Newcastle University

Gastro-oesophageal reflux, aspiration and anti-reflux surgery in a human lung transplant population

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Author's statement

The author acknowledges the practical assistance of a number of individuals to carry out the work. The author is personally responsible for:

-part of the study design

-ethics application

-R&D approval

-grant applications

-recruitment of patients

-consenting patients

-collecting clinical data on patients

-organising and co-ordinating patients' investigations

-performing manometry and pH-impedance

-interpreting manometry and pH

-being available during these tests for assistance

-50% of the ELISAs

-collecting the gastric juice samples

-50% of the analysis of gastric juice samples

-transporting samples to microbiology

-co-ordinating the analysis of the microbiology samples

-performing the cell stimulation experiments

-performing the viability assays

-developing the MUC5AC plate ELISA

-co-ordinating referrals for fundoplication

-collecting clinical data on the fundoplication cohort

-administrating the quality of life questionnaires

-performing statistical analyses of the data

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Thesis abstract

Introduction

Asymptomatic gastro-oesophageal reflux and aspiration may be associated with allograft dysfunction post lung transplant. Early anti-reflux surgery has been advocated in selected patients and may improve long-term survival. Little has been published on this topic and the current evidence supporting this is flawed. The understanding of the pathophysiology of aspiration in lung transplant recipients is currently limited. This study reports a prospective analysis of reflux/aspiration immediately post-transplantation to date and its subsequent management.

Methods

Lung transplant recipients were recruited over 12 months. At one and six months post-transplantation, patients completed a reflux symptom index (RSI) questionnaire for symptoms of extra-oesophageal reflux and underwent objective assessment for reflux (manometry & pH/impedance). Testing was performed with subjects on maintenance proton pump inhibitor. Bronchoalveolar lavage fluid was assessed for pepsin, bile salts, interleukin-8 and neutrophils. Laparoscopic fundoplication was performed on selected patients. Subsequent laboratory based work was performed to determine the composition of gastric juice and to assess the effects of aspiration on primary bronchial epithelial cells and HT29-MTX goblet cells.

Results

18 patients with a median age of 46 years (range 22-59) were studied. Manometry was abnormal in 8/18 (44%) patients. Seventeen patients completed 24 hour pH-impedance measurements. 12 of 17 (71%) had evidence of GORD on pH-impedance monitoring. 3 of 12 (25%) of patients had exclusively weakly-acid reflux. A statistically significant correlation existed between proximal reflux events and neutrophilia at one month (n=13)(Spearman correlation r=0.52, p=0.03). Pepsin was detected in BALF signifying aspiration. Bile salts were rarely detected using 3 separate assays [sensitivity 0.1μmol/l]. The prevalence of reflux increased over the first six months post-transplant despite a reduction in immunosuppression and normal lung function. Nine patients have subsequently undergone fundoplication for severe

or symptomatic reflux. No major complications occurred. This was associated with improved quality of life and decreased symptoms. Laboratory work gave useful background information on pepsin and bile salts. Mean levels in gastric juice were 380µg/ml (range 0-3892) for pepsin and 50µmol/l (range 0-8000) for bile salts. Microaspiration may lead to primary bronchial epithelial cell damage and death.

Conclusion

Reflux/aspiration is prevalent early post-operatively. Pepsin but rarely bile salts were detected in the lavage fluid suggesting pepsin to be a more common biomarker of aspiration. This study suggests that the causes for reflux are not all related to adverse thoracic changes and immunosuppression as surprisingly, despite a lack of a significant increase in immunosuppression levels, reflux indices increased over the first six months. Laboratory based work provides background information on the use of biomarkers and suggests aspiration could lead to cell death. Fundoplication is safe in selected patients and improved quality of life and GORD symptoms. Further studies are required to assess the effects on lung function and survival.

Presentations

1) ***AGN Robertson,*** *C Ward, JP Pearson, AJ Fisher, JH Dark, PA Corris, SM Griffin.* "The role of oesophageal impedance measurement and markers of aspiration in the detection of extra-oesophageal reflux disease and in the development of allograft dysfunction in human lung transplant recipients." "The OESO Research Grant Competition". World Organization for the Specialized Studies on the Diseases of the Oesophagus 9th World Congress, April 2008, Monaco.

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3) The Ella Forster Memorial Award Presentation 2009:
"The detection and management of aspiration secondary to gastro-oesophageal reflux in the immediate post lung transplantation period." Presented by myself.

4) ***AGN Robertson***, *SM Griffin, C Ward, JP Pearson, AJ Bredenoord, JH Dark, AJ Fisher, J Lordan, PA Corris.* "Aspiration in the immediate post lung transplantation period." Poster presentation. International Society for Heart & Lung Transplantation Annual Scientific Meeting April 2009, Paris.

5) ***AGN Robertson***, *C Ward, JP Pearson, JH Dark, P Corris, J Shenfine, D Karat, SM Griffin.* "The detection and management of aspiration secondary to gastro-oesophageal reflux in the immediate post lung transplantation period." North of England Surgical Society Registrar's Prize Competition. Annual Clinical Meeting, June 2009, Newcastle.

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7) ***AGN Robertson***, *C Ward, JP Pearson, AJ Bredenoord, AJ Fisher, J Lordan, PA Corris, JH Dark, SM Griffin.* Gastro-oesophageal reflux and aspiration in the immediate post lung transplantation period. The Association of Upper Gastro-Intestinal Surgeons of Great Britain Annual Scientific Meeting, September 2009, Nottingham.

8) *S. Parikh, B. Verdon,* ***AGN Robertson****, C. Ward, P. W. Dettmar, J. P. Pearson.* Biomarkers of reflux into the airways. European Students' Conference. October 2009, Berlin.

9) *S. Parikh, B Verdon,* ***AGN Robertson****, C Ward, PW Dettmar, JP Pearson.* "Bile Acid and Pepsin-Reliable and Quantifiable Markers of Reflux?" Poster presentation. GASTRO 2009, Joint Meeting of the United European Gastroenterology Federation/World Gastroenterology Organisation /World Organisation of Digestive Endoscopy (OMED)/British Society of Gastroenterology, London

10) ***AGN Robertson.*** Getting involved in research. Lecture to the University of Edinburgh Surgical Society. Edinburgh, November 2009.

11) ***AGN Robertson***, *C Ward, T Small, J Lordan, AJ Fisher, AJ Bredenoord, PA Corris, J Dark, JP Pearson, SM Griffin.* "Longitudinal Changes in Gastro-Oesophageal Reflux from three months to six months post lung transplantation." Poster presentation. Society of Academic and Research Surgery Annual Conference January 2010, London.

12) ***AGN Robertson.*** Gastro-oesophageal reflux, aspiration and anti-reflux surgery in a human lung transplant population. Invited presentation OESO 10th World Congress. Boston 2010.

13) ***A Robertson***, *A Krishnan, M Griffin, J Pearson, C Ward, P Corris, J Dark, J Shenfine, D Karat.* Initial experience of anti-reflux surgery in lung transplant recipients in a European centre. The Association of Upper Gastro-Intestinal Surgeons of Great Britain Annual Scientific Meeting, Poster Presentation September 2010, Oxford.

14) *A Krishnan,* ***A Robertson,*** *M Griffin, J Pearson, C Ward, P Corris, J Dark, J Shenfine, DK Karat.* The safety and feasibility of anti-reflux surgery in lung transplant recipients: The initial experience in a European centre. Poster presentation. 18th United European Gastroenterology Week, October 2010, Barcelona.

15) ***AGN Robertson,*** *C Ward, A Krishnan, JP Pearson, PA Corris, JH Dark, J Shenfine, DK Karat, SM Griffin.* Gastro-oesophageal reflux, aspiration and laparoscopic fundoplication post-lung transplantation. University of Edinburgh School of Surgery Day. Clinical Presentation. December 2010. Edinburgh.

16) *A Krishnan,* ***AGN Robertson,*** *J Shenfine, DK Karat, S M Griffin.* Laparoscopic fundoplication slows deterioration of lung function post-lung transplant. E-Poster of distinction. The Association of Surgeons of Great Britain & Ireland, May 2011, Bournemouth.

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Publications

Peer-reviewed publications

*1) **AGN Robertson***, LJ Dunn, J Shenfine, D Karat, SM Griffin. Randomized clinical trial of laparoscopic total (Nissen) versus posterior partial (Toupet) fundoplication for gastro-oesophageal reflux disease based on preoperative oesophageal manometry (Br J Surg 2008; 95: 57-63). British Journal of Surgery. 2008; Vol 95 (6):799.

2) ***AGN Robertson**, J Shenfine, C Ward, JP Pearson, JH Dark, PA Corris, SM Griffin.* A call for standardisation of antireflux surgery in the lung transplantation population. Transplantation 2009; Vol 87 (8): 1112-4.

3) ***AGN Robertson**, SM Griffin, DM Murphy, JP Pearson, JH Dark, PA Corris, C Ward.* Targeting allograft injury and inflammation in the management of post-lung transplant Bronchiolitis Obliterans Syndrome (Invited review). American Journal of Transplantation 2009; 9 (6): 1272-8.

4) ***AGN Robertson**, C Ward, JP Pearson, T Small, J Lordan, AJ Fisher, AJ Bredenoord, J Dark, SM Griffin, PA Corris.* Longitudinal changes in gastro-oesophageal reflux from three months to six months post lung transplantation. Thorax 2009; Vol 64 (11): 1005-1007.

5) ***AGN Robertson**, C Ward, JP Pearson, PA Corris, JH Dark, SM Griffin.* Lung transplantation, gastroesophageal reflux, and fundoplication. Annals of Thoracic Surgery 2010; Vol 89 (2): 653-660.

Book Chapters

1) *JP Pearson, S Parikh,* ***AGN Robertson,*** *R Stovold, IA Brownlee.* Chapter 4 Pepsins. in: Effects, Diagnosis and Management of Extra-Esophageal Reflux. Editors: Nikki Johnston and Robert J. Toohill. ©2010 Nova Science Publishers, Inc. ISBN:978-1-61668-177-7.

2) *SM Griffin,* ***AGN Robertson***. Prophylactic antireflux surgery in lung transplantation. In "Difficult Decisions in Thoracic Surgery: An Evidence Based Approach." (Second Edition) Editor M Ferguson. Springer-Verlag 2010 ISBN.

Abstract publications

1) ***AGN Robertson****, SM Griffin, C Ward, JP Pearson, AJ Bredenoord, JH Dark, AJ Fisher, J Lordan, PA Corris.* "Qualitative and quantitative assessments of aspiration in the immediate post lung transplantation period." Thorax, 2008; 63 (Suppl VII): A10-11.

2) ***AGN Robertson****, SM Griffin, C Ward, JP Pearson, AJ Bredenoord, JH Dark, AJ Fisher, J Lordan, PA Corris.* "Aspiration in the immediate post lung transplantation period." Journal of Heart and Lung Transplantation 2009; 28 (2) (Supp 1): A S106.

3) ***AGN Robertson****, C Ward, JP Pearson, AJ Bredenoord, AJ Fisher, J Lordan, PA Corris, JH Dark, SM Griffin.* "Aspiration secondary to gastro-oesophageal reflux but not duodenal reflux occurs in the immediate post lung transplantation period." Transplant International 2009; 22 (Supp 2): 218.

4) ***AGN Robertson****, C Ward, JP Pearson, AJ Bredenoord, AJ Fisher, J Lordan, PA Corris, JH Dark, SM Griffin.* Gastro-oesophageal reflux and aspiration in the immediate post lung transplantation period. British Journal of Surgery 2009; 96 (Supp 6): 30.

5) *S. Parikh, B Verdon,* ***AGN Robertson****, C Ward, PW Dettmar, JP Pearson.* Bile acid and pepsin-reliable and quantifiable markers of reflux? Gut 2009; 58 (Supp II): A431.

6) ***A Robertson****, A Krishnan, M Griffin, J Pearson, C Ward, P Corris, J Dark, J Shenfine, D Karat.* Initial experience of anti-reflux surgery in lung transplant recipients in a European centre. British Journal of Surgery 2010 95 (Supp 5): 24.

7) *A Krishnan,* ***A Robertson,*** *M Griffin, J Pearson, C Ward, P Corris, J Dark, J Shenfine, DK Karat.* The safety and feasibility of anti-reflux surgery in lung transplant recipients: The initial experience in a European centre. Gut 2010, 59 (Suppl III) A340.

8) ***AGN Robertson***, *C Ward, T Small, J Lordan, AJ Fisher, AJ Bredenoord, PA Corris, J Dark, JP Pearson, SM Griffin.* Longitudinal changes in gastro-oesophageal Reflux from three months to six months post lung transplantation. British Journal of Surgery 2010 95 (Supp 6): 46.

9) *A Krishnan,* ***AGN Robertson,*** *J Shenfine, DK Karat, S M Griffin.* Laparoscopic fundoplication slows deterioration of lung function post-lung transplant. British Journal of Surgery 2011 (in Press).

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Awards

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Abbreviations

A Grade	Pathological grade of acute rejection
ABO	Blood groups A, B and O.
ABTS	2,2`-Azino-bis(3-ethylbenzothiazoline-6-)sulfonic acid
B Grade	Pathological grade of inflammation
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCT	Bolus clearance time
BMI	Body mass index
BOS	Bronchiolitis obliterans syndrome
BSA	Bovine serum albumin
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ERS	European Respiratory Society
ESLD	End stage lung disease
FEF_{25-75}	Expiratory flow rate from 25-75% exhalation
FEV_1	Forced expiratory volume in 1 second
FiO_2	Fraction of inspired oxygen
FVC	Forced vital capacity
GIQLI	Gastro-intestinal quality of life index
GORD	Gastro-oesophageal reflux disease
HLA	Human leukocyte antigen
HLT	Heart lung transplant.
IL	Interleukin
ISHLT	International Society for Heart and Lung Transplant
ITU	Intensive therapy unit
LPR	Laryngo-pharyngeal reflux
LSLT	Left single lung transplant
M	Molar
mg/ml	Milligrams/millilitre

µg/ml	Micrograms per millilitre
MHC	Major histocompatibility complex
MII	Multichannel intraluminal impedance
Ml	Millilitres
mm	Millimetres
MMF	Mycophenolate mofetil
mmHg	Millilitres of mercury
µmol/l	Micro moles per litre
MUC	Mucin
NAD/NADH	Nicotinamide adenine dinucleotide
NBT	Nitrotetrazolium blue
ng/l	Nano grams per litre
nm	Nanometres
^{o}C	Degrees centigrade
paO_2	Arterial pressure of oxygen
PBS	Phosphate buffer solution
PEG	Percutaneous endoscopic gastrostomy
PFT	Pulmonary function test
pg/ml	Picograms/millilitre
PPI	Proton pump inhibitor
r.p.m	Revolutions per minute
RSI	Reflux symptom index
RSLT	Right single lung transplant
SAP	Symptom association probability
SI	Symptom index
SLT	Single lung transplant
SSI	Symptom sensitivity index
SSLT	Single sequential lung transplant
T-cell	Thymus derived lymphocyte
3-α HSD	3-α hydroxysteroid dehydrogenase

1. Introduction

1.1. Gastro-oesophageal reflux disease

Gastro-oesophageal reflux disease (GORD) is defined as any symptomatic condition or histopathological alteration secondary to retrograde movement of gastric contents into the oesophagus. It is common in the general population. Eleven percent of Americans experience symptoms of daily reflux, and 33 % experience these over a 72 hour period (Wise and Murray 2007). In lung transplant recipients, gastro-oesophageal reflux disease and chronic aspiration have been linked to the development of BOS and this process may be prevented by fundoplication (Davis, Lau et al. 2003). More severe GORD has been suggested to be associated with decreased FEV_1 and increased frequency and severity of BOS. This is a fairly recent concept and was first suggested in 1990 (Reid, McKenzie et al. 1990).

There is a suggested high prevalence of reflux disease in patients with asthma, cystic fibrosis, pulmonary fibrosis, COPD, BOS-associated pneumonia and diffuse bronchiolitis in the non-lung transplant population, (Davis, Lau et al. 2003; Young, Hadjiliadis et al. 2003; Cantu, Appel et al. 2004; Casanova, Baudet et al. 2004; D'Ovidio, Mura et al. 2005; Ward, Forrest et al. 2005; D'Ovidio and Keshavjee 2006; Sweet, Patti et al. 2007; Blondeau, Dupont et al. 2008; Gasper, Sweet et al. 2008). Gastro-oesophageal reflux may contribute to pulmonary pathophysiology, e.g. in asthma; cystic fibrosis and pulmonary fibrosis (Cantu, Appel et al. 2004; Havemann, Henderson et al. 2007). The Japanese have proposed an entity- diffuse aspiration bronchiolitis. This has been described in elderly patients with dementia who suffer from chronic aspiration (Teramoto, Matsuse et al. 1999). There is an even higher prevalence of gastro-oesophageal reflux disease post-transplantation (D'Ovidio and Keshavjee 2006).

Clinical studies have also suggested a link between GORD and BOS (Davis, Lau et al. 2003). Anti-reflux surgery, especially early fundoplication, may have a role in preventing BOS and prolonging survival (Appel and Davis 2004).

1.1.1. Pre-operative reflux in patients with chronic advanced lung disease

Extra-oesophageal reflux is increased in chronic advanced lung disease. There is a high prevalence of foregut motility problems and GORD in patients with end-stage lung disease (ESLD) including interstitial lung disease, pulmonary fibrosis and cystic fibrosis (D'Ovidio, Singer et al. 2005).

Seventy two percent of pre-transplant patients have decreased lower oesophageal sphincter pressure (D'Ovidio, Singer et al. 2005). Thirty three to forty seven percent of patients have oesophageal body dysmotility and impaired peristalsis (D'Ovidio, Singer et al. 2005; Sweet, Herbella et al. 2006). In total, almost 80% of these patients have oesophageal dysmotility and or a hypotensive lower oesophageal sphincter (D'Ovidio, Singer et al. 2005). Sweet et al, in a study of end stage lung disease patients, suggests that 55% of patients with reflux had a hypotensive lower oesophageal sphincter compared with 26% of patients without reflux (Sweet, Herbella et al. 2006). Impaired oesophageal peristalsis was associated with reflux and respiratory symptoms (Sweet, Herbella et al. 2006).The amplitude of peristalsis in the distal oesophagus is lower in GORD positive patients (Sweet, Herbella et al. 2006).

Forty four percent of these patients had prolonged gastric emptying for solids and 24% for liquids (D'Ovidio, Singer et al. 2005). The combination of a defective lower oesophageal sphincter and delayed gastric empting leads to an increase of abnormal reflux findings (D'Ovidio, Singer et al. 2005; Sweet, Herbella et al. 2006). In a study by Sweet et al., 17 patients underwent gastric emptying studies due to symptoms of delayed gastric emptying: post-prandial bloating, fullness, nausea and vomiting. Most of these patients (16 of 17) had abnormal distal oesophageal acid exposure. Four of these 16 had delayed liquid emptying and ten had delayed solid emptying (Sweet, Herbella et al. 2006). However, as only symptomatic patients were tested, the conclusions that can be drawn from this data are limited.

There is a high prevalence (63-68%) of GORD in patients with end stage pulmonary disease awaiting lung transplant (Cantu, Appel et al. 2004; Sweet, Herbella et al. 2006). D`Ovidio et al (2005) report a lower prevalence- 38%. In that study, PPIs were only stopped for 5 days prior to the assessment. PPIs can affect acid secretion for upto 10 days. Therefore, the prevalence of GORD may be underestimated (Sweet, Herbella et al. 2006). Twenty to thirty seven percent of patients have documented proximal reflux on ambulatory pH monitoring. Proximal oesophageal reflux is more dangerous, as it predisposes to microaspiration. There is conflicting data whether proximal reflux events in ESLD patients occur mainly in the upright (Sweet, Herbella et al. 2006) or in the supine position (D'Ovidio, Singer et al. 2005). Interestingly, although rare,

patients with normal distal reflux but abnormal proximal reflux were encountered (Sweet, Herbella et al. 2006).

It is unclear whether GORD causes pulmonary pathology or whether disordered pulmonary function leads to GORD. There is evidence to suggest that in patients with COPD, episodes of reflux may be associated with a drop in arterial oxygen saturation (Casanova, Baudet et al. 2004).

The presence of reflux in ESLD may be related to an increased negative intrathoracic pressure and increase positive abdominal pressure. Lung hyper-expansion may interfere with the oesophageal hiatus in the crura and also the lower oesophageal sphincter pressure (Linden, Gilbert et al. 2006).

1.2. Lung transplantation

Lung transplantation has been performed since 1963 (Cantu, Appel et al. 2004). It is now a life saving treatment for end-stage lung disease (Hosenpud, Bennett et al. 1998; Davis, Lau et al. 2003; Cantu, Appel et al. 2004; D'Ovidio and Keshavjee 2006). Transplants performed in Newcastle have a one year survival of 82-84% and a 5 year survival of 60%. Forty percent of lung transplant recipients at the Freeman Hospital Cardiothoracic Transplant Unit now survive for 10 years. The survival rate continues to improve (Rutherford, Fisher et al. 2005) and is comparable with the International Society for Heart and Lung Transplantation registry (ISHLT 2010). The main indications for lung transplantation are chronic obstructive pulmonary disease (COPD) (45%), pulmonary fibrosis (16%) and cystic fibrosis (14%). Primary pulmonary hypertension has decreased as an indication for transplant due to improvements in its medical management (Appel and Davis 2004). In the early post-operative period, mortality is commonly due to infection and primary graft failure. Over a longer time period- several years, bronchiolitis obliterans syndrome is a major cause of morbidity and mortality (Appel and Davis 2004).

Lung transplant survival is reduced when compared to heart, liver and kidney transplants (Appel and Davis 2004). This may occur as the lungs are exposed to the external environment. The process of transplantation involves denervating the donor lung, reducing the cough reflex and muco-ciliary clearance (Veale, Glasper et al. 1993). This attenuates the protective mechanisms of the lung against infection and aspiration.

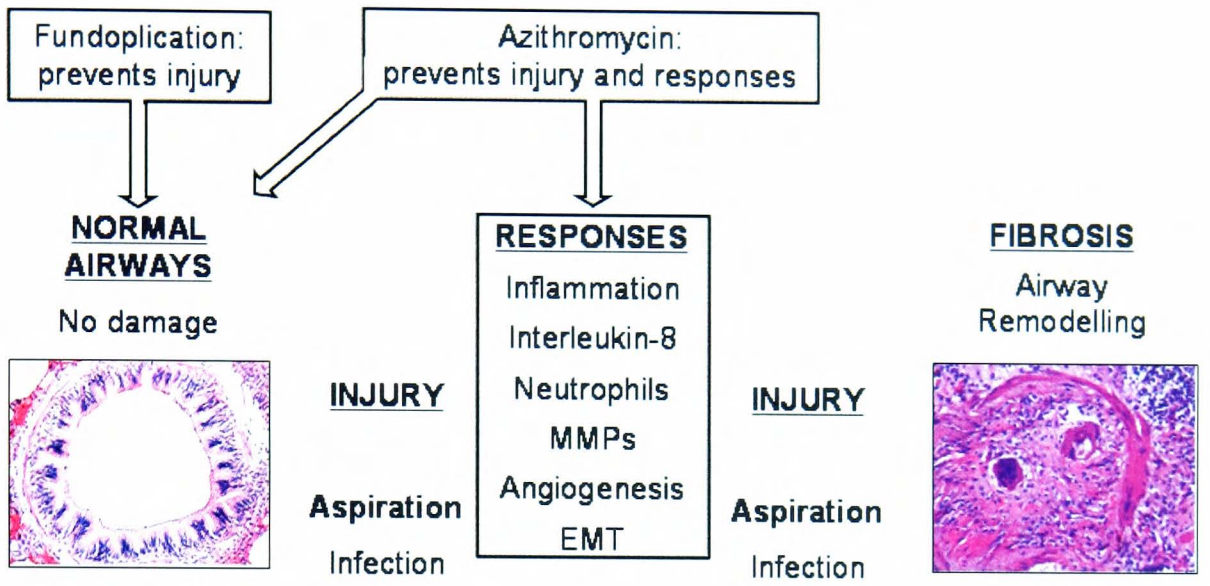
1.3. Long-term complications

1.3.1. Bronchiolitis obliterans syndrome

Death post-lung transplant is commonly due to chronic allograft dysfunction otherwise known as obliterative bronchiolitis (Davis, Lau et al. 2003; Cantu, Appel et al. 2004). Obliterative bronchiolitis is likely to be the pathological process of chronic rejection and is diagnosed on open lung biopsy. Although open biopsy is the gold standard, it is very invasive. Usually transbronchial biopsies are performed but due to sampling problems these are not reliable in diagnosing obliterative bronchiolitis.

