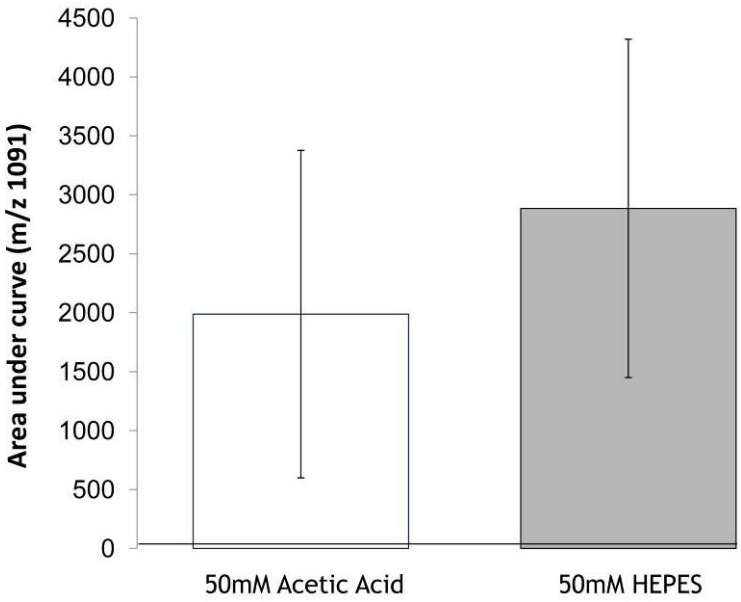

Figure 6.7 Pepstatin agarose bead binding of porcine pepsin A is not affected by alteration of sample pH.

Porcine pepsin (500pmol) (Sigma, UK) dissolved in acidic (pH 3) and neutral (pH7.4) solutions was incubated with 2µl resuspended pepstatin A agarose beads (Sigma, UK) for 1 hour at RT. Following bead trypsin digestion, MALDI-TOF MS analysis was performed on the digest solution, using an UltrafleXtreme MALDI TOF/TOF mass spectrometer and Flex Analysis software (Bruker). Two experimental replicates were performed and each sample was analysed in quadruplicate. The mean area under the curve of m/z 1091 peak was compared for acidic and neutral pH conditions (limitations discussed in Section 6.7.1). No significant difference was found (data presented as mean and standard deviation, p=0.696).

6.7.2 Bead binding capacity

The aim of this experiment was to establish pepstatin A agarose bead (Sigma, UK) binding capacity under optimised experimental conditions.

Increasing volumes (2-40µl) of resuspended pepstatin A agarose beads (as described in Section 6.6) were added to 500pmol porcine pepsin (Sigma, UK) in solutions of increasing concentration, enabling equal total sample volume (50µl) across all experimental conditions. Following incubation and centrifugation, the supernatant fraction was removed from each bead pellet and precipitated using 15% TCA (v/v), as described in Section 2.5.3, to allow loading of the complete supernatant sample for SDS-PAGE gel analysis (as described in Section 2.5.1). Any unbound pepsin would be visualised by this technique.

The experiment was repeated in triplicate and 35µl of pepstatin A agarose beads was required to consistently bind 500pmol porcine pepsin (**figure 6.8 (b)**).

6.7.3 Pepsin autolysis does not occur during the time taken to incubate the sample with pepstatin A agarose beads.

The aim of this experiment was to check that there was no loss of pepsin protein (through autolysis) during the time spent incubating the sample with pepstatin A agarose beads (one hour). Variations in pepsin activity curves have been observed and it has been suggested that this is due to variation in the method of measuring activity and the origin of the pepsin (Piper & Fenton, 1965). Therefore, although pepsin autolysis is unlikely at pH7.4, I sought confirmation of this within the following experimental protocol.

Binding of two aliquots of 500pmol porcine pepsin A to 16µl pepstatin A agarose beads was performed, as previously described in Section 6.6. The supernatant fraction was removed from the beads post centrifugation and TCA precipitated (15% v/v), as previously described (Section 2.7.2). The beads and supernatant fractions were loaded onto a polyacrylamide gel for SDS-PAGE gel analysis, with a 500pmol porcine pepsin control. The entire experiment was also performed without the addition of pepsin, as a negative control. Densitometry analysis was performed on protein bands post Coomassie R-250 staining, using TotalLab Quant software.

Figure 6.8 (a) demonstrates that the starting amount of pepsin was fully accounted for at the end of the experiment (it was either attached to the beads or remained in the supernatant). Therefore, no obvious autolysis had occurred.

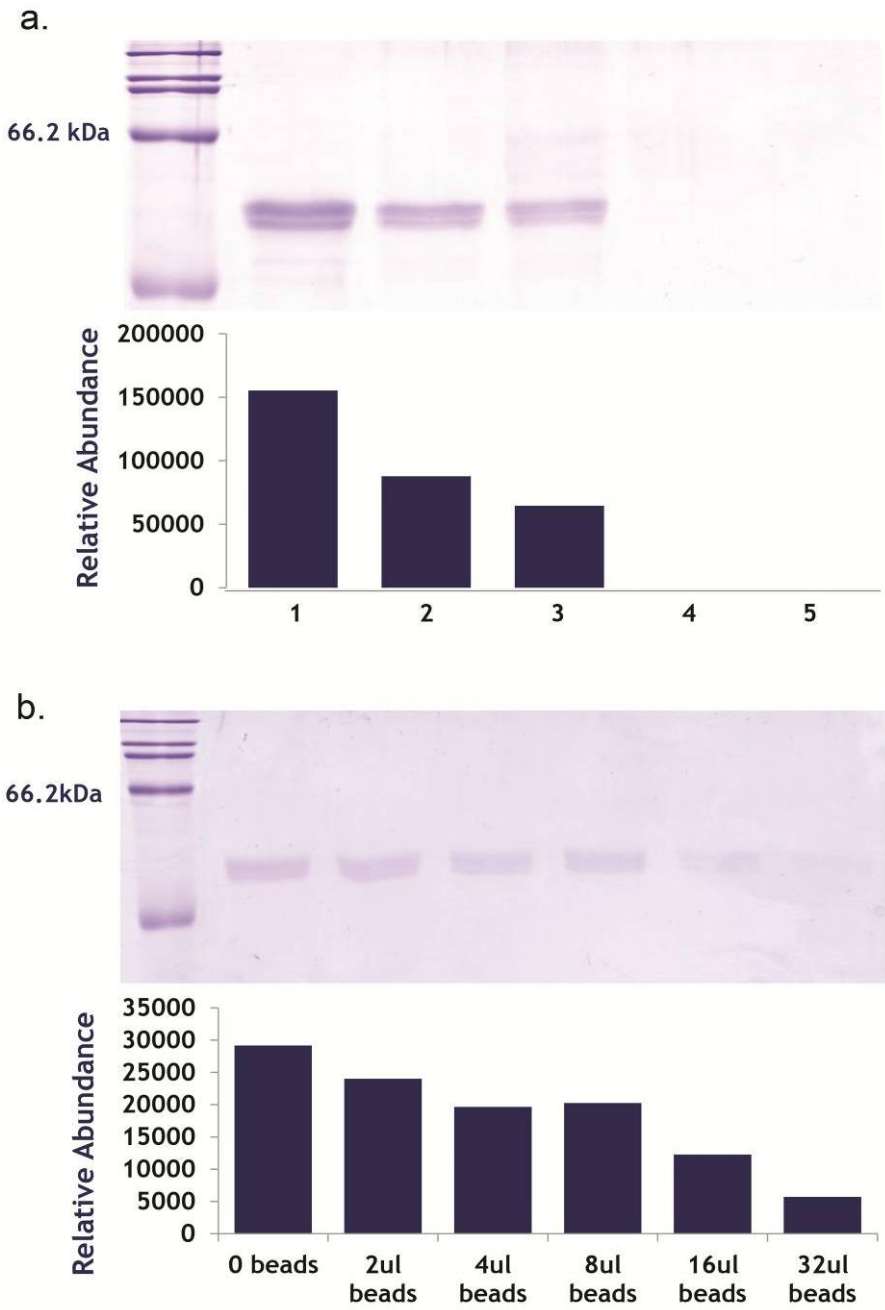

Figure 6.8 a. SDS PAGE gel (and accompanying band densitometry) to demonstrate that all pepsin protein was accounted for within the bead binding experiment (no degradation). b. SDS PAGE gel (and accompanying band densitometry) showing the effect of increasing volumes of pepstatin agarose beads on porcine pepsin A retrieval.

a. Porcine pepsin A (500pmol in 50µl 50mM HEPES, pH7.4) was incubated with 16µl pepstatin A agarose beads (Sigma, UK). The washed beads (2) and the supernatant protein (3) were analysed by SDS-PAGE gel. A 500pmol pepsin control was included (1) as well as bead and supernatant controls from an identical experiment with no pepsin added (4 & 5, respectively). Densitometry analysis was performed using TotalLab Quant software. The sum density of the bands visualised in lanes 2 and 3 was equal to the band density in lane 1, i.e. the starting amount of porcine pepsin A protein was fully accounted for (on the beads or in the supernatant) at the end of the incubation period.
b. Pepstatin A agarose beads (2-40 µl, Sigma, UK) were added to 500pmol porcine pepsin A in solutions of increasing concentrations, allowing equal total sample volume across all samples. Following incubation, supernatants were analysed by SDS-PAGE gel analysis (looking for residual 'unbound' protein) and densitometry was performed using TotalLab Quant software. Following three experimental repeats, it was concluded that 35µl of pepstatin A agarose beads were needed to consistently bind 500pmol porcine pepsin.

6.7.4 Pepstatin-pepsin binding is not disrupted by prolonged exposure to pH conditions similar to those in the human lung

The aim of this experiment was to investigate whether prolonged exposure of porcine pepsin A to pH 7.4 (24 hours) would disrupt subsequent binding with pepstatin A agarose beads. This was important to establish, as pepsin A may be present in the pulmonary pH environment for several hours prior to sampling.

500pmol porcine pepsin A (in 50µl 50mM HEPES, pH 7.4) was incubated for 24 hours. The pH of the solution was checked at the beginning and end of the incubation period. Following this, the same binding experiment as detailed previously (Section 6.6) was performed, using 35µl re-suspended pepstatin A agarose beads for pepsin retrieval. Subsequently, the beads, supernatant and a 500pmol control were analysed by SDS-PAGE analysis.

Figure 6.8 demonstrates that 35µl pepstatin A agarose beads are able to completely bind 500pmol porcine pepsin A, even after exposure to pH 7.4 for 24 hours. This result is in keeping with the literature that pepsin is held in a 'static' state at pH 7.4 and is not denatured until pH >8 (Nikki Johnston et al., 2007).

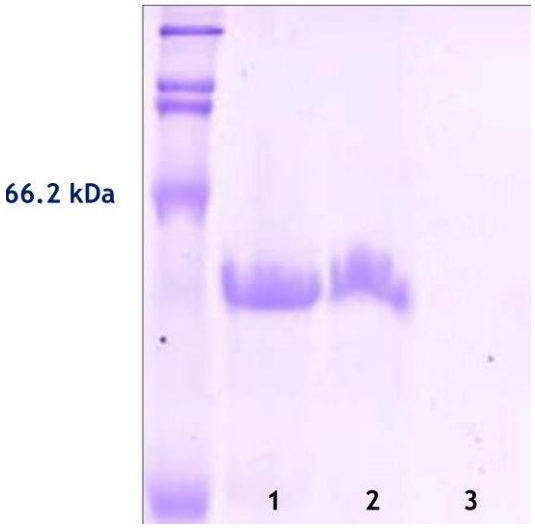

Figure 6.9 Exposure of porcine pepsin A to pH 7.4 for 24 hours does not preclude subsequent complete binding of 500pmol pepsin to 35µl pepstatin agarose beads.

This SDS PAGE gel image shows bands of equal density in lane 1 and 2 for (1) 500pmol porcine pepsin loaded directly onto the gel and (2) pepstatin agarose A beads loaded onto the gel post incubation with 500pmol porcine pepsin A pre-exposed to pH 7.4 for 24 hours. There is no band visible in lane three (supernatant of porcine pepsin A solution post incubation with pepstatin A agarose beads), indicating that all pepsin was retrieved by the pepstatin A agarose beads.

6.7.5 Pepstatin agarose A beads are capable of retrieving physiological levels of porcine pepsin A in water and BAL matrix

The aim of this series of experiments was to establish whether porcine pepsin A, at physiologically relevant levels (based on currently available literature), could be retrieved by pepstatin A agarose beads, in water and also in BAL matrix (McNally et al., 2011).

Experiment 1: The first experiment involved repeating the experiment performed in Section 6.6 but with much lower levels of pepsin (dissolved in 50µl 50mM HEPES). The amounts of pepsin used were 100ng (2.9pmol), 50ng (1.45pmol) and 20ng (580fmol). A smaller volume (5µl) of pepstatin agarose beads was used, as the potential pepsin load was smaller. Beads were incubated in the solutions as previously described (Section 6.6). Following this, the beads and the remaining supernatant were loaded onto a 12% precast Tris-glycine gel (Biorad) for subsequent Western blot (WB) analysis (as described in Section 2.5.2.1), to assess bead pepsin retrieval. A positive control for each pepsin amount was also loaded.

Figure 6.10 (1) demonstrates pepstatin A agarose bead retrieval of physiologically relevant levels of pepsin in 50mM HEPES (pH 7.4).

Experiment 2: A similar experiment was repeated in BAL matrix. Porcine pepsin A (2.9pmol) was spiked into (a) 20µl water (b) 20µl 1:2 pre-diluted BAL (c) 20µl 1:5 pre-diluted BAL and (d) 20µl 1:10 pre-diluted BAL. The retrieval experiment was repeated using 5µl pepstatin A agarose beads, as previously described (Section 6.6). A 2.9pmol porcine pepsin A control and the beads and supernatants from all conditions were analysed by porcine pepsin WB, as previously described (Section 2.5.2.1).

Figure 6.10 (2) demonstrates that retrieval of 2.9pmol pepsin was incomplete in BAL matrix at 1:2 dilution but improved with further BAL matrix dilution.

Experiment 3: Three 50µl aliquots of three different BAL samples were taken and diluted 1:10. One aliquot of each BAL sample was spiked with 2.9pmol pepsin and one was unspiked. The third was set to one side to run directly on

the WB gel. Pepstatin A agarose bead binding experiments were performed on unspiked and spiked BAL samples, as previously described (Section 6.6). The washed beads and the BAL supernatant were then collected post bead incubation and analysed by porcine pepsin WB analysis, as previously described (Section 2.5.2.1). The supernatants were precipitated with 15% (final v/v) TCA to load onto the gel. The volume of pepstatin agarose A beads needed for efficient pepsin retrieval was increased to 15µl during optimisation of this experiment.

Figures 6.10 (3a) and (3b) demonstrate that 15µl pepstatin agarose A beads could efficiently retrieve 2.9pmol porcine pepsin from three clinical samples. A small amount was left behind in one sample and this may need further optimisation, using MS quantification of pepsin retrieval. Retrieval and detection of an unidentified protein, of a slightly higher molecular weight than pepsin, was demonstrated in all samples. This unidentified protein was not human pepsin (Chapter 5, Section 5.4.2.1) and was non-specifically bound to the beads (Section 6.8.5). Variable (sample dependent) non-specific binding of porcine pepsin antibodies to other unidentified BAL proteins was seen during these experiments, the extent of non-specific binding increasing with higher BAL protein load (further discussed in Chapter 5).

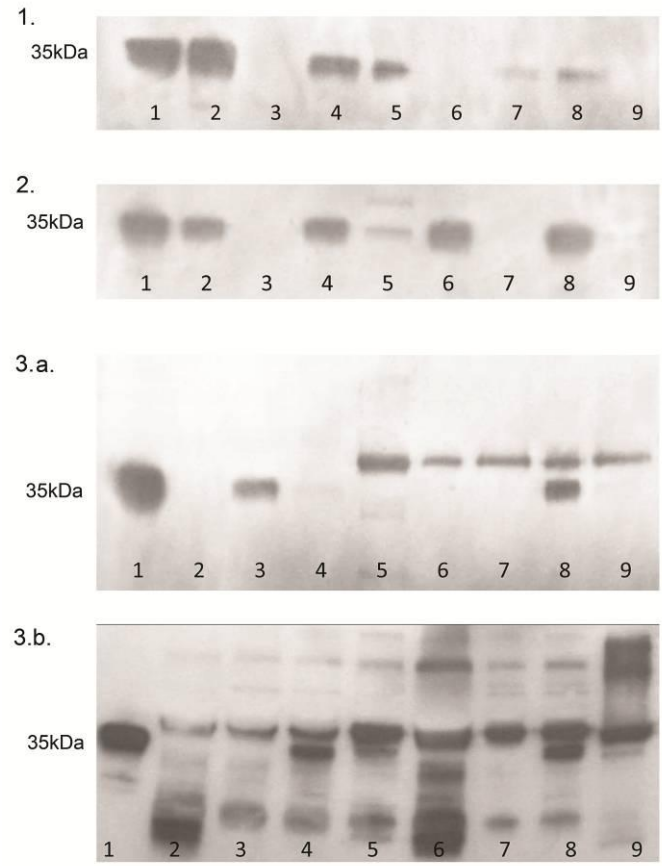

Figure 6.10 Series of WB images demonstrating recovery of physiologically relevant levels of porcine pepsin A under various conditions

1. Pepstatin A agarose beads are capable of recovering physiologically relevant levels of porcine pepsin A in 50mM HEPES buffer

Methods described in text. Lanes: 1 - 2.9pmol porcine pepsin, 2 - washed pepstatin agarose A beads post incubation with 2.9pmol porcine pepsin (in 50mM HEPES), 3 - pepsin solution supernatant post incubation with beads. Lanes 4-6, as for lanes 1-3 with 1.45pmol porcine pepsin. Lanes 7-9, as for lanes 1-3 with 580fmol porcine pepsin.

2. Pepstatin A agarose beads are capable of recovering small amounts of porcine pepsin in BAL. The efficacy of this process is improved by BAL dilution.

Methods described in text. Lanes: 1 – 2.9pmol porcine pepsin, 2 - pepstatin agarose beads post incubation with 2.9pmol porcine pepsin in 50mM HEPES, 3 – Porcine pepsin/50mM HEPES solution supernatant post incubation with beads, 4 - pepstatin agarose beads post incubation with 2.9pmol porcine pepsin in BAL diluted 1:2, 5 – Porcine pepsin/BAL (1:2 diluted) solution supernatant post incubation with beads, Lanes 6-7 as for 4-5 except 1:5 diluted BAL, Lanes 8-9 as for 4-5 except 1:10 diluted BAL.

3. Efficient pepstatin A agarose bead recovery of pepsin from BAL is demonstrated in three inflammatory, proteinaceous BAL samples (1:10 diluted).

(a) Methods as described in text. Lanes: 1 – 2.9pmol porcine pepsin control, 2 – beads alone, 3 – 2.9pmol pepsin on beads in water, 4 – water supernatant post incubation with beads, 5 – P80 direct on gel, 6 – P80 non-spiked on beads, 7 – P80 non-spiked supernatant post incubation with beads, 8 - P80+2.9pmol porcine pepsin on beads, 9 - P80+2.9pmol pepsin supernatant post beads.
(b) Methods as described in text. Lanes: 1 – 2.9pmol porcine pepsin control, 2 – 100µl P30 direct on gel, 3 – P30 on beads, 4 - P30+2.9pmol porcine pepsin on beads, 5 - P30+2.9pmol pepsin supernatant post beads, 6 – 100µl P71 direct on gel, 7 – P71 on beads, 8 - P71+2.9pmol pepsin on beads, 9 - P71 +2.9pmol pepsin supernatant post beads.