In 1993 Bronchiolitis obliterans syndrome (BOS) was defined as the clinical equivalent of obliterative bronchiolitis (Hadjiliadis, Duane Davis et al. 2003). The BOS score, based on lung function, is of great value. BOS is a significant process which leads to decreased quality of life and increased mortality. BOS normally develops between 6 months & 2 years post transplantation (Palmer, Miralles et al. 2000). It affects 50-60% of patients at 5 years post-transplantation. BOS accounts for 30% of deaths after the 3rd post-operative year and its associated survival is only 30-40%, 5 years after its onset. The 5 year post-transplantation survival is 20-40% lower than average in patients with BOS (D'Ovidio, Mura et al. 2005). It is a leading cause of late graft failure (Zheng, Walters et al. 2000). The pathology behind this process involves progressive fibrosis of the small airways leading to complete obstruction with sclerosis of the airways, intimal thickening and destruction of the pulmonary vasculature.

Figure 1-1: Model of non-alloimmune lung allograft injury and inflammation in BOS pathogenesis from Robertson et al Am J Trans 2009



(Robertson, Griffin et al. 2009) (Artwork by IA Brownlee)

Pathologically there are two different types- an acellular type with a concentric fibrosing picture limited to the terminal bronchioles and a focal cellular process which is associated with aspirated food content and foreign body-type giant cells in the alveolar spaces (Abernathy, Hruban et al. 1991; D'Ovidio, Mura et al. 2005; D'Ovidio and Keshavjee 2006). Clinically this is accompanied by a decreased FEV_1 and progressive dyspnoea. The accepted ISHLT definition of BOS is a decrease FEV_1 from the best post-operative function, in the absence of anastomotic stricture, infection, bronchitis or other complications (Estenne, Maurer et al. 2002). BOS scores are shown in Table 1-1 (Estenne and Hertz 2002). The revised score from 2002 includes a new grade of BOS: BOS 0p. This is beneficial as it allows the identification and early treatment of deteriorating lung function.

BOS is thought to be mediated by both alloimmune and non-alloimmune factors. Risk factors for BOS include number, time to and severity of acute rejection (a process characterised by T-lymphocyte infiltration of the allograft), HLA mismatch, cytomegalovirus, and other viral infections, age of patient, ischaemic time, and single lung transplant. More putative associations include GORD with aspiration (Appel and Davis 2004; Cantu, Appel et al. 2004; Ward, Forrest et al. 2005; D'Ovidio and

Keshavjee 2006). It has recently been suggested that BOS is a heterogeneous condition with neutrophilic reversible allograft dysfunction representing a patient subset, which may have important therapeutic implications. An implication of this model, is that the definition of BOS, which currently is described as irreversible may need to be revised (Vanaudenaerde, Meyts et al. 2008).

Table 1-1 Bronchiolitis obliterans syndrome (BOS) scores

1993 classification		**2002 classification**	
BOS 0	FEV_1: 80% or more of baseline	FEV_1: >90% of baseline and FEF_{25-75} >75% of baseline	BOS 0
		FEV_1: 81-90% of baseline and FEF_{25-75} ≤75% of baseline	BOS 0p
BOS 1	FEV_1: 66-80% of baseline	FEV_1: 66-80% of baseline	BOS 1
BOS 2	FEV_1: 51-65% of baseline	FEV_1: 51-65% of baseline	BOS 2
BOS 3	FEV_1: ≤50% or more of baseline	FEV_1: ≤50% or more of baseline	BOS 3

(Estenne and Hertz 2002; Estenne, Maurer et al. 2002)

1.3.2. Reflux post-lung transplant

GORD with potential aspiration, as determined by an abnormal pH study, is prevalent after lung transplantation (70-75%) (Davis, Lau et al. 2003; Hadjiliadis, Duane Davis et al. 2003). Post-transplantation remodelling of the chest and oesophagus may help to recreate the lower oesophageal sphincter and reduce reflux (D'Ovidio, Singer et al. 2005). This benefit may be off set, by suspected predisposing factors such as immunosuppressive medication, vagal nerve damage (leading to delayed gastric emptying) and the high pre-operative prevalence of reflux disease (Hadjiliadis, Duane Davis et al. 2003; D'Ovidio and Keshavjee 2006). pH monitoring is becoming routine practice in several transplant units due to the high incidence of post transplant reflux and the potential improvement in lung function post fundoplication (Davis, Lau et al. 2003). Few studies exist which assess longitudinal data on GORD in lung transplant recipients.

One study demonstrated an increase in the prevalence of reflux from 35% pre-transplantation to 65% post-transplantation (Young, Hadjiliadis et al. 2003). An abnormal test was defined as an acid contact time of greater than 3%. Acid contact time increased by a mean of 3.7% post-lung transplant. The greatest increase in acid contact time was in the supine position where the increase was 6.3%. Most of these

patients were asymptomatic. The changes in acid contact time were not always explained by changes in oesophageal manometry or gastric emptying studies. This suggested a multifactorial aetiology to this condition (Young, Hadjiliadis et al. 2003). A strong correlation exists between distal and proximal pH monitoring (Davis, Lau et al. 2003). Many patients with evidence of reflux on distal pH monitoring will have proximal reflux.

Another study of 43 patients showed that mean oesophageal acid exposure time was 10%. Thirty of 43 patients had abnormal tests in total. Twenty four of 43 had abnormal tests in the upright position and 29 of 43 were abnormal when supine. This cohort of patients with GORD consisted of asymptomatic and symptomatic patients (Hadjiliadis, Duane Davis et al. 2003). A further study suggests that of lung transplant recipients with GORD, a third will have ineffective oesophageal motility (Davis, Shankaran et al. 2010).

GORD is associated with worse pulmonary function tests in the post-transplant population (Hadjiliadis, Duane Davis et al. 2003). Over half of patients in this study had allograft dysfunction based on FEV_1 measurements- 9 had BOS1, 7 had BOS2 and 10 had BOS 3. Seventy six percent of patients with allograft dysfunction had an abnormal oesophageal pH study compared to 59% of patients without allograft dysfunction. A negative correlation existed between the severity of total/upright acid reflux and FEV_1 (Hadjiliadis, Duane Davis et al. 2003).

Another study by D'Ovidio evaluated reflux post-lung transplantation, using a 2 probe pH monitor. Abnormal distal or proximal pH was present in 32% of patients at 3 months and 53% at 12 months (D'Ovidio, Mura et al. 2006). This suggests a worsening of reflux over the first year post-transplant. The frequency and severity of reflux, especially the upright acid exposure time, is associated with chronic allograft dysfunction. Proximal oesophageal reflux is also associated with decreased lung function (Hadjiliadis, Duane Davis et al. 2003).

A further study has suggested that 48% of lung transplant patients have reflux after the first year post-transplant. Almost a third of these patients had exclusively weakly acid reflux (pH>4) (Blondeau, V. Mertens et al. 2008). A further study using pH

impedance supports this prevalence of weakly acid reflux (27%) (King, Iyer et al. 2009). There is evidence suggesting a link between non-acid reflux on pH/impedance testing and aspiration (Blondeau, Mertens et al. 2009). The study from Harefield Hospital suggested that total reflux detected by impedance is a risk factor for BOS whereas oesophageal acid exposure was not (King, Iyer et al. 2009). The presence of reflux, rather than the pH of reflux, may be the important issue. Thus PPI therapy is excluded as an anti-reflux therapeutic option in lung transplant recipients. An important implication of this study is that impedance may be important in GORD assessment post-lung transplantation.

A small study from Australia suggests that many transplant patients experience reflux and obstructive sleep apnoea overnight. This may be important as studies in a lung transplant population showed the upper oesophageal sphincter has no intrinsic tone during sleep and there is a predisposition to reflux (Shepherd, Chambers et al. 2008).

Post transplant pH studies have had to be performed after discontinuing anti-acid therapy (Hadjiliadis, Duane Davis et al. 2003). Adopting a pH based approach to reflux, potentially ignores weakly acidic or non-acid reflux. This may be physiologically important especially if this leads to aspiration in this vulnerable population (Stovold, Forrest et al. 2007). It would be of interest to assess patients whilst on PPI therapy and to evaluate non-acid or weakly acidic reflux. Combined Impedance/pH studies provide the opportunity to do this (Hirano 2006).

1.4. Causes of post-operative reflux

1.4.1. Pre-operative reflux

There is a high prevalence of GORD (63-68%) in patients with end stage lung disease (Cantu, Appel et al. 2004; Sweet, Herbella et al. 2006).

1.4.2. Vagal nerve damage

The recipient pneumonectomy requires meticuluous haemostasis. The vagal nerves are at risk from direct trauma and electrocautery. Injuries often occur near the lung hila. Both nerves lie posterior-inferiorly in the mediastinum to the lung root. The right vagus nerve is in apposition to the trachea. The left lies in the interval between the

common carotid and the subclavian artery. At the lung roots nerve branches are given off to the pulmonary plexus (Au, Hawkins et al. 1993).

It is important to preserve the vagus, phrenic and recurrent laryngeal nerves. However this may prove difficult and maintaining haemostasis has a higher priority.

Biomechanical vagal damage leads to delayed gastric emptying and dysmotility of the distal third of the oesophagus, promoting reflux post-transplantation (Au, Hawkins et al. 1993).

The physiological consequences of vagotomy on the oesophagus have been studied in animals. The vagus innervates the striated muscle of the oesophagus. The effects are dependent on the proportion of striated muscle present. Dysphagia may result from a complete vagotomy. (Au, Hawkins et al. 1993).

Complete vagotomy results in complete atonia. Partial vagotomy enhances liquid gastric emptying and delays solid gastric emptying as it disrupts receptive relaxation of the stomach and leads to increased intragastric pressure. The increased liquid emptying is mainly dependent on an antro-duodenal pressure gradient. By disturbing antral motility solid emptying is slowed (Au, Hawkins et al. 1993).

The lower oesophageal sphincter is under neural (vagal) and hormonal control. Vagotomy in dogs and cats affects the lower oesophageal sphincter resting tone and may induce spasm. In humans delayed gastric emptying predisposes to GORD. Evidence of oesophageal dysmotility and delayed gastric emptying is a manifestation of a complete vagotomy.

Modification of surgical technique can decrease the risk of vagal injury and thus reduce morbidity. This involves circumspect or bipolar diathermy and stapling. Risk to the vagal nerve is minimized by performing an alternative operation- bilateral sequential lung transplantation. Thus dissection of the distal trachea, subcarinal and posterior mediastinum can be avoided and the vagus can be preserved (Au, Hawkins et al. 1993).

1.4.3. Post-operative gastroparesis

Up to 90% of patients have delayed gastric emptying post-lung transplant (D'Ovidio and Keshavjee 2006; D'Ovidio, Mura et al. 2006).

A study by Au et al. involved patients post heart-lung transplantation. A radioisotope of technetium was used to perform gastric emptying studies for liquids and solids to

evaluate foregut dysmotility. Symptoms of dysmotility- flatulence, nausea and reflux were common. Evidence of foregut dysmotility and vagal damage (delayed gastric emptying) were also common post transplantation. Thirty percent (3/10) of patients had grossly delayed liquid/solid emptying compatible with complete vagotomy. Six patients had delayed liquid emptying but normal solid emptying. This finding is unusual and the opposite of what is expected post vagotomy. The physiological mechanisms behind this are unknown (Au, Hawkins et al. 1993). When compared to a heart-lung transplant, a single lung transplant or a single sequential lung transplant requires less extensive dissection and thus less risk of vagal nerve damage.

Several other studies have shown delayed gastric emptying to be prevalent post lung transplant (23-91%) (Hadjiliadis, Duane Davis et al. 2003; Young, Hadjiliadis et al. 2003; D'Ovidio, Mura et al. 2006). Gastroparesis did not necessarily predispose patients to reflux as determined by pH monitoring (Young, Hadjiliadis et al. 2003). The above evidence suggests gastric dysmotility is common post-lung transplantation.

1.4.4. Transplant medication

Gastrointestinal complications are common post-transplantation, often due to immunosuppressant therapy (Lubetkin, Lipson et al. 1996; Nunes, Lucey et al. 1999; Gautam 2006). After renal transplant, 20% of patients develop gastrointestinal complications (Ponticelli, Passerini et al. 2005) and 8% of renal patients have been reported to have upper gastrointestinal complications (Logan, Morris-Stiff et al. 2002). These may be related to side effects of medication or infection. Nausea, vomiting and dyspepsia (83%) are common (Ponticelli, Passerini et al. 2005; Ekberg, Kyllonen et al. 2007). Forty seven percent of renal transplant patients report reflux symptoms (Ekberg, Kyllonen et al. 2007). These may be related to gastroparesis from the gastrotoxicity of calcineurin inhibitors, steroids and mycophenolate mofitil (Austin, Gougoutas et al. 2000; Ponticelli, Passerini et al. 2005). Viral gastric infection may also affect gastric motility (Austin, Gougoutas et al. 2000). Gastroparesis has been documented post-transplantation in lung, renal and bone marrow transplant patients (Au, Hawkins et al. 1993; Eagle, Gian et al. 2001; Logan, Morris-Stiff et al. 2002).

1.4.5. Post-pneumonectomy reflux

Pneumonectomy with and without transplantation, has been associated with oesophageal dysfunction (Suen, Hendrix et al. 1999; Mitchell, Hazelrigg et al. 2006). Reflux has been associated with pneumonectomy in the non-transplant situation (Kopec, Irwin et al. 1998). This may be due to anatomical changes, local trauma, traction on the oesophagus, vagal injury or diaphragmatic complications (Kopec, Irwin et al. 1998; Suen, Hendrix et al. 1999; Berry, Friedberg et al. 2006; Mitchell, Hazelrigg et al. 2006).

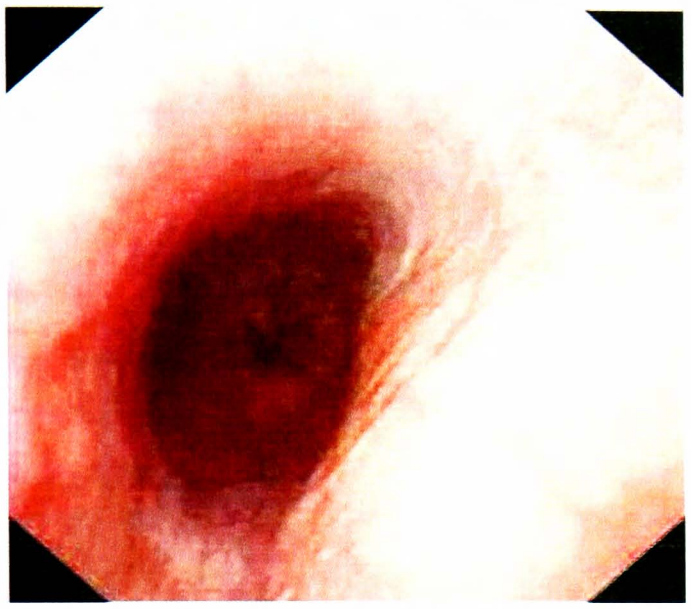
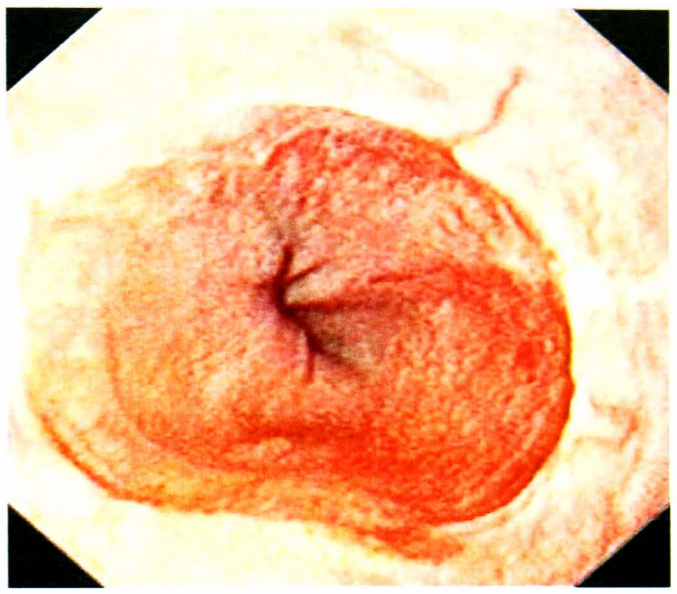
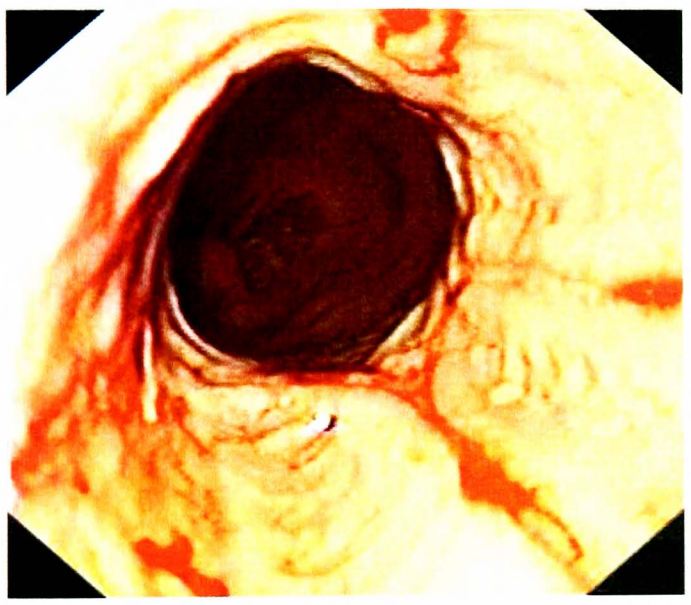
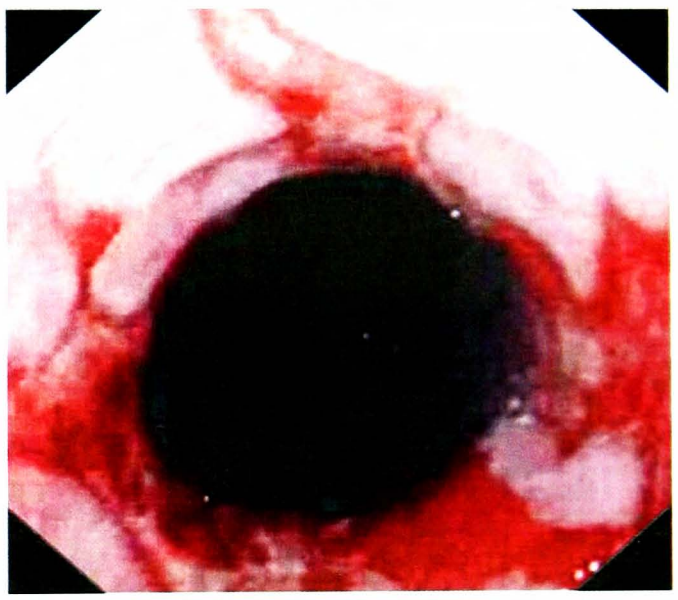
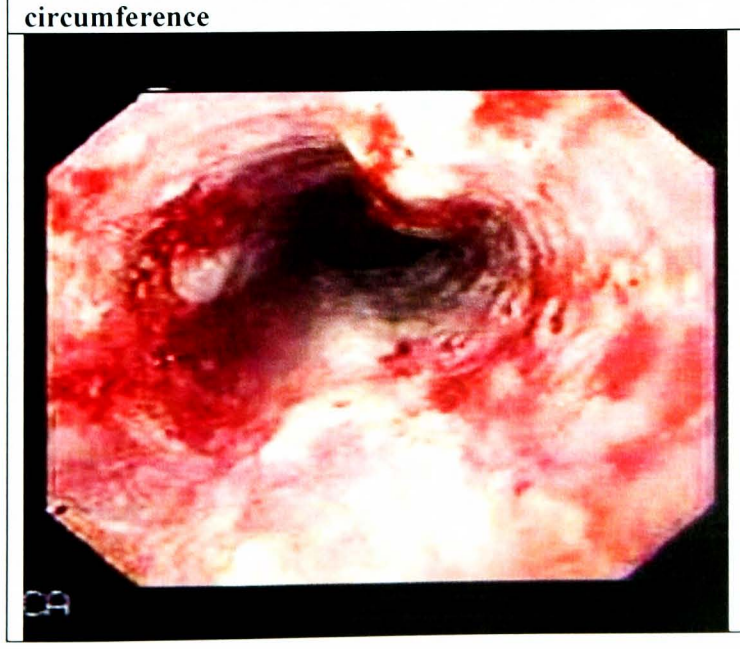
1.5. Detection of reflux

Many methods have been developed to detect and quantify GORD. Most have been aimed at all patients but only some have been specific to lung transplant recipients.

1.5.1. Endoscopy

Flexible endoscopy is often performed early in the management of reflux symptoms. It is performed to exclude malignancy, achalasia and strictures and can diagnose oesophagitis. Endoscopy allows histological samples to be taken. A third of patients with a normal oesophagus on endoscopy will have pathological reflux (Lundell, Dent et al. 1999).

Figure 1-2 Endoscopic views of oesophagus and Los Angeles grades of oesophagitis (Lundell, Dent et al. 1999)

Grade 0 Normal oesophagus	Grade 1 Mucosal break ≤ 5 mm in length
	
Grade 2 Mucosal break > 5mm	**Grade 3 Mucosal break continuous between > 2 mucosal folds**
	
Grade 4 Mucosal break ≥75% of oesophageal circumference	
	

1.5.2. Ambulatory pH testing

Ambulatory pH monitoring has been used for many years to evaluate GORD. It has previously been called the "gold-standard" (Hirano 2006). pH monitoring- the measurement of H^+ ions- is very useful for assessing acid reflux. (Wise and Murray 2007). Measurements are based on the time that the pH of the oesophagus is less than 4. This is detected by applying a probe 5cm above the lower oesophageal sphincter (Hirano 2006). However, there are several disadvantages. Its main shortcoming is its inability to detect or acknowledge weakly acid and non-acid reflux. It is also unable to measure the proximal extent of reflux. Dual channel pH monitors have been designed to measure proximal and distal reflux.

1.5.3. Bravo capsule

To remove the technical difficulties of nasal catheterisation, the Bravo Capsule (Medtronic, Minneapolis, MN, USA) has been developed. This is a wireless pH probe which is attached to the lower oesophageal mucosa during endoscopy or by using a dedicated catheter. Its advantages are its tolerability and the fact it allows recording for over 24 hours (Hirano 2006).

1.5.4. Bilitec

The Bilitec 2000 (Medtronic, Minneapolis, MN, USA) device only measures bile reflux (Hirano 2006). A specific diet has to be used. Refluxate can get stuck in the sensor opening overestimating bile exposure. There can be difficulties with dietary compliance. The detection of bile refluxate is important. It may be better achieved by the biomarker approach assessing levels in the bronchoalveolar lavage fluid.

1.5.5. Multichannel intraluminal impedance

Standard pH monitoring may underestimate the degree of reflux. Therefore oesophageal impedance was developed and has a growing role in the detection of reflux (Wise and Murray 2007). Convergences of improvements in catheter technology and computer software in the last decade have increased the availability of multichannel intraluminal impedance (MII), an exciting technology that is very sensitive in the detection of reflux. The direction and the proximal extent of liquid and gas reflux events can be accurately measured by MII (Wise and Murray 2007). It is becoming the gold standard for assessment of reflux (Bredenoord 2008).

Theory, validation, intra-observer variability & reproducibility

Impedance is inversely proportional to electrical conductivity and cross sectional area of the lumen. It is studied using a catheter with multiple spaced, pairs of cylindrical metal rings connected in circuits to the lumen of the tubular organ. Each paired ring circuit has a voltmeter outside the body. As boluses pass, there are changes in impedance recordings. Gases cause a sharp rise in impedance, with rapidly decreasing conductivity. Fluids (food, water and gastric contents) decrease impedance by connecting circuits between electrodes (Wise and Murray 2007).

Before a fluid or food bolus passes, the oesophagus is empty and the impedance is intermediate reflecting the intermediate conductivity of the oesophageal mucosa. Whilst a fluid bolus passes, impedance is low. After it has passed, impedance is again intermediate (Figure 1-3). These changes in impedance occur when the bolus is between a pair of electrodes. Liquid reflux will drop impedance by 50% in 2 consecutive sensors. Gas reflux is defined as a retrograde, simultaneous rise in impedance to >3,000 ohms (Wise and Murray 2007). Initially impedance was measured in the lumen of the gastrointestinal tract and has been validated by barium radiographs in anaesthetised cats (Sifrim, Silny et al. 1999).

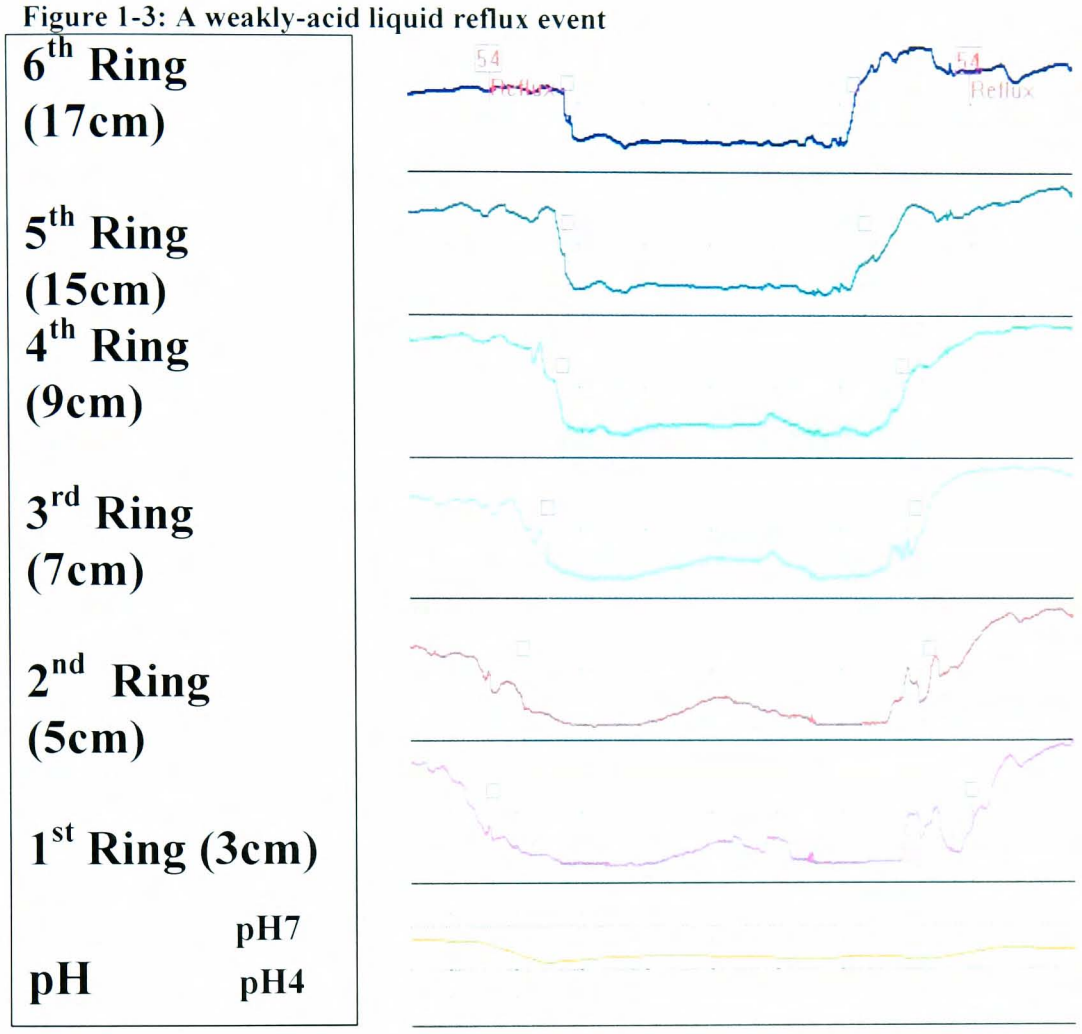
Multichannel intraluminal impedance collects data samples at high frequency rates, 50 Hz. This enables it to determine the direction of the bolus. Swallowed liquids can be distinguished from reflux events and swallowed air can be distinguished from "belched" air (Wise and Murray 2007).

There is some intra- and inter-individual variability with impedance measurements. Bredenoord et al evaluated 20 healthy volunteers, 2 weeks apart. They found that there was more variability between different subjects by >50%, than within the same subjects measured at different times (Bredenoord, Weusten et al. 2005; Wise and Murray 2007).

Refluxate can be acid, weakly acid or weakly alkaline and can be composed of liquid, gas or a mixture of the two. Patients with pathological GORD, have more acid events and fewer non-acid and weakly acid reflux events when compared to normal subjects. Pure gas reflux is a non-acidic event (Wise and Murray 2007). Gas reflux often occurs

whilst in the left lateral decubitus position, and liquid reflux tends to occur in the right lateral decubitus position (Wise and Murray 2007).

Figure 1-3: A weakly-acid liquid reflux event



Legend: This picture shows a combined pH/impedance trace. The bottom reading is of pH, measured by the pH sensor located 5cm above the lower oesophageal sphincter. As the reading does not drop below pH 4, it shows that this is a weakly-acidic event. The traces above the bottom reading represent the impedance values from 3,5,7,9,15 and 17cm (1st ring to 6th ring) above the lower oesophageal sphincter. The traces measure electrical impedance within the oesophagus. The sequential drop in impedance from the 1st to the 6th ring, demonstrates a reflux event reaching the proximal oesophagus.

"Some" reflux is physiological, with an oesophageal acid exposure of <4.5% being considered normal (DeMeester, Wang CI et al. 1980). In a "normal" population (72 healthy volunteers with a mean age of 35 years, with no known gastrointestinal disease or history of thoracic or abdominal surgery), a study showed that on average there will be 40 reflux events per 24 hours (Zerbib, des Varannes et al. 2005). After a standardised liquid meal, most events were mixed gas and liquid reflux events (Wise and Murray 2007). Two thirds of reflux events are non-acidic or weakly-acidic events (Wise and Murray 2007).

Impedance allows detailed evaluation of refluxate and evaluation in patients on PPI therapy (Wise and Murray 2007). Proton pump inhibitors have been shown not to decrease reflux events but render the events non-acid or weakly acid. Thus, PPIs do not prevent reflux (Wise and Murray 2007). There is evidence to suggest that PPIs may not reduce the volume of gastric secretions (Verdu, Viani et al. 1994). A study of pH monitoring of 250 patients on PPI therapy showed 3.8% to have an abnormal study. Impedance showed that weakly acid events were just as common after proton pump inhibitor therapy as acid events prior to acid suppression. The acid levels detected were greatly reduced but impedance showed that reflux events were just as common (Wise and Murray 2007). At least a third of reflux events are weakly alkaline or weakly acidic. These episodes may elicit symptoms (Sifrim 2005; Sifrim, Dupont et al. 2005). The association between atypical extra-oesophageal symptoms with reflux is controversial. A study, using pH-impedance, was performed on 10 subjects with symptomatic reflux. Half of the patients have a temporary association with reflux and cough. A causative link has yet to be proven (Wise and Murray 2007).

Standard definitions have been created for acid reflux, superimposed acid reflux, weakly acid reflux (Figure 1-3) and weakly alkaline reflux on the basis of combined pH/impedance measurements (Table 1-2). Oesophageal and extra-oesophageal symptoms can be related to less acid reflux (Shay, Tutuian et al. 2004; Sifrim 2004; Sifrim, Castell et al. 2004; Zerbib, des Varannes et al. 2005). The distinction between "acidic", "weakly-acidic" and "non-acid" is artificially created on the basis of pH and is of limited importance. All refluxate if aspirated will be damaging to the lungs regardless of pH.

Table 1-2: Standard definitions for reflux events

Acid reflux	Refluxate of gastric juice which reduces the pH<4
Superimposed acid reflux	Further refluxate of gastric juice before the pH has recovered to >4.
Weakly acid reflux	Refluxate of gastric juice when the pH remains between 4-7.
Weakly alkaline reflux	Refluxate of gastric juice when the nadir pH is greater than 7

(Shay, Tutuian et al. 2004; Sifrim 2004; Sifrim, Castell et al. 2004; Zerbib, des Varannes et al. 2005)

Weakly acid reflux events often occur near meal times. If there is prolonged gastric emptying, patients experience an increase in weakly acid reflux and a decreased acid reflux (Sifrim, Castell et al. 2004). Weakly acid refluxate causes less heartburn when compared to acid reflux, but patients may suffer regurgitation or chronic cough. (Sifrim, Castell et al. 2004).

1.5.6. Comparison of pH monitoring to impedance

Acid reflux events, detected by impedance appear to be shorter, as neutralisation of acid takes longer than the clearance of oesophageal volume. There is a higher detection rate of reflux events with impedance compared to pH monitoring. In one study, Impedance detected 96% of reflux events compared to 28% detected by pH study using acid reflux event definition. Non-acid and weakly acid reflux events are common in normal subjects and those with GORD (Sifrim 2004; Wise and Murray 2007).

The Porto consensus on the detection of reflux stated that reflux is best evaluated by a combination of impedance and pH monitoring (Hirano 2006). This is in agreement with the British Society of Gastroenterology guidelines (Bodger and Trudgill 2006).

1.5.7. *Reflux questionnaires*

Questionnaires have been designed to detect symptoms suggestive of both oesophageal and extra-oesophageal reflux. These have been used to assess severity of symptoms and responses to treatment.

Symptoms do not always correlate with objective assessments of reflux (Young, Hadjiliadis et al. 2003; D'Ovidio, Singer et al. 2005; Hartwig, Appel et al. 2005; King, Iyer et al. 2009). In one study, there was no difference in the prevalence of abnormal pH studies in end-stage lung disease patients reporting or not reporting symptoms. There was no relationship between the severity of symptoms and the DeMeester score (Sweet, Herbella et al. 2006). This is a method for analysing acid reflux based on the number of reflux events and the duration of reflux events (pH <4) within a 24 hour period (Johnson, Demeester et al. 1974). 14-33% of patients had asymptomatic distal reflux (Young, Hadjiliadis et al. 2003; D'Ovidio, Singer et al. 2005; Sweet, Herbella et al. 2006). The symptoms of reflux may be absent in patients post-transplant (Hartwig, Appel et al. 2005; Sweet, Herbella et al. 2006). This hyposensitive condition may be partially due to damage to the vagal nerve.

Little is known of the value of extra-oesophageal reflux questionnaires in assessing reflux in lung transplant recipients. The DeMeester Reflux Questionnaire is a validated straightforward tool to assess basic reflux symptoms (DeMeester, Wang CI et al. 1980). It is based on a score of 0-3 for symptoms of reflux, regurgitation and dysphagia. The DeMeester Reflux Questionnaire has been useful in assessing the responses to treatment of both medical and surgical patients however it has never been validated in lung transplant recipients.

Laryngopharyngeal reflux does not always cause classical heartburn or oesophagitis. Signs and symptoms of laryngopharyngeal reflux include hoarseness, vocal fatigue, excessive throat clearing, globus pharyngeus, chronic cough, post-nasal drip and dysphagia. Several laryngopharyngeal reflux questionnaires have been designed. One validated questionnaire which focuses on extra-oesophageal reflux symptoms is the reflux symptom index (RSI) (Figure 2.1). This is a 9 item questionnaire (Belafsky, Postma et al. 2002). An alternative LPR questionnaire is the laryngopharyngeal reflux-health related quality of life questionnaire (Carrau, Khidr et al. 2005). This was

considered for our study. As it is a 43 point questionnaire, we favoured the more concise RSI questionnaire for ease of administration. The RSI is easily administered and highly reproducible. It was validated on 25 laryngopharyngeal reflux patients and 25 controls. A limitation of this questionnaire is that 5 points can be attributed to heartburn. Thus, the RSI is not limited to extra-oesophageal reflux symptoms but can be elevated in patients with typical reflux symptoms. A RSI score of greater than 13, is abnormal (Belafsky, Postma et al. 2002). As this is predominantly based on extra-oesophageal reflux questions, it may have a role in assessing lung transplant recipient. There is no literature to support this suggestion.

Quality of life is a concept which is subjective and not directly measurable (Yano, Sherif et al. 2009). In 1948, the World Health Organisation released a consensus definition of quality of life as a complete state of physical, psychological and social health and not merely the absence of disease. Questionnaires need to cover physical function, symptoms experienced, social function, role performance, subjective feeling of well-being and emotional state (Kirk 1986; Eypasch, Williams et al. 1995; Yano, Sherif et al. 2009). A diversity of questionnaires exist and are both generic and system/disease specific. The gastrointestinal quality of life index (GiQLI) was developed by Eypasch in German. This has been subsequently translated into English and French (Eypasch, Williams et al. 1995). It is a well established, tested and validated tool which has been shown to be reproducible (Eypasch, Williams et al. 1995; Yano, Sherif et al. 2009). It is system specific (Eypasch, Williams et al. 1995). Both the gastro-oesophageal reflux disease health-related quality of life questionnaire and short form- 36 are useful and patient centred. They do not address the gastrointestinal system alone. Combined, the questionnaires work well but require the patient to fill out two separate forms. The quality of life in reflux and dyspepsia questionnaire was also considered but was dismissed as it focuses towards the foregut rather than the whole gastrointestinal system (Wiklund, Junghard et al. 1998). The GIQLI is a single form that is a good alternative to using the gastro-oesophageal reflux disease health-related quality of life questionnaire and short form- 36 (Eypasch, Williams et al. 1995; Yano, Sherif et al. 2009). The use of GIQLI is recommended for the assessment of anti-reflux surgery by the European Association for Endoscopic Surgery and has been validated for this purpose (Korolija, Sauerland et al. 2004).

1.6. Aspiration techniques

Aspiration studies have been carried out in the stomach, oesophagus and lungs. Historically oesophago-gastric aspiration studies have proven reflux occurs. Now these studies have been applied for a different reason. Bronchoalveolar lavage technique can now be analysed for gastrointestinal contents.

GORD may deleteriously influence lung allografts in several ways. It may trigger bronchoconstriction via a vagal reflex. Lung transplant recipients have denervated lungs and the oesophagus may also have undergone denervation due to vagal damage. Bronchoconstriction secondary to vagal reflex is unlikely to be a mechanism of injury in this population. Extra-oesophageal reflux may lead to microaspiration and lung injury (Linden, Gilbert et al. 2006). Microaspiration is difficult to define. We propose that it means the aspiration of small volumes of gastric contents into the lungs causing subclinical damage. This does not lead to aspiration pneumonia. Microaspiration may lead to epithelial damage, stimulation of cytokine production, inflammation, graft failure and may lead to BOS. Post-transplantation there are impaired pulmonary defence mechanisms: cough and muco-ciliary clearance (Veale, Glasper et al. 1993). Muco-ciliary clearance has been shown to be less than 15% of normal (Veale, Glasper et al. 1993). These factors may lead to a prolonged and increased contact between reflux material and the lung parenchyma (Young, Hadjiliadis et al. 2003; Cantu, Appel et al. 2004; D'Ovidio, Mura et al. 2005; Ward, Forrest et al. 2005; D'Ovidio and Keshavjee 2006). The cough reflex has been shown to improve over the first year post-transplant (Duarte, Terminella et al. 2008), but it is unknown whether muco-ciliary clearance improves or not.

Little is known about the role of aspiration during swallow. A recent study by Atkins (2007), shows that 64% of lung transplant recipients aspirate during swallowing, 78% of these patients were asymptomatic. This is associated with a prolonged hospital stay and increased episodes of severe rejection (Atkins, Trachtenberg et al. 2007). In studies on rats by Duke University exposure of the lung allograft to gastric juice lead to grade 4 acute rejection, characterised by monocyte infiltration, fibrosis and lung destruction. Aspiration has been shown to increase $CD8^+$ T-cells. T-cell activation is involved in acute rejection (Hartwig, Appel et al. 2006; Stovold, Forrest et al. 2007). Furthermore chronic aspiration in rats is associated with obliterative bronchiolitis (Li, Hartwig et al. 2008). Further animal models of aspiration suggest lung damage is independent of pH. It could even be damaging at pH >7, suggesting acid

neutralization therapy to be inadequate in the treatment of reflux (Downing, Sporn et al. 2008). There are limitations to these animal models and no studies have been performed looking at microaspiration (Robertson, Shenfine et al. 2009). A further study looking at aspiration in miniature swine has suggested that aspiration may increase fibrosis, obliterans bronchiolitis and infection. There was an increased shedding of allograft alloantigens and increased activity of the indirect alloimmune response. This is where the host antigen presenting cells present donor processed MHC peptides to the immune system (Meltzer, Weiss et al. 2008). One hypothesis to explain this may be that aspiration leads to increased cell death and breakdown, MHC peptides are released and picked up by host antigen presenting cells and immunity is then triggered. Aspiration may introduce bacterial infection into the allograft. Little evidence supports this and no single organism has been implicated. Aspiration studies could be carried out in BALF and gastric fluid to search for gastric microflora.

The danger signal hypothesis, first suggested by Matzinger (Matzinger 1994) may explain the link between aspiration and chronic rejection. It suggests that the immune system's main priority is not the recognition of foreign material but of material that is injurious and harmful (Matzinger 1994; Matzinger 2002). It suggests that tissues undergoing stress, damage or abnormal death processes will release endogenous danger signals which will activate dendritic cells. This then triggers both innate and specific immunity (Gallucci, Matzinger et al. 2001). Thus injury to the allograft may trigger both inflammation and rejection.

The Toll-like 4 receptor is a membrane receptor highly expressed on alveolar macrophages and airway epithelia which detects antigens and stimulates innate immunity. Studies have suggested that activation of innate immunity via the Toll-like 4 receptors may also activate specific immunity. There is growing evidence to suggest that stimulation of the Toll-like 4 receptor by external factors, including lipopolysaccharide, may stimulate the specific immunity and lead to inflammation and acute rejection (Palmer, Burch et al. 2003; Palmer, Burch et al. 2005; Garantziotis, Palmer et al. 2007).

The end results of injuries leading to BOS are fibrosis and airway remodelling. The fibroblasts which effect fibrosis may originate from recruited circulating fibrocytes and through *in situ* airway proliferation. It has been suggested by our group that

fibroblasts may originate from injured epithelium through Epithelial Mesenchymal Transition (EMT) (Ward, Forrest et al. 2005). EMT is recognised to occur in organogenesis, metastasis and chronic rejection of other transplant organs. It is denoted by loss of epithelial markers and up-regulation of mesenchymal properties. Reflux injury is associated with the loss of E-cadherin in the oesophageal epithelium of patients with Barrett's oesophagus. This loss is more marked in oesophageal adenocarcinoma (Bailey, Biddlestone et al. 1998). It occurs despite the presence of defences such as carbonic anhydrase, evolved in the oesophagus to protect against reflux. The *airway* epithelia without such defences may be expected to be more vulnerable to aspiration injury. It is reasonable to hypothesise that microaspiration may directly injure the allograft leading to EMT, fibrosis and BOS. A translational implication of this is that treatments of GORD may reduce microaspiration, epithelial injury and EMT thus impacting favourably on BOS (Robertson, Ward C et al. 2010).

1.7. Biomarkers of aspiration

1.7.1. Pepsin

Pepsin, a proteolytic enzyme, is secreted by chief cells located in gastric pits in the stomach as a precursor pepsinogen (Wallace 1989). The reported "normal" concentration of pepsin in gastric juice in people without PPI therapy is 100-600μg/ml (Wallace 1989; Gotley, Morgan et al. 1991; Balan, Jones et al. 1996). Pepsin has been used as a marker of extra-oesophageal reflux in bronchiectasis and cystic fibrosis. It is a potential marker of gastric aspiration (Ward, Forrest et al. 2005). Several papers have been published using pepsin as a biomarker of extra-oesophageal reflux with glue ear and as a marker of aspiration with lung disease and pulmonary damage (Tasker, Dettmar et al. 2002; Tasker, Dettmar et al. 2002; Ufberg, Bushra et al. 2004; Ward, Forrest et al. 2005).

Assay variability, in general, can be a serious problem (Haslam, Baughman et al. 1999) and the analysis of results for pepsin between units is varied (Table 1-3). Pepsin can be measured using an ELISA and also as an activity assay (Badellino, Buckman et al. 1996). Gastric juice contains 100-600μg/ml of pepsin(Wallace 1989; Gotley, Morgan et al. 1991). Alveolar fluid is diluted one hundred to two hundred fold by bronchoalveolar lavage. If neat gastric juice was aspirated then the alveolar fluid would contain approximately 100-600μg/ml. With a hundred to two hundred fold dilution of gastric juice this would then become 0.5-6μg/ml. Some papers published have a lower limit of detection of 1μg/ml (Metheny, Chang et al. 2002). This cut off would most likely miss aspiration events. Further variability arises over the exact volume of lung fluid (approximately 1-2ml) in each patient- the volume instilled in the BAL and the volume of BAL recovered from the lung. These variables can explain some of the differences in levels of biomarkers reported in the BAL fluid. Comparing results from units can be difficult (Haslam, Baughman et al. 1999).

Table 1-3: Summary of pepsin levels in aspiration studies

Study	Instilled volume	Pepsin levels
(Ward, Forrest et al. 2005)	180ml	35-1375ng/ml
(Stovold, Forrest et al. 2007)	180ml	0-51.7ng/ml
(Blondeau, V. Mertens et al. 2008)	100ml	0-2000ng/ml
(Starosta, Kitz et al. 2007)	Unknown	0-2500ng/ml

Pepsin is a general marker of aspiration in lung transplant patients (Blondeau, V. Mertens et al. 2008). Bronchoalveolar lavage (BAL) pepsin levels in clinically stable lung transplants were shown to be up to one hundredfold higher when compared to controls (109ng/ml vs <1ng/ml) suggesting gastric aspiration (Ward, Forrest et al. 2005). Levels detected were 10-1,000 times higher than serum reference range (Ward, Forrest et al. 2005). Pepsin was still detected even after treatment with a PPI. This further supports the hypothesis that prophylactic PPIs will suppress symptoms, but there may still be occult aspiration of gastric contents (Ward, Forrest et al. 2005).