6.7.6 Rapigest, heat, reduction, alkylation and trypsin digestion are required for complete release of pepsin from pepstatin A agarose beads

The aim of this experimental series was to establish the conditions required for complete removal of porcine pepsin A from pepstatin A agarose beads.

Experiment 1: The first experiment used trypsin digestion alone for > 18 hours, at 37^0C and with a protein:trypsin ratio of 25:1. Four 500pmol aliquots of porcine pepsin A were each bound to 35µl pepstatin agarose beads, as previously described. Trypsin digestion was performed on two aliquots and 25mM AmBic buffer alone was added to the other two aliquots (digestion negative controls). Two 500pmol porcine pepsin A aliquots were also digested directly in solution, using equal protein:trypsin ratios and equal digest volumes to those used for the bead samples. Following centrifugation, the digest solution was removed and stored (following addition of 0.5% (v/v) TFA to stop the reaction). A 500pmol pepsin control and the two sets of non-digested and digested beads were analysed directly by SDS page gel (as described in Section 2.5.1), to assess any residual pepsin left on the pepstatin agarose A beads. The bead digest supernatants and in solution 500pmol pepsin digests were assessed by MALDI-TOF analysis (as described in Section 2.8.3). A 16pmol Glu-1-fibrinopeptide B (Glu-fib) spike was diluted 1:10 in 9.5µl of each digest solution. Glu-fib:pepsin ratios were then assessed and compared to assess m/z 1091 ion retrieval in the bead digests compared to the in solution digests.

The SDS-PAGE gel and MALDI-TOF supernatant analyses highlight incomplete digestion of pepsin from beads by trypsin proteolysis **(Figures 6.11 1(a) and 2).**

Experiment 2: Six bead samples with 500pmol porcine pepsin attached were produced, as previously. A bead digestion pre-step was added to the previous protocol. A proprietary surfactant (0.1% (final v/v in 25mM AmBic) Rapigest™, Waters, UK), which enhances proteolysis, was added to the previous protocol for four samples. Two of these samples were incubated, with agitation, at 80^0C and two samples were incubated at 95^0C for five minutes. Pepsin contains four disulphide bonds and therefore, samples were also reduced and alkylated. All four samples underwent reduction with 3.3mM dithiothreitol for 10 minutes at 60^0C. Alkylation was with 9.6mM iodoacetamide for 30 minutes at RT in the

dark. Finally, sequencing grade trypsin (Sigma, UK) was added (protein:trypsin ratio 25:1). All samples were digested overnight for 18 hours at 1200rpm in an ependorf shaker at 37^{0}C . The final two samples were 'undigested' in 50µl AmBic as a negative control. Following processing (as previously described), beads were run on an SDS PAGE gel and supernatants were assessed by MALDI-TOF analysis.

A reduction in Glu-fib:pepsin ratio was seen with this digestion protocol. Increased temperature during incubation with Rapigest™ (95^{0}C) appeared to have a beneficial effect (**Figure 6.11 1(b**)). However, despite this, the SDS PAGE gel still showed some residual pepsin bound to the beads.

Experiment 3**:** The final experiment involved increasing the Rapigest™ incubation time to 10 minutes (95^{0}C). Samples were also directly agitated (through vortexing and brief centrifugation to return the beads to the bottom of the tube) on two occasions during the 18 hour digestion period. Samples were analysed as previously described.

Figures 6.11 1(c) and 2 show that the increased Rapigest™ incubation time resulted in complete digestion of porcine pepsin A from pepstatin A agarose beads.

6.7.7 Optimised bead digestion protocol

Add 22.5µl 25mM AmBic to washed beads with 2.5µl 1% rapigest. Incubate at 95^{0}C for 10 minutes with brief vortexing twice during incubation.

Add 16.5µl AmBic and then 2.5µl DTT (9.2mg in 1ml 25mM AmBic). Incubate at 60^{0}C for 10 minutes with direct agitation after 5 minutes.

Add 2.5µl IAN (33mg in 1ml) then incubate in the dark at RT for 30 minutes with agitation half way through.

Add trypsin at 25:1 (protein:trypsin) and digest overnight for at least 18 hours with two direct agitations with vortexing.

Following digestion, centrifuge at 6000rpm for 5 minutes. Remove the digest supernatant and add 0.5% TFA (final v/v). Incubate at 37^{0}C for 30 minutes.

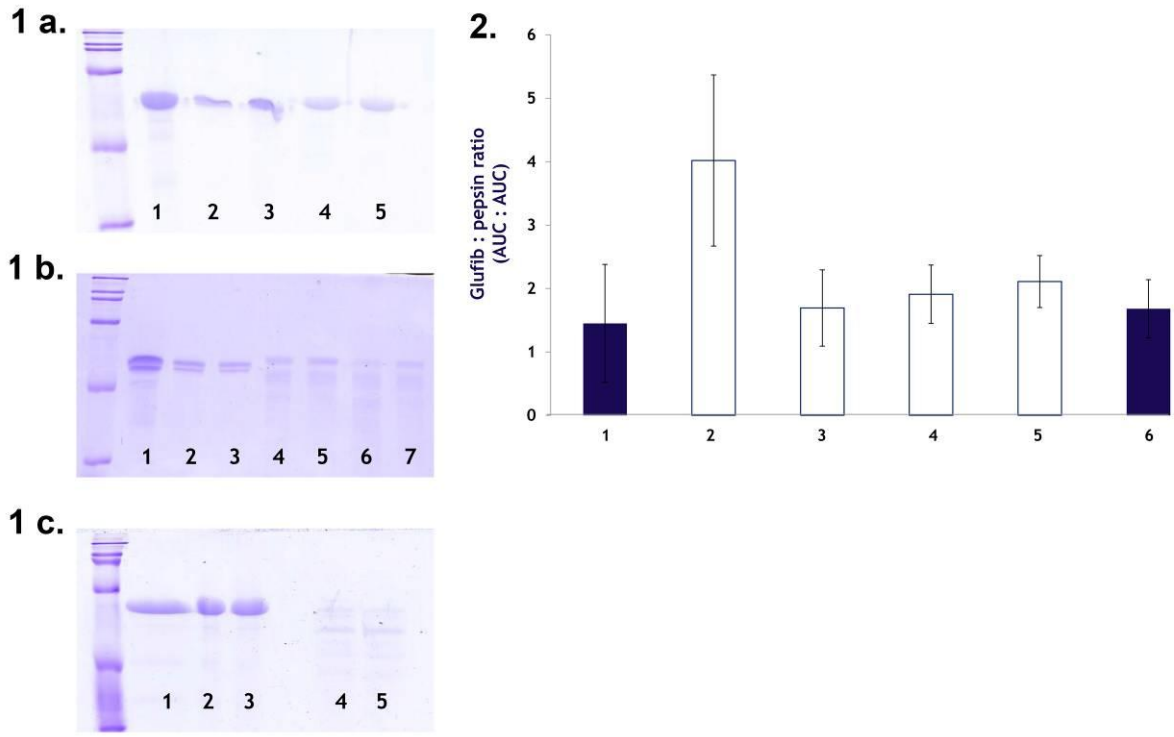

Figure 6.11 Complete removal of porcine pepsin A from pepstatin agarose beads is achieved with 10 minutes incubation in 0.1% Rapigest at 95^{0}C, reduction and alkylation and then trypsin digestion at a ratio 25:1 (protein:trypsin) for >18 hours, with agitation.

Methods as described in text.

(1a) Lane 1 – 500pmol pepsin, lanes 2+3 – 500 pmol pepsin on beads, lanes 4+5 – beads + residual pepsin post trypsin digestion.

(1b) Lane 1 - 500pmol pepsin, lanes 2+3 - 500pmol pepsin on beads (digestion negative), lanes 4+5 - residual pepsin on beads post 0.1% Rapigest at 80^{0}C for 5 minutes, reduction & alkylation and trypsin digestion, lanes 6+7 – residual pepsin on beads post 0.1% Rapigest at 95^{0}C for 5 minutes, reduction & alkylation and trypsin digestion.

(1c) Lane 1 – 500pmol pepsin, lanes 2+3 – 500pmol pepsin on beads, lanes 4+5 - 500pmol pepsin on beads post 0.1% Rapigest at 95^{0}C for 10 minutes, reduction & alkylation and trypsin digestion.

(2) Bar chart to show mean (SD) glu-fib:pepsin ratio (area under curve:area under curve). Lane 1 - in solution digest 500pmol porcine pepsin, lane 2 - bead digest post trypsin digestion alone, lane 3 - addition of Rapigest (0.1%) 80^{0}C for 5 minutes, lane 4 - addition of Rapigest (0.1%) 80^{0}C for 5 minutes and reduction and alkylation, lane 5 - addition of Rapigest (0.1%) 95^{0}C for 5 minutes and reduction and alkylation, lane 6 - addition of Rapigest (0.1%) 95^{0}C for 10 minutes and reduction and alkylation

6.8 Identification of pepsin by tandem mass spectrometry

6.8.1 Detection of porcine and human pepsin by LC-MSMS analysis

The aim of this experiment was to confirm MS detection of the two predicted 'identification' peptides for both porcine and human pepsin.

Porcine pepsin A (Sigma, UK) and human pepsinogen 1 (Cell Sciences, US) were digested in solution, using sequencing grade trypsin (protein:trypsin, 25:1), calculated to give a final digest concentration of 1000fmol/µl. Following dilution, 100fmol of each was loaded onto a nanoAcquity UPLC™ system (Waters MS Technologies, Manchester, UK) and eluted over a 1 hour gradient. MSMS analysis was performed using a Synapt G1 or Synapt G2 mass spectrometer, as described in Section 2.8.5.

The chromatograms in **Figure 6.12** confirm that the predicted 'identification' peptides were observed for porcine pepsin and for human pepsin. All peptides are easily observed at 100fmol **however**, there appears to be a higher sensitivity for the C terminus peptides VGLAPVA (porcine) or ANNQVGLAPVA (human). It was noted that the VGLAPVA was singly charged whereas ANNQVGLAPVA was doubly charged.

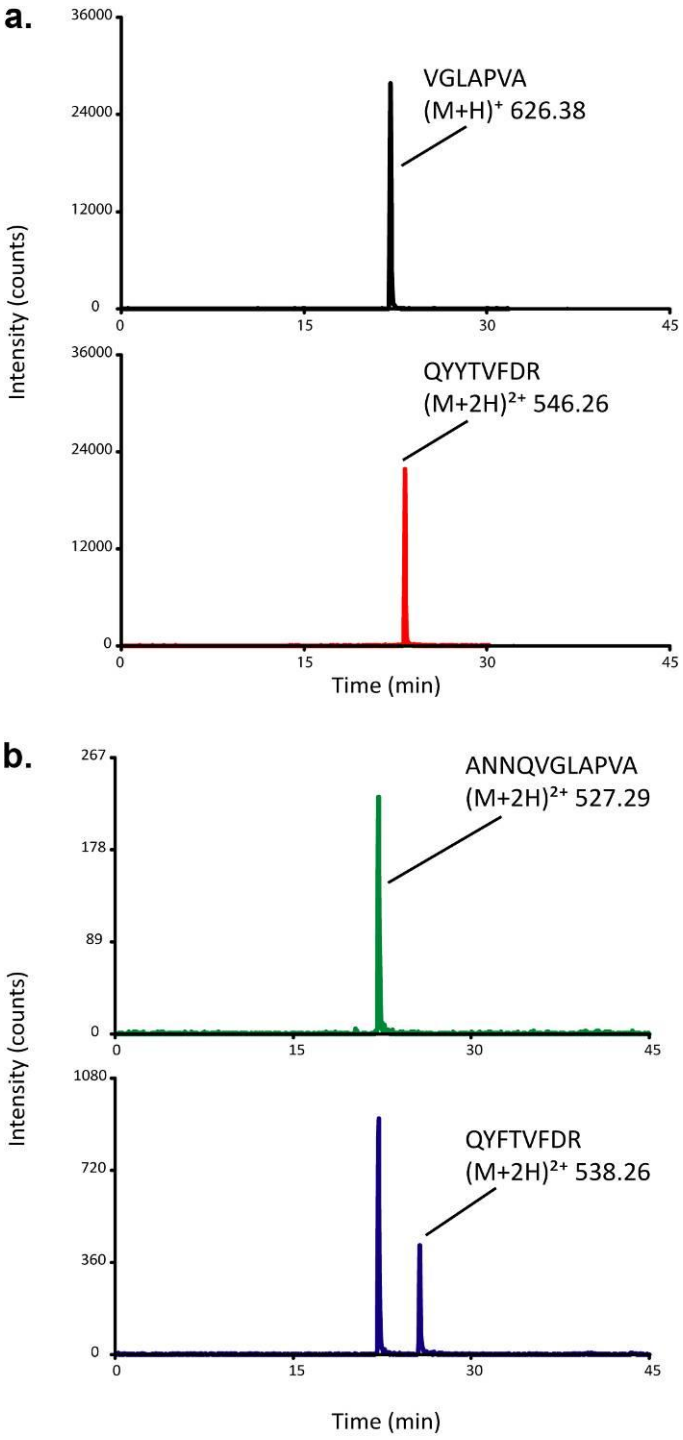

Figure 6.12 (a) Porcine and (b) Human pepsin 'identification' peptides are visible when loading 100fmol onto column for LC-MSMS analysis using a nanoAcquity UPLC™ system and Synapt G1 or Synapt G2 mass spectrometers (both Waters MS Technologies, Manchester, UK)

a. Following in solution digestion, 100fmol porcine pepsin (Sigma) was loaded onto a nanoAcquity UPLC™ system (Waters MS Technologies, Manchester, UK) and eluted on a 1 hour gradient. A Synapt G2 Mass spectrometer (Waters) was used for analysis. Predicted peaks relating to VGLAPVA (m/z 626.38 ($[M+H]^+$)) and QYYTVFDR (m/z 546.26 ($[M+2H]^{2+}$)) were seen at 22 and 23 minutes, respectively. Extracted ion chromatograms shown (created using Origin software)
b. Following in solution digestion, 100fmol human pepsinogen 1 (Cell Sciences) was loaded onto a nanoAcquity UPLC™ system (Waters MS Technologies, Manchester, UK) and eluted on a 1 hour gradient. A Synapt G1 Mass spectrometer (Waters) was used for analysis. Predicted peaks relating to ANNQVGLAPVA (m/z 527.28 ($[M^+2H]^{2+}$)) and QYFTVFDR (m/z 538.26 ($[M^+2H]^{2+}$)) were observed at retention times of 22 and 25 minutes, respectively. Extracted ion chromatograms shown.

6.8.2 Building an MRM assay for pepsin identification

MRM methods were built (including generation of fragment peptide lists and collision energies) using Skyline (MacCoss Lab Software). The fragment peptide lists for the precursor peptides targeted for detection of porcine and human pepsin are listed in **Figure 6.13**. A minimum of five fragment peptides were selected for each precursor. Fifteen data-points were acquired over a 15 second chromatographic peak and the minimum dwell time was 50 milli-seconds. The collision energies (eV) suggested by Skyline for fragmentation of the precursor peptides were human pepsin; QYFTVFDR – 19, ANNQVGLAPVA – 38, porcine pepsin; QYYTVFDR – 19, VGLAPVA – 22. LC-MSMS methods are detailed in Section 2.8.6. The Xevo™ TQ triple quadrupole mass spectrometer (Waters) was used for MS analysis.

Transition	QYYTVFDR	VGLAPVA	QYFTVFDR	Transition	ANNQVGLAPVA
y2	+290.1459	+189.1234	+290.1459	**b8**	+768.39
y3	+437.2143	+286.1761	+437.2143	**B8-NH_3**	+751.37
y4	+536.2827	+357.2132	+536.2827	**b7**	+697.36
y5	+637.3304	+470.2973	+637.3304	**B7-NH_3**	+680.33
y6	+800.3937	+527.3188	+784.3988	**b9**	+865.45
y7	+963.4571		+947.4621	**y3**	+286.17
	Porcine Pepsin Peptides		**Human Pepsin Peptides**		

Figure 6.13 Fragment ion transitions for porcine pepsin peptides QYYTVFDR and VGLAPVA and human pepsin peptides QYFTVFDR and ANNQVGLAPVA.

Fragment ion transition lists were generated using Skyline software (MacCoss Lab Software).

6.8.3 100fmol porcine pepsin A is detectable by LC-MSMS using MRM transitions, following bead retrieval from BAL matrix

Having established that the proposed 'identification' peptides are detectable by LC-MSMS analysis, the aim of this experiment was to establish whether 100fmol porcine pepsin A could be retrieved from BAL matrix onto pepstatin agarose beads and detected by selected ion monitoring for precursor and predicted fragment ion transitions (Skyline, MacCoss Labs), using the Xevo™ TQ triple quadrupole mass spectrometer (Waters).

Therefore, 2.9pmol porcine pepsin A was spiked into a 100µl BAL sample. The BAL sample was diluted 1:10 with HPLC water and incubated with 15µl pepstatin agarose beads for 1 hour, as previously described (Section 6.7.5). Trypsin bead digestion was performed in a final volume of 50µl (optimised protocol detailed in Section 6.7.7), theoretically yielding 57fmol/µl porcine pepsin peptides if bead retrieval was complete. The bead digest solution (2µl, approximately 100fmol porcine pepsin peptides) was loaded for LC-MSMS analysis. MRM transitions were programmed using Skyline software. The collision energies used were QYYTVFDR – 19 eV, VGLAPVA – 22 eV.

Figure 6.14 shows a chromatogram which illustrates detection of the porcine pepsin peptide QYYTVFDR ion (m/z 538.26) at 23 minutes and accompanying detection of five predicted fragment ions of this precursor at the same retention time. Five predicted fragment ions of the VGLAPVA precursor ion were identified at 22 minutes (chromatogram not shown). The next step in creation of an MRM assay would be to insert time windows to scan only for QYYTVFDR precursor and fragment ions at 23 minutes and VGLAPVA ions at 22 minutes.