Further studies using a pepsin(ogen) ELISA were performed to evaluate the levels of pepsin in the BAL samples (Stovold, Forrest et al. 2007).
36 lung transplant patients in three equal groups: clinically stable; acute vascular rejection and BOS were studied. BAL pepsin levels were increased in transplant patients compared to control volunteers (8.3 vs 1.1 ng/ml) ($p=0.02$). BAL pepsin was raised in lung transplant patients without BOS showing that pepsin can be present without airflow limitation (Stovold, Forrest et al. 2007). Detection of pepsin, as evidence of aspiration, is present even in those on proton pump inhibitor therapy.
The highest levels were present in patients with acute A2+ histological rejection. These patients also had the highest grades of inflammation on pathology. This is important and further supports the hypothesis that there may be interaction between alloimmune and non-alloimmune factors suggesting a link between acute rejection and aspiration (Stovold, Forrest et al. 2007). There was no statistical significant difference in pepsin levels between stable and BOS patients although levels in BOS patients were higher. BAL pepsin levels were similar in those on or off PPI therapy. There was no relationship between time post-transplant and pepsin levels (Stovold, Forrest et al. 2007).

1.7.2. Bile salts

Bile salts (aka bile acids) are water soluble steroids synthesised in the liver by hepatocytes during the catabolism of cholesterol. They are a major component of the bile secreted by liver (Jenkins and Hardie LJ 2008). Bile salts are normally conjugated with glycine or taurine before secretion (Klokkenburg, Hoeve et al. 2009). Their role is to aid digestion and absorption of lipids in the small intestine. They are strongly cytotoxic and associated with gastrointestinal malignancy (Jenkins and Hardie LJ 2008). The main bile acids present are the glycine and taurine conjugates (Table 1-4) (Janowitz, Swobodnik et al. 1990; Jenkins and Hardie LJ 2008). Bile salts are later resorbed in the ileum and colon (Klokkenburg, Hoeve et al. 2009). Bile acids exist as mixtures and due to their detergent status, they will influence each other's solubility. For example, taurine conjugates are strong sulphonic acids, which can protonate other bile acids. This allows other bile acids to enter the epithelium without any regard for established solubilities (Jenkins and Hardie LJ 2008).

Table 1-4: Composition of bile and biochemical properties

Bile acid	Water solubility	pKa	% in bile
Free bile acids			
Cholic acid	Poorly soluble	5.2	Trace
Deoxycholic acid	Poorly soluble	6.2	Trace
Chenodeoxycholic acid	Poorly soluble	6.2	Trace
Glycine conjugates			
Glycocholic acid	Poorly soluble	3.8	30
Glycodeoxycholic acid	Poorly soluble	4.8	15
Glycochenodeoxycholic acid	Poorly soluble	4.3	30
Taurine conjugates			
Taurocholic acid	Very soluble	<2	10
Taurodeoxycholic acid	Very soluble	<2	10
Taurochenodeoxycholic acid	Very soluble	<2	5

modified from (Jenkins and Hardie LJ 2008)

Duodenogastric reflux is a physiological event especially in the post-prandial (Klokkenburg, Hoeve et al. 2009) and early morning periods (Byrne, Romagnoli et al. 1999). Decreasing gradients of bile concentration have been reported from the pre-pylorus to the oesophagus (Klokkenburg, Hoeve et al. 2009), suggesting dilution of bile salts over distance.

Pancreatic and biliary secretions may be cytotoxic both to gastro-oesophageal mucosa and also to pulmonary epithelium (Henderson, Fung et al. 1975; Oelberg, Downey et al. 1990). Unconjugated bile acids may pass the cell membrane in a non-ionised lipophilic form at pH 3-6. After entering the cell they become ionised due to high intracellular pH and are trapped inside the cell. Bile acids may reach intracellular levels eight times higher than luminal levels. This injures cells and their tight junctions and may makes cells susceptible to other injuries (Jenkins and Hardie LJ 2008; Klokkenburg, Hoeve et al. 2009).

Various methods have been reported to detect bile salts. A common method is the 3α hydroxylase method described by Fausa & Skalhegg (Fausa and Skalhegg 1974). This assay is not affected by pH but the presence of food or colourants can interfere with results (Collins, Watt et al. 1984). There is some contention about the lower limit of detection of photospectrometric assays; Collins et al suggested 62.5μmol/L (Collins, Crothers et al. 1985), Klokkenburg et al claims 5μmol/l (Klokkenburg, Hoeve et al. 2009), Biostat, who produce the commercially available assay claim a lower limit of detection 1μmol/L and the Leuven group have claimed an accuracy of 0.2μmol/L (Blondeau, Dupont et al. 2008; Blondeau, V. Mertens et al. 2008). These levels are lower than serum bile salt levels (<8μmol/L) (D'Ovidio, Mura et al. 2005). One group have found this type of assay to be unreliable (Gotley, Morgan et al. 1990). The presence of 3α hydroxyl groups and sterol molecules interferes with and cause cross reactivity with the dehydrogenase enzyme and this assay. In normal serum, other 3α hydroxysteroids are present in less than a few nmol/L (Klokkenburg, Hoeve et al. 2009) and in one study lavage samples contaminated by blood had less bile salts present (Klokkenburg, Hoeve et al. 2009).

There is a wide variation of intra-gastric bile salt concentrations reported between individuals and at varying times. Intra-gastric levels have been reported between 0-

13,000μmol/l (Schindlbeck, Heinrich et al. 1987; Gotley, Morgan et al. 1990). Normal intra-gastric levels have been reported at <100-700μmol/l (Collins, Watt et al. 1984). 90% of people will have intra-gastric bile salts concentrations of less than 250μmol/l (Gotthard, Bodemar et al. 1985). Intra-gastric levels up to 34,256μmol/l have been reported post-gastrojejunostomy (Watt, Sloan et al. 1984). No data exists of intra-gastric bile salt levels in lung transplant recipients.

There was no significant difference between fasting and post-prandial intra-gastric bile levels in one study (Collins, Crothers et al. 1985). Nine of these patients had levels >200μmol/l. Of these 9, seven had a pH < 3.5, showing high concentration of bile salts can be present in acidic refluxate (Collins, Crothers et al. 1985).

A study compared levels of intra-gastric bile salts in controls, patients with duodenal ulcers, those undergoing highly selective vagotomy, polya partial gastrectomy, truncal vagotomy and pyloroplasty, truncal vagotomy and gastrojejunostomy. This showed patients with duodenal ulcers had increased intra-gastric bile salt concentrations pre-operatively. Post-operatively patients who had undergone polya partial gastrectomy, truncal vagotomy and pyloroplasty, truncal vagotomy and gastrojejunostomy had increased intra-gastric bile salts. Those who underwent a highly selective vagotomy had decreased intra-gastric bile salts. Highly selective vagotomy preserves the pylorus and the antropyloroduodenal complex, whereas a complete vagotomy and pyloroplasty will not (Dewar, King et al. 1982).

In summary intragastric bile acid concentrations are very variable between patients and throughout the day. Increased levels are seen in patients who have undergone surgery to disrupt the pylorus and antropyloroduodenal complex.

Reported levels of bile salts in the oesophagus range from 0 to greater than 10,000μmol/L although most studies report a low median level (3.5-5.1μmol/L) (Kauer, Peters et al. 1997; Klokkenburg, Hoeve et al. 2009). A quarter of patients had no bile salts detectable in the oesophagus and levels greater than 1,000μmol/L are rare (Gotley, Morgan et al. 1991). Bile reflux often occurred on a background of acidic reflux pH 4-7(Kauer, Peters et al. 1995).

The artificial distinction between "acid" and "bile" reflux is a common misunderstanding. Whilst pure "bile" (or duodenal) reflux may occur post-gastrectomy, virtually all duodenal reflux events will combine with gastric refluxate by mixing with gastric contents. When bile salts are detected in the oesophagus on a background of a higher pH, likely explanations are PPI use or elevation of gastric pH by food or bicarbonate from the duodenum. It must be remembered that the detection of elevated bile salts signifies gastric as well as duodenal reflux.

In a further study, Kauer et al. assessed distal oesophageal aspirates for the presence of bile salts. Distal oesophageal bile salts were increased in the supine position and in the post-prandial period. Bile salts were present in 58% of normal controls and 86% of patients with GORD. The bile detected in the oesophagus consisted of 60% glycocholic acid 16% glycodeoxycholic acid, 15% glycochenodeoxycholic acid and remainder 10% taurocholic acid, taurodeoxycholic acid, taurchenodeoxycholic acid and glycolithocholic acid.
In summary, oesophageal levels of bile salts are variable. Although the majority of oesophageal bile salt concentrations appear low, levels have been reported up to 15,000μmol/l.

Bile salt levels have been analysed in the saliva of patients. Levels detected in cystic fibrosis patients, pre-transplant have been reported at a median of 3.3μmol/l (Range 2.4-6.1) and in patients with GORD a median of 1.23μmol/l (Range 1.2-2.3). Chronic cough patients have a lower reported level 0.72μmol/l (0.2-1.2) (Blondeau, Dupont et al. 2008).

In a study by De Corso et al, patients undergoing Billroth II gastrectomy or total gastrectomy revealed 17/52 (32.6%) of patients having bile in saliva. Controls were negative for bile salts. A correlation existed between salivary bile, bilirubin, pepsinogen and laryngeal damage, suggesting extra-oesophageal reflux may be associated with laryngeal damage. Concentrations of bile salts have been documented with a mean of 1μmol/l (range 0.5-5).

The median level reported from a single study of middle ear effusion were 17.7μmol/L (5.9-40.9μmol/l) (Klokkenburg, Hoeve et al. 2009). These were three to

twenty times higher than serum levels (Klokkenburg, Hoeve et al. 2009). The median level reported in middle ear is similar to oesophageal levels but the maximal levels reported are lower.

Bile salts in the bronchoalveolar lavage fluid are markers of duodenal gastro-oesophageal reflux and aspiration(D'Ovidio, Mura et al. 2005). BOS is associated with abnormal pH, the presence of bile salts in the BALF and duodeno-gastro-oesophageal aspiration. 50% of patients with abnormal pH studies and 20% of patients with normal pH studies post-transplant had bile acids in the BALF. This may be significant as the presence of bile acids in the bronchoalveolar district, may decrease the time to the development of BOS significantly. 70% of patients with high levels of bile acids (>8μmol/ml) in their BALF samples have been proposed to develop BOS within 12 months (D'Ovidio, Mura et al. 2006).

Bile acid aspiration is associated with severe pulmonary injury (Henderson, Fung et al. 1975; D'Ovidio, Mura et al. 2005). Bile aspiration is cytotoxic, disrupts the cellular membrane and alters cationic permeability, as demonstrated in vitro on Type II pneumocytes (Oelberg, Downey et al. 1990; D'Ovidio, Mura et al. 2005; D'Ovidio and Keshavjee 2006). In the stomach, bile acids break the mucosa barrier. In the lungs they may disrupt the mucus layer and their detergent effect may disrupt the lipids in the surfactant. They may also cause direct injury to Type II pneumocytes that are responsible for surfactant protein and phospholipids production and homeostasis. Bile salts may also lead to down-regulation of innate immunity receptors on monocytes and macrophages (D'Ovidio, Mura et al. 2006). It has been shown in rabbits that bile salts cause decreased macrophage function by decreasing phagocytosis and LPS mediated cytokine production. Interferon-mediated signal transducers may be down-regulated by bile salts (D'Ovidio, Mura et al. 2006).

Bile aspiration is thought to disrupt the regional innate immunity. This encourages local infection and affects the balance of innate and adaptive immunity. Paradoxically this may lead to an up-regulation of and a more aggressive adaptive immunity as well as encouraging infection. (D'Ovidio, Mura et al. 2006). This immune response maybe augmented via damage to the surface epithelial cells (Davis, Lau et al. 2003).

The presence of bile salts has been associated with elevated neutrophils, IL-8 and the presence of bacteria, fungi, lower levels of pulmonary surfactant and higher inflammatory scores on transbronchial biopsy (D'Ovidio, Mura et al. 2006; Vos. Blondeau et al. 2008). There are lower levels of surfactant surface proteins A & D, (collectins) which are opsonins and regulate cytokine production. These proteins are involved in the cross-talk between innate and adaptive immunity (D'Ovidio, Mura et al. 2006). There are decreased levels of dipalmitoylphosphatidyl-choline and phosphatidyl-l-glycerol phospholipids, which play a role in maintaining the pulmonary epithelium and local innate immunity. There is increased lipid sphingomyelin (a membrane related phospholipid), which further supports the evidence of the cytotoxic effects of bile acids. This damages phospholipids and leads to alterations in the prospective mucosal barriers (D'Ovidio, Mura et al. 2006).

A prospective study of 120 lung transplant patients evaluated bronchoalveolar lavage bile salts, interleukins, differential cell counts, microbiology testing, trans bronchial biopsies and BOS scores. (D'Ovidio, Mura et al. 2005). The median score for bile acids in BOS negative patients (0.3μmol/L) was lower than in BOS positive patients (1.6μmol/L) ($p=0.002$). Patients with early BOS (developed within one year) had higher levels of bile acids (2.6μmol/L) than those with late BOS (developed after one year) (0.8μmol/L) ($p=0.02$) (D'Ovidio, Mura et al. 2005). Bronchiolitis obliterans syndrome positive patients had significantly higher levels of IL-8 (121pg/ml vs 64.5) (D'Ovidio, Mura et al. 2005).

Bile acid levels were divided into 3 groups: high >8μmol/L 9.3% (10/107), low 0.1-8μmol/L 57% (61/107) or none 0μmol/L 34% (36/107). Patients with BOS had higher levels than those without. Of the two types of onset of BOS, levels of bile acids in the bronchoalveolar district seem to predispose to early BOS. IL-8 was also increased in correlation with increased bile acids and neutrophils. There was a correlation between bile acids and neutrophils. There was also a correlation between bile acids, IL-8 and early development of BOS (D'Ovidio, Mura et al. 2005). The relationship between bile salts and BOS is further supported by Blondeau (Blondeau, V. Mertens et al. 2008).

In a study by D'Ovidio et al., the median bile salts level in those with positive biopsies for inflammation (1.1μmol/L) was higher than those with a negative biopsy

(0.2μmol/L). Patients with positive microbiology samples had higher levels of bile salts (0.7μmol/L) than those with negative samples (0.3μmol/L). Higher bile acid levels were associated with increased fungal growth (0.75 versus 0.36μmol/L). Cytomegalovirus status was not affected by bile salt levels (D'Ovidio, Mura et al. 2005).

The median IL-8 was 118 pg/ml in the high bile acid group, 107 pg/ml in the low group and 61 pg/ml in the group with no bile salts. Neutrophils in the high group (5%) were elevated when compared with the low group (2%) and for those with no bile acids (2%) (D'Ovidio, Mura et al. 2005).

A further recent study in patients with cystic fibrosis suggests an increase in duodenal gastro-oesophageal reflux and aspiration post-transplantation (40% versus 60%). However the numbers are small and this was not a longitudinal study (Blondeau, Dupont et al. 2008). A summary of bile salt levels detected in several studies is shown in Table 1-5.

Table 1-5: Summary of reported bile salt levels reported in the upper and lower airways

Study	Fluid	Instilled volume	Bile salt levels
(D'Ovidio, Mura et al. 2005)	BALF	Unknown	0-32 μmol/l
(D'Ovidio, Mura et al. 2006)	BALF	Unknown	0->3.5 μmol/l
(Blondeau, V. Mertens et al. 2008)	BALF	100ml	0-0.8 μmol/l
(Vos, Blondeau et al. 2008)	BALF	100ml	0.1-3.7 μmol/l
(Blondeau, Dupont et al. 2008)	BALF sputum	100ml	1.2-6.1 μmol/l
(Blondeau, Mertens et al. 2009)	BALF	100ml	0.4-1.5 μmol/l
(Klokkenburg, Hoeve et al. 2009)	Middle Ear	0-0.5ml	5.9-40.9 μmol/l
(Starosta, Kitz et al. 2007)	BALF	Unknown	0.6-5.4 μmol/l

1.7.3. Trypsin

Trypsin is a protease secreted by the pancreas into the duodenum and can be used as a marker of duodeno-gastro-oesophageal reflux. It has been suggested that most of the active trypsin refluxed into the stomach, may be degraded by pepsin and cannot pass through the acid environment to reach the oesophagus. In one study, trypsin was found in 17 of 365 gastric juice aspirates and only 4 specimens had levels >20μg/ml. All of these samples had a pH >4.6. This suggests that trypsin may be a less useful indicator of aspiration and injury (Gotley, Morgan et al. 1991).

1.8. Biomarkers of inflammation

1.8.1. Neutrophils

Neutrophils are likely to be associated with chronic rejection and contain potent inflammatory mediators. These include proteases, acid hydrolases and low molecular weight cationic proteins. Reactive oxygen metabolites induce parenchyma cell injury and extracellular matrix degradation. This may lead to pulmonary fibrosis (Zheng, Walters et al. 2000).

The pathological mechanisms of BOS are unclear but involve T-cells, macrophages and the adaptive immunity. Little consideration has previously been given to the innate immunity. Persistent neutrophilic inflammation is associated with fibrosing and inflammatory pulmonary conditions including pulmonary fibrosis, asbestosis and also severe asthma. Increased neutrophils & IL-8 in the BALF have been implicated with BOS and increased mortality (Zheng, Walters et al. 2000). Alveolar neutrophilia has been proposed as a predictor of mortality (Henke, Golden et al. 1999; D'Ovidio and Keshavjee 2006).

Chronic inflammation affects all 3 compartments: the airway wall, lung parenchyma, and BAL fluid. Zheng et al (2000) performed a study investigating airway neutrophilia post-lung transplantation. Neutrophils were found beneath the epithelium, in the epithelium and in the lamina propria. The BALF neutrophil count was 557 neutrophils/mm^2 for BOS, 450 neutrophils/mm^2 for stable lung transplant patients and 220 neutrophils/mm^2 for normal controls (Zheng, Walters et al. 2000).

There was neutrophil accumulation in the airway walls of lung transplant patients with and without BOS. These levels were significantly higher when compared to normal controls. BALF neutrophils and IL-8 were increased in both groups but higher levels were present in those with BOS. There was a positive correlation between wall and BAL neutrophils (Zheng, Walters et al. 2000). There is also an association between elevated BAL neutrophils, increased IL-8 concentrations and BOS (Zheng, Walters et al. 2000).

Henke, (1999) evaluated the median levels of neutrophils in the BAL samples as a predictor of mortality. Neutrophil levels were lower in survivors (2% of BAL

leukocytes), compared to non-survivors (7% of BAL leukocytes). Deaths were due to BOS, infection or non-pulmonary causes. High neutrophil counts in lavage fluid are a suggested predictor of increased mortality. Neutrophils are also a marker for acute rejection (Henke, Golden et al. 1999).

A neutrophilic response to epithelial injury from pathogens or aspiration may constitute a final common pathway, linking impaired defence mechanisms, infection, aspiration, inflammation, airway remodelling and BOS (Walters, Reid et al. 2008). It is increasingly recognised that epithelia may be both a target for injury and play a role in the damage process, including airway scarring. Epithelial mesenchymal transition (EMT) is a response to injury in which epithelial cells transform into fibroblasts. This potentially indicates a direct link between activation and injury of epithelium with subsequent fibrosis, airflow limitation and BOS (Ward, Forrest et al. 2005; Robertson, Griffin et al. 2009).

1.8.2. Interleukin 8

Interleukin 8 is a marker of injury and is produced by many cells including epithelial cells, fibroblasts, smooth muscle cells, endothelial cells and alveolar macrophages in response to injury. It is an important chemokine in pulmonary pathology. Not only does it have a role in leukocyte trafficking especially neutrophils, but it also stimulates angiogenesis and has a direct stimulatory effect on lung mesenchymal and parenchymal cells. IL-8 is also a mucin secretagoge (Zheng, Walters et al. 2000; Strieter 2002).

The mechanisms of BOS appears to involve IL-8 and neutrophils (D'Ovidio, Mura et al. 2005). BOS positive patients had significantly higher mean levels of IL-8 (121 versus 64.5 pg/ml). There was no difference for IL-8 levels between early and late BOS patients (D'Ovidio, Mura et al. 2005). BAL IL-8 levels are highest in BOS patients, then stable lung transplant patients, then normal controls (Zheng, Walters et al. 2000). Immunostaining has localised IL-8 to peribronchial lesions in OB. Therefore it may contribute to the development of BOS through its neutrophil attracting and angiogenic role. It has multiple inflammatory and immunological activities and may also lead to airway remodelling. This could be another mechanism involved in the pathophysiological process of BOS (Zheng, Walters et al. 2000).

1.9. Airway mucus and goblet cells

Airway pathologies involving chronic airflow limitation or neutrophilia and suppuration can lead to mucus hypersecretion. In lung transplant there is a possibility for disordered mucus homeostasis and this may be problematic (Veale, Glasper et al. 1993). To date, little research has been performed on this topic. Mucus also plays a role in other pulmonary pathologies including CF, COPD and asthma. Respiratory mucus is produced from the secretions of submucosal tracheobronchial glands and epithelial goblet cells. Epithelial surfaces are lined by mucus which consists of water, ions, glycoproteins (mucins), proteins and lipids. The mucins may be secretory or membrane tethered. Mucus is involved in muco-ciliary defence and the innate immune defence system. In the respiratory tract, it protects the airway against pathogens and environmental toxins by trapping and clearing particles. It has an antibacterial effect and humidifies the inspired air (Rose and Voynow 2006). Hypersecretion of mucus contributes to the morbidity of airways diseases, predisposes to respiratory infections and contributes to airflow obstruction and patient discomfort. It is associated with increased mortality (Kim 1997).

In health, there is little mucus in the lungs. The amount is governed by production and clearance by cough and ciliary activity (Kim 1997).

Hypersecretion of mucus may lead to the accumulation of mucus. An increased volume may be beneficial to combat infection or detrimental and lead to airway obstruction with enhanced deposition of inhaled particles in the tracheobronchial tree (Kim 1997).

Mucin levels are increased in airway disease and lead to increased airway obstruction. Inflammatory/immune response mediators activate mucin gene regulation and airway remodelling including goblet cell hyperplasia. These changes are sustained and an increase in mucin production may contribute to airway obstruction (Rose and Voynow 2006).

The effects of aspiration on the respiratory mucus layers are complex and not fully understood. Pepsin and bile salts will disrupt this layer and expose the epithelium. They may also lead to an up-regulation of mucus secretion leading to airway obstruction. The overall changes are unknown.

Mucins are highly glycosylated macromolecules. They are characterised by numerous tandem repeats containing proline. They are high in serine and /or threonine residue,

the sites of O-glycosylation. Mucins are complex glycoproteins with a large molecular weight (2-20x10^6 Daltons) and high carbohydrate content: 50-90% content/weight. Mucins are characterised by the MUC protein backbone produced from MUC genes. Transcripts have 1.1-15 kilobases and proteins have several hundred to eleven thousand amino acids in their backbone (10-50% of weight) (Rose and Voynow 2006).

Of the 18 types of mucins, MUC5AC and MUC5B are the two major mucins found in the airway. In health, goblet cells produce MUC5AC and glandular mucosal cells produce MUC5B and MUC8. MUC5B is expressed in goblet cells as a marker of disease but this has also been reported in healthy individuals. MUC7 mucin is produced from the mucosal and serosal cells in salivary glands. It is also found in 15-20% of normal individuals where it is produced from localised subsets of serous cells in submucosal glands of airway tissue (Jackson 2001; Rose and Voynow 2006). In health, goblet cells and submucosal glands are present in the large airways and are sparse in the periphery with few or none in the small non-cartilaginous airways (Jackson 2001). Terminal and respiratory bronchioles are not cleared by cough and do not possess the same muco-ciliary clearance capacity of the large airways.

1.9.1. Mucus secretion

Exposure to cytokines and leukocytes may trigger mucus secretion. Injurious stimuli including bacteria, lipopolysaccharide, a Gram negative bacterial endotoxin, smoke, matrix metalloproteinases, neutrophil elastase, reactive oxygen species, triphosphates (markers of cell injury), bacterial by-products and growth factors have been shown to increase mucin production. These may work directly or via stimulation of leukocytes (Kim 1997; Jackson 2001). In vitro studies have shown lipopolysaccharide to increase MUC5AC, MUC5B and IL-8 (Smirnova, Guo et al. 2003). This study suggests that goblet cells, via IL-8 and mucins secretion in response to lipopolysaccharide, are an important part of mucosal immunity.

Mediators triggering mucin release result in hypersecretion within minutes via the secretory cascade. This protects the lungs from infection and damage but overproduction may be deleterious (Rose and Voynow 2006).

Previous sections of this introduction have proposed the case that GORD and aspiration are important injuries post-lung transplant. The paucity of longitudinal data and data from the early post-transplant period has created the opportunity for this thesis. As a result of these clinical suspicions, therapeutic strategies have been proposed to treat GORD in the hope of improving lung function and survival.

1.10. Treatments of GORD in lung transplant recipients

Historically, peptic ulcer disease has been associated with transplant recipients due to high dose steroid immunosuprresion use. PPIs have an important role in these patients to reduce the incidence and sequelae of ulceration and in the symptomatic relief of heartburn (Logan, Morris-Stiff et al. 2002). PPIs have no effect on the lower oesophageal sphincter and will not prevent reflux events. Although they reduce the acidity of gastric contents and perhaps the volume of contents, this may not be important. As BALF pepsin is detectable in patients both on and off proton pump inhibitor, it is thought that prophylactic PPIs do not prevent aspiration of gastric contents in lung transplant recipients (Hartwig, Appel et al. 2005; Ward, Forrest et al. 2005; Wise and Murray 2007). The pH of aspirated contents does not influence pulmonary damage in an animal model (Downing, Sporn et al. 2008). Treatment with PPI therapy may have a deleterious side effect by increasing intragastric pH leading to an increase of bacterial flora. This may potentiate the effects of aspiration and introduce bacteria into the lungs (Verdu, Viani et al. 1994).

Alginates are popular in the symptomatic management of dyspepsia and GORD. They work by creating a raft in the stomach to prevent reflux into the oesophagus ((Klinkenberg-Knol, Festen et al. 1995). No evidence supports their role in preventing reflux and aspiration in lung transplant recipients.

Promotility agents may, however, be of benefit by preventing or reducing reflux. Azithromycin has been shown to improve airflow limitation even in those patients with longstanding BOS (Yates, Murphy et al. 2005). Azithromycin, a macrolide, has multiple beneficial activities: anti-inflammatory, antibacterial and promotility (Arts, Caenepeel et al. 2005; Murphy, Forrest et al. 2007; Gottlieb, Szangolies et al. 2008). The presence of GORD symptoms predicts a favourable outcome of treatment. This

improvement in lung function may be partially through an amelioration of GORD (Gottlieb, Szangolies et al. 2008) and a reduction of aspiration.

1.11. Anti-reflux surgery

Anti-reflux surgery has been used as a treatment for extra-oesophageal reflux (Westcott, Hopkins et al. 2004) and has been performed in the setting of end stage lung disease (Tsai, Peters et al. 1996; Linden, Gilbert et al. 2006; Gasper, Sweet et al. 2008; Gasper, Sweet et al. 2008). The first documented case of GORD as a reversible cause of decreasing lung allograft function was reported in 2000 by Palmer et al. After anti-reflux surgery the patient had improved pulmonary function tests and resolution of bronchial inflammation (Palmer, Miralles et al. 2000).

A key paper was published in 2003 by Davis et al. This suggests that anti-reflux surgery may lead to increased survival and improved lung function post-transplantation, by preventing lung damage through aspiration. There is less evidence for the effectiveness of surgery in advanced disease as there may already be irreversible pathological scarring (Davis, Lau et al. 2003; D'Ovidio and Keshavjee 2006). This study involved 43 patients undergoing antireflux surgery post-lung transplantation. The predominant procedure was laparoscopic Nissen's fundoplication. 10 patients had abnormal gastric emptying and 9 of these had further surgery to improve gastric drainage. Fundoplication was performed on the basis of abnormal pH studies, but occasionally due to other factors: reflux demonstrated on barium swallow, after a repeat transplant in which graft failure was secondary to chronic aspiration and for recurrent aspiration (Davis, Lau et al. 2003).

FEV_1 increased significantly by an average of 24% post-fundoplication and greater than 80% of patients had an increase in FEV_1 after fundoplication surgery. Those free from BOS before fundoplication were free from this after surgery. 77% (10/13) of patients with BOS-1 improved post surgery. 43% of patients with BOS-2 improved but only 17% of BOS-3 patients improved. This shows that the decrease in lung function is reversible but the further advanced BOS is, the less there is to be gained. (Davis, Lau et al. 2003). Survival was significantly better in patients with no reflux after transplant compared to those with reflux: 3 year survivals were 91% versus 82% and 5 year survivals were 77% versus 48% (Davis, Lau et al. 2003).

1.11.1. *Timing of surgery: a role for fundoplication before lung transplant?*

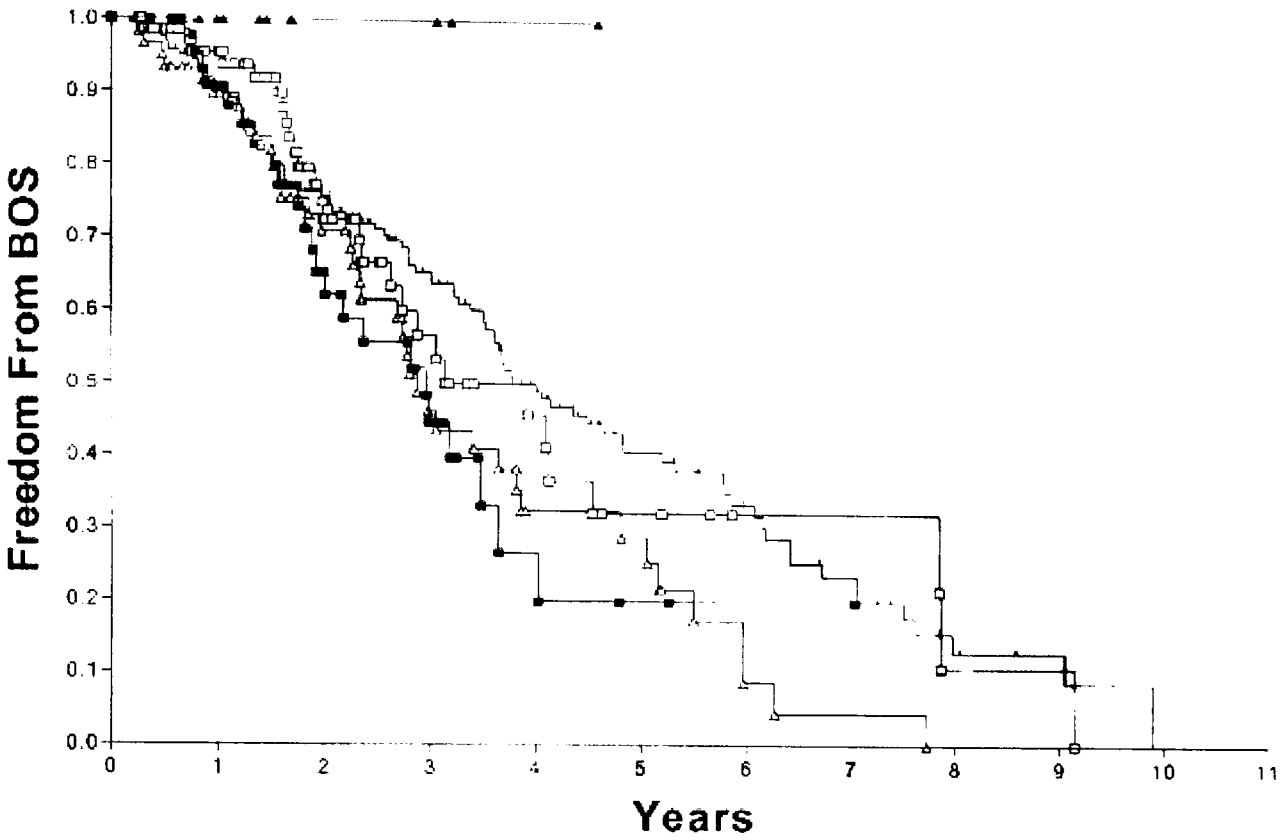
Introduction of fundoplication has not been systematic, but has been considered in patients with end stage lung disease (Linden, Gilbert et al. 2006; Gasper, Sweet et al. 2008). There is a risk of morbidity and mortality and some patients derive little benefit. There are potential benefits to performing fundoplication before transplant. This allows immediate protection from microaspiration, a decreased risk of peri-operative aspiration and may allow an improvement, stabilisation or reduced decline in function (Linden, Gilbert et al. 2006). Small series of antireflux surgery in patients with end-stage lung disease have been described. In one series, there was no statistical significant decrease in pulmonary function over 15 months post-operatively, although 4 patients died before lung transplant (2-19 months) due to progressive respiratory failure. One patient with pulmonary fibrosis had a significant improvement in FEV_1 from 77% of predicted to 103%. He subsequently had decreased oxygen requirements, and was taken off the transplant list. Patients with pulmonary fibrosis who underwent fundoplication, had decreased oxygen requirements, when compared to those who had no surgery (Linden, Gilbert et al. 2006). The second series demonstrates that anti-reflux surgery can be safe in the pre-lung transplant (n=15) and post-lung transplant (n=17) population (Gasper, Sweet et al. 2008).

1.11.2. *Early versus late fundoplication*

Davis et *al's* earlier work from Duke University suggested that the decreased FEV_1 post-transplant was reversible if fundoplication was performed early. If treated later this may not be successful as irreversible fibrosis may have developed. Cantu et al (2004) carried out a study to evaluate the effect of early versus late fundoplication. Fundoplication was performed if pH studies showed a total oesophageal acid exposure time of >10% or there was an unexplained decrease in FEV_1 (Cantu, Appel et al. 2004). Laparoscopic Nissen's fundoplication was the procedure of choice unless oesophageal dysmotility was present. If present, then a Toupet procedure was performed (Cantu, Appel et al. 2004). Seventy six patients underwent fundoplication. All post-transplant patients were divided into 5 groups: Normal pH study; reflux with no fundoplication; reflux and early fundoplication (within 90 days); reflux and late fundoplication (after 90 days) and unknown reflux status. Figure 1-4 shows those who were free from BOS at 1 and 3 years. There is a significant difference between those

who underwent early fundoplication and the other groups (p=0.01) (Cantu, Appel et al. 2004).

Figure 1-4: Freedom from bronchiolitis obliterans syndrome (Cantu, Appel et al. 2004)



Patients at risk at year	0	1	2	3	4	5	6	7	8	9	10
Normal pH Study	47	44	33	20	10	4	2	1	1	0	0
Reflux no Surgery	79	68	49	28	15	10	5	3	3	1	1
Reflux Early Surgery	14	12	8	5	3	1	0	0	0	0	0
Reflux Late Surgery	62	57	44	30	17	10	8	2	1	0	0
Unknown	180	158	131	97	72	44	29	20	12	4	3

	Freedom from BOS	1 year	3 year
•	No reflux (n=47)	91%	62%
□	Reflux & no surgery (n=79)	92%	60%
▲	Reflux & early fundoplication (n=14)	100%	100%
Δ	Reflux & late surgery (n=62)	90%	47%
⊥	Unknown (n=180)	90%	66%

There was no significant difference between groups for episodes of acute rejection. Survival however, was significantly better (p=0.02) after one year with patients who underwent early fundoplication (100%), when compared to the rest of the patients

(90-98%). This difference was more pronounced at 3 years (p=0.03) (Table 1-6) (Cantu, Appel et al. 2004).

Table 1-6: Patient survival at 3 years (Cantu, Appel et al. 2004)

	% Survival
Reflux & early fundoplication (n=5)	100%
No reflux (n=20)	71%
Reflux & late surgery (n=30)	86%
Reflux & no surgery (n=26)	69%
Unknown (n=197)	66%

A survival advantage was shown in patients undergoing early fundoplication, even when compared to those with a normal pH study. This may be partly due to a "normal" pH study containing patients with mild reflux (7.9%) (physiological values for acid exposure are <4.2% (Johnson, Demeester et al. 1974). This suggests that any degree of reflux may be deleterious to this patient group. Patients with advanced BOS have a lesser chance of improvement with surgery because the later stages of this disease are irreversible (Cantu, Appel et al. 2004).

There were several serious flaws and significant limitations to this study of Cantu *et al*. Firstly it was a retrospective study with a non-random analysis open to significant bias. Those with reflux, who did not undergo fundoplication, may have been excluded from treatment due to significant co-morbidity, explaining their increased mortality. The early fundoplication cohort underwent their transplants towards the end of the study. Their survival advantage may be due to general improvements in post-transplant management and increased clinical experience. Finally the numbers at risk at each time point were extremely small in the early fundoplication group (i.e. n=5 at 3 years). Slight changes in the prevalence of BOS or mortality in the early group (e.g. n=1) would massively affect the overall results and conclusions of this study (Cantu, Appel et al. 2004). This groups most recent data presented at the ISHLT suggests that in patients undergoing early fundoplication (n=67) there is a lower incidence of BOS at 1 year (15.9% versus 47.7%) when compared to patients undergoing late fundoplication (p<0.0001) (Balsara, E. Cantu et al. 2008). A recent study of late fundoplication (mean time to surgery 768 days post-transplant) suggests late intervention may stabilise lung function and slow decline but does not improve FEV_1 (Burton, Button et al. 2009). The overall evidence supporting this practice is limited and flawed.

1.11.3. Choice of procedure

Open approaches to anti-reflux surgery have excellent long term success rate (25 year success rate of 70-80%) in controlling reflux (Luostarinen, Isolauri et al. 1993). The laparoscopic approach, first performed in 1991, is now the procedure of choice and has been shown to be as successful in the control of reflux as open procedures in the medium to long-term (Kelly, Watson et al. 2007). Laparoscopic surgery requires increased operative time, but has the advantage of shorter hospital stay, lower operative morbidity and faster time to recovery when compared to open procedures (Darling, Deschamps et al. 2005). These benefits are important in lung transplant recipients. Most of the evidence in the non-transplant population is based upon Nissen fundoplication and the evidence supporting tailoring the wrap (Watson, Jamieson et al. 1999; Stewart, Watson et al. 2004; Baigrie, Cullis et al. 2005; Rice, Watson et al. 2006; Guerin, Betroune et al. 2007; Booth, Stratford et al. 2008; Cai, Watson et al. 2008; Fein, Bueter et al. 2008; Strate, Emmermann et al. 2008) and routine division of the short gastric vessels (Luostarinen and Isolauri 1999; Blomqvist, Dalenback et al. 2000; O'Boyle, Watson et al. 2002; Yang, Watson et al. 2008) is limited. None of these trials are relevant in the context of lung transplant recipients. Published studies in the lung transplant population favour laparoscopic Nissen fundoplication (Cantu, Appel et al. 2004; Hartwig, Appel et al. 2005).

1.11.1. Morbidity & mortality

O'Halloran et al (2004) compared the results of 28 lung transplant recipients undergoing uncomplicated laparoscopic Nissen fundoplication with 63 non-transplant patients. No peri-operative deaths occurred (O'Halloran, Reynolds et al. 2004). Compared to the non-transplant population there were no significant differences in the intra-operative data. (O'Halloran, Reynolds et al. 2004). The transplant population had an increased length of stay and a higher readmission rate, due to transplant co-morbidity (O'Halloran, Reynolds et al. 2004). Only one lung transplant death post-fundoplication has been reported (Burton, Button et al. 2009). The patient had a pre-operative FEV_1 of 30% predicted and developed chronic vascular rejection and pneumonia, dying 17 days post-operatively (Burton, Button et al. 2009). Reported complications include pneumonia, urinary tract infections, nausea, ileus and dysphagia (Hartwig, Appel et al. 2005). Specific problems include temporary dysphagia, nausea (Hartwig, Appel et al. 2005), gas bloat and flatulence.

Results suggest that fundoplication may retard the development of BOS, and extend survival (Cantu, Appel et al. 2004). Several fundamental questions remain unanswered however including: how should one confirm aspiration? and what are the indications for anti-reflux surgery (D'Ovidio and Keshavjee 2006)? In particular, the criteria for selection to surgery are yet to be defined and vary greatly from unit to unit. It may be the case that some reflux is physiological, but safe levels, are unknown. Most of the available data supporting anti-reflux surgery in lung transplant recipients is derived from a single centre; however, other centres are actively studying the role of fundoplication. The current data from different units and even from the same unit is conflicting and although there are some early promising studies (Table 1-7) we suggest that there is a need for appropriate trials, and solid evidence based guidelines (Robertson, Shenfine et al. 2009).

Table 1-7: Summary of published studies on fundoplication pre- and post-lung transplant

Author/ date	Unit	Number of patients undergoing fundoplication	Outcome	PFTs	Survival	Operative mortality
Lau 2002	D	18	Feasibility	Improved	n/a	0%
Davis 2003	D	43	Survival	Improved	Improved	0%
O'Halloran 2004	D	28	Safety	Improved	n/a	0%
Cantu 2004	D	76	Survival	Improved	No change	0%
Benden 2005	GOSH	5	Paediatric	No change	No change	0%
Linden 2006	H	19	Pre-transplant lung function	Slowed decline in some patients	No change	0%
Gasper 2007	UCSF	32	Safety pre & post transplant	n/a	n/a	0%
Balsara 2008	D	184	BOS	Improved	n/a	0%
Burton 2009	M	21	QoL	Slowed decline in some patients	n/a	1/21

Key to table: D= Duke University, GOSH= Great Ormond Street Hospital, H= Harvard University, UCSF= University of California, San Francisco, QoL= quality of life

Aims

-To identify gastro-oesophageal reflux and aspiration occurring within in the first month post-lung transplantation

-To evaluate longitudinal changes in gastro-oesophageal reflux and aspiration in the first six months post-lung transplantation

-To analyse gastric juice for biomarkers of aspiration and presence of bacteria

-To investigate the effects of pepsin and mixed gastric juice on goblet and bronchoepithelial and cells in vitro

-To evaluate the effects of anti-reflux surgery on reflux symptoms and quality of life in lung transplant recipients

2. Methods

2.1. General study design

Patients undergoing lung transplantation at the Freeman Hospital, Newcastle, were studied in a longitudinal manner to test for the presence of reflux. Their lung allografts were under standard surveillance using bronchoscopy, bronchoalveolar lavage samples and pulmonary function tests.

From 1st November 2007 to 1st November 2008 all newly transplanted lung recipients were approached and asked if they wished to participate in the study. Patients were recruited even if they had undergone pre-transplant fundoplication as it was unknown if the lung transplant would disrupt the integrity of the fundoplication. Patients, therefore, had the potential to have pathological reflux in the post-transplant period. We were unable to calculate a sample size for this study due to the absence of current data. Therefore, this is a descriptive study.

Our protocol was to assess for GORD at one, three and six months post lung transplantation, using a validated extra-oesophageal reflux questionnaire, manometry and pH/impedance measurements. These assessments were performed around similar time periods as bronchoscopy and pulmonary function tests. However, exact practice was tailored to suit individual patients. Patients were assessed on their routine proton pump inhibitor therapy. Routine practice was for lansoprazole 30mg once daily. If patients were symptomatic on once daily dose then the dose was doubled. PPI twice daily was not routinely prescribed as no evidence exists to suggest this reduces microaspiration. Results were then compared with markers of aspiration and inflammation in the bronchoalveolar lavage samples, microbiology, pathological rejection scores and pulmonary function tests.

2.2. Ethical approval

Ethical approval was obtained from County Durham & Tees Valley 2 Research Ethics Committee (Appendix 5). Trust Research & Development approval was granted by the Newcastle Upon Tyne Hospital Trust Research & Development Department (Appendix 5).

2.3. Clinical assessment

Patients had their case notes reviewed on enrolment to the study to establish patient demographics, indication for transplant, co-morbidities and current medication. The patients were clinically followed up for 6 months.

2.4. Consent & information

Patients were recruited in the post-transplant period, once they were beginning to recover. Before enrolment, patients were given information sheets and an explanation regarding the study. They were given up to a week to contemplate the study and discuss this with the transplant team. After a period of time, patients were asked if they wished to participate in the study and written consent was obtained.

2.5. Reflux symptom index questionnaire

The reflux symptom index (RSI) questionnaire, which includes laryngopharyngeal reflux symptoms, was used. This was a straight forward 9 point questionnaire, which has been designed and validated by J Koufman's group in the USA at Wake Forest University School of Medicine (Belafsky, Postma et al. 2002). The questionnaire allowed patients to score their symptoms of reflux from 0-5. The 9 areas of interest are shown in Figure 2-1. Once completed, a total RSI score was calculated. This was deemed positive if greater than 13.

Figure 2-1: Reflux symptom index questionnaire

Within the **last Month** how did the following problems affect you	0 = No Problem 5 = Severe Problem					
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
Excess throat or postnasal drip	0	1	2	3	4	5
Difficulty swallowing food, liquids or pills	0	1	2	3	4	5
Coughing after you eat or after lying down	0	1	2	3	4	5
Breathing difficulties or choking episodes	0	1	2	3	4	5
Troublesome or annoying cough	0	1	2	3	4	5
Sensation of something sticking in your throat or a lump in your throat	0	1	2	3	4	5
Heartburn, chest pain, indigestion or stomach acid coming up	0	1	2	3	4	5
	RSI					

2.6. Oesophageal manometry

Patients underwent manometry after a minimum 4 hour fast for solids and at least 2 hours for liquids (Bodger and Trudgill 2006). Immunosuppression medications were not omitted, but imbibed with a small volume of water, at least 3 hours before manometry. A 3.9mm single catheter, eight lumen water perfused manometry system was used (Mediplus Limited, Buckinghamshire, United Kingdom). This catheter had 4 radial ports and 4 lateral ports spaced 5 centimetres apart. Only the 4 lateral ports were used to measure oesophageal pressures. The catheter assembly was connected to a standard four channel compressed air pneumo-hydraulic low compliance perfusion pump. Distilled water was perfused at a constant rate of 0.6ml/s. This was connected to a Polygraf transducer (Meditronics Synectics, Stockholm, Sweden) on a Windows compatible desktop computer (Dresner 2001).

2.6.1. Standard technique

Informed consent was obtained. Patients were seated and the catheter was passed horizontally through the nares into the nasopharynx (Bodger and Trudgill 2006). Then patients were asked to tilt their head forward, put their chin on their chest and to take lots of small swallows via a "bendy straw". This technique, helps the catheter progress through cricopharyngeus into oesophagus. The tube is then passed into the stomach to 70cm from the nares. The patient then was asked to lie in a recumbent or semi-recumbent position, as this is the ideal position for water perfused manometry. Patients often had difficulty lying completely supine, as they had recently undergone major thoracic surgery and also many patients had not lain flat for years due to their respiratory co-morbidity. The manometry catheter was calibrated with the "zero" point being at the patient's sternal angle. These points were not thought to influence results significantly. The presence of all 4 channels in the stomach were confirmed by a positive deflection in all 4 channels in response to inspiration (Evans and Buckton 1997).