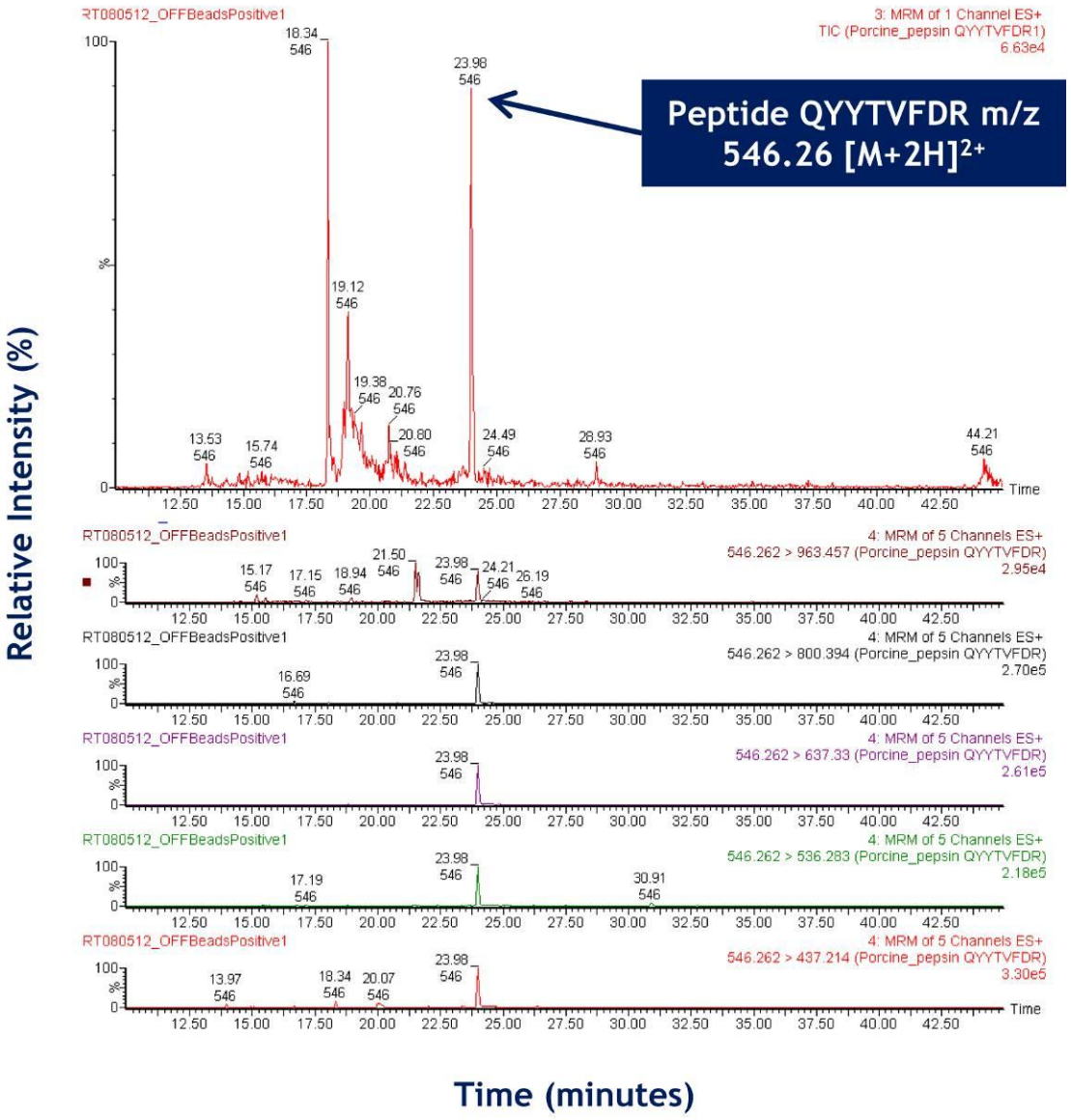

Figure 6.14 2.9pmol porcine pepsin spiked into 100µl BAL, retrieved onto pepstatin agarose beads and digested off beads according to an optimised protocol is clearly identifiable by LC-MRM-MS

Analysis of 2µl bead digest (theoretically 114fmol porcine pepsin) on Xevo^{TM} TQ MS (Waters) by selected ion monitoring for precursor peptide ion 538.26 (QYYTVFDR) and it's predicted fragment ions. Chromatogram shows a peak at 23.98 minutes for precursor ion m/z 538.26. Five predicted fragment ion peaks were detected at the same retention time.

6.8.4 100fmol human pepsin is detectable LC-MRM-MS analysis

The aim of this experiment was to visualise the predicted fragment ions of the peptides QYFTVFDR and ANNQVGLAPVA by programming an MRM transition experiment, using the Xevo TQ triple quadripolar mass spectrometer (according to methods described in Section 2.8.6). An in solution trypsin digest was performed on human pepsinogen 1 (Cell Sciences, US) and 100fmol was loaded onto the LC column.

Figure 6.15 displays identification of peptide ANNQVGLAPVA at 22 minutes with detection of 4 fragment peptides and identification of peptide QYFTVFDR at 27 minutes with detection of 6 fragment peptides. Completion of MRM assay development would include insertion of time windows.

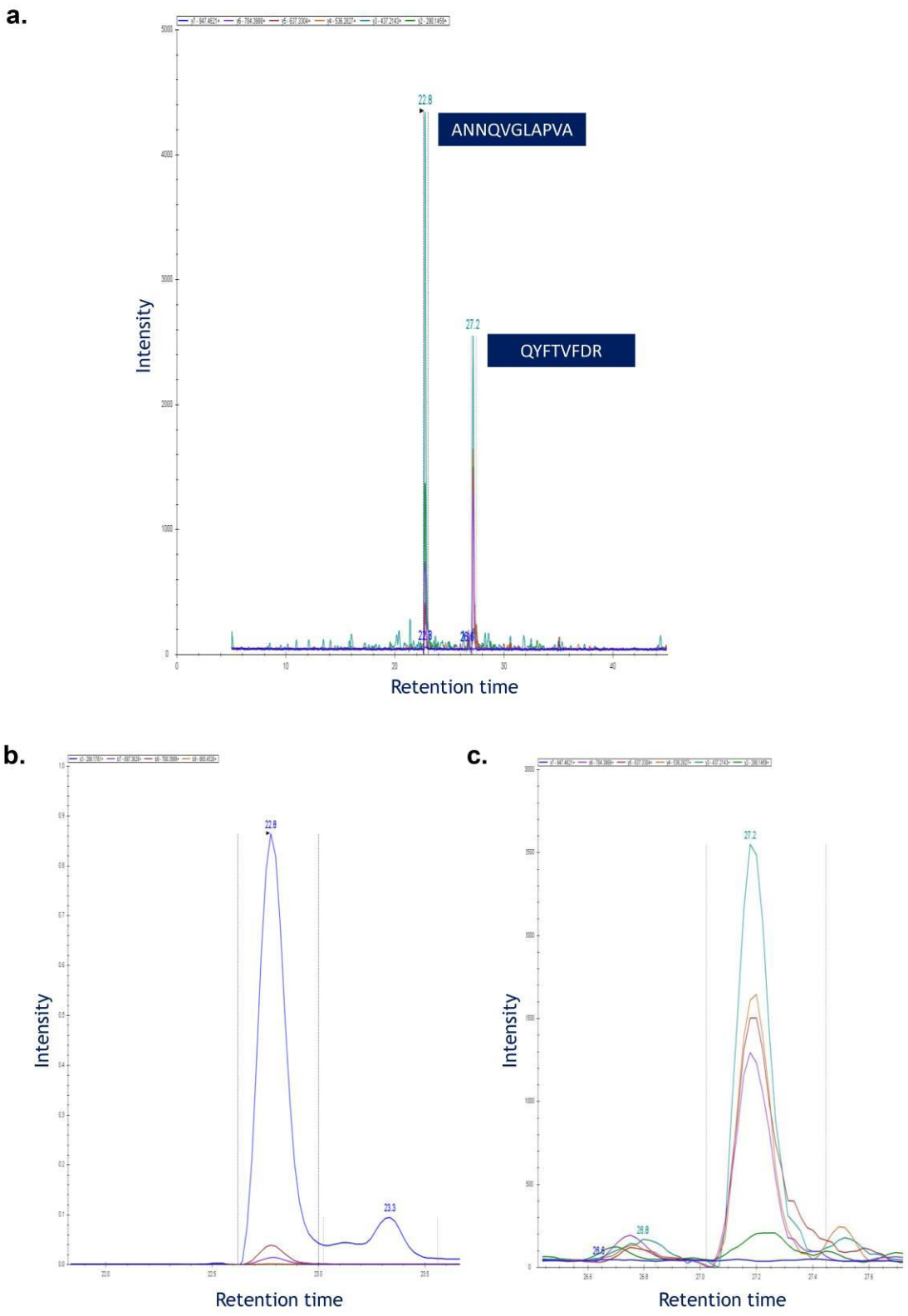

Figure 6.15 100fmol of trypsin digested human pepsinogen 1 (Cell Sciences) is detectable by LC-MSMS (Xevo TQ) using MRM transitions for the two 'identification' peptides ANNQVGLAPVA and QYFTVFDR and their predicted fragment ions.

The main chromatogram **(a.)** displays clear peaks at 22 minutes (m/z 527.28) and 27 minutes (m/z 538.26) relating to the precursor m/z values for the 'identification' peptide precursor ions. The smaller chromatograms display the pattern of fragment ion peak intensity detected for each peptide (**(b.)** ANNQVGLAPVA, **(c.)** QYFTVFDR).

6.8.5 Improvement in bead washing protocol enables increased sample loading

The next stage would be to identify human pepsin extracted from patient samples (known to contain pepsin as shown by human pepsin WB (Chapter 5)). In an initial experiment, 100µl of sample P8 (strongly positive by WB (results shown in **Figure 5.3**)) was taken, diluted 1:10 and pepsin retrieval was attempted as described in Section 6.8.3. Pepsin was not detected on LC-MSMS analysis (Xevo^{TM} TQ). A possible explanation would be that the levels were below the lower limit of detection. Sample loading could not be increased because LC-MSMS analysis (Synapt G1) revealed a high total ion count, indicating large amounts of non-specifically bound protein were present in the bead digest. Therefore, the aim of the following set of experiments was to improve the bead washing procedure, in order to enable increased sample loading for Xevo^{TM} TQ analysis.

Experiment 1: Four 50µl aliquots of the same BAL sample were spiked with 2.9pmol porcine pepsin (Sigma) and diluted 1:10, pre incubation with 15µl pepstatin A agarose beads. Post centrifugation, the supernatant was removed from all samples, as previously. Two bead samples were set aside to analyse directly by SDS PAGE gel. The other two bead samples were washed five times with 500µl 200mM NaCl. Between each wash, the beads were re-pelleted by centrifugation and the wash supernatant was removed and TCA precipitated (15% final v/v) for analysis. The first set of samples were analysed by SDS PAGE gel and the second set were analysed by porcine pepsin WB.

Figures 6.16 (1a and b) illustrates that 200mM NaCl washes remove non-specifically bound protein but do not displace porcine pepsin from pepstatin agarose beads. No benefit was demonstrated in performing more than three washes.

Experiment 2: Four 50µl aliquots of the same BAL sample (diluted 1:10) were incubated with 15µl pepstatin agarose beads, as previously. Following pelleting and supernatant removal, the bead samples were washed three times with either (a) 500µl 200mM NaCl or (b) 500µl ultra-pure water. Each wash was kept for SDS PAGE gel analysis, as previously. The beads, pre- and post- three washes were also analysed.

Figure 6.16 (2) demonstrates that three 200mM NaCl washes are more effective in removing non-specifically bound protein from pepstatin agarose beads than three water washes.

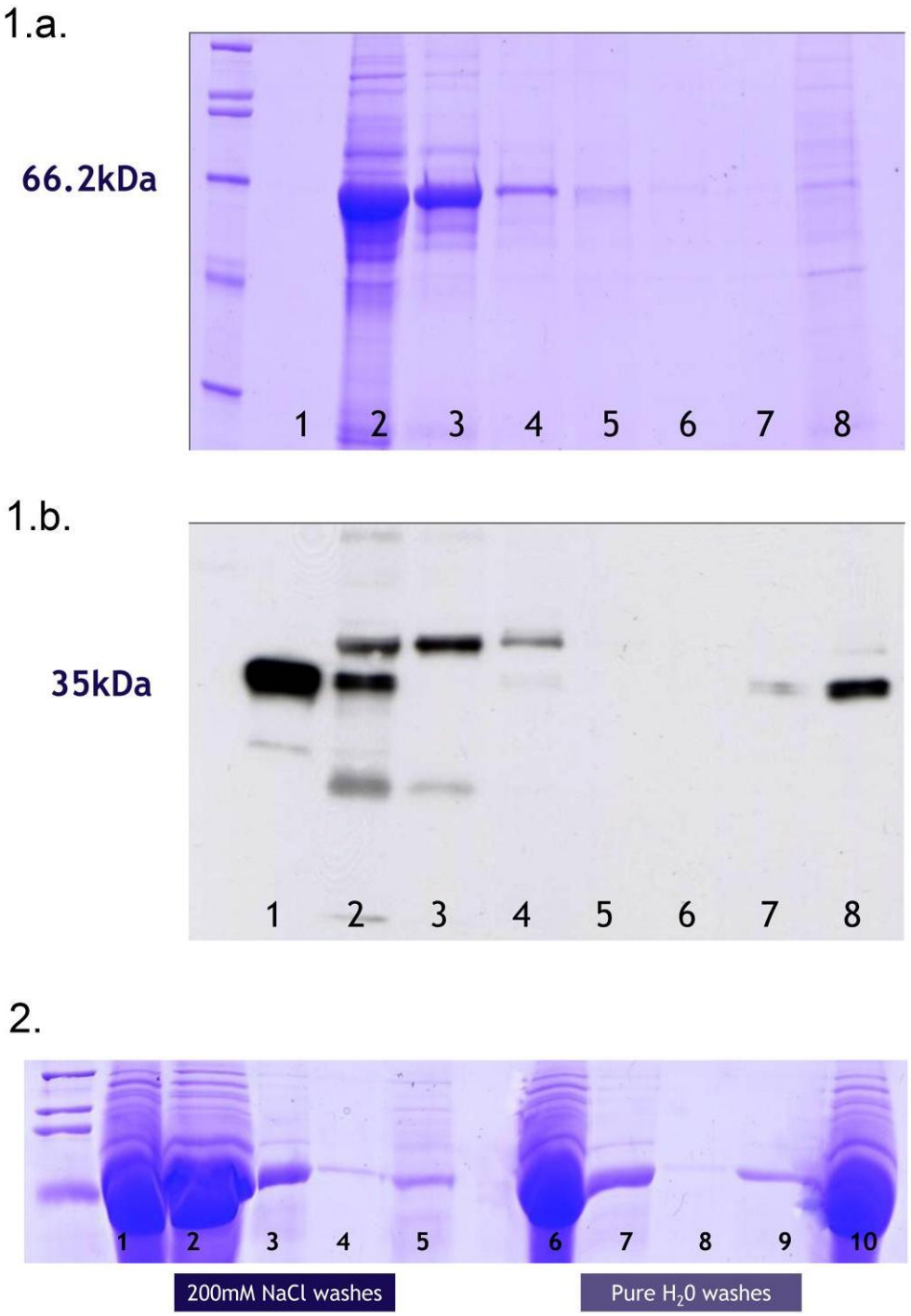

Figure 6.16 (1) SDS PAGE gel and WB images to show that repeated 200mM NaCl pepstatin agarose bead washes do not displace pepsin from pepstatin agarose beads. (2) SDS PAGE gel image to show 200mM NaCl washes are more effective in removing non-specifically bound protein from pepstatin agarose beads than three HPLC H_20 washes.

(1) For methods see text. **(a.)** SDS PAGE gel analysis.and **(b.)** porcine pepsin WB analysis**.** Lane1 – 2.9pmol porcine pepsin, Lane 2 - BAL +2.9pmol porcine pepsin on unwashed beads, lane 3 - wash1, lane 4 - wash 2, lane 5 - wash 3, lane 6 - wash 4, lane 7 - wash 5, lane 8 – BAL + 2.9pmol porcine pepsin on beads post washing.
(2) For methods see text. Lane 1 – BAL on beads pre wash, lane 2 – 200mM NaCl wash 1, lane 3 – 200mM NaCl wash 2, lane 4 – 200mM NaCl wash 3, lane 5 – BAL on beads post 3x 200mM NaCl washes, lane 6 –BAL on beads pre wash, lane 7 – H_20 wash 1, lane 8 – H_20 wash 2, lane 9 – H_20 wash 3, lane 10 – BAL on beads post 3x H_20 washes.

6.9 Discussion

In this chapter, the feasibility of using an immobilised inhibitor to recover pepsin with an active site from paediatric BAL samples was demonstrated. Specifically, a pepsin inhibitor (pepstatin A) immobilised on agarose beads was shown to effectively isolate pepsin from the patient sample BAL matrix. Pepsin-pepstatin binding and release protocols were optimised, using porcine pepsin A as a surrogate for human pepsin 3b (which is not commercially available). Two unique peptides to porcine pepsin A (post trypsin digestion) were identified for the purposes of experimental MS optimisation as well as two human pepsin A peptides, which were demonstrated to be unique to human pepsin A and detectable by MS techniques. Successful bead retrieval of a porcine pepsin A spike in paediatric BAL matrix was shown, and subsequent digestion and detection by LC-MSMS with selected ion monitoring. This now needs to be achieved with human pepsin in patient samples. Following this, an MRM method could be further optimised and a labelled peptide based quantification method included.

The major challenges in the preliminary stages of this work surrounded the various potential effects of BAL sample matrix on pepstatin function and bead circulation. Early experiments established that probable pulmonary pH conditions and known BAL sample pH ranges do not inhibit pepstatin-pepsin binding (Fischer & Widdicombe, 2006). This is in keeping with previous literature where despite structural changes in pepsin exposed to conditions above pH 4, continued pepstatin binding ability has been demonstrated (unless the protein has been irreversibly denatured by pH conditions above pH8.5) (Campos & Sancho, 2003). The sticky, mucin rich consistency of BAL samples also posed challenges for both efficient bead circulation and bead function (due to non-specific protein binding) (Spicer & Martinez, 1984). Sample dilution experiments established that a 1:10 dilution of even the most challenging PICU samples allowed almost complete bead retrieval of a porcine pepsin spike. Difficulties with non-specific protein binding meant the number of beads required for complete pepsin retrieval was increased in BAL compared to HEPES, likely due to other proteins coating the bead surface. Sodium chloride solution was effective in subsequently removing the majority of this non-specifically bound protein

from the beads through interferance with electrostatic and ionic interactions (Thermo Scientific, 2009).

It was perhaps unsurprising that optimisation of a protocol for release of pepsin from pepstatin was challenging, considering that following binding, the physical conformation of pepsin alters to enclose the inhibitor tightly within it's active site (**Figure 6.1**) (Fujinaga et al., 1995). Use of heat, a proprietary surfactant (to aid proteolysis) and reduction and alkylation (to break the disulphide bonds known to be present in pepsin) enabled subsequent complete trypsin proteolysis of pepsin from the beads.

Porcine pepsin spike retrieval from BAL and subsequent detection using LC-MSMS MRM transition monitoring was demonstrated. However, human pepsin peptides were not detected in a strongly pepsin positive BAL sample (by WB analysis). There are various potential explanations for these incongruous results, which will require further investigation. The first explanation may be that the amount of human pepsin A retrieved was below the lower limit of detection for the new assay. The limit of detection will need to be established for both 'identification' peptides in BAL matrix, ideally using a labelled peptide solution, which has been accurately quantified.

The effects of BAL sample matrix on bead function were considered however, direct effects of other substances found within the lung on pepsin structure are also possible. Various proteases are known to be present and active in the lung, which could potentially cause post-translational modification of pepsin (Juillerat-Jeanneret, Aubert, & Leuenberger, 1997; Kimura et al., 2005; Van Der et al., 1999). Carboxypeptidases, for example, could potentially cleave one or two amino acids from the pepsin A C terminus peptide ANNQVGLAPVA *in vivo*, thus altering the 'identification' peptide of interest (Nagae et al., 1993). Additionally, an example of a chemical modification that could affect the peptide QYFTVFDR *in vitro* (post digestion) is cyclisation of N-terminal glutamate to pyro-glutamate, resulting in a 17.03 Da loss in mass and alteration of chromatographic retention time (Reimer et al., 2011). All potential *in vivo* and *in vitro* modifications need to be considered and carefully interrogated. Such peptide modifications will pose additional complications to building a quantitative pepsin assay using labelled peptide standards. All modified peptide

formats which are identified would need to be individually quantified because of the potential for variability in the extent of protein or peptide modification between samples; for example, due to the variable presence of pulmonary proteases *in vivo* in health and disease (Dragović et al., 1995).