2.6.2. Lower oesophageal sphincter

Using the standard stationary pull through technique (Bodger and Trudgill 2006), the catheter assembly was withdrawn by 1cm every 30seconds (Zaninotto, DeMeester et al. 1988). Inspiration and wet swallow of 5ml were performed. As this was performed 1 month post lung transplant, the technique was modified to suit the patient's ability

to cope with the procedure. This did not compromise evaluation of the lower oesophageal sphincter and oesophageal peristalsis. The lower oesophageal sphincter was defined as the high pressure zone at the lower end of the oesophagus. The length, resting pressure position and response to swallows were calculated manually, with the aid of the Polygraf computer programme. The lower oesophageal sphincter end expiratory pressure was defined as the difference between basal tone pressure and the average of the end-expiratory resting pressures found in each port whilst in the high pressure zone. This was measured in millimetres of mercury (mmHg). The degree of sphincter relaxation to a 5ml water swallow was observed (Bodger and Trudgill 2006). The respiratory inversion point was difficult to define as patients had difficulty with forced inspiration and expiration. However it has been suggested that this represents a respiratory artefact and failure to define it did not affect assessment (Bredenoord 2006).

2.6.3. Oesophageal motility

Ten "wet" swallows were performed to assess oesophageal motility. Motility was evaluated for normal peristalsis, simultaneous contractions or aperistalsis. Two techniques were used. Initially manometry was carried out performing swallows at one centimetre intervals. Mean distal and proximal amplitudes were calculated as an average of peristaltic amplitudes between 3-8cm and 13-18cm above the lower oesophageal sphincter respectively. Latterly all ten swallows were performed with the distal port 5cm above the lower oesophageal sphincter. Mean distal oesophageal peristaltic amplitude was calculated based on the average of all swallows performed at 5cm. Mean proximal peristaltic amplitudes were based on the average of all swallows performed at 15cm above the lower oesophageal sphincter. Traces were analysed in depth and divided into the following categories (Table 2-1).

Table 2-1: Classification of oesophageal peristalsis

Normal peristalsis	Normal peristalsis >70% of the time
Mild ineffective oesophageal motility	Abnormal peristalsis 30-70% of the time
Severe ineffective oesophageal motility	Normal peristalsis <30% of the time
Aperistalsis	Abnormal peristalsis 100% of the time
Diffuse oesophageal spasm	>10% of swallows simultaneous with mean amplitudes over 30mmHg
Nutcracker oesophagus	Mean amplitude of peristalsis >180mmHg
Hypertonic lower oesophageal sphincter	>45mmHg but relaxing
Hypotonic lower oesophageal sphincter	<10mmHg
Achalasia	Hypertonic LOS, absent or incomplete relaxations >70-80% of the time. Simultaneous contractions or aperistalsis in the oesophageal body

(Evans and Buckton 1997; Spechler and Castell 2001; Bodger and Trudgill 2006; Fox, Bredenoord et al. 2008; Pandolfino, Ghosh et al. 2008)

2.6.4. Cricopharyngeus

The cricopharyngeus was identified to determine the length of the oesophagus. It was defined as the high pressure zone at the proximal oesophagus, which demonstrated relaxation on swallowing.

2.7. Ambulatory impedance/pH studies

After oesophageal manometry, combined 24 hour ambulatory pH impedance was performed. Proton pump inhibitors were not discontinued.

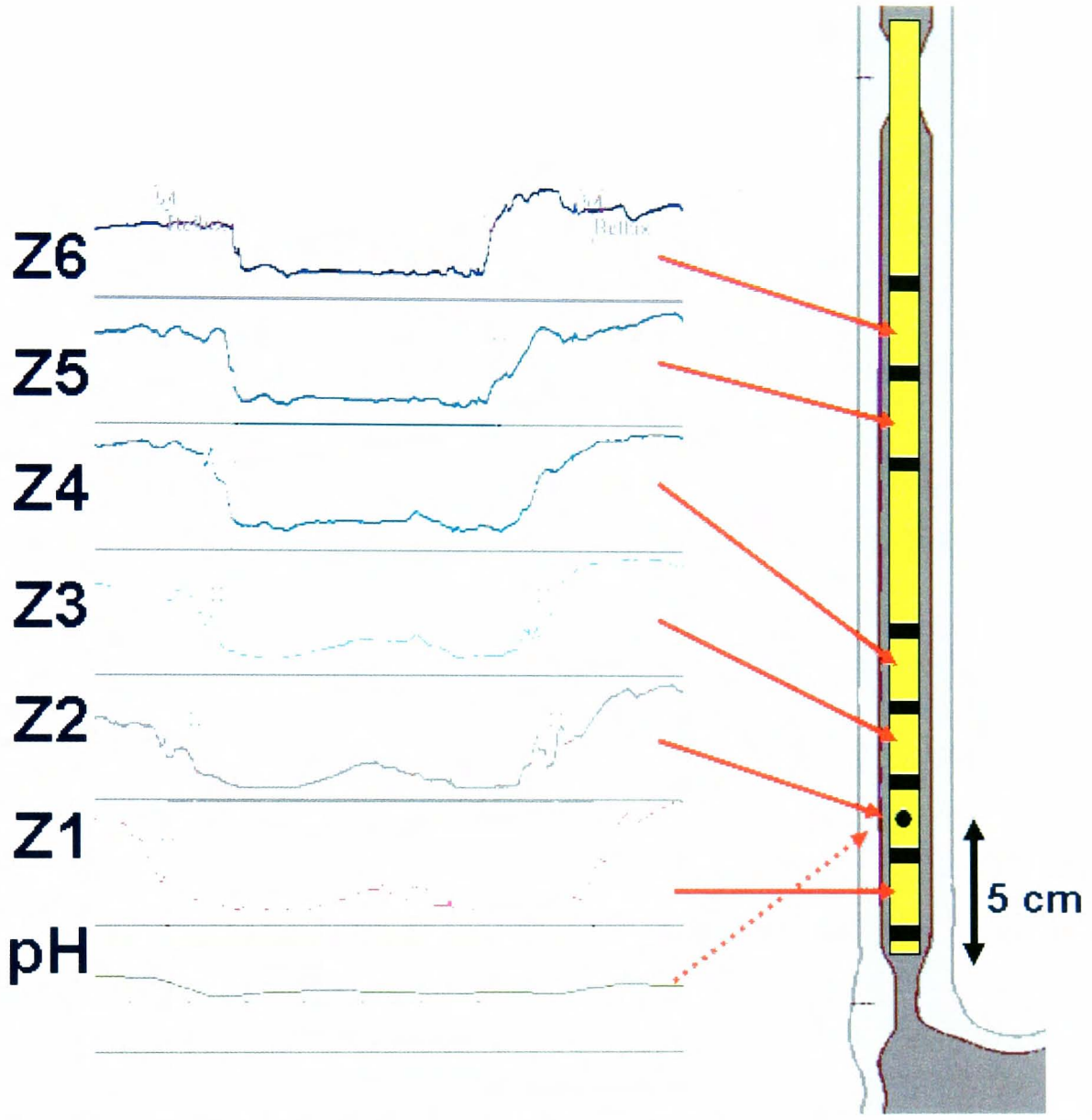
Ambulatory impedance/pH was performed using the Medical Measurement System and Ohmega Device (Ohmega Utrecht, The Netherlands). A Phersiflex Z61A\ZNIS-8R catheter was used. This is a 1.9mm catheter with 8 ring, 6 channel system with a single pH probe at 5cm. Channnels were located at 3,5,7,9,15 & 17cm.This allowed the proximal extent of the reflux to be determined.

The catheter was connected to the Ohmega device and calibrated in a standard fashion. After a ten minute pre-soak the pH probe was calibrated using standard buffer solutions of pH 4 & 7 at room temperature. The impedance catheter was

inserted into the oesophagus using standard technique, described above (2.6.1) to place the end of the catheter at the upper border of the lower oesophageal sphincter and the pH probe 5cm above the upper border of the lower oesophageal sphincter (Figure 2-2) (Bodger and Trudgill 2006).

Patients were encouraged to maintain their habitual eating habits during the pH-impedance monitoring period. Patients were instructed to record symptoms (cough, something in the throat, heartburn), meals and position (erect or supine) using the Ohmega device. They also were given a simple, standardized patient diary to complete. After 24 hours the recording was complete and the ambulatory Ohmega device was connected to a Windows compatible computer with the MMS software loaded on the computer. The data was uploaded to the MMS programme. The electronic diary was verified with the paper diary and edited appropriately. The trace was then analysed manually with the aid of the software.

Figure 2-2 Diagram of pH/impedance catheter within the oesophagus and the subsequent trace



Legend: This diagram shows the pH-impedance catheter within the oesophagus. The pH probe lies 5cm above the lower oesophageal sphincter and there are multiple rings for the impedance measurements (Z1-6). The trace on the left hand side shows pH at the bottom. The sequential drop in impedance from Z1-Z6 shows a proximal reflux event, which is subsequently cleared by the oesophagus.

2.7.1. *pH analysis*

pH results were analysed and values compared with normal values described by Johnson and DeMeester. An abnormal study was defined as a pH less than 4 for more than 4.5% of the duration of the study (Johnson, Demeester et al. 1974). No normal values exist for patients on PPI therapy, therefore standard normal values were used.

2.7.2. *Impedance analysis*

Impedance traces were analysed visually with the aid of the software. Reports were verified by Dr Arjan Bredenoord, Gastroenterologist, Holland, who is a world expert on pH-impedance traces. Values were compared with normal European values determined by Zerbib. An abnormal study was defined as volume exposure >1.2% (Zerbib, des Varannes et al. 2005).

2.7.3. *Comparison of symptoms to reflux events*

24 hour pH/impedance recording has the advantage of allowing the software to compare patient symptoms to reflux events.

2.7.4. *Symptom index*

This is calculated using the number of symptomatic episodes associated with reflux events as a percentage of the total symptomatic episodes. 50% is the optimum threshold for a positive result (Bredenoord, Weusten BLAM et al. 2005; Bredenoord 2006).

2.7.5. *Symptom sensitivity index*

This accounts for the limitation of the symptom index. It is calculated as the number of reflux events associated with symptoms as a percent of acid reflux events. It is positive if over 10% (Bredenoord, Weusten BLAM et al. 2005; Bredenoord 2006).

2.7.6. *Symptom associated probability*

This is a statistical attempt to utilise all the data. It is calculated by dividing the test into two minute intervals and determining when reflux or symptoms occur.

The data is then evaluated using a Fisher exact test of the following 4 distributions:

Symptoms & reflux	No symptoms & reflux
Symptoms & no reflux	No symptoms & no reflux

The test then evaluates whether the distribution occurs by chance. If the level is over 95% then the test is positive.

The role of SI has been verified by clinical studies and there is evidence for its clinical value in predicting response to proton pump inhibitor and fundoplication (Bodger and Trudgill 2006). Symptom associated probability utilises all parameters and provides a better insight into the relationship between symptoms and reflux (Bredenoord, Weusten BLAM et al. 2005; Bredenoord 2006).

2.7.7. Overall pH-impedance analysis

Overall analyses were interpreted to identify if patients had pathological reflux. Key distal reflux indices were oesophageal acid exposure and oesophageal volume exposure. The key proximal reflux index is proximal reflux events. Oesophageal acid exposure was the percentage of time that the pH is less than 4, 5cm above the lower oesophageal sphincter during a 24 hour period (normal <4.5%). Oesophageal volume exposure was defined as the percentage of time that impedance detects refluxate within the oesophagus over a 24 hour period (normal <1.2%). Proximal reflux events were impedance events reaching 17cm above the lower oesophageal sphincter. Patients were deemed to have distal reflux if either the oesophageal acid exposure or the oesophageal volume exposure were abnormal. If oesophageal volume exposure was abnormal on a background of normal oesophageal acid exposure then it was deemed that the patient had weakly acidic reflux. If patients had more than 17 proximal reflux events over a 24 hour period then they were deemed to have abnormal proximal reflux.

2.8. Bronchoscopy

Bronchoscopy was routinely performed at one week, one, three, six and twelve months post lung transplantation. It was also performed if there was deteriorating lung function, suspicion of rejection or infection. After receiving informed consent, up to 10mg intravenous midazolam was administered to cause adequate sedation. Topical application of 4% lignocaine to the nose, pharynx, larynx and below the vocal cords in 1ml aliquots, was used as required to create local anaesthesia. The maximum dose given was 7mg/kg body weight. Oxygen saturations were monitored by oximetry. Supplemental oxygen was administered. Bronchoscopy was then performed in a supine position via the nasal/oral route. A 4.9mm external diameter, 2mm internal diameter fibre-optic bronchoscope was passed through the mouth or nares. The endoscope is then guided through the vocal cords and trachea. The bronchial anastomosis was subsequently inspected and then the bronchoscope was passed into the lingular bronchus or the bronchus of the right middle lobe of the transplanted lung (Ward, Forrest et al. 2005; Stovold, Forrest et al. 2007).

2.9. Bronchoalveolar lavage

Bronchoalveolar lavage was performed in a standardized manner in accordance with ERS guidelines (Haslam, Baughman et al. 1999). Three samples of 60ml of sterile saline were injected into the lobe. The fluid was then retrieved. The retrieved BAL fluid sample was then split. Samples were sent for clinical microbiology and the rest was taken for research purposes. Microbiology was assessed in a standardized fashion. This is described later in detail (Section 2.11). Differential cell counts were made on Giemsa-stained cyto-centrifuge preparations. Cell free BAL supernatants were prepared by centrifugation; aliquots were snap-frozen by immersion in liquid nitrogen and stored at -80°C for research purposes (Section 2.12).

2.10. Pathology

Transbronchial biopsies were obtained from the allografts using fluoroscopy. Five to seven biopsies were taken at each bronchoscopy and sent immediately to pathology to undergo urgent processing. On arrival the samples underwent microwave fixation using 10% formalin. They then underwent standard histological processing using paraffin and then subsequent staining with haematoxylin and eosin to assess acute vascular and airway inflammation. These were then assessed according to revised standardised ISHLT criteria (Table 2-2) by two specialised pathologists (Yousem 1996; Stewart, Fishbein et al. 2007). Samples were also stained in PAS to exclude viral and fungal infections and Gram stain to detect bacterial pathogens.

At our centre, grade A2 or above is treated as being clinically significant. This would result in alteration in patient management, such as an increase in steroid dose.

Table 2-2: Revision of the 1996 working formulation for the standardisation of nomenclature in the diagnosis of lung rejection (Yousem 1996; Stewart, Fishbein et al. 2007)

A: Acute rejection

Grade	Rejection	Histological criteria
A0	None	No evidence of mononuclear cell infiltration, haemorrhage or necrosis.
A1	Minimal	Scattered infrequent perivascular mononuclear infiltrates in alveolated lung parenchyma.
A2	Mild	More frequent perivascular mononuclear infiltrates surrounding venules & arterioles, recognisable at low magnification.
A3	Moderate	Easy recognizable cuffing of venules and arterioles by dense perivascular mononuclear cell infiltrates associated with endothelialitis, eosinophils and neutrophils.
A4	Severe	Diffuse perivascular, interstitial & airspace infiltrates of mononuclear cells with prominent alveolar pneumocyte damage and endothelialititis.
Ax	Ungradeable	Ungradeable due to sampling problems, infection. tangential cutting, artefact etc.

(Yousem 1996; Stewart, Fishbein et al. 2007)

B: Airway inflammation: lymphocytic bronchiolitis

Grade	Rejection	Histological criteria
B0	None	No evidence of bronchiolar inflammation.
B1R (B1& B2)	Low grade	Mononuclear cells within the submucosa of the bronchioles which can be infrequent & scattered or forming a circumferential band.
B1 (1996)	Minimal	Rare scattered mononuclear cells within the submucosa.
B2 (1996)	Mild	Circumferential bands of mononuclear cells.
B2R (B3& B4)	High grade	Mononuclear cells in the submucosa appear larger and activated, with greater numbers of eosinophils and plasma cytoid cells, in addition, there is evidence of epithelial damage in the form of necrosis and metaplasia & marked intra-epithelial lymphocytic infiltration. In its most severe form there is epithelial ulceration, fibro-purulent exudate, cellular debris and neutrophils.
B3	Moderate	A dense band-like infiltrate of activated mononuclear cells in the lamina propria of bronchi/bronchioles including activated lymphocytes and eosinophils with evidence of epithelial damage in the form of necrosis, metaplasia & marked intra-epithelial lymphocytic infiltration.
B4	Severe	A dense band-like infiltrate of activated mononuclear cells in bronchi and/or bronchioles associated with dissociation of epithelial cells from the basement membrane, epithelial ulceration, fibrinopurulent exudates containing neutrophils, and epithelial cell necrosis.
BX	Ungradeable	Ungradeable due to sampling problems, infection, tangential cutting, artefact etc.

(Yousem 1996; Stewart, Fishbein et al. 2007)

C: Chronic airways rejection: obliterative bronchiolitis

Grade	Rejection	Histological criteria
C0	None	No evidence of obliterans bronchiolitis.
C1*	Obliterans bronchiolitis	Dense eosinophilic hyaline fibrosis in the sub-mucosa of membranous and respiratory bronchioles, resulting in partial or complete luminal occlusion.

*Note: Transbronchial biopsy is an insensitive method for detecting obliterative bronchiolitis. The clinical use of PFTs and the Bronchiolitis obliterans syndrome are the preferred methods of diagnosing and monitoring chronic airways rejection.
(Yousem 1996; Stewart, Fishbein et al. 2007)

D: Chronic vascular rejection

Chronic vascular rejection	Fibrointimal thickening of arteries and veins. Diagnosed by open biopsy.

(Yousem 1996; Stewart, Fishbein et al. 2007)

2.11. Clinical microbiology

Bronchoalveolar lavage samples were processed, cultured and analysed at the Department of Microbiology at the Freeman Hospital using standardized techniques. All samples were analysed by trained staff using appropriate containment and safety procedures in accordance with Freeman Hospital accredited standard operating procedures.

2.11.1. Culture of BAL samples

On arrival, samples were verified and were taken to the category 3 suite and placed in the safety cabinet. Initially samples were centrifuged for 10 minutes at 3,000r.p.m.. The supernatant was removed as lignocaine is inhibitory to legionella. 20ml of sterile deionised water was then added to the residue and vortexed. The sample was then centrifuged again and the supernatant removed. After a further vortex, the sample was ready for culture.

Lavages were then cultured neat on appropriate media by adding 10µl of sample to plates and spreading for single colonies.

Patients with cystic fibrosis had their lavages diluted 5µl in 10ml sterile water and then inoculated onto a chocolate agar plate for further microbial assessment, including *Pseudomonas*. Plates were then incubated as per standard protocol (Table 2-3).

Table 2-3: Microbiology protocol for BALF analysis

Clinical conditions	Standard media	Incubation			Cultures read	Target organisms
Culture neat only		Temp (°C)	Atmosphere	Time		
	Horse blood agar	35-37	5-10% CO2	24-48h	Daily	S. Pneumoniae M. Catarrhalis S. Aureus Other organisms in pure growth may be significant
	Chocolate agar with Bacitracin	35-37	5-10% CO2	24-48h	Daily	*Haemophilus* sp *Enterobacteriaceae* *Pseudomonas* sp *Capnoocytophagia*
	Cysteine Lactulose Electrolyte Deficient	35-37	Air	24-48h	Daily	*Enterobacteriaceae*, *Pseudomonas* sp.
	Legionella media	35-37	CO2	10 days	Daily	*Legionella* sp *Nocardia*
	Gram stain					Any organisms and cellular examination
	Cultures sent to Health Protection Agency for tuberculosis culture					*Mycobacteria*
	Sabaraud medium	35-37	Air	24-48h. Can be extended to 5 days	Daily	*Candida* sp *Aspergillus* sp Other fungi
Cystic fibrosis patients						
	Cepacia media	35-37	Air	5 days	Daily	B. cepacia B gladioli
	Mannitol trehalose salt agar/ aztreonam blood agar	35-37	Air	24-48h	Daily	S Aureus

2.12. Bronchoalveolar lavage processing

Bronchoalveolar lavage fluid was processed to measure the volume of fluid received to count the total number of cells and prepare cytospins.

The BAL fluid was stored at 4°C for up to a maximum of 1 hour before processing. In the class 2 cabinet the BAL fluid was filtered through a layer of gauze into 2x 50ml centrifuge tubes. The volume was measured and recorded. Samples were centrifuged at 1250rpm for 6 mins at 4°C. The supernatant was then decanted into 2x 50ml centrifuge tubes, taking care not to disturb the cell pellet. This supernatant was centrifuged at 2500rpm for 6 mins at 4°C, before being divided: 600µl in microcentrifuge tubes and 4x 5ml in 5 ml centrifuge tubes. 50ml of Dulbecco's PBS was added to the cell pellet to give an opaque suspension and it was then mixed gently. The total cell concentration was calculated using an improved Neubauer counting chamber, counting the cells in 4 large squares. The volume was adjusted to give a final cell concentration of 0.5million cells /ml. Cytospins were then prepared using 100µl of re-suspended cells at 300 rpm for 3 minutes at room temperature. Cytospins were then fixed in acetone at room temperature for 10 minutes and allowed to air dry. The remaining cytospins were air dried overnight, wrapped in cling film and stored at -20°C. After preparation of the cytospins was complete, the cell suspension was re-centrifuged. The supernatant was discarded and the cell suspension was stored at -20°C until transfer to -80°C freezer.

This process resulted in:

25x 600µl aliquots of acellular BAL fluid stored at -80°C.

4x 5ml aliquots of acellular BAL fluid

1x cytospins acetone fixed with and stained with Geimsa

5x cytospins air dried, wrapped and stored at -20°C

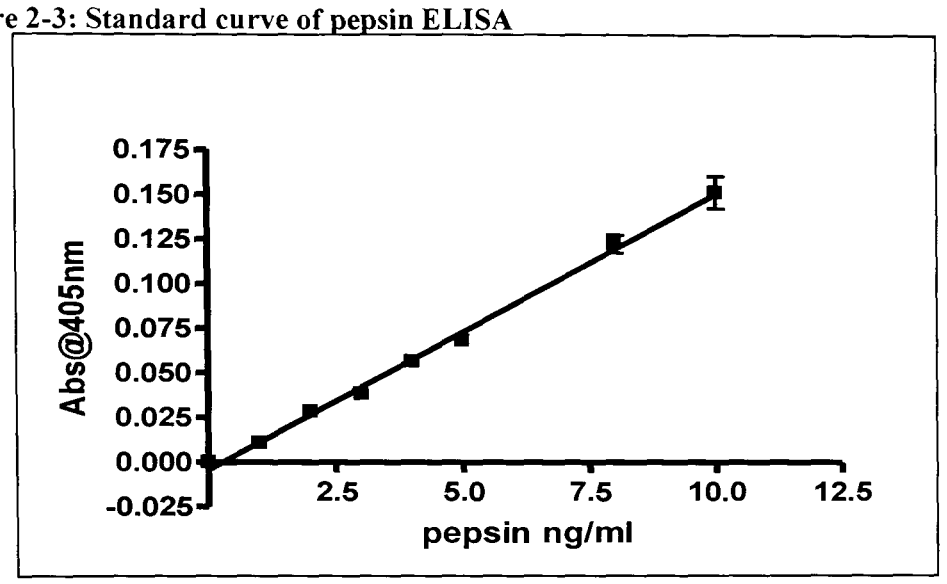
6x cell pellets stored at -80 °C

2.13. Biomarkers

2.13.1. Pepsin/pepsinogen ELISA

The pepsin assay used was developed and extensively calibrated, tested and verified (Stovold 2009). 100µl of standards diluted in phosphate buffered saline (PBS) or 20µl of sample, added to 80µl of PBS were added to coat a 96 well microplate Maxisrop, Nunc). PBS consisted of 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5 mM KH2PO_4, pH7.2-7.4, 0.2µm filtered. The plate was sealed and incubated overnight at room temperature. Each well was aspirated and washed with 400µl wash buffer (0.05% Tween 20 in PBS pH 7.2-7.4, R&D Systems) repeating the process twice for a total of three washes, followed by two more washes of 1% PBS. The plate was then blocked by adding 300µl of block buffer (1% bovine serum albumin in PBS) to each well and incubated at room temperature for 1 hour. Aspiration and wash were repeated. Primary antibody (antipepsin, Biodesign International, USA) was diluted to working concentration (1 in 2000) in reagent buffer (0.1% BSA, 0.05% Tween 20 in PBS) and 100µl was added to each well. The plate was then covered with parafilm and incubated for 2 hours at room temperature. Aspiration and wash were repeated. 100µl of the secondary detection antibody (horse radish peroxidase-conjugated anti sheep/goat antibody, Sigma, UK), diluted in reagent dilutant (1 in 10,000). was then added to each well. This was then covered with a new adhesive strip and incubated for 2 hours at room temperature. Aspiration and wash were repeated. 100µl of substrate solution (2,2'-azino-bis(3-ethylbenzothiazoline-6-) sulfonic acid) was then added to each well. This was incubated for 20 minutes at room temperature, avoiding direct light. 100µl of stop solution (1% sodium dodecyl sulphate) was added to each well. The plate was gently tapped to ensure thorough mixing. Optical density of each well was determined immediately using a microplate reader set to 405nm (Figure 2-3) (Stovold 2009). Negative controls were analysed. These samples were analysed identically apart from omitting the primary antibody. In addition a correction factor of (x2) was used to correct for the difference in primary antibody affinity to human compared to pig pepsin (Stovold 2009).

Figure 2-3: Standard curve of pepsin ELISA



2.13.2. *Bile salt assays*

Spectrophotometric

Initially the Bioquant commercially available enzymatic assay (Bioquant, San Diego, CA, USA) was assessed. This system is based on the principle that in the presence of NAD^+, the enzyme 3-α hydroxysteroid dehydrogenase (3-α HSD) converts bile acids to 3-keto steroids and NADH. The NADH formed reacts with nitrotetrazolium blue (NBT) to form a formazan dye in the presence of diaphorase enzyme. The dye formation is monitored by measuring absorbance at 540nm and is directly proportional to the bile acids concentration in the serum sample.

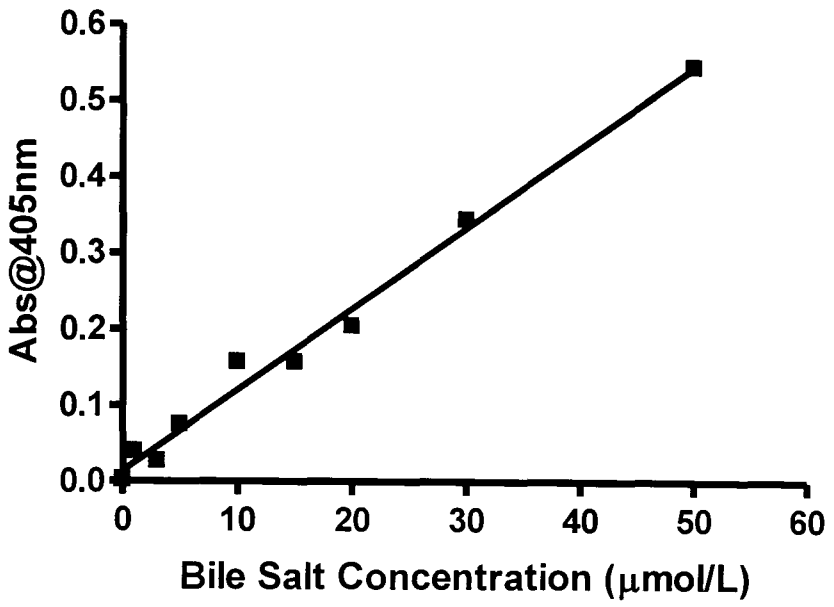
The kit was supplied with a standard solution of bile acid- 35μmol/L- and several standard curves were performed. These showed that the lower limit of quantitation was 5μmol/l. This quantitation is contrary to previous reports from other units which have stated that this assay is accurate down to <0.2μmol/l (Blondeau, V. Mertens et al. 2008).

Because of the large dilution of any bile acids in the lung produced by the use of 180ml of saline in the lavage procedure, I tried to find an assay with a greater sensitivity. Another commercial assay which claimed a sensitivity/detection limit of 1μmol/l (Biostat, Stockport, UK) was assessed. This assay is based on the fact that 3α-hydroxysteroid dehydrogenase, in the presence of Thio NAD^+, converts bile acids to 3-keto steroids and Thio-NADH. This process is reversible. In the presence of excess NADH, enzyme cycling is efficient, and the rate of Thio-NADH formation can be quantified using photospectrometry at 405nm (Turley, Dietschy et al. 1978).

Reagents were warmed to room temperature before analysis. 270μl of reagent 1 (which consisted of Na_2HPO_4 15g/l, NaN_3 0.3g/l, EDTA 1mM and Thio-NAD 2.5g/l) was added to coat a 96 well microplate. To this was added 4μl of samples, standard or control. In house standards were constructed from mixtures of 0.8% bile made up of 50% cholic acid, 30% chenodeoxycholic acid, 15% deoxycholic acid and 5% lithocholic acid dissolved in methanol (concentrations 0-200μmol/l, range 0, 20, 40, 100 & 200μmol/l). Control was reagent 2, heated to denature the 3-α HSD enzyme. Samples were incubated at 37°C for 3 minutes and absorbance was read at 405nm.

BALF samples were measured undiluted. In our hands, this assay had a lower limit of detection of 2μmol/l (Figure 2-4).

Figure 2-4: Standard curve of Biostat bile salt assay



Analytical mass spectrometry

Because BALF bile salts were likely to be essentially undetectable by spectrophotometric based approaches, a more sensitive tandem mass spectrometry method was used at a nationally accredited external laboratory, blind to the study; Sheffield Children`s Hospital, UK. Tandem mass spectrometry is a technique that allows the analysis of metabolites and proteins in blood samples. It permits simultaneous examination of a large number of materials. Mass spectrometry is a technique that measures the mass of substances (molecular weight). Tandem mass spectrometry involves two mass spectrometers performed in sequence. The first pass spectrometer tests a single molecular mass (precursor ions) from nebulised samples. Then these ions are passed through a "collision cell" and molecules are bombarded with high energy argon gas. This fractures the molecules and fragments are passed through a second spectrometer. Different compounds fragment uniquely in different ways. If a mass of a molecule and of its fragments are known then the identity of the molecule can be inferred (Sweetman 1996; Berger 1999; Mushtaq, Logan et al. 1999). Concentrations of glycodeoxycholate, glycocholate, taurodeoxycholate, taurocholate glycochenodenodeoxycholate and taurochenodeoxycholate, which are prototypical physiologically relevant bile salts (making up approximately 95% of total human bile

salts), were measured (Sweetman 1996; Berger 1999; Mushtaq, Logan et al. 1999). Estimations of total bile salt concentration were calculated from the arithmetic sum of the individual bile salt concentrations. The lower limit of detection limit was 0.1μmol/l.

This procedure was further modified to improve the lower limit of detection to 1nmol/l as follows:
450μl of BALF was added to 10ml of distilled water containing 150μl of deuterated taurocholate (internal standard). This solution was loaded onto a C18SPE column (Supelco LC-18) washed with 5ml water and 2ml hexane. The bile salts were eluted with 10ml of methanol and evaporated to dryness. They were then reconstituted in 1ml of 90% acetonitrile. 30μl was injected directly onto tandem mass spectrophotometry with 50% acetonitrile as running buffer. The bile salts were measured using negative ion mode and multiple reaction monitoring scans, giving a sensitivity down to 1nmol/l.

2.13.3. *Interleukin-8*

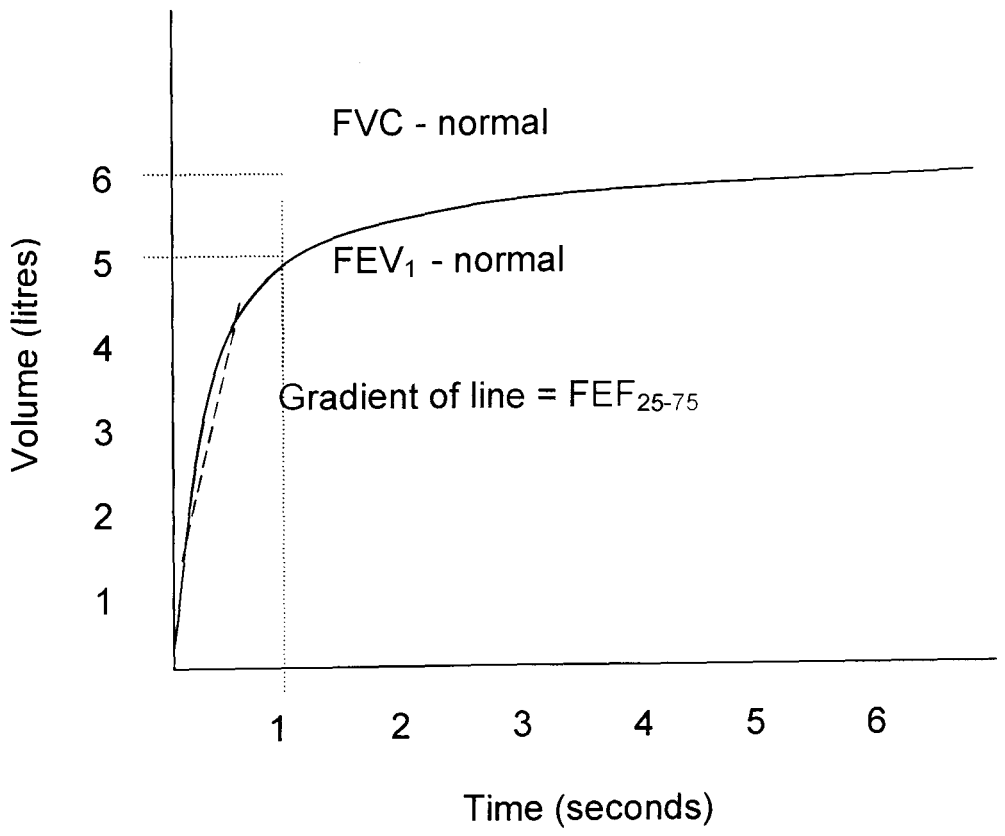
A standard indirect DuoSet ELISA was used to evaluate IL-8 levels (R&D Systems, USA). 100µl of capture antibody (capture antibody was antibody from R&D Systems) was diluted to working concentration (1 in 100) in reagent buffer (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (20mM Trizma base, 150mM NaCl) . pH 7.2-7.4, 0.2µm filtered) and 100µl was added to each well to coat a 96 well microplate overnight. Each well was aspirated and washed with 400µl wash buffer (0.05% Tween™ 20 in phosphate buffer solution pH 7.2-7.4, R&D Systems) repeating the process twice for a total of three washes. The plate was then blocked by adding 300µl of block buffer (1% bovine serum albumin in phosphate buffer solution) to each well and incubated at room temperature overnight. Aspiration and wash were repeated.

100µl of standards diluted in PBS or 10µl of sample added to 90µl of PBS. The plate was then covered with an adhesive strip and incubated for 2 hours at room temperature. Aspiration and wash were repeated. 100µl of the detection antibody diluted in reagent dilutant (1 in 10,000), was then added to each well. This was then covered with a new adhesive strip and incubated for 2 hours at room temperature. Aspiration and wash were repeated. 100µl of substrate solution (1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (tetramethylbenzidine)) was then added to each well. This was incubated for 20 minutes at room temperature, avoiding direct light. 50µl of stop solution 3M (H_2SO_4) was added to each well. The plate was gently tapped to ensure thorough mixing. Optical density of each well was determined immediately using a microplate reader set to 450nm. Negatives were also performed. These samples were analysed identically apart from omitting the primary antibody. The lower limit of detection from this assay was 10pg/ml.

2.14. Pulmonary function tests

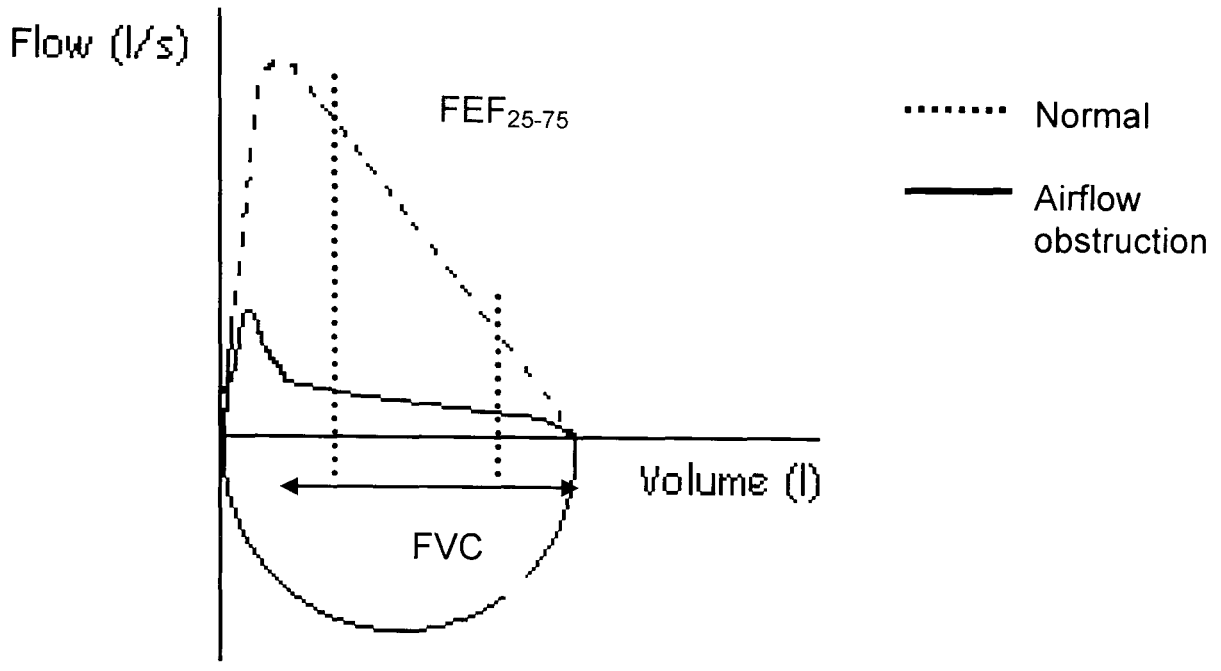
Patients underwent pulmonary function tests (PFTs) performed by clinical physiologists using standard European Respiratory Society guidelines (Miller, Crapo et al. 2005; Miller, Hankinson et al. 2005). During the test, patients were seated. A mouthpiece and nose clip prevented escape of airflow during expiration. After a few breaths, enabling the patient to relax, the patient was then asked to take a maximal breath in, followed by a hard, fast breath out to full expiration. To achieve accurate, reproducible tests the patient must ensure that the expiration is both forceful and prolonged. This test was repeated for a minimum of three and a maximum of eight times as per the American Thoracic Society/European Respiratory Society recommendations to ensure precision and reproducibility (Miller, Hankinson et al. 2005). Simple spirometry was used to give a print out of volume against time from which the FEV_1 and FVC could be taken. The FEF_{25-75} can be calculated from the volume-time graph by taking the point 25% and 75% of the vital capacity and drawing a line between the two points. The gradient of this line gives the mid expiratory flow FEF_{25-75}. This is demonstrated in Figure 2-5.

Figure 2-5: Volume –time graph for a normal subject



Legend: The gradient of the red line shows the FEF_{25-75}.

Figure 2-6: Volume –time graph for normal subject and subject with airflow obstruction.



Legend: The FEF_{25-75} is the flow rate over the mid expiratory flow range, from 25% to 75% of the forced vital capacity. From this figure it can be demonstrated that the FEF_{25-75} would be greatly reduced in a subject with airflow obstruction. Thus a drop in FEF_{25-75} is diagnostic for BOS.

2.14.1. Flow volume curves

The flow-volume curves were measured using a Collins Owl body plethysmography and Raptor software using a pneumotach to give a flow signal (Figure 2-6). These were then integrated to give volume (Table 2-4):-

FEV_1, forced expiratory volume in one second

FVC, forced vital capacity

FEV_1/FVC, the FEV_1 to FVC ratio

FEF_{25-75}, forced expiratory flow between 25% and 75% of the FVC

(Miller, Hankinson et al. 2005)

Table 2-4: Definitions of pulmonary function tests

FVC	Maximal volume of air exhaled with maximally forced effort from a maximal inspiration, expressed in litres at body temperature and ambient pressure saturated with water vapour (BTPS)
FEV_1	Maximal volume of air exhaled in the first second of a forced expiration from a position of full inspiration, expressed in litres at BTPS
FEV_1/FVC	Ratio of FEV_1 as a percentage of FVC
FEF_{25-75}*	Mean forced expiratory flow between 25 and 75% of FVC. Also known as the maximal mid-expiratory flow. This index is taken from the blow with the largest sum of FEV_1 and FVC

*Note this is highly dependent on the validity of FVC measurement and the level of expiratory effort (Miller, Hankinson et al. 2005).

2.14.2. Bronchiolitis obliterans syndrome

Bronchiolitis obliterans syndrome (BOS) scores were calculated in patients after 6 months, based on the ISHLT guidelines (Estenne, Maurer et al. 2002). (Table 1-1). In summary, BOS scores can be calculated as a drop in FEV_1 from the baseline (i.e. the best post-transplant scores) in the absence of other causes (e.g. acute rejection, infection, anastomotic stricture) (Estenne, Maurer et al. 2002). However, the post-operative PFTs often continue to rise and BOS can only be demonstrated at 6 months post-transplantation (Estenne, Maurer et al. 2002).

2.15. Methodology for analysis of gastric juice and cell stimulation experiments

2.15.1. Gastric juice

Ethical approval was obtained from County Durham & Tees Valley 2 Research Ethics Committee. Trust Research & Development approval was granted by the Newcastle Upon Tyne Hospital Trust Research & Development Department. After informed consent was obtained, gastric juice was collected from routine endoscopies both of lung transplant recipients and also from routine endoscopies on non-transplant patients on and off PPI. The sample population was heterogeneous with significant variance in demographics, pathology and individual PPI use. This method was chosen to maximise sample numbers and include all potential patients for analysis. Before use, endoscopes were thoroughly cleaned and processed in accordance with national British Society of Gastroenterology guidelines (Allison, Bradley CR et al. 2008) - manual cleaning followed by automated disinfection for 30 minutes. Enzymatic agents (Endozime) followed by Steris Hamo PAA containing peracetic acid, detergent) were used to fully decontaminate the endoscopes. Endoscopies were performed using a fibre-optic endoscope after midazolam (1-5mg) or xylocaine throat spray. Gastric juice was aspirated endoscopically from the gastric lumen and gastric juice was collected in a trapper (Pennine Healthcare, UK). Samples were then purified, by filtering and removing large food particles, before being analysed for pH, pepsin, trypsin, bile salts and microbiology. The pepsin and trypsin activity assays were described below. pH was analysed using a glass electrode and a pHmeter. Samples of gastric juice were sent to microbiology to be cultured for pathogens.

2.15.2. Pepsin activity assay

This assay was previously developed and validated (Stovold 2009). It was derived from an assay by Hutton et al (a modification of Lin et al. (Lin, Means et al. 1969)) and relied on the production of new N-terminal amino groups that are formed during proteolytic hydrolysis of the substrate succinyl albumin. The new amino groups reacted with trinitrobenzylsulphonic acid (TNBS, Fluka Biochemika, Buchs. Switzerland), generating trinitrophenyl (TNP) derivatives. These were then estimated spectrophotometrically (Hutton et al, 1986). 200µl of sample from gastric juice or standard (0-2µg) were added to test tubes in triplicate. 500µl of substrate (8mg/ml

succinyl albumin in HCl adjusted to pH 2 using 1M HCl) was added to each tube and the tubes were mixed, covered and incubated at 37°C for 1 hour. After incubation, the reaction was stopped by adding 500µl of 4% (w/v) $NaHCO_3$ followed by 500µl 0.05% (w/v) TNBS solution (0.05% trinitrobenzylsulphonic acid in deionised water). Subsequently the tubes were mixed and placed in a waterbath at 55°C for 10 minutes to allow the colour to develop. After 10 minutes 500µl of 10% sodium dodecyl sulphate (SDS, w/v) followed by 500µl 1M HCl was added and the tubes were mixed, covered and left to stand at room temperature for 1 hour. The tubes were then read on a spectrophotometer at 340nm. Negative controls were produced by adding substrate to sample immediately before the $NaHCO_3$ step (Stovold 2009).

2.15.3. Bile salt assay

These were analysed with the Biostat assay described in Section 2.13.2.

2.15.4. Trypsin activity assay

Quantification was carried out via an N-terminal assay for proteolytic activity (Sunderland 2003) with the following modifications:

For the trypsin standard (porcine, pancreatic trypsin, Fluka Biochemika), a concentration range of 0-2.5µg/ml was used, (0-0.5µg trypsin in 200µl phosphate buffered saline, pH 7.4). Negative controls contained denatured inactive trypsin having been heat-treated for five minutes in a 100°C waterbath. The trinitrobenzene sulfonic (TBNS, Fluka Biochemika) acid had to be pre-washed to remove aniline derivatives and thereby reduce its background colour. The protocol for TNBS preparation from AM Sunderland (Chapter 2, Section 6, page 42) (Sunderland 2003) was slightly modified: One and a half millilitres of TNBS was mixed with 10mg activated charcoal and centrifuged at 5000r.p.m. for 10 minutes (minispin plus, eppendorf centrifuge). Then the supernatant was simply taken off with a Pasteur pipette, not being passed through a syringe filter as stated in the protocol. The levels of trypsin in pancreatic juice are approximately 0.3mg/ml. Therefore a series of dilutions of gastric juice (1 in 10 to 1 in 50) were made assuming a range of possible levels of duodenal reflux (Sunderland 2003).

2.15.5. Microbiology

This protocol was designed, performed and written by Dr John Perry, Clinical Microbiologist Freeman Hospital. 10µl of aspirate was inoculated onto three plates of Columbia blood agar (Oxoid Ltd., Basingstoke, UK). The plates were incubated at 37°C for 72 hours in three different atmospheric conditions: in air, under strict anaerobic conditions and under micro-aerophilic conditions. The plates were examined daily and each distinct colony type was subcultured on the same medium to obtain a pure culture for further investigation. In the first instance, pure subcultures were investigated using Gram stain and simple biochemical tests including tests for oxidase and catalase. This allowed a presumptive identification and led to further analysis and identification to species-level. For example, suspect *Enterobacteriaceae* (Gram negative rods, catalase positive, oxidase negative, facultatively anaerobic) were identified by using the API 20 E biochemical kit (which comprises 20 biochemical tests). Similarly, suspect *Pseudomonas* or *Acinetobacter* were identified using the API 20 NE kit. Species characteristic of mouth flora such as *Neisseria* species and alpha haemolytic *Streptococci* were not identified to species level.

2.15.6. Cell studies

2.15.7. Goblet cells

The goblet cell line HT29-MTX, a human colon carcinoma-derived mucin secreting goblet cell line, kindly provided by Dr. Thecla Lesuffleur (INSERM U178, France) was grown in Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Sigma) at 37°C in a 10% CO_2/90% air atmosphere. For maintenance $3x10^6$ cells were seeded in a collagen coated T25 flask (Vitrogen 100; Cohesion™, USA) in 10ml of medium. The medium was changed every second day until the cells reached confluence. Once at least 80% confluence had been achieved, cells were passaged for maintenance, using trypsin 0.0125% in 0.53mM EDTA (Sigma, UK) in Ca^{2+}, Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS) (Sigma,UK). This occurred on average every 15 days. For the experiments, cells were seeded and grown in 6 and 24 well plates (Sigma,UK). 0.5ml of cell suspension, with a concentration of $9\text{-}10x10^5$cells/ml, was used in our experiments (Smirnova, Birchall et al. 2002; Stovold 2009). Cells had been

characterised in the laboratory, by their resistance to methotrexate and by the presence of secretory granules staining positive for the mucins MUC5AC and MUC5B.