The effect of freeze-thawing samples on subsequent retrieval of pepsin using pepstatin has also not been investigated. This could be tested by spiking BAL with purified human pepsin 3b and attempting to retrieve this pre- and post-freezing. It is possible that the use of pepstatin may be restricted to fresh sample analysis. Finally, we have assumed that human pepsin 3b will behave in the same way as porcine pepsin with regard to pepstatin agarose bead interactions. This seems likely, due to the high degree of homology between these two proteins, but will need to be tested following production of purified human pepsin 3b from gastric juice (A. T. Jones & Roberts, 1992).

In further development of a quantitative LC-MRM-MS assay, the challenges of absolute quantification using MS technology will need to be considered. These include variable ionisation, chromatographic behaviour and sample preparation factors including protein mis-cleavage, which can be influenced by factors such as temperature and length of the sample preparation (Lubec & Afjehi-Sadat, 2007). Some of these challenges can be overcome through use of matched stable isotope labelled peptides as internal standards for quantification. These peptides should behave identically to the biological peptide of interest during chromatographic processing and ionisation therefore, reducing the influence of 'run-to-run' variability (Bantscheff, Schirle, Sweetman, Rick, & Kuster, 2007). Inconsistencies introduced during sample preparation need to be assessed via technical replicate testing and subsequent critical analysis of possible points of inconsistency in the sample preparation protocol.

Despite these challenges, the work presented clearly demonstrates the validity of bead based affinity techniques to extract pepsin from BAL matrix. Pepstatin agarose offers a promising option for affinity extraction but other options such as antibody based techniques could be considered if insurmountable challenges arise in future work. LC-MRM-MS quantification assay development is challenging but potentially could provide a reliable, transparent gold standard with which to make quantitative measurements of pepsin in BAL. Should pepsin prove to be a

useful clinical biomarker, simple bedside test development would be the eventual aim. The likelihood of eventual successful return to BAL pepsin analysis using simpler immunological methods of analysis (such as ELISA) is increased by the addition of a 'sample clean up' step using bead-based methods for affinity enrichment, as outlined in this chapter. However, in view of the various limitations of immunological analysis, validation by quantitative LC-MRM-MS methods would be a valuable tool.

6.10 Conclusions

Based on the evidence presented within this chapter, the use of a bead based inhibitor affinity technique for the extraction of pepsin from paediatric BAL samples is valid. Subsequent development of a quantitative LC-MRM-MS assay would offer a gold standard for identification and quantification of pepsin in BAL. Based on Western blot and MS data generated in this thesis, I believe that an optimised inhibitor affinity enhanced LC-MRM-MS assay will have adequate sensitivity for measurement of pepsin in bronchial lavage samples. Large patient studies making accurate quantitative measurements of BAL pepsin in health and disease are essential to assess whether pepsin is a useful biomarker of reflux-aspiration.

Chapter 7 – Effects of mild pH alteration and pepsin exposure on airway epithelial cells

7.1 Introduction

Chronic aspiration results in recurrent lower respiratory tract infections and ultimately leads to pulmonary fibrosis and bronchiectasis, the end stage pathology commonly observed in children with severe ND (Piccione et al., 2012; Seddon & Khan, 2003). However, the mechanism of aspiration related lung injury is poorly understood. Following aspiration, the airway epithelium is the first to encounter inhaled refluxate. Whilst having important barrier functions, the airway epithelium also plays a key role in coordinating the airway immune response to environmental insult, through the secretion of pro-inflammatory, anti-microbial and regulatory mediators and direct interaction with other airway cells involved in generating the immune response (Kato & Schleimer, 2007; Polito & Proud, 1998). Airway epithelial cells (AECs) also play direct roles in airway remodelling through release of specific mediators, such as TGFβ-1 (a key driver of fibrosis) and direct interaction with the mesenchymal unit, sometimes leading to structural changes within the airway wall, characterized by smooth muscle hypertrophy, basement membrane thickening, angiogenesis and sub-mucosal fibrosis (Davies, 2009; Hackett et al., 2009). Direct investigation of the primary AEC response to elements of the refluxate is therefore central to increasing our understanding of the mechanisms of aspiration related lung injury and the resulting clinical outcomes, ultimately leading to development of new therapeutic targets.

The majority of previous literature has focussed on the effects of unphysiological acid lung injury: this has been extensively studied in both animal and *in vitro* models (Amigoni et al., 2008; Beck-Schimmer et al., 2003; Bonnans, Fukunaga, Levy, & Levy, 2006; Bonnans et al., 2007; Nagase et al., 2000; Segal et al., 2007). However, the acidity of gastric aspirate once it reaches the lower airway is probably much less extreme, particularly in the context of chronic micro-aspiration, the commonest clinical scenario. Combined oesophageal and tracheal pH studies have been performed in children and adults with asthma, cystic fibrosis, post stroke and when ventilated on intensive care (Beck-Schimmer et al., 2003; Clayton et al., 2006; Donnelly, Berrisford, Jack, Tran, & Evans, 1993; Hue et al., 1996; Jack et al., 1995; Ledson, Wilson, Tran, & Walshaw, 1998). Moderate falls in tracheal pH associated with marked falls in oesophageal pH were reported in all these studies, lasting for prolonged periods in some subjects

and sometimes correlated with other parameters of respiratory morbidity. However, it is notable that falls in tracheal pH below pH 4 are rarely reported (most studies using pH 5.5 as their cut off to define tracheal aspiration) and therefore, the clinical relevance of studies involving direct instillation of acid into the lungs or onto AECs is debatable. However, study of the effects of mild pH alteration on AECs is important and relevant in children with ND, both in the context of exogenous acid inhalation (following aspiration of gastric contents) and also in the context of possible endogenous airway pH alteration, associated with chronic lower airway inflammation (as discussed in Section 7.2.2).

The role of other non-acid elements of the gastric refluxate (such as pepsin and bile acids) in aspiration-related injury is unclear, and the question of whether reflux aspiration is equally or more damaging than direct aspiration of saliva is relevant to clinical practice. Various groups have investigated the effects of bile acids on the airway and have shown induction of AEC production of TGF ß1, IL-8, and connective tissue growth factor (CCN2) and also increases in cell membrane permeability and decreasing cell viability in type II pneumocytes (Oelberg, Downey, & Flynn, 1990; Perng et al., 2007; Perng et al., 2008). The effect of pepsin on AECs has been studied less extensively. A recent study showed that exposure of AECs to unphysiological levels of pepsin resulted in inflammation and cell death, and that these effects increased at lower pH (Bathoorn et al., 2011). However, it is essential to consider the physiological norm in order to generate meaningful results. We know that airway pH decreases moderately in inflammatory and infective disease states (as discussed in Section 7.2.2) and possibly following reflux-aspiration (Fischer & Widdicombe, 2006) (Carpagnano et al., 2004). It is thus possible that pepsin, as an acidic protease, has potential to cause greater injurious effects in the airways of individuals with chronic airway diseases, whether they have acid reflux-aspiration or not. However, the pH of the lining fluid may also be less relevant in the context of pepsin activity. Johnston *et al*. recently found that pepsin causes laryngeal epithelial damage, even at pH 7 (Samuels & Johnston, 2009). The authors speculated that this was because it is reactivated in low pH intracellular compartments such as late endosomes and the Golgi apparatus.

In this chapter, AEC response is studied following exposure to physiologically relevant concentrations of pepsin (according to available literature) at both a

neutral and mildly acidic pH, using a submerged AEC culture model. AEC expression (mRNA and protein) of IL-8, IL-6 and TGFβ-1 is assessed and cytotoxicity and cell viability are monitored by measurement of lactate dehydrogenase (LDH) activity and trypan blue staining.

7.1.1 Pepsin concentration of the epithelial lining fluid

In vivo, direct contact of inhaled substances with AECs occurs via the AEC lining fluid (ELF). Therefore, *in vitro* experiments should aim to mimic physiologically relevant ELF pepsin concentrations in cell culture media. Gastric juice is estimated to contain 20-260μg/ml pepsin, variable according to meal times and gastric pathology (Pearson & Roberts, 2001). Aspiration of large amounts of gastric juice is uncommon, the usual scenario being chronic small volume aspiration. In this situation, small amounts of gastric juice travel to the top of the oesophagus where it is diluted in saliva before travelling down the trachea into the lungs, where it becomes further diluted in ELF. The process of bronchial lavage collection produces a sample with a further diluted (up to one hundred times) representation of the ELF (Rennard et al., 1986). Measurements of pepsin in BAL samples have ranged from sub 10ng/ml levels to over 1000ng/ml (Abd El-Fattah, Maksoud, Ramadan, Abdalla, & Aziz, 2007; McNally et al., 2011; N. A. Metheny et al., 2006; Stovold et al., 2007). These quantifications may be inaccurate (as discussed in Chapter 5), however they provide the best available estimation of ELF pepsin levels post aspiration. Therefore, taking account of BAL dilution, cell growth media pepsin concentrations of 1 and 10μg/ml were used in this study.

7.1.2 Airway pH

Consideration of airway pH is essential when studying the effects of pepsin on AECs, as pepsin enzymatic activity is pH dependent (Nikki Johnston et al., 2007). The normal ELF pH remains difficult to measure, due to the susceptibility of sampling techniques to cause irritation and trigger sub-mucosal gland secretion. However, review of the available evidence suggests it may be mildly acidic (Fischer & Widdicombe, 2006; J.W., 2005). A tightly regulated ELF pH is important for airway health (Fischer & Widdicombe, 2006). Airway acidification can occur through exogenous acid inhalation, gastro-oesophageal reflux/aspiration or through altered endogenous pH homeostasis. It is

hypothesised that ELF pH self-regulation may be part of an innate host defence strategy in chronic inflammatory/infective disease states (Fischer & Widdicombe, 2006). Paediatric and adult exhaled breath condensate studies have demonstrated abnormal airway acidity, particularly at times of exacerbation, in asthma and cystic fibrosis which improves with treatment (J. F. Hunt et al., 2000; Tate, MacGregor, Davis, Innes, & Greening, 2002).

Both exogenous and endogenous airway acidification may be relevant in children with severe ND, who have a high incidence of GORD plus chronic lower airway inflammation (as documented in Chapter 4). Known detrimental effects of acidification include impaired ciliary function and increased mucous viscosity (Clary-Meinesz, Mouroux, Cosson, Huitorel, & Blaive, 1998; Holma & Hegg, 1989; Holma, Lindegren, & Andersen, 1977). However, studies investigating the potential direct inflammatory effects of mild acidification on human AECs have been limited.

7.1.3 Outcome measures

AEC expression of three important chemokines/cytokines (IL-8, IL-6 and TGFβ-1) was chosen as the outcome measure for cell culture experiments in this chapter. The relevance of IL-8 and TGFβ-1 in the investigation of aspiration lung disease was discussed in Sections 4.1.1 and 4.1.2. In Chapter 4, both of these proteins were found to be raised in BAL collected from children with severe ND, compared to healthy controls.

Interleukin-6 (IL-6) is a multifunctional cytokine with a range of both pro and anti-inflammatory actions within the lung (M. Yu, Zheng, Witschi, & Pinkerton, 2002). Produced by a variety of respiratory cells (monocytes, macrophages, T cells, B cells, fibroblasts and AECs), IL-6 has direct actions on T and B cells, as well as inducing production of acute phase proteins (Chung, 2001). Anti-inflammatory actions include inhibition of cellular apoptosis, inhibition of other pro-inflammatory cytokines, stimulation of metalloproteinase inhibitor production and anti-oxidant actions (M. Yu et al., 2002). More specifically, IL-6 levels have been shown to be raised in the sputum of adults and children with non-cystic fibrosis bronchiectasis and Bathoorn *et al* demonstrated direct

induction of AEC IL-6 expression following stimulation with unphysiological levels of pepsin at a low pH (Osika et al., 1999) (Bathoorn et al., 2011).

7.1.4 Cytotoxicity and cell viability

Cell supernatant lactate dehydrogenase (LDH) was used for monitoring cytotoxicity. LDH is a cytoplasmic enzyme which is present in most cell types and catalyses the conversion between pyruvate to lactate and the inter-conversion of NADH to NAD+ through hydrogen ion transfer (Drent, Cobben, Henderson, Wouters, & Van Dieijen-Visser, 1996). This cytoplasmic enzyme is only released on breakdown of the cell membrane and therefore, it can be used as a measure of cell death. Cell viability was also assessed by trypan blue staining, another measure of cell wall integrity. Cells differentially take up this stain if the cell wall barrier is compromised. The percentage of viable cells can be assessed by light microscopy (K. H. Jones & Senft, 1985).

7.2 Aims

In order to assess the effects of pepsin and mild pH alteration on AECs, the chapter aims were to expose BEAS-2B cells (in a submerged culture model) to media of neutral and mildly acid pH, with and without pepsin, for a range of time-points and subsequently assess:

1) Cell morphology by light microscopy

2) Cell viability and cytotoxicity by trypan blue staining and measurement of LDH activity in cell culture supernatants

3) AEC mRNA expression of IL-8, IL-6 and TGFβ-1 by real time quantitative PCR (Taqman assays) and subsequent protein secretion through measurement of IL-8, IL-6 and TGFβ-1 protein by ELISA assay in cell culture supernatants

7.3 Specific methods

BEAS-2B cells were grown to 70% confluence in 96 well plates, as described in Section 2.7. Bronchial epithelial cell growth media (BEGM) pH was adjusted to pH 6.5 or pH 6 using a 1 molar solution of hydrochloric acid. Porcine pepsin A (Sigma Aldrich, UK) was also added to aliquots of BEGM media at pH 7.4, pH 6.5 and pH 6 to a final concentration of 1 or 10µg/ml. Cell media was replaced with BEGM at pH7.4, pH6.5 or pH6 with either no pepsin, 1µg/ml pepsin or 10µg/ml pepsin for up to 24 hours. A protein control (1µg/ml BSA in BEGM, pH7.4) was used as a negative control for the pepsin media conditions, to assess any non-specific AEC response to alteration of the media protein concentration.

Cells were harvested at 2, 6 and 12 hours and IL-8, IL-6 and TGFβ-1 mRNA expression was assessed using quantitative RT-PCR (Taqman gene expression assays), as described in Section 2.6.3. Positive and negative controls were used, as described in the methods. Cell supernatants were collected at 2, 12 and 24 hours and IL-8, IL-6 and TGFβ-1 protein secretion was measured by ELISA (R&D Systems), as detailed in Section 2.4. Cytotoxicity was assessed using an LDH activity assay, as described in Section 2.4.6. Cell viability was assessed using trypan blue staining and visualisation by light microscopy. All experiments were run in triplicate and at least three experimental repeats were performed for each condition.

The potential effect of acidic pH on protein degradation and ELISA function was assessed by spiking equal amounts of the protein of interest (IL-8, IL-6, TGF-B1) into media of varying pH (pH7.4, pH6.5 or pH6) and incubating at 37^0C for 12 hours. The samples were subsequently assayed and spike retrieval was compared between pH conditions.

7.3.1 BEAS-2B cell culture model

Cell culture is a process where cells are grown outside of their natural environment. The conditions for cell growth are tightly controlled in terms of temperature, CO_2 concentration and hormonally supplemented growth media. Cultures can either be established from primary cells (taken directly from the host) or using cell lines. Cell lines originate from primary cells and have become immortalised through mutation or modification using foreign genes, commonly

introduced using a virus or plasmid. This process inhibits the cell's ability to initiate senescence, meaning it will proliferate indefinitely under suitable conditions (Shay, Wright, & Werbin, 1991). The cell line chosen for this study was the BEAS-2B cell line (available from the European Cell Culture Collection). This cell line originated from bronchial epithelial cells isolated from patients with non-malignant conditions. The cells were infected with a replication-defective SV40/adenovirus 12 hybrid and cloned.

The two major variations in cell culture are 2D (monolayer) or 3D (air-liquid interface culture) models. The normal, differentiated bronchial epithelium is a columnar, pseudo-stratified layer which contains ciliated, goblet, and basal cells (Parker et al., 2010). In monolayer culture, cells grow on a plastic surface in 2D and remain undifferentiated. Therefore, the mucociliary functions of the *in vivo* airway epithelium are not expressed. However, if cells are grown at air liquid interface (on inserts), differentiation occurs leading to expression of all the cell types in the functional bronchial epithelium, thus giving a closer representation of *in vivo* conditions (Dvorak, Tilley, Shaykhiev, Wang, & Crystal, 2011). The 3D model also enables the cells to interact, form gap junctions, signal and migrate as they would in *vivo (Abbott, 2003)*. However, monolayer cultures provide a good starting point to study early cellular responses to novel mechanisms of injury and therefore, submerged monolayer cultures were used in this study.

7.4 Results

7.4.1 Morphological changes

Morphological changes in cell appearance were noted on visualisation by phase contrast light microscopy. An elongated cell shape was often, but not universally, noted following exposure of cells to pH 6 from the 6 hour time-point onwards (**Figure 7.1**).

7.4.2 Cell viability

Cell viability was monitored by adding a small volume (30µl) of 1:5 diluted trypan blue stock solution to each experimental well at 2, 6, 12 and 24 hours. Cells were visualised by light microscopy, using a phase contrast lens. No consistent fall in cell viability was noted in any experimental condition up to the 12 hour time-point (**Figure 7.2**). However, by 24 hours cell viability was variable (**Figure 7.3**). Formal viability cell counts were not recorded.

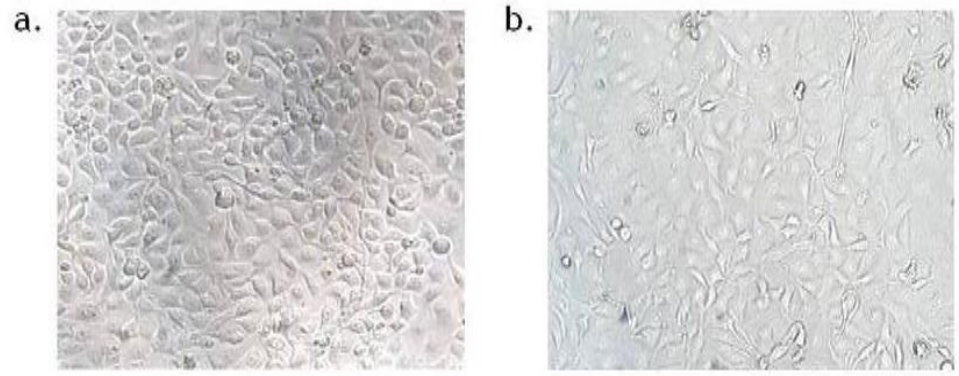

Figure 7.1 Images of (a) control BEAS-2B cells and (b) BEAS-2B cells exposed to pH 6 for 12 hours.

BEAS-2B cells were grown to 70% confluence in 96 well plates and exposed to conditions of variable pH (pH 7.4, pH 6.5 or pH 6) +/- 1-10µg/ml pepsin. Cells were visualised at 2-24 hours using a light microscope with a phase contrast lens. An elongated cell shape was often (but not universally) noted in cells exposed to pH 6 from the 6 hour time-point onwards.

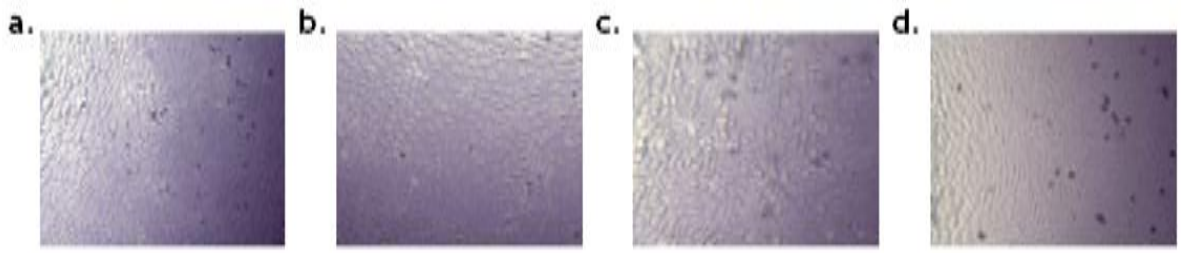

Figure 7.2 Images of (a) control BEAS-2B cells, (b) BEAS-2B cells exposed to 10µg/ml pepsin, (c) BEAS-2B cells exposed to pH6, (d) BEAS-2B cells exposed to pH6 +10µg/ml pepsin. Images shown were recorded at the 12 hour time-point, post trypan blue staining.

BEAS-2B cells were grown to 70% confluence in 96 well plates and exposed to various conditions of altered pH (pH 7.4, pH 6.5 or pH 6) +/- 1-10µg/ml pepsin. Cell media was removed at 2-24 hours and replaced with a dilute solution of trypan blue stain. Cells were visualised using a light microscope with a phase contrast lens. Images shown demonstrate no obvious change in cell viability up to the 12 hour time point in any condition.

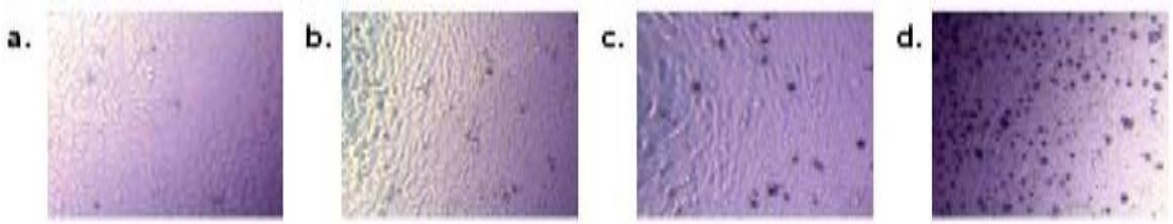

Figure 7.3 Images of (a) control BEAS-2B cells, (b) (c) and (d) BEAS-2B cells exposed to pH6 +10µg/ml pepsin 24 hours (post trypan blue staining) in three separate experiments.

BEAS-2B cells were grown to 70% confluence in 96 well plates and exposed to control media or pH6 + 10µg/ml pepsin for 24 hours. Cell media was removed at 24 hours and replaced with a dilute solution of trypan blue stain. Cells were visualised using a light microscope with a phase contrast lens. Images shown demonstrate a high degree of inter-experiment variation in cell viability by 24 hours in the cells exposed to pH 6 conditions.

7.4.3 Interleukin-8 gene and protein expression

IL-8 mRNA expression was significantly up-regulated (compared to control cells) following 2 hours of exposure to pH 6.5 (mean 2.8x, p=0.002), pH 6.5 + 1µg/ml pepsin (mean 3.24x, p=0.013) and pH 6.5 + 10µg pepsin (mean 4.2x, p=0.002), with a trend towards an additional effect of pepsin. Up-regulation of IL-8 mRNA was also seen at pH 6 (2.4x, p=0.001), pH 6 + 1µg pepsin (2.5x, p=0.047) and pH 6 + 10µg pepsin (3.35x, p=0.121). IL-8 mRNA expression had returned to control levels in all pH 6.5 conditions by 12 hours, whereas those cells exposed to pH 6 further up-regulated IL-8 mRNA expression (pH 6, 6.5x, p=0.005; pH 6 + 1µg pepsin, 6.5x, p=0.005; pH 6 + 10µg, 9.5x, p=0.081). No statistically significant effects of pepsin exposure at a neutral pH were seen **(Figure 7.4)**.

A trend towards down regulation of IL-8 protein secretion in those cells exposed to pH 6 conditions was observed at the 2 hour time-point, reaching statistical significance in the pH 6 + 1µg/ml pepsin condition (60.1% of control levels, p=0.043). However, IL-8 protein secretion then increased (relative to control) in cells exposed to pH 6 conditions and this reached statistical significance for pH 6 + 1µg pepsin (139.0% of control levels, p=0.040) and pH 6 + 10µg pepsin (172.4% of control levels, p=0.011) at 12 hours. No corresponding increased IL-8 protein secretion was observed in conditions exposed to pH 6.5, despite up-regulation of IL-8 mRNA at 2 hours **(Figure 7.4)**.

7.4.4 Interleukin-6 gene and protein expression

IL-6 mRNA expression was significantly increased compared to control cells following two hours exposure to pH 6.5 (2.5x, p=0.019), and a similar trend was observed in those cells exposed to pH 6.5 with pepsin. No additive effect of pepsin was observed. Interestingly, following continued exposure, the same cells had down-regulated IL-6 mRNA expression (compared to control cells) by 12 hours (pH 6.5 + 1µg/ml pepsin, 0.62x, p=0.044; pH 6.5 + 10µg/ml pepsin, 0.64x, p=0.005). There was a trend towards up-regulation of IL-6 mRNA expression in all cells exposed to pH 6 conditions. There did not appear to be any additional effect of pepsin exposure. There was also a trend towards a dose-wise increase

in IL-6 mRNA expression at two hours post exposure to pepsin at a neutral pH **(Figure 7.5)**.

IL-6 protein secretion (expressed as % control) was significantly down regulated by two hours in conditions of altered media pH (pH 6.5, 70.29%, p=0.013; pH 6.5 + 10µg/ml pepsin, 77.97%, p=0.004; pH 6, 31.68%, p=0.014; pH 6 + 1µg/ml pepsin, 32.34%; pH 6 + 10µg/ml pepsin, 31.47%, p=0.009). IL-6 protein secretion continued to be significantly down regulated in all cells exposed to pH6 at 12 hours (pH 6, 50.9%, p=0.001; pH 6 + 1µg/ml , 49.9%, p=0.006; pH 6 + 10µg/ml, 51.9%, p=0.007) and at 24 hours (pH 6, 31.6%, p<0.001; pH 6 + 1µg/ml pepsin, 31.8%, p=0.005; pH 6 + 10µg/ml pepsin, 35.8%, p=0.001). A dose wise trend towards increased IL-6 protein secretion was observed on exposure to pepsin at a neutral pH at the 2 hour time-point, although this did not reach statistical significance **(Figure 7.5)**.

7.4.5 TGF-B1 gene and protein expression

No differences in TGFβ-1 mRNA expression were observed at two hours, compared to controls cells. However, by twelve hours, TGFβ-1 mRNA was up-regulated in all conditions at pH 6.5 and pH 6, reaching statistical significance in two conditions (pH 6.5 + 10µg/ml pepsin, 1.69x, p=0.043; pH6, 3.08x, p=0.043). There was no additional up-regulation in mRNA with the addition of pepsin **(Figure 7.6)**.

TGFβ-1 protein secretion was not detectable in any condition until twelve hours post media change. At this time-point, TGFβ-1 protein secretion was significantly down regulated (relative to control cells) in all cells exposed to an altered pH (pH 6.5, 50.2%, p=0.039; pH 6.5 + 1µg/ml pepsin, 30.6%, p=0.029; pH 6.5 + 10µg/ml pepsin, 42.2%, p=0.022; pH 6, 41.4%, p=0.001; pH 6 + 1µg/ml pepsin, 37.5%, p<0.001; pH 6 + 10µg/ml pepsin, 33.3%, p=0.016). This pattern continued at 24 hours exposure (pH 6.5, 66.4%, p=0.003; pH 6.5 + 1µg/ml, 64%, p=0.008; pH 6.5 + 10µg/ml, 65.24%, p=0.010; pH 6, 39.21%, p=0.001; pH6 + 1µg/ml, 44.8%, p=0.010; pH6 + 10µg/ml, 41.8%, p=0.006) **(Figure 7.6)**.

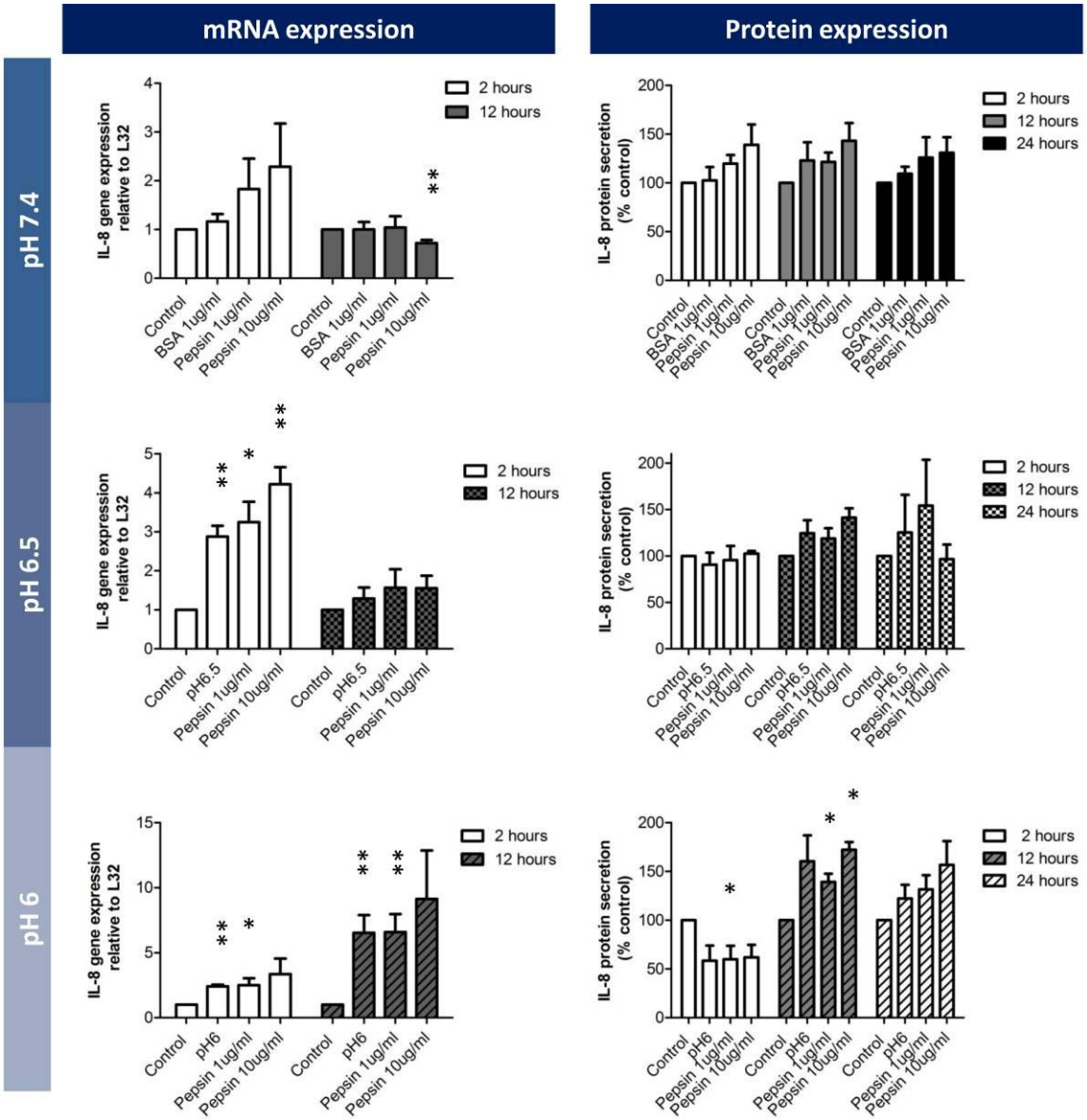

Figure 7.4 Bar charts to show BEAS-2B cell IL-8 mRNA expression (relative to L32) following 2 and 12 hours exposure to pH 6.5 or pH 6 +/- pepsin and the corresponding IL-8 protein secretion at 2, 12 and 24 hours.

BEAS-2B cells were grown to 70% confluence in 96 well plates and exposed to conditions of mildly altered pH (pH 6.5 or pH 6) +/- 1-10µg/ml pepsin for up to 24 hours. At 2 and 12 hours, cells were harvested for mRNA analysis. mRNA analysis was performed using Taqman gene expression assays (Applied Biosystems, UK) and all IL-8 mRNA expression was normalised to the ribosomal L32 housekeeping gene, using the comparative CT method for analysis. IL-8 protein secretion was measured in supernatants at 2, 12 and 24 hours using commercially available ELISA kits (R&D systems, UK). Mean (SEM) is displayed. * = $p<0.05$, ** = $p<0.01$.

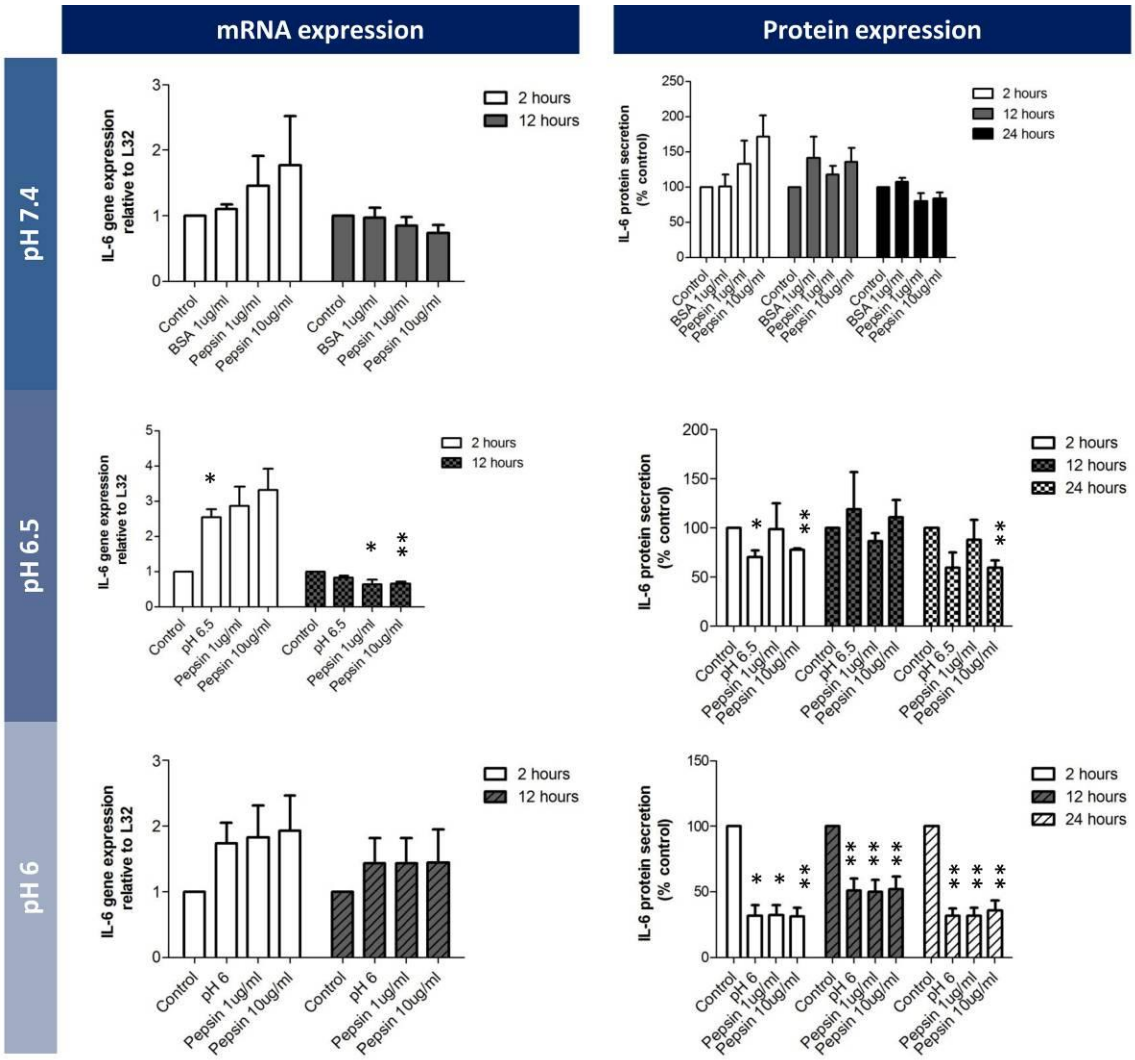

Figure 7.5 Bar charts to show BEAS-2B cell IL-6 mRNA expression (relative to L32) following 2 and 12 hours exposure to pH 6.5 or pH 6 +/- pepsin and the corresponding IL-6 protein secretion at 2, 12 and 24 hours.

BEAS-2B cells were grown to 70% confluence in 96 well plates and exposed to conditions of mildly altered pH (pH 6.5 or pH 6) +/- 1-10µg/ml pepsin for up to 24 hours. At 2 and 12 hours, cells were harvested for mRNA analysis. mRNA analysis was performed using Taqman gene expression assays (Applied Biosystems, UK) and all IL-6 mRNA expression was normalised to the ribosomal L32 housekeeping gene, using the comparative CT method for analysis. IL-6 protein secretion was measured in supernatants at 2, 12 and 24 hours using commercially available ELISA kits (R&D systems, UK). Mean (SEM) is displayed. * = $p<0.05$, ** = $p<0.01$.

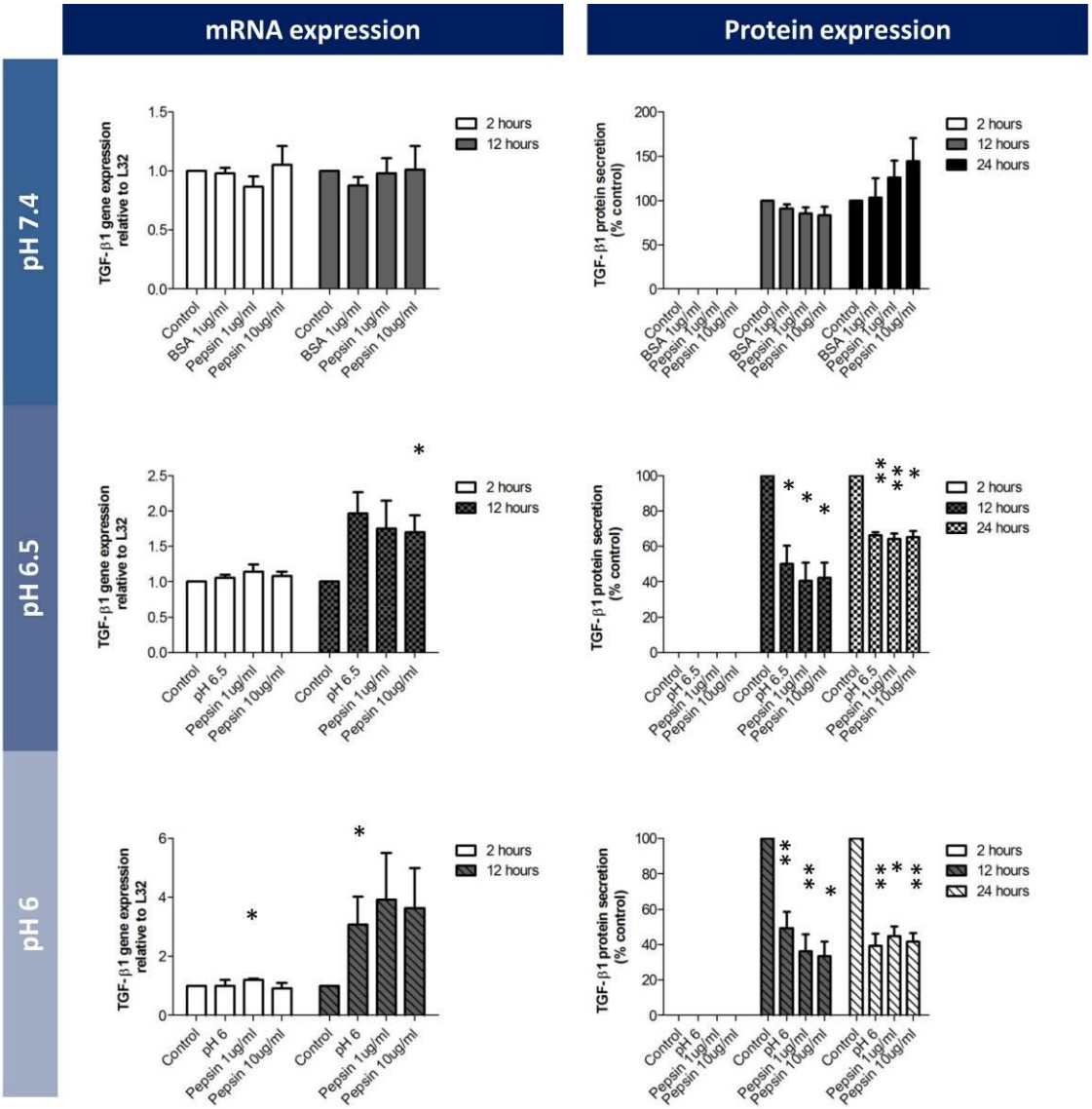

Figure 7.6 Bar charts to show BEAS-2B cell TGFβ-1 mRNA expression (relative to L32) following 2 and 12 hours exposure to pH 6.5 or pH 6 +/- pepsin and the corresponding TGFβ-1 protein secretion at 2, 12 and 24 hours.

BEAS-2B cells were grown to 70% confluence in 96 well plates and exposed to conditions of mildly altered pH (pH 6.5 or pH 6) +/- 1-10µg/ml pepsin for up to 24 hours. At 2 and 12 hours, cells were harvested for mRNA analysis. mRNA analysis was performed using Taqman gene expression assays (Applied Biosystems, UK) and all TGFβ-1 mRNA expression was normalised to the ribosomal L32 housekeeping gene, using the comparative CT method for analysis. TGFβ-1 protein secretion as measured in supernatants at 2, 12 and 24 hours using commercially available ELISA kits (R&D systems, UK). Mean (SEM) are displayed. * = p<0.05, ** = p<0.01.

7.4.6 Cytotoxicity

No significant differences in cell supernatant LDH activity were observed for any experimental condition at 2, 6 or 12 hours exposure (**Figure 7.7 (a)**).

7.4.7 Effects of pH alteration on ELISA function

Experiments were conducted to investigate whether the down-regulation of cytokine protein secretion observed was due to ELISA dysfunction or protein degradation, secondary to pH alteration. A known amount (250pg) of each cytokine protein measured (IL-8, IL6 and TGF-B1) was spiked into BEGM at both pH 7.4 and pH 6. This was incubated at 37^0C, 5% $C0_2$ for 12 hours. It was then assayed as normal and spike retrieval was compared between unadjusted and pH adjusted BEGM media. No significant differences in spike retrieval were observed (**Figure 7.4 (b)**).The experiment was repeated twice and all samples were assayed in duplicate.

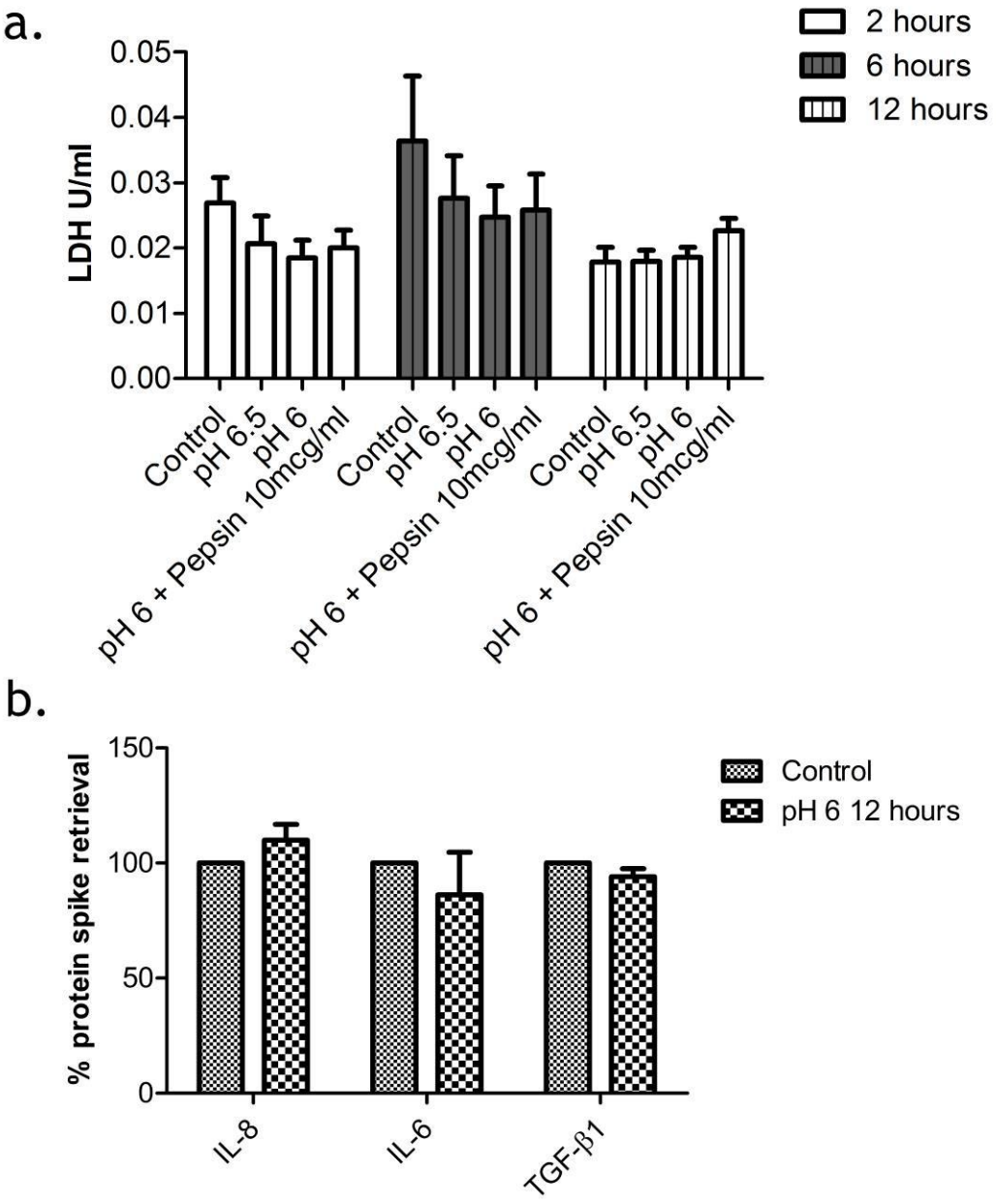

Figure 7.7 Bar charts to show (a) LDH activity (U/ml) in cell culture supernatants following exposure of BEAS-2B cells to pH 6.5, pH 6 or pH 6 + 10µg/ml pepsin. (b) % spike retrieval following incubation of equal amounts of cytokine protein (IL-8, IL-6 and TGFβ-1) at normal media pH (pH 7.4) and pH 6 for 12 hours.

(a) BEAS-2B cells were grown to 70% confluence in 96 well plates and exposed to conditions of mildly altered pH (pH 6.5 or pH 6) +/- 1-10µg/ml pepsin for up to 24 hours. At 2, 6 and 12 hours, cell culture supernatants were collected for LDH activity analysis (Roche, UK). No difference in LDH activity was observed following exposure of cells to any experimental condition. Selected results are presented. Mean (SEM) is displayed.

(b) Equal amounts (250pg) of each cytokine protein (IL-8, IL-6 and TGFβ-1) were incubated in BEGM media at normal pH (pH 7.4) and pH 6 for 12 hours, at 37^0C and 5% CO_2. Samples were then assayed by ELISA and spike retrieval was compared between conditions. No differences were observed and therefore, it was concluded that no protein degradation or ELISA dysfunction had occurred due to alteration in media pH.

7.5 Discussion

In this chapter, the BEAS-2B cell response to pepsin exposure, with or without mild alteration in cell media pH was examined. Pepsin exposure (1-10µg/ml) at normal growth media pH (7.4) did not induce statistically significant effects on IL-8, IL-6 or TGFβ-1 mRNA or protein expression. However, BEAS-2B cells were sensitive to mild acidification, significantly up-regulating TGFβ-1, IL-8 and IL-6 gene expression. Corresponding increase in cytokine protein secretion was not observed and in some cases, was down regulated. It was difficult to assess the effects of pepsin exposure at a mildly acidic pH due to the marked pH driven effect on protein expression however, a trend towards a dose dependent increase in IL-8 mRNA and protein expression was observed. No significant cytotoxicity (as assessed by cell supernatant LDH activity) or decrease in cell viability (as assessed by trypan blue staining) was observed in any experimental condition for a period of up to 12 hours, in keeping with previous literature (D. S. Cohen, Palmer, Welch, & Sheppard, 1991) (Ichimonji et al., 2010). Continued up-regulation of gene expression between 2 and 12 hour time-points also adds supportive evidence of continuing cell viability. No pH induced protein instability was observed and therefore, this did not provide an explanation for the seemingly down-regulated cytokine protein results.

The most striking responses were those triggered by mild alteration in media pH. Gene expression of all three cytokines was up-regulated, with various peaks in differential cytokine mRNA expression observed at different time-points and pH levels. It is unclear from our experiments whether the observed increase in cytokine mRNA expression is due to increased transcription or decreased degradation of mRNA. However, it does appear that the effects of extra-cellular acidification are not cytokine specific, although differential signalling is observed at different time-points and pH levels. Patterns of cytokine expression are likely to be variable between cell types, as observed by previous researchers studying TNF-alpha expression following acid stimulation (Heming et al., 2001). It may be of interest to conduct multiplex array mRNA analysis in future experiments to further characterise the AEC response. This was the approach taken by Johnston *et al* when studying the effects of pepsin exposure on laryngeal epithelial cells (N. Johnston, Wells, Samuels, & Blumin, 2009).

Interestingly, corresponding up-regulation of cytokine protein secretion was not necessarily observed and indeed TGFβ-1, IL-6 and IL-8.were all significantly down-regulated from as early as two hours post stimulation at pH 6. However, this down-regulation was selectively transient for IL-8 protein secretion, which subsequently increased over control levels at later time-points, reaching statistical significance in selected conditions at 12 hours.

Various potential explanations for cytokine protein down regulation can be found within the literature. A previous study of LPS induced TNF-alpha protein expression in alveolar macrophages demonstrated increased mRNA expression at pH 5.5 compared to pH 7.4, but decreased extracellular TNF-alpha protein expression. The authors investigated potential effects of pH on protein stability and cell viability. However, as in this study, they concluded that protein instability or reduced cell viability did not explain their findings. However, on measuring *intra-cellular* TNF-alpha protein, they found significantly increased levels of retained synthesised cytokine at pH 5.5 (Heming et al., 2001). They proposed a mechanism of pH stress, causing inhibition of protein transfer from the Golgi network to the plasma membrane and interference with membrane exocytic and endocytic transport pathways. Cosson *et al* elegantly demonstrated this pH sensitive mechanism in fibroblasts (on reduction of fibroblast cytosol pH to pH 6.8) in the 1980s (Cosson, De Curtis, Pouyssegur, Griffiths, & Davoust, 1989). The observed reversibility of this mechanism implies that stimulation and recovery experiments would be interesting in any future extension of our study, particularly if intra-cellular cytokine levels were found to be raised compared to extra-cellular levels on further investigation.

In contrast, other researchers found increased IL-6 and connective tissue growth factor mRNA which was accompanied by increased protein secretion in human airway smooth muscle cells exposed to pH 6.3 for 16 hours (Ichimonji et al., 2010; Matsuzaki et al., 2011). This highlights potential differences in response to acidity between cell types. The authors acknowledged previous findings of down-regulated cytokine protein secretion on exposure of cultured cells to acidic conditions, and proposed that these differences may be due to differential cellular expression of proton sensing G-protein coupled receptors. They demonstrated that knockdown of the proton-sensing ovarian cancer G-protein-

coupled receptor (OGR 1) in airway smooth muscle cells attenuated the cellular inflammatory response to acidic conditions (Matsuzaki et al., 2011) Ichimonji, 2010). The potential for differential response of different respiratory cell types to extracellular acidification suggests that once the primary bronchial epithelial cell response has been characterised, co-culture models may be useful in studying terminal remodelling effects on the airway epithelium.

An alternative explanation for the observed response could be post-transcriptional regulation of cytokine protein production. This could potentially occur through regulation of mRNA processing, mRNA export and localization, mRNA decay or mRNA translation (Paul Anderson & Kedersha, 2009). Noxious environmental triggers (including alteration in extracellular pH) could trigger a cellular 'stress response' in which selective mRNA protein translation is halted in order to conserve energy for cellular repair. This response is known to be under the control of various eIF2α kinases (Paul Anderson & Kedersha, 2008; Johno et al., 2012). One example of an eIF2α kinase is PERK (PKR-like endoplasmic reticulum kinase). This is a resident endoplasmic reticulum (ER) protein, activated following accumulation of unfolded or mis-folded proteins in the endoplasmic reticulum (ER) lumen (responsible for the production and folding of secretory proteins) (Schroder & Kaufman, 2005). In an attempt to restore homeostasis, the stressed ER triggers the 'unfolded protein response' in which protein transcription and translation is halted, production of molecular chaperones involved in the process of protein folding are up-regulated (such as heat shock proteins and foldases) and ER associated degradation of unfolded proteins is increased. If resolution is not achieved, apoptotic pathways are ultimately triggered (Schroder & Kaufman, 2005). It has been suggested that post transcriptional control of cytokine production, via mechanisms such as these, may play a role in the control of inflammation (P. Anderson, 2008; Kitamura, 2011). Interestingly, Cohen *et al* documented depressed overall protein synthesis with specific up-regulation of stress proteins, following transient mild acidification of guinea pig AECs. Further investigation of cellular stress markers at early experimental time-points may be helpful in understanding the mechanisms involved in the human bronchial epithelial cell response to mild alterations in pH. Also, further detailed study of the pathways

leading to cell death (with continued insult) at later time-points would be informative.

Once mechanisms of pH injury have been fully investigated, therapeutic targets can be explored. Hunt *et al* explored one such target, investigating intrinsic buffering mechanisms within AECs. They demonstrated increased glutaminase activity after exposure of human AECs to pH 5.6, leading to increased pH buffering through the production of ammonia. Interestingly, this self-protective cellular response to acidic stress was found to be inhibited by inflammatory cytokines (JOHN F. HUNT et al., 2002). This work demonstrated one of many potential mechanisms of cellular defence against acid stress. However, overall the investigation of therapeutic targets in this field has been limited to date.

Correlation between pepsin and lower airway inflammatory markers has been found in clinical studies, providing justification for study of the direct effects of pepsin exposure on the airway epithelium. Bathoorn *et al* documented inflammatory effects of pepsin at low pH on AECs. However, the concentrations of pepsin and levels of acidity used in their stimulation studies were not physiologically relevant. Mertens *et al* conducted limited studies of AEC response to pepsin exposure at a neutral pH and found no effect on IL-8 protein secretion. However, Johnston *et al* found increased inflammatory cytokine mRNA expression on multiplex analysis post exposure of laryngeal epithelial cells to pepsin at a neutral pH (Samuels & Johnston, 2009). They subsequently documented cytotoxicity (by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay) and up regulation of genes involved in stress and toxicity (N. Johnston et al., 2009). The pepsin levels used in these experiments were appropriately higher than those used in our study, as it is likely that the contents of gastric refluxate are further diluted by the point of contact with the airway epithelium. We found no statistically significant effect of pepsin at neutral or mildly acidic pH on TGFβ-1 or IL-6 gene or protein expression. However, a consistent dose wise trend was observed at neutral and mildly acidic pH on IL-8 mRNA and protein expression, although this did not reach statistical significance. It would be interesting to perform a wider array analysis, as performed by Johnston et al, to fully investigate the effects of pepsin on the AECs. It would also be useful to examine intracellular cytokine

protein content, in view of the marked pH related effects on cytokine protein secretion and the discussion presented above. Therefore, although no significant effects were observed in this study, further study of the response of AECs to pepsin exposure at a neutral and mildly acidic pH is justified.

Ideally, this experimental series should be repeated on primary AECs of paediatric origin and in an air-liquid interface culture model. Marked differences in immunological response between BEAS-2B cells and primary epithelial cells have been noted by our group in the context of viral infection, however, this has not been explored in the field of pH injury (Fonceca, Flanagan, Trinick, Smyth, & McNamara, 2012). There are always limitations to translating *in vitro* findings into an *in vivo* context. However, multilayer air-liquid interface culture models provide a closer representation of the *in vivo* airway epithelium than monolayer cultures, as discussed in Section 7.3.1. A key limitation of this study is the lack of mucus secreting cells in the 2D culture model. Mucus plays an important role in buffering changes in airway pH and studies have shown increased mucus viscosity in response to pH alteration: potentially another self-protective strategy of AECs (Holma & Hegg, 1989). Also, although monolayer cultures can be used in studying early cellular responses, multi-layer ALI culture models are more appropriate for the study of airway remodelling and repair (Hackett et al., 2009). There are various reasons for this, including the limited lifespan of monolayer cultures: squamous terminal differentiation and consequent falls in viability occur when monolayer cultures reach confluence. Also, epithelial cell death and sloughing may form an important part of the injury and repair process and this cannot be examined in a monolayer model. Finally, differences in cell viability observed at 24 hours may have been due to slight variations in cellular confluence at the beginning of the experiment. Cell-cell interaction and adhesion is important for epithelial cell defence and this variable would have increased uniformity in an ALI culture model (Heijink et al., 2010). If different cell culture models are to be used in future experiments, intracellular pH will need to be considered. Multiple studies have established that extracellular pH does not directly reflect intracellular pH (Furukawa, Nakamura, & Okabe, 1997) and it is possible that intra-cellular buffering capacity may vary between cell types and cell culture models. This would therefore require further

characterisation in order to understand the thresholds at which acid stress injury occurs in AECs, and also to comment on differences in results between models.

Effects of pH alteration on the constituents of the growth media need to be considered. Again, a solution to this potential confounder lies in using an air-liquid interface culture model, where normal growth media would be used on the basal side of the epithelium, whilst the apical side would be exposed to a solution of appropriate ELF osmolarity and altered pH. Finally, addition of hydrochloric acid to growth media may have altered both the media osmolarity and chloride concentration. Previous researchers have explored potential effects of adjusting the pH of DMEM media using hydrochloric acid. Significant alteration in osmolarity was only identified at pH 2 and below (Ishizuka et al., 2001). Therefore, this is unlikely to have been a confounder in our study. However, chloride concentration may have affected the results and this could be further explored by using glacial acetic acid to adjust media pH and comparing to results to those obtained when using hydrochloric acid.

7.6 Conclusions

Beas-2B cells are sensitive to mild acidification, up-regulating IL-8 and IL-6 gene expression by two hours and TGFβ-1 mRNA expression by 12 hours. However, corresponding cytokine protein release is generally not observed and in some cases, is down regulated. This did not appear to be due to protein degradation or reduction in cell viability. There are various potential mechanistic explanations which require further investigation. Exposure of AECs to pepsin did not induce statistically significant effects in terms of IL-8, IL-6 or TGFβ-1 expression. However, extended investigation of the potential effects of AEC pepsin exposure at a neutral or mildly acidic pH is justified.

This preliminary evidence suggests that mild alterations of airway pH may directly contribute to the patho-physiology of inflammatory airway diseases and may also be relevant in reflux-aspiration related lung disease. This area requires further detailed scientific and clinical study as a potential novel therapeutic target

Chapter 8 - Concluding Remarks and Future Work

8.1 Background

Respiratory disease is a major cause of morbidity and mortality in children with severe ND (Seddon & Khan, 2003). The evidence base in this area is limited, but these children have a shortened life expectancy and respiratory pathology is the leading cause of death (J. L. Hutton, 2006; Jane L. Hutton, 2008; Reid et al., 2012; Strauss et al., 1997; Thomas & Barnes, 2010). The daily burden of respiratory symptoms is poorly documented, however, children with severe ND are at high risk of prolonged, complicated hospital admissions and high re-admission rates, impacting on quality of life (Mahon & Kibirige, 2004; Young et al., 2011). This has considerable healthcare cost implications: in 2006, 29% of US children's hospital expenditure was on inpatient care of children with ND and at least 10% of these admissions were for respiratory problems (Berry et al., 2012).

Clinical management of this patient group is challenging, due to the complex background aetiology of the respiratory disease and the lack of high quality evidence on which to base clinical interventions (Seddon & Khan, 2003; Waespi et al., 2000). This contributes to the 'reactionary' approach to care discussed by Berry *et al (Berry Jg & et al., 2011; Berry et al., 2012)*. Aetiological factors include poor cough, muscle weakness, scoliosis, poor nutrition and sleep apnoea. Gastro-oesophageal reflux and direct or indirect (reflux) aspiration are likely to be major contributory factors, but diagnosis is difficult due to the challenges of clinical assessment and limitations of diagnostic testing. Consequently, clinical decision-making is challenging both for clinicians and parents, particularly when symptomatic management of two potentially indistinguishable conditions (for example, reflux or direct aspiration) is distinct and furthermore, potentially invasive.

Novel biomarkers of reflux and direct aspiration, such as BAL pepsin and alpha amylase, have been proposed, but some of the available evidence is conflicting, particularly with regard to BAL pepsin (Trinick, Johnston, et al., 2012). Interestingly, these biomarkers have not previously been studied in this particularly high-risk patient group.

The clinical outcomes and underlying patho-physiology of aspiration lung disease remain ill-defined, primarily due to limitations in diagnostic testing. Clinical

associations have been shown between aspiration and bronchiectasis and a cycle of recurrent aspiration leading to repeated respiratory tract infection, resulting in chronic inflammatory changes and ultimately bronchiectasis or lung parenchymal damage has been proposed (Piccione et al., 2012; Seddon & Khan, 2003). Elements of the refluxate (such as pepsin or altered pH) may be directly damaging to the airway epithelium (the first pulmonary barrier to encounter inhaled substances) and therefore, study of the direct immunological response of AECs to elements of the refluxate is directly relevant to furthering our understanding of the patho-physiology of aspiration lung disease and developing new therapeutic targets.

8.2 Daily respiratory symptomatology and levels of lower airway inflammation are high in children with severe ND, even at times of stability

Data presented within this thesis documents (for the first time) details of the type and character of daily respiratory symptoms, lower airway inflammation and microbiology in individuals with severe ND. Even at a time of stability, children with severe ND often have significant respiratory symptoms, particularly at night, the consequences of which affect them and their families. Respiratory symptoms appeared to relate to airway neutrophilia in Elective-ND patients. There was also a trend for greater airway neutrophilia and respiratory symptoms in those with lower airway microbial rDNA positivity, suggesting that lower airway infection may play a significant role. This requires further study in a much larger population in order to draw firm conclusions, as discussed in Chapter Four.

The co-morbid complexity of this patient group was highlighted in Chapter Three. Prospective, interventional studies are needed to fully assess the detailed interactions of co-morbidities and respiratory symptoms in these children. Marked variation was observed in the use of respiratory interventions (such as prophylactic antibiotics and anti-cholinergic medications). Interestingly, in this study the majority of ND patients were not under the care of a respiratory paediatrician, despite high levels of chronic respiratory symptoms. Those who were under specialist care often required home oxygen or non-invasive ventilation, suggesting that referral for a respiratory paediatric opinion occurs

late in the disease process, again highlighting a reactionary rather than prophylactic approach to care. Future studies should focus on the outcomes of early respiratory intervention, specifically in terms of child and family quality of life.

The use of a standardised respiratory symptom score to document respiratory symptoms has been shown to be useful in other paediatric groups (Powell et al., 2002; Trinick, Southern, et al., 2012). However, this is the first time it has been studied in children with ND. The LRSQ-Neuro has the potential to be used both to screen for patients who may benefit from specialist respiratory assessment or as a possible outcome measure in future prospective clinical trials. However, further psychometric evaluation will be required, particularly with regard to sensitivity to change and relevance of scores to longer-term respiratory outcomes (Cella et al., 2007; Turner et al., 2007). Early, tailored respiratory assessment of children with severe ND and chronic respiratory symptoms may be useful and well-designed clinical trials are needed to determine the risks and benefits of various prophylactic therapies in this group. Useful, patient/family relevant outcome measures will be central to the success of such studies.

8.3 BAL tests (current and novel) for reflux aspiration are limited: an affinity enhanced mass spectrometry BAL pepsin assay may provide a new gold standard

The finding of no BAL LLM positivity was surprising in this high-risk patient group. However, the lack of test specificity dictates a high cut off for positive reporting by cytopathologists which, amongst other limitations (as discussed in Chapter Five), restricts the use of LLMs as a diagnostic test (Reid-Nicholson et al., 2010).

The lack of consensus within the literature regarding the utility of pepsin as a biomarker of reflux aspiration is perhaps partially explained in Chapter Five. There are various potential explanations for the finding of conflicting results between two published pepsin ELISAs and WB techniques. Certainly, no firm conclusions can be drawn about pepsin as a biomarker of reflux aspiration until an accurate method of BAL pepsin detection and equally importantly, quantification is validated due to occasional detection of pepsin in

asymptomatic controls. The crucial questions are: i) whether there are significant differences in BAL pepsin load in those who are symptomatic and have other evidence of pathological reflux aspiration; ii) if pathological reflux aspiration is definable using BAL pepsin levels, what are the longer term clinical outcomes of this and how do clinical interventions influence these (studied longitudinally)?

The data presented in Chapter Six confirms the feasibility of using a bead based inhibitor affinity enrichment followed by LC-MRM-MS analysis to create a 'gold standard' assay for pepsin identification and quantification. Further work is needed to validate this approach in more detail and there is potential for use of a similar assay to study GORD in salivary samples. This could allow accurate quantitative measurement of salivary or BAL pepsin in health and disease, something that is essential to assess whether pepsin is a useful biomarker of reflux or reflux aspiration, respectively. Should pepsin prove to be a useful biomarker, future development of a simpler bedside assay is conceivable, using MS as a 'gold standard' for validation.

8.4 BAL alpha-amylase activity is a promising biomarker of direct aspiration

Data presented within Chapter Five suggests that BAL alpha-amylase activity may be a promising biomarker of direct aspiration. Significantly increased alpha amylase activity levels were found in Elective-ND patients compared to healthy controls, suggesting this may be a useful biomarker of chronic direct aspiration. Importantly, the correlation between BAL alpha amylase activity and lower airway inflammatory markers in Elective-ND patients suggests that the degree of chronic aspiration may be directly linked to lower airway pathology in these patients.

BAL alpha-amylase activity does not differentiate direct and reflux aspiration. However, it may potentially be interpreted alongside specific biomarkers of reflux aspiration in determining the relative contributions of direct and reflux aspiration in this group. The question of whether alpha-amylase is directly damaging to AECs is interesting and it may be that other elements of the aspirate are also relevant, including gastric and bacterial contents. Future

studies should focus initially on establishing normative BAL alpha amylase activity levels in health and disease, both in the paediatric and adult age groups. Further studies defining longitudinal clinical outcomes related to BAL alpha amylase activity and accompanying microbiology would be of great interest, as supported by the relationship of BAL alpha-amylase activity and microbial rDNA positivity observed in this study. Interventional studies of, for example, surgical salivary management options, with measurement of BAL alpha-amylase pre-operatively and careful study of respiratory outcomes post-operatively, would also be very informative in assessing interventional treatment outcomes.

8.5 Mild pH alteration is damaging to the airway epithelium and may provide a promising therapeutic target

Data presented in Chapter Seven documents BEAS-2B cell sensitivity to mild acidification, up-regulating IL-8 and IL-6 gene expression following 2 hours exposure and TGF Beta 1 mRNA expression by 12 hours. Interestingly, corresponding cytokine protein release was generally not observed and in some cases was significantly down-regulated. There are many potential underlying mechanisms to explain this response, including interference with membrane exocytic and endocytic pathways, differential cellular expression of proton sensing G-coupled receptors and post transcriptional regulation of cytokine protein production as part of a cellular 'stress response'.

Mild airway pH alteration may theoretically occur in children with severe ND: i) as a result of chronic inflammation caused by recurrent aspiration as well as other contributory factors to lower airway disease; or ii) as a direct result of reflux aspiration. It would be useful to investigate this, taking direct measurements of airway epithelial lining pH in this group compared to healthy controls once appropriate technology allows (current limitations are discussed in Chapter Seven).

Further investigation of the cellular mechanisms of injury following mild pH alteration (as well as the effects of exposure to other elements of the aspirate) is important in the exploration of novel therapeutic targets for aspiration lung disease. This approach may be critical in children with ND, given that it may be

impossible to completely control aspiration in this group. Targeted management of lower airway damage secondary to aspiration is therefore paramount and will only be possible with further exploration of the specific mechanisms behind AEC aspirate induced injury. An alternative approach to investigating the effects of chronic aspiration on the lower airway may be to perform proteomic analyses on BAL samples from children with and without high risk of aspiration (based on clinical evidence and BAL biomarkers), documenting broader differences in pro/anti-inflammatory and pro-resolution proteins. This data may help to inform and direct further research both in vitro and vivo.

8.6 Study Limitations

Various limitations of this study are discussed in greater detail within the individual chapters. However, it is important to further consider ways in which the study could be improved, should it be repeated. As discussed in Chapter Three, achievement of study recruitment targets is essential to ensure the study is adequately powered to detect differences between study groups. If the study were to be repeated, it would be useful to have a recruitment team or potentially consider multi-centre recruitment in order to achieve recruitment targets. It would also be essential to try and include a non-respiratory PICU cohort in order to further investigate aspiration risk pre/post PICU admission. Ideally, all samples should be processed fresh by pre-optimised assays. This would be particularly important for any microbiology work, where ideally conventional culture as well as 16S PCR techniques (+/- human DNA degradation) would be used. Any further cell culture work should be conducted in an air-liquid interface model which would allow isolated apical exposure to elements of the aspirate (as in vivo) and also allow key cellular defences, such as tight junction integrity and mucus production, to play their role.

Appendix 1

Proforma – Group 1 (PICU-ND)

Study Number ……

AH Number ……

Initials ……

Date of birth ……

Weight .....

Gender Male ☐ Female ☐

Contact number ..……

Consultant ……

Cerebral Palsy GMFC group 4 ☐ 5 ☐

Medical Diagnoses
……
……
……

History of atopic symptoms
……

Medication
……
……

Gastrostomy Yes ☐ No ☐

Any oral feeds? Yes ☐ No ☐

Details……...

Anti reflux surgery previously? Yes ☐ No ☐

Details……...

Previous videofluoroscopy Yes ☐ No ☐

Details……..

Previous oesophageal biopsy Yes ☐ No☐

Results……..

Date/time of BAL/brushings/blood samples ……

Sample numbers ……

Hours since onset of illness ……

Hours ventilated ……

History of Current Illness

……
……
……

Current FiO2 ……..……

Oxygenation Index ……

Ventilatory support ……

CXR changes ……...

ETT Cuffed ☐ Uncuffed ☐

Proforma – Group 2 (Elective-ND)

Study Number ……

AH Number ……

Initials ……

Date of Birth ……

Weight ……

Gender Male ☐ Female ☐

Contact number ……

Consultant ……

Cerebral Palsy GMFC group 4 ☐ 5 ☐

Medical Diagnoses
……
……
……

History of atopic symptoms
……
……

Medication
……
……

Date/time of BAL/brushings/blood samples ……
Sample numbers ……

Had videofluoroscopy? Yes ☐ No☐
Results
……
……

Had oesophageal biopsy? Yes ☐ No☐
Results
……
……

Pepsin as a biomarker of pulmonary aspiration in children with CP
Proforma – Group 3 (Healthy Controls)

Study Number ……

AH Number ……

Initials ……

Date of Birth ……

Gender Male ☐ Female ☐
Weight ……

Contact number ……

Consultant ……

Medical Diagnoses
……
……
……

Procedure
……

History of atopic symptoms
……
……

Medication
……
……

Date/time of BAL/brushings/blood samples ……

Sample numbers ……

Appendix 2

Parent/Guardian Information Document

(For parents/guardians of children attending hospital for elective surgery)

"Looking for a test of pulmonary aspiration in children with cerebral palsy"

You and your child are being invited to take part in a research study. Your child is having a general anaesthetic. During the anaesthetic we would like to collect some samples that would not otherwise be possible. Before you decide whether or not you wish 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. Ask us if there is anything that is not clear or if you would like more information.

What is the study about?

One in 500 children is born with cerebral palsy, a condition caused by damage to the brain before, during or soon after birth. Children with cerebral palsy are particularly prone to food and fluids aspirating (going down the wrong way) into their lungs. Every year, thousands of children with cerebral palsy are admitted to hospital with chest problems because of aspiration. Unfortunately the tests we have to diagnose this condition are not very good.

We are trying to find out whether a protein called pepsin, which is normally found in the stomach, can be used as a test for aspiration by looking to see whether it is present in lung fluid from children with and without cerebral palsy and with and without chest problems. By looking at the cells which line the airways, we also want to find out what effect pepsin has in the chest. Our long-term aim is to find ways of decreasing aspiration so that we can help children with respiratory disease, but to do this, we first need a good diagnostic test.

Why has my child been chosen?

Your child has been chosen because they have no chest problems but are attending hospital for a routine operation.

What will happen if I agree for my child to take part?

- You will be asked to complete a short questionnaire about any chest symptoms your child may have had over the past three months and about any medicines they might be taking
- As part of their surgery, a drip will be put in your child's arm so that the anaesthetic can be given. Through this drip, blood will be taken and tested to see whether your child is allergic to anything. No extra needles will be involved.
- When your child has had the anaesthetic and is asleep, we will wash out their lungs using a small amount of salt-water and brush some cells from the surface of the airways. Your child will not feel anything when this is going on.

Are there any disadvantages to my child taking part in this study?
Our group has performed hundreds of these tests without any serious problems. The only disadvantage from brushing cells from the airways is that some children occasionally get a mild, dry cough afterwards. The cough is very short-lived and makes no difference to recovery from the operation.

Will I receive any results from the tests you do?
If you wish, we can contact you with the results of the allergy tests following surgery.

One of the tests we do on the lung fluid looks for special cells called lipid laden macrophages. We have previously used this as a test for aspiration, but it is not a very good test. However, should your child have lots of these cells we would want to discuss the results with you and your child's doctor.

Are there any benefits to my child taking part in this study?
No, there are none. While this study will not benefit your child directly, it may help us to understand more about childhood respiratory disease and help us to find treatments for other children in the future.

Will my child's GP be informed of their participation in this study?
Your child's GP will not be informed unless you wish otherwise.

Do I have to take part?

No. It is up to you and your child (where possible) to decide whether or not to take part. You are both free to withdraw from the research at any time and without giving a reason. Your decisions about this will not affect the standard of care your child receives.

If you would like to take part, you will be given this information sheet to keep and be asked to sign a consent form. If your child is able to understand the research and is happy to take part, they can write their name on an "assent" form, if they want to.

Will the information on my child be kept confidential?
Yes, all personal information will be kept confidential and secure. Only people involved in the study will have access to the information. This study will be published in a medical journal but it won't be possible to identify individual children or parents from what is written. This study has been approved by the Liverpool Children's Research Ethics Committee, which includes lay members representing the local community.

What if you have any problems or would like further information about the study?
You can either contact:

Dr Paul McNamara,
Institute of Child Health,
Royal Liverpool Children's NHS Trust,
Eaton Rd,
Liverpool, L12 2AP
email: mcnamp@liv.ac.uk

The Patient Advisory Liaison Service (PALS)
Royal Liverpool Children's NHS Trust,
Eaton Rd,
Liverpool, L12 2AP
Tele: 0151 252 5161
Email: pals@alderhey.nhs.uk

Patient Information Sheet

(For children aged 13-16 attending hospital for elective surgery)

"Looking for a test of pulmonary aspiration in children with cerebral palsy"

We are asking if you would agree to take part in a research project called "Looking for a test of pulmonary aspiration in children with cerebral palsy". Before you decide if you want to join in, it's important to understand why the research is being done and what it will involve for you. So please read this leaflet carefully. Talk about it with your family, friends, doctor or nurse if you want to.

What is the study about?

One in 500 children is born with cerebral palsy, a condition caused by damage to the brain before, during or soon after birth. Children with cerebral palsy are particularly prone to food and fluids aspirating or going down the wrong way, into their lungs. Every year, thousands of children with cerebral palsy are admitted to hospital with chest problems because of aspiration. Unfortunately the tests we have for this condition are not very good.

We are trying to find out whether a protein called pepsin which is normally found in the stomach, can be used as a test for aspiration. By looking at the lung fluid in the airways of children with and without cerebral palsy, we want to find out whether pepsin can be used as such a test. By looking at the cells which line the airways, we also want to find out what effect pepsin and other stomach contents have on the lung. Our long-term aim is to find ways of decreasing aspiration so that we can help children with respiratory disease, but to do this, we first need a good diagnostic test.

Why have I been chosen to take part?

You have been chosen because you have no chest problems but are attending hospital for a routine operation.

What will happen if I agree to take part?

- One of your parents/guardians will be asked to fill out a short questionnaire about any chest symptoms you might have and about any medicines you might be taking.
- When you go for your operation, a drip will be put in your arm so that the anaesthetic drugs can be given. Through this drip, we will take some blood to test to see whether you are allergic to anything. No extra needles will be involved. If you or your parents wish, we can contact you with the results of these tests following your operation.
- When you are asleep, we will wash out some fluid from your lungs using a small amount of salt-water and also brush some cells from the surface of the airways. You will not feel this happen.

Are there any disadvantages to my taking part in this study?
Our group has performed hundreds of these tests without any serious problems. The only disadvantage from brushing cells from the airways is that some children occasionally get a mild, dry cough afterwards. The cough is very short-lived and makes no difference to recovery from the operation.

Are there any benefits to my taking part in this study?
No, there are none. While this study will not benefit you directly, it may help us to understand more about childhood respiratory disease and help us to find treatments for other children in future.

Will I receive any results from the tests you do?
If you or your parents wish, we can contact you with the results of the allergy tests following your operation.

One of the tests we do on the fluid from your lungs looks for special fat filled cells. We have previously used this as a test for aspiration, but it is not a very good test. However, should you have lots of these cells we would want to discuss the results with you, your parents and your doctor.

Will my doctor be informed of their participation in this study?
Your doctor will be not be informed unless you wish otherwise.

Do I have to take part?

No, it is up to you. If you do:

- Your doctor will ask you to sign a form giving your consent or assent
- You will be given a copy of this information sheet and your signed form to keep.
- You are free to stop taking part at any time during the research without giving a reason. If you decide to stop this will not affect the care you receive.

Will any information collected be kept confidential?
Yes, all personal information will be kept confidential and secure. Only people involved in the study will have access to the information. This study will be published in a medical journal but it won't be possible to identify you or your parents/guardians from what is written.

What if you have any problems or would like further information about the study?
You can either contact:

Dr Paul McNamara,
Institute of Child Health,
Royal Liverpool Children's NHS Trust,
Eaton Rd,
Liverpool, L12 2AP
email: mcnamp@liv.ac.uk

The Patient Advisory Liaison Service (PALS)
Royal Liverpool Children's NHS Trust,
Eaton Rd,
Liverpool, L12 2AP
Tele: 0151 252 5161
Email: pals@alderhey.nhs.uk

Patient Information Sheet

(For children aged 6-12 attending hospital for elective surgery)

"Looking for a test of pulmonary aspiration in children with cerebral palsy"

What is the study about?

Some children have a condition called cerebral palsy. These children are at risk of food and drinks going down the wrong way (or aspirating) onto their chest. Every year, lots of children with cerebral palsy come into hospital with chest problems because of this problem. Unfortunately, the tests we have for aspiration are not very good.

We are trying to find out whether something made in the stomach called pepsin, can be used as a test for aspiration by looking at the lining cells and lung fluid from the airways of children. Our long-term aim is to find ways of stopping aspiration to help children with respiratory disease but before we can do this, we need a good test.

Why have I been chosen to take part?

You have been chosen because you have nothing wrong with your chest but you are coming in to hospital for an operation.

What will happen if I agree to take part?

- When you go for your operation, a plastic tube will be put in your arm so that medicines can be given. This happens for all operations. We will take some blood from this tube and test it to see if you are allergic to anything. There are no extra needles in this study.
- When you are asleep, we will wash out some fluid from your chest. We will also use a very small brush to brush some cells from your chest. You will not feel either of these things happen.

Do I have to take part?

- No, it is up to you.

Are there any disadvantages to my taking part in this study?

The only thing that might happen is that you could get a slight cough afterwards which won't last very long.

Will joining in help me?

No, probably not, but the information we get might help treat other young people with chest problems with better medicines in future.

Will my doctor be informed of their participation in this study?

Your doctor will not be informed unless you wish otherwise.

Will any information collected be kept private?

Yes, all medical details will be kept private. This study will be published but it won't be possible to identify you from what is written.

What if I don't want to do the research anymore?

If at any time you don't want to do the research anymore just tell your parents, doctor or nurse. You are free to stop taking part at any time without giving a reason.

What if you have any problems or would like further information about the study?

You can either contact:

Dr Paul McNamara,
Institute of Child Health,
Royal Liverpool Children's NHS Trust,
Eaton Rd,
Liverpool, L12 2AP
email: mcnamp@liv.ac.uk

The Patient Advisory Liaison Service (PALS)
Royal Liverpool Children's NHS Trust,
Eaton Rd,
Liverpool, L12 2AP
Tele: 0151 252 5161
Email: pals@alderhey.nhs.uk

Patient Information Sheet

(For children aged less than 6 years attending hospital for elective surgery)

"Looking for a test of pulmonary aspiration in children with cerebral palsy"

*Note to reader: Please read the whole sheet to yourself before reading it to your child

- Some children have trouble with their chests and with breathing
- Your doctors want to find out why this happens and how best to make it better
- They want to learn from children with and without chest problems
- You have been chosen because you have nothing wrong with your chest

What does it involve?
- Your doctors want to collect a small sample from your chest when you have your operation
- They also want to do a blood test
- You won't feel either of these things happen

What do I have to do?
We are asking you to say "Yes" or "No" to the question "Can we collect a blood sample and some samples from your chest when you are asleep for your operation?"

Do I have to say yes?
No, you don't.

What will happen if I say no?
Nothing. No one will mind and you will be treated just the same. If you change your mind, just tell your mum.

What shall I do now?
Talk with your mum or dad about what you are being asked to do. Your doctors and nurses are happy to answer any questions you have.

Parent/Guardian Information Document

(For parents/guardians of children with cerebral palsy attending hospital for elective surgery)

"Looking for a test of pulmonary aspiration in children with cerebral palsy"

You and your child are being invited to take part in a research study. Your child is having a general anaesthetic. During the anaesthetic we would like to collect some samples that would not otherwise be possible. Before you decide whether or not you wish 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. Ask us if there is anything that is not clear or if you would like more information.

What is the study about?

One in 500 children is born with cerebral palsy, a condition caused by damage to the brain before, during or soon after birth. Children with cerebral palsy are particularly prone to food and fluids aspirating (going down the wrong way) into their lungs. Every year, thousands of children with cerebral palsy are admitted to hospital with chest problems because of aspiration. Unfortunately the tests we have to diagnose this condition are not very good.

We are trying to find out whether a protein called pepsin, which is normally found in the stomach, can be used as a test for aspiration by looking to see whether it is present in lung fluid from children with and without cerebral palsy and with and without chest problems. By looking at the cells which line the airways, we also want to find out what effect pepsin has in the chest. Our long-term aim is to find ways of decreasing aspiration so that we can help children with respiratory disease, but to do this, we first need a good diagnostic test.

Why has my child been chosen?

Your child has been chosen because they have cerebral palsy and are attending hospital for a routine operation.

What will happen if I agree for my child to take part?

- You will be asked to complete a short questionnaire about your child and about any medicines they might be taking
- As part of their surgery, a drip will be put in your child's arm so that the anaesthetic can be given. Through this drip, blood will be taken and tested to see whether your child is allergic to anything. No extra needles will be involved. If you wish, we can contact you with the results of these tests following surgery.
- When your child has been anaesthetised and is asleep, we will wash out their lungs using a small amount of salt-water and also brush some cells from the surface of the airways. Your child will not feel anything when this is going on.

Are there any disadvantages to my child taking part in this study?
Our group has performed hundreds of brushings without any serious problems. The only disadvantage from brushing cells from the airways is that some children occasionally get a mild, dry cough afterwards. The cough is very short-lived and makes no difference to recovery from the operation.

Are there any benefits to my child taking part in this study?
No, there are none. While this study will not benefit your child directly, it may help us to understand more about childhood respiratory disease and help us to find treatments for other children in the future.

Will I receive any results from the tests you do?
If you wish, we can contact you with the results of the allergy tests following surgery.

One of the tests we do on the lung fluid looks for special cells called lipid laden macrophages. We have previously used this as a test for aspiration, but it is not a very good test. However, should your child have lots of these cells we would want to discuss the results with you and your child's doctor.

Will my child's GP be informed of their participation in this study?
Your child's GP will not be informed unless you wish otherwise.

Do I have to take part?

No. It is up to you to decide whether or not to take part. You are free to withdraw from the research at any time and without giving a reason. Your decisions about this will not affect the standard of care your child receives.

If you would like to take part, you will be given this information sheet to keep and be asked to sign a consent form.

Will the information on my child be kept confidential?
Yes, all personal information will be kept confidential and secure. Only people involved in the study will have access to the information. This study will be published in a medical journal but it won't be possible to identify individual children or parents from what is written. This study has been approved by the Liverpool Children's Research Ethics Committee, which includes lay members representing the local community.

What if you have any problems or would like further information about the study?
You can either contact:

Dr Paul McNamara,
Institute of Child Health,
Royal Liverpool Children's NHS Trust,
Eaton Rd,
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Liverpool, L12 2AP
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Email: pals@alderhey.nhs.uk

Parent/Guardian Information Document

(For parents/guardians of children with cerebral palsy on the intensive care unit)

"Looking for a test of pulmonary aspiration in children with cerebral palsy"

You are being invited to take part in a research study. Your child is on the paediatric intensive care unit at the moment and has a tube helping them to breath. We would like to collect some samples from your child's chest through the tube. Before you decide whether or not you wish 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. Ask us if there is anything that is not clear or if you would like more information.

What is the study about?

One in 500 children is born with cerebral palsy, a condition caused by damage to the brain before, during or soon after birth. Children with cerebral palsy are particularly prone to food and fluids aspirating (going down the wrong way) into their lungs. Every year, thousands of children with cerebral palsy are admitted to hospital with chest problems because of aspiration. Unfortunately the tests we have to diagnose this condition are not very good.

We are trying to find out whether a protein called pepsin, which is normally found in the stomach, can be used as a test for aspiration by looking to see whether it is present in lung fluid from children with and without cerebral palsy and with and without chest problems. By looking at the cells which line the airways, we also want to find out what effect pepsin has in the chest. Our long-term aim is to find ways of decreasing aspiration so that we can help children with respiratory disease, but to do this, we first need a good diagnostic test.

Why has my child been chosen?

Your child has been chosen because they have cerebral palsy and chest problems and are on the intensive care unit.

What will happen if I agree for my child to take part?

- You will be asked to complete a short questionnaire about respiratory symptoms your child might have had in the past and about any medicines they might have been taking
- As part of being on the intensive care unit, your child will need to have blood tests taken. When this is done some extra blood will be taken to see whether your child is allergic to anything. No extra needles will be involved. If you wish, we can contact you with the results of these tests. In future, using the same blood sample, we may also test for genes which may be important in respiratory disease.
- At the moment your child has a tube down helping them to breathe. As part of your child's routine care, fluid from this tube and your child's chest is regularly washed out and thrown away. We will take some of this fluid back to the laboratory and test it for levels of pepsin. We will also use a very small brush to brush up some cells from the airways through the tube.

Are there any disadvantages to my child taking part in this study?
If your child is sedated, then there will be no disadvantages from taking part in the study. If your child is not sedated, the brushing technique and the suction of fluid from your child's chest may cause them to cough immediately afterward.

Are there any benefits to my child taking part in this study?
No, there are none. While this study will not benefit your child directly, it may help us to understand more about childhood respiratory disease and help us to find treatments for other children in the future.

Will I receive any results from the tests you do?
If you wish, we can contact you with the results of the allergy tests following surgery.

One of the tests we do on the lung fluid looks for special cells called lipid laden macrophages. We have previously used this as a test for aspiration, but it is not a very good test. However, should your child have lots of these cells we would want to discuss the results with you and your child's doctor.

Will my child's GP be informed of their participation in this study?
Your child's GP will not be informed unless you wish otherwise.

Does my child have to take part?

No. It is up to you to decide whether or not to take part. You are free to withdraw from the research at any time and without giving a reason. Your decisions about this will not affect the standard of care your child receives.

If you would like to take part, you will be given this information sheet to keep and be asked to sign a consent form.

Will the information on my child be kept confidential?
Yes, all personal information will be kept confidential and secure. Only people involved in the study will have access to the information. This study will be published in a medical journal but it won't be possible to identify individual children or parents from what is written. This study has been approved by the Liverpool Children's Research Ethics Committee, which includes lay members representing the local community.

What if you have any problems or would like further information about the study?
You can either contact:

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Royal Liverpool Children's NHS Trust,
Eaton Rd,
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Email: pals@alderhey.nhs.uk

Appendix 3

Respiratory Questionnaire

Study No : ……

Clinic Date : ……

Consultant : ……

Date ……

Name : ……

Sex : male female (please circle)

Date of birth : ……

Age (months) : ……

The following questionnaire asks questions about your child and what has been happening to him/her over the last **three months**.

Please could you fill in the questionnaire by putting a circle around your response to each question.

It is important that every question is answered, even if your child has been perfectly well, with no problems at all.

Thank you.

Name of Child **Study Number**

A) During the day (when awake) in the last three months:

i) My child has had wheezing (whistling noise coming from the chest):

Every day *most days* *some days* *a few days* *not at all*

ii) My child has had a cough:

Every day *most days* *some days* *a few days* *not at all*

iii) My child has had a rattly chest:

Every day *most days* *some days* *a few days* *not at all*

iv) My child has been short of breath:

Every day *most days* *some days* *a few days* *not at all*

B) During the night (when asleep) in the last three months:

i) My child has had wheezing (whistling noise coming from the chest):

Every night *most nights* *some nights* *a few nights* *not at all*

ii) My child has had a cough:

Every night *most nights* *some nights* *a few nights* *not at all*

iii) My child has had a rattly chest:

Every night *most nights* *some nights* *a few nights* *not at all*

iv) My child has been short of breath:

Every night *most nights* *some nights* *a few nights* *not at all*

v) My child has snored:

Every night *most nights* *some nights* *a few nights* *not at all*

Name of Child **Study Number**

C) How many colds has your child had in the last three months:

None *one* *two* *three* *more than three* *always has a cold*

If the answer to the above question is *'none'* continue to questions in Section D:

When my child has had a COLD in the last three months:

i) My child has had wheezing (whistling noise coming from the chest):

Every cold *most colds* *some colds* *a few colds* *not at all with colds*

ii) My child has had a cough:

Every cold *most colds* *some colds* *a few colds* *not at all with colds*

iii) My child has had a rattly chest:

Every cold *most colds* *some colds* *a few colds* *not at all with colds*

iv) My child has been short of breath:

Every cold *most colds* *some colds* *a few colds* *not at all with colds*

D) When my child does NOT have a COLD, in the last three months:

i) My child has had wheezing (whistling noise coming from the chest):

Every day *most days* *some days* *a few days* *not at all*

ii) My child has had a cough:

Every day *most days* *some days* *a few days* *not at all*

iii) My child has had a rattly chest:

Every day *most days* *some days* *a few days* *not at all*

iv) My child has been short of breath:

Every day *most days* *some days* *a few days* *not at all*

Name of Child **Study Number**

E) These next three questions are about other problems your child may have had. Over the last three months:

i) My child has had noisy breathing that does not seem to come from the chest:

Every day *most days* *some days* *a few days* *not at all*

ii) My child has had fast breathing:

Every day *most days* *some days* *a few days* *not at all*

iii) My child has had noisy breathing that appears to come from the throat or back of the throat:

Every day *most days* *some days* *a few days* *not at all*

Name of Child **Study Number**

F) The next four questions are on how your child's chest symptoms actually affect HIM or HER over the last three months:

i) My child's chest symptoms have affected my child's feeding, eating or tolerance of feeds:

Every day *most days* *some days* *a few days* *not at all*

ii) My child's chest symptoms have woken up my child:

Every night *most nights* *some nights* *a few nights* *not at all*

iii) My child's chest symptoms have made my child unusually tired:

Every day *most days* *some days* *a few days* *not at all*

G) The next four questions are on how your child's chest symptoms actually affect YOU and YOUR family's life the last three months:

i) My child's chest symptoms have limited my activities:

Every day *most days* *some days* *a few days* *not at all*

ii) My child's chest symptoms have resulted in adjustments being made to our family life:

Every day *most days* *some days* *a few days* *not at all*

iii) My child's chest symptoms have disturbed our sleep:

Every night *most nights* *some nights* *a few nights* *not at all*

iv) I have been worried about my child's chest symptoms:

Every day *most days* *some days* *a few days* *not at all*

Appendix 4

The last five questions are about other problems that your child may have experienced in the last month:

i) My child coughs after he/she eats or when he/she is laid down:

Every day *most days* *some days* *a few days* *not at all*

ii) My child is short of breath after he/she eats or when he/she is laid down:

Every day *most days* *some days* *a few days* *not at all*

iii) My child seems to be in pain/uncomfortable after/with eating:
Every day *most days* *some days* *a few days* *not at all*

iv) My child has choking episodes:

Every day *most days* *some days* *a few days* *not at all*

v) My child frequently vomits or regurgitates food or liquid:

Every day *most days* *some days* *a few days* *not at all*

vi) *Do any of the above symptoms coincide?*
.....
.....

Appendix 5

Sample	Container	Species	PCR signal C(T)	Signal Strength	Identity [%]	Coverage [%]
1	S/N	Staphylococcus aureus	32	+	100	100
		Corynebacterium accolens	31	+	99.7	100
	UMD	Staphylococcus aureus	28	++	100	100
		Corynebacterium accolens	28	++	99.7	100
2	S/N	Streptococcus mitis group	24	+++	99.8	100
		Rothia mucilaginosa	27	++	98.4	100
	UMD	Streptococcus mitis group	20	+++	99.8	100
		mixed sequence	22	+++		
3	S/N	PCR negative				
		Bifidobacterium breve	35	+	100	100
	UMD	Enterococcus villorum/ratti/hirae/durans/faecium	33	+	100	100
		Bifidobacterium breve	32	+	99.5	100
4	S/N	PCR negative				
	UMD	PCR negative				
5	S/N	PCR negative				
	UMD	Moraxella Catarrhalis/non liquefacians	35	+	100	100
6	S/N	Dolosigranulum pigrum	35		99.7	97
		Candida parapsilosis	32	+	99.6	100
	UMD	Dolosigranulum sp.	28	++	91	99
		Candida parapsilosis	28	++	99.6	100
C(T) value:	<25: +++					
	<30: ++					
	>31: +					

Comparison of SepsiTest™ results for paired samples stored in recommended UMD tubes versus BAL supernatant fractions stored in Eppendorf tubes

SepsiTest results for BAL samples which were divided into two and stored either as ‘whole’ (unprocessed) BAL in the recommended UMD tubes or as supernatant fractions in Eppendorf tubes (S/N).

Appendix 6

Preparation of Maldi Standards for the Ultraflex

Standard	Sigma	Amount in vial	H_2O (µl)	Conc. pmol/µl	* Std (µl)	* H_2O (µl)	Individual Conc. pmol/µl	Mass No. $[M+H]^+$
Des Arg Bradykinin	B-1901	1mg	1000	1105	5	455	12	904.47
Angiotensin	A-9650	1mg	1000	771	10	630	12	1296.69
Neurotensin	N-6383	1mg	1000	597	10	490	12	1672.92
ACTH 1-17	A-2407	1mg	1000	477	10	390	12	2093.09
ACTH	A-0673	1mg	1000	405	15	495	12	2465.20
ACTH 7-38	A-1527	250µg	250	270	30	420	18	3657.93

- Reconstitute standards in MilliQ water to give a concentration of 1mg/ml.
- *Dilute each standard, further, as shown above. The final concentration of each should be 12 pmol/µl, except for ACTH 7-38, which should be 18 pmol/µl.
- Combine all 6 standards 1:1:1:1:1:1 (120µl of each) and mix thoroughly.
- Freeze 10µl aliquots.
- When mixed with 10µl of matrix, the final concentration of each standard is 1 pmol/µl (except ACTH 7-38, which is 1.5 pmol/µl).

Details of calibration standards for Ultraflex (MALDI-Tof) analysis

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