2.15.8. Epithelial cells

Epithelial cells were retrieved from bronchoscopy of lung transplant recipients. Ethical approval had been obtained from the Local Research Ethics Committee and all patients gave informed consent.

2.15.9. Bronchial epithelial cell isolation and culture from brushings

Routine bronchoscopy was performed as described in Section 2.8. Single-sheathed nylon cytology brushes were used to collect bronchial brushings from subsegmental bronchi and samples were placed in Dulbecco's PBS. These suspended samples were centrifuged for 5 minutes at 1000rpm and the cell pellet was re-suspended in 2ml of basal epithelial growth medium (BEBM); Clonetics (Cambrex), San Diego, Ca, USA) together with BEGM single quots (Clonetics). Penicillin 50U/ml, streptomycin 50mg/ml, gentamicin 50mg/ml and amphotericin B 50ng/ml were the final antimicrobial concentrations in the culture medium throughout the process (Forrest, Murphy et al. 2005). Cells were characterised in the laboratory by the identification of epithelial markers including cytokeratin.

Cell suspensions were put in to a $25cm^2$ dish pre-coated with collagen (Vitrogen 100, cohesion, Palo Alto CA, USA) and placed in a carbon dioxide incubator ($37^oC/5\%$ CO_2). After the first 48 hours a further 3ml of supplemented medium was added with subsequent exchanges every 48 hours, until the primary bronchial epithelial cells (PBECs) reached confluence. Once confluent, PBECs were passaged using trypsin, which was neutralised using an equal volume of RPMI supplemented with 10% of FCS. PBECs were then put into 10ml of culture medium to Vitrogen (Cohesion) coated $75cm^2$ flasks or to eight chamber slides (Lab-Tek, Nunc, Naperville, IL, USA: Chamber-1). These were cultured to 80-95% confluence (Aseeri 2007; Brodlie 2009; Stovold 2009). 0.5ml of cell suspension, with a concentration of $7\text{-}8x10^5$cells/ml, was used in our experiments (Smirnova, Birchall et al. 2002; Stovold 2009).

2.15.10. Cell passage

As cells neared confluence in cell culture dishes, passage was performed. 2.5ml of trypsin was added and incubated at 37°C for 2-4minutes, then 5-10ml of RPMI media was added to re-suspend cells. Cells were centrifuged for 5 minutes at 1000rpm to create pellets. 12ml of epithelial media was subsequently added and mixed gently. 24 well plates were then seeded with 0.5ml of cell suspension per well (Stovold 2009).

2.15.11. Cell stimulation

Once cells had reached 80-95% confluence on the 24 well plates, they were rested for 24 hours with the addition of serum free medium (BEBM, penicillin, streptomycin, gentamicin without singlequots). Cells were subsequently stimulated with pepsin/gastric juice in resting media.

Goblet and epithelial cells were stimulated with porcine pepsin at concentrations of 25µg/ml, 50µg/ml, 100 µg/ml in 500µl DEMEM serum free, Sigma, UK or BEBM, without singlequots, Lonza, Switzerland respectively). For both goblet and epithelial cells the experiments were carried out on two repeated cultures with five repeated wells, giving an overall experiment number of n=10. Goblet cells were incubated for 72 hours; epithelial cells were incubated for 48hours. Control stimulations were constructed by incubating cells in dilutant vehicle alone (resting serum free medium). Samples were analysed for viability at 48 hours, as it was felt if cells had not experienced significant death, then cells would be unlikely to die between 48-72 hours. The lack of viability data at 72 hours would not affect the interpretation of the IL-8 and MUC5AC results. Media was collected for IL-8 and MUC5AC measurements at 24 and 48 hours from epithelial cell culture. Media was collected for IL-8 and MUC5AC measurements at 24, 48 and 72 hours from goblet cell culture.

Initially we endeavoured to stimulate both goblet and epithelial cells with gastric juice. The goblet cell lines were infected due to contamination in the incubator and we were unable to carry out these experiments.

Epithelial cells were then stimulated with gastric juice from transplant and non-transplant patients with dilutions 1/1,000 to 1/10,000 (gastric juice: DEMEM) in 500µl DEMEM serum free, Sigma UK or BEBM, without singlequots, Lonza Switzerland respectively) for 24 hours. Three samples were chosen. Sample one was

chosen as it was from a lung transplant recipient with low pH, high pepsin and bile salt levels and bacterial colonisation. Samples two and three were from non-transplant patients. Sample two was chosen as it had a high pH and was colonised with bacteria and fungi. Sample three was chosen as it had a low pH and no bacterial colonisation. These three samples were used to see if they would cause different effects on the epithelial cells. Control stimulations were produced by incubating cells in dilutant vehicle alone (resting serum free medium). These epithelial cell experiments were carried out on one culture with seven repeated wells, giving an overall experiment number of n=7. Epithelial cells were analysed for viability at 24 hours and media was collected for IL-8 production at 24 hours. These were analysed at this time point as PBECs in previous experience are more susceptible to damage compared to cell lines.

2.15.12. Interleukin-8

The IL-8 concentrations were measured using a commercial ELISA described earlier (2.13.3).

2.15.13. Mucin MUC5AC

100µl of standards diluted in PBS or 20µl of sample, added to 80µl of PBS were added to coat a 96 well microplate Maxisrop, Nunc). The plate was sealed and incubated overnight at room temperature. Each well was aspirated and washed with 400µl wash buffer (0.05% Tween 20 in PBS pH 7.2-7.4, Sigma, UK) repeating the process twice for a total of three washes. The plate was then blocked by adding 300µl of block buffer (1% bovine serum albumin in PBS) to each well and incubated at room temperature for 1 1/2 hour. Aspiration and wash were repeated. Primary antibody (antiMUC5AC (NCL-H^M-45MI), Sigma, UK) was diluted to working concentration (1 in 150) in reagent buffer (0.1% BSA, 0.05% Tween 20, Sigma, UK, and 100µl was added to each well. The plate was then covered with parafilm and incubated for 2 hours at room temperature. Aspiration and wash were repeated. 100µl of the secondary detection antibody (horse radish peroxidase-conjugated anti-goat antibody, Sigma, UK), diluted in reagent dilutant (1 in 10,000), was then added to each well. This was then covered with parafilm and incubated for 2 hours at room temperature. Aspiration and wash were repeated. 100µl of substrate solution (2.2`-Azino-bis(3-ethylbenzothiazoline-6-) sulfonic acid) was then added to each well. This was incubated for 30 minutes at room temperature, avoiding direct light. 100µl of stop

solution (1% sodium dodecyl sulphate) was added to each well. The plate was gently tapped to ensure thorough mixing. Optical density of each well was determined immediately using a microplate reader set to 405nm (Stovold 2009). Negative controls were also analysed. These samples were analysed identically apart from omitting the primary antibody.

2.15.14. Viability assay

This assay has been described by Stovold (Stovold 2009). The viability of both the goblet and epithelial cells was measured using the Cell Titerblue assay (Promega, Madison, WI, USA).

This assay relies on the reduction reactions in the viable cell reducing resazurin, a dark blue compound in the Titerblue reagent, to resorufin which is pink. Resorufin has an absorbance maximum of 573nm compared to resazurin, 605nm. Viability is based on a ratio of these two absorbances OD573/D605. The higher the ratio, the greater number of viable cells.

Challenge media was removed from the cells and stored at -20°C for further analysis. Titerblue reagent (Sigma, Gillingham, UK), was mixed directly with the goblet and epithelial cell media (20µl TiterBlue for every 100µl DMEM, Sigma UK or BEBM, Lonza Switzerland) and the cells were incubated under standard conditions for 2-4h. Absorbance was then measured at 560nm on a spectrophotometer. Negative controls were also performed by fixing cells for 10 minutes in ice-cold methanol prior to adding the Titerblue reagent. As dead cells have no reducing potential, the reagent should not change colour, indicating that nothing present in media alone is responsible for the colour change (Stovold 2009).

A second basic method was used for several experiments. This was based on a Trypan blue (Sigma, UK) stain. After removal and storage of media, 10ul of 0.4% Trypan blue was added to the cell culture for 2 minutes. One hundred cells were counted and it was recorded how many of these were stained with the dye. Viable cells exclude Trypan blue, remaining clear, whereas dead cells take up the dye and are stained blue.

2.16. Statistical analyses

The relevant statistical analyses were carried out using Graphpad Prism 4.0 (San Diego, CA, USA). Due to the small sample sizes non-parametric tests were predominantly used. In chapter 3, the analyses were performed using non-parametric Spearman rank correlation tests and non-parametric Mann-Whitney, unpaired t-tests. In chapters four and six, the analyses were performed using non-parametric Wilcoxon paired t-tests. In chapter 5, the statistical analyses of the results of the gastric juice samples were performed using Mann-Whitney analysis and Fisher's exact test. The statistical analyses of the cell stimulation experiments were performed using non-parametric one-way analysis of variance with a post-hoc Mann-Whitney analysis. Comparison of the cell viability tests was performed using a Bland-Altman analysis.

3. *Identification of gastro-oesophageal reflux and aspiration in the first month post-lung transplantation*

3.1. Abstract

Background

Chronic allograft dysfunction occurs frequently in lung transplant recipients. Reflux and asp iration may occur post-lung transplant and may be injurious to the allograft. Nothing is known about the prevalence of GORD and aspiration in the first month post-transplant.

Aims

This study aimed to identify gastro-oesophageal reflux and aspiration in the first month post-lung transplantation.

Methods

Lung transplant recipients were recruited over a 12 months period (November 2007-October 2008). At approximately one month post-transplantation, patients completed a Reflux Symptom Index (RSI) questionnaire for symptoms of extra-oesophageal reflux and underwent objective assessment for reflux (manometry & pH-impedance). Testing was performed with subjects on maintenance PPI. BALF was assessed for pepsin and bile salts, IL-8 and neutrophils. Microbiology samples, rejection scores and PFTs were analysed.

Results

18 patients with a median age of 46years (range 22-59) were studied. Manometry was abnormal in 8/18 (44%) patients. 12 of 17 (71%) had evidence of reflux on pH-impedance. 25% of patients had exclusively weakly-acidic reflux. A weak correlation existed between RSI score and proximal reflux events. Pepsin was detected in 11/15 BALF samples signifying gastric aspiration (median 18ng/ml, range 0-43). Bile salts were rarely detectable, using spectrophotometry/dual mass spectrometry (2/15) [sensitivity 0.1μmol/l]. (One of these was just above the level of detection). BALF IL-8 (1,057pg/ml Range 156-15,559) and neutrophil levels were elevated (11% Range 1-63%). A correlation existed between number of proximal reflux events and BALF neutrophilia (Spearman Correlation r=0.52, p=0.03).

Conclusion

Reflux/aspiration is prevalent early post-operatively and proximal reflux events correlate with BALF neutrophilia, which is linked to allograft dysfunction and mortality.

3.2. Introduction

It has been demonstrated that chronic aspiration, secondary to extra-oesophageal reflux, may contribute to BOS and up to 75% of patients may have demonstrable gastro-oesophageal reflux disease (GORD) following lung transplantation (D'Ovidio and Keshavjee 2006; King, Iyer et al. 2009). Anti-reflux surgery may be associated in this population with an increased survival and improved lung function (Hartwig, Appel et al. 2005). More recent data stress the role of early fundoplication in preventing the development of BOS (Cantu, Appel et al. 2004).

Most studies of reflux have either been pre-transplantation or at least 3 months post-transplant. None have assessed recipients for reflux in the immediate post-transplant period. If the aspiration contributing to BOS begins early post-transplant, then an important question is whether reflux and aspiration are present within the first month post-transplant.

This chapter aimed to identify gastro-oesophageal reflux and aspiration in the first month post-lung transplantation.

3.3. Methods

Patients undergoing lung transplantation at the Freeman Hospital were studied at one month post-transplant to test for the presence of GORD. Their lung allografts were under standard surveillance using bronchoscopy, bronchoalveolar lavage samples and pulmonary function tests. From 1st November 2007 to 1st November 2008 all newly transplanted lung recipients were approached to be recruited into the study.

Our protocol was to assess for GORD at one month post lung transplantation, using a validated extra-oesophageal reflux questionnaire, manometry and pH/impedance measurements. These assessments were performed around similar time periods as bronchoscopy and pulmonary function tests. However exact practice was tailored to suit individual patients. Patients were assessed on their routine proton pump inhibitor therapy. Results were then compared with markers of aspiration and inflammation in the bronchoalveolar lavage samples, microbiology, pathological rejection scores and pulmonary function tests as described in chapter two.

3.4. Results

3.4.1. Demographics

Forty five patients received lung transplants between October 23rd 2007 and October 23rd 2008. Forty patients were approached to participate (five patient died in ITU). Twenty three patients agreed to participate, seventeen patients declined to take part. Of the initial 23, 5 patients dropped out, one as he was afraid of the test, another could not tolerate manometry, and three gave no reason. Eighteen patients were therefore studied (Figure 3-1) (12 women, 6 men) with a median age of 42 years (range 22-59 years). Indications for transplant were cystic fibrosis (10), lymphangioleiomyomatosis (2), severe asthma (1), asthma/COPD (1), asthma/pulmonary fibrosis (1), COPD (2), Histiocytosis X (1). 13 patients had suppurative lung disease at the time of transplant. Demographics are shown in Table 3-1.

Figure 3-1: Consort diagram of patient recruitment

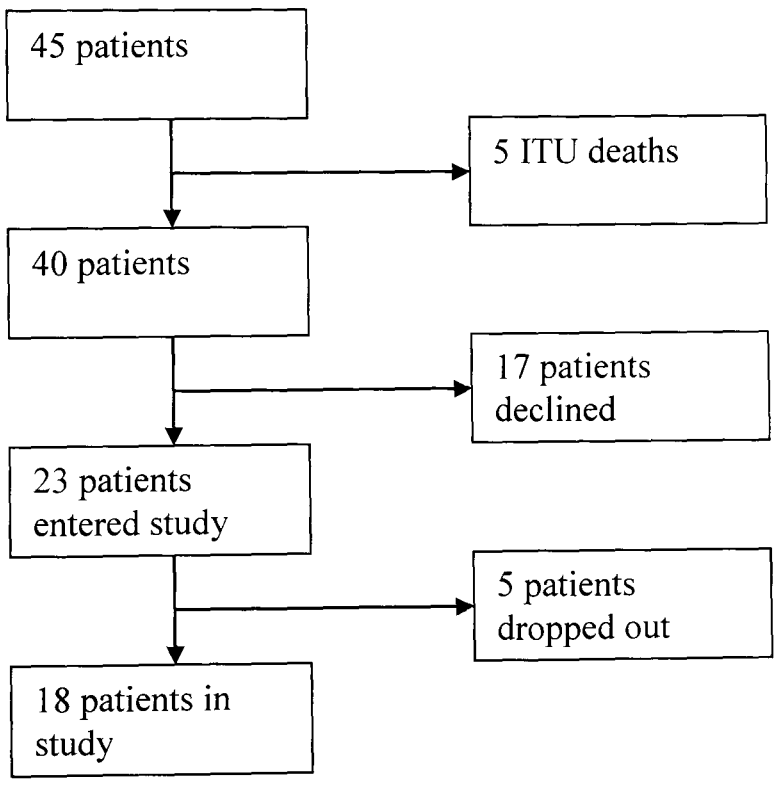


Table 3-1: Demographics of study patients

Age -Median -Range	 42 years 22-59 years
Sex -Male -Female	 6 12
Underlying pathology -Cystic fibrosis - Lymphangioleiomyomatosis -COPD -COPD/asthma -Severe asthma -Pulmonary fibrosis/asthma -Histiocytosis X	 10 2 2 1 1 1 1
Transplant -SSLT -LSLT -RSLT -HLT	 15 1 2 0

Legend: SSLT= Single sequential lung transplant, LSLST= Left single lung transplant, RSLT= Right single lung transplant, HLT= Combined heart lung transplant.

3.4.2. Immunosuppression

All patients were treated with a combination of cyclosporin/tacrolimus; mycophenolate mofetil/azathioprine; prednisolone. No patients were given azithromycin during this study.

3.4.3. Pre-operative diagnoses of GORD

Six patients have had pre-operative diagnoses of GORD. One of which had this confirmed by pH study and had pre-operative Nissen's fundoplication.

3.4.4. Proton pump inhibitor therapy

100% of patients were started on proton pump inhibitors. The various medications and doses are listed in Table 3-2.

Table 3-2: Proton pump inhibitor therapy on recruitment to the study

lansoprazole 30mg od	12
lansoprazole 15mg od	1
lansoprazole 30mg bd	2
omeprazole 20mg od	1
omeprazole 20mg bd	1
rabeprazole 20mg od	1

3.4.5. Oesophageal manometry

18 patients underwent oesophageal physiology tests within the first month post-transplant. Overall 44% (8/18) had abnormal oesophageal physiology. No complications were attributed to manometry or pH/impedance monitoring. Manometry was performed approximately one month post transplant.

Lower oesophageal sphincter

The median lower oesophageal sphincter length was 2.75cm (2-5.25cm). Sphincter pressure was normal in the majority of patients (14/18) with an average sphincter pressure of 23mmHg (Range 9.4-91.1mmHg). One had a hypotonic sphincter and three had a hypertonic sphincter. The median LOS nadir pressure was 1.2mmHg (Range -12.3 to 21.7) with a median percentage relaxation of 93.3% (Range 69.9-100%).

Oesophageal peristalsis

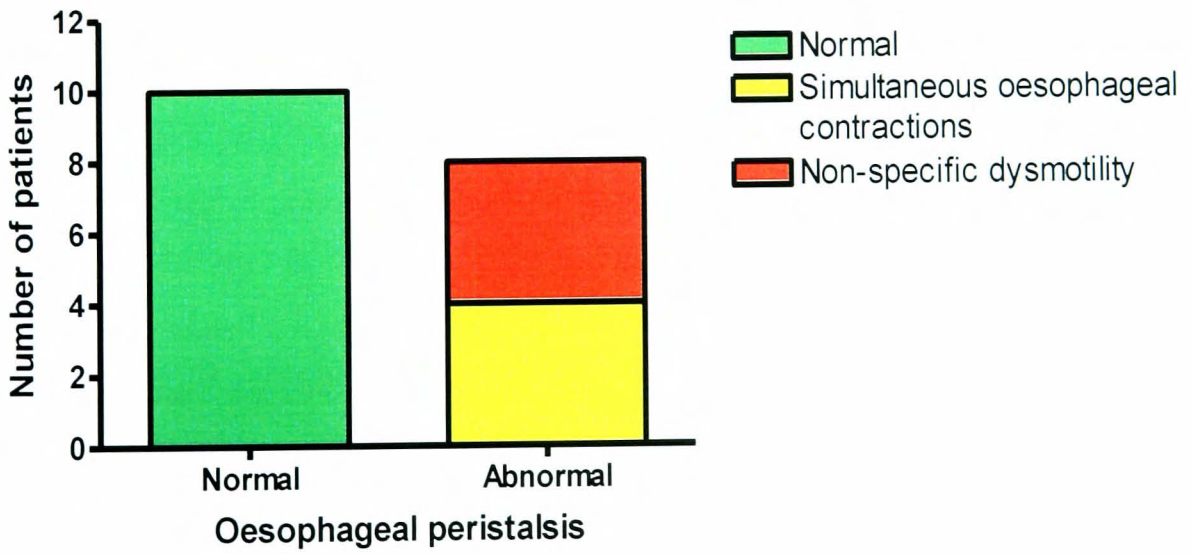
The median percentage of normal swallows was 90% (Range 0-100%). In total ten patients had normal peristaltic activity (one had hypertonic oesophageal peristalsis, characterised by high pressure oesophageal peristaltic amplitudes), four patients had ineffective oesophageal motility (two mild, one severe and one had an aperistaltic oesophagus), four patients had simultaneous oesophageal contractions in >20% of swallows (Figure 3-2).

Table 3-3: Oesophageal peristaltic amplitudes

	Median (mmHg)	Range (mmHg)	Normal Values (mmHg)
Maximum oesophageal Amplitude	156.2	58.3-602.7	
Minimum peristaltic amplitude	18.75	0-54.5	
Average peristaltic amplitude	67	29.3-303.5	30-180
Distal oesophageal amplitude (5cm above the lower oesophageal sphincter)	64.9	26.3-482.6	30-180
Proximal oesophageal amplitude (15cm above the lower oesophageal sphincter)	58.9	12.1-128.6	30-180

Median peristaltic amplitudes are shown in Table 3-3. One of eighteen patients had a hypotonic distal oesophagus, fifteen had a normotonic distal oesophagus and two had a hypertonic distal oesophagus. Four of eighteen had a hypotonic proximal oesophagus, fourteen had a normotonic proximal oesophagus and none had a hypertonic proximal oesophagus. All four patients with a hypotonic proximal oesophagus had a normotonic distal oesophagus.

Figure 3-2: Oesophageal peristalsis



3.4.6. *Reflux data*

18 patients underwent assessments for reflux post-lung transplant. One patient had their probe placed too distally. This was apparent after analysis of the tracing. The other seventeen were therefore analysed. 71% (12/17) had pathological GORD.

Reflux symptom index scores

Five patients had positive reflux symptom index (RSI) scores. Twelve patients had negative RSI scores. Median RSI score was 10 (range 0-32). Three patients with a positive RSI had pathological proximal reflux; two patients with a positive RSI had no pathological proximal reflux. Five patients with a negative RSI score had abnormal proximal reflux and seven patients with a negative RSI had proximal reflux within normal limits (<17) (Table 3-4). A breakdown of scores is shown in Appendix 9.

Table 3-4: The predictive value of the RSI score

	Proximal reflux	No proximal reflux	
RSI positive	3	2	PPV=60%
RSI negative	5	7	NPV= 58.3%
	Sensitivity= 38%	Specificity= 78%	

PPV= Positive predictive value, NPV=Negative predictive value

pH-impedance results

All seventeen patients successfully underwent 24 hour recordings. 12 of 17 (71%) patients had pathological distal reflux as determined by either an abnormal acid exposure or oesophageal volume exposure. A summary of median reflux indices is shown in Table 3-5. The patient with pre-operative fundoplication had no reflux. Of the 12 with reflux nine had evidence of acid and weakly acid reflux; three had exclusively weakly acid reflux (25%) (Figure 3-3). Eight of the seventeen had abnormal proximal oesophageal reflux (47%). Of these eight, seven had evidence of distal reflux and one had no evidence of pathological distal reflux. Most reflux events were in the upright position 66 (25-130) versus 11 (1-37) supine. This was true of proximal reflux events (upright 15 (3-47) versus supine 1 (0-17)).

Table 3-5 Key pH/Impedance results

	Median	Range	Normal values	Number of patients with abnormal results
Acid exposure (%) (percentage of time that pH <4, 5cm above the LOS in 24hrs)	**4.8**	**1-79.9**	**<4.5**	**9/17**
Oesophageal volume exposure (%)(percentage of time that impedance detects refluxate within the oesophagus in 24hrs)	**1.6**	**0.71-5.48**	**0.4-1.2**	**12/17**
No of reflux events	**72**	**27-147**	**(25-58)**	**12/17**
-Acid reflux events	**25**	**2-90**	**(10-35)**	**7/17**
-Weakly acid reflux events	**38**	**5-140**	**(5-18)**	**12/17**
-Non acid reflux events	**0**	**0-3**	**(1-7)**	**0/17**
Bolus clearance time (secs)	**15s**	**8-26.5s**	**(8-13)**	**11/17**
Proximal reflux events	**17**	**4-54**	**(4-17)**	**8/17**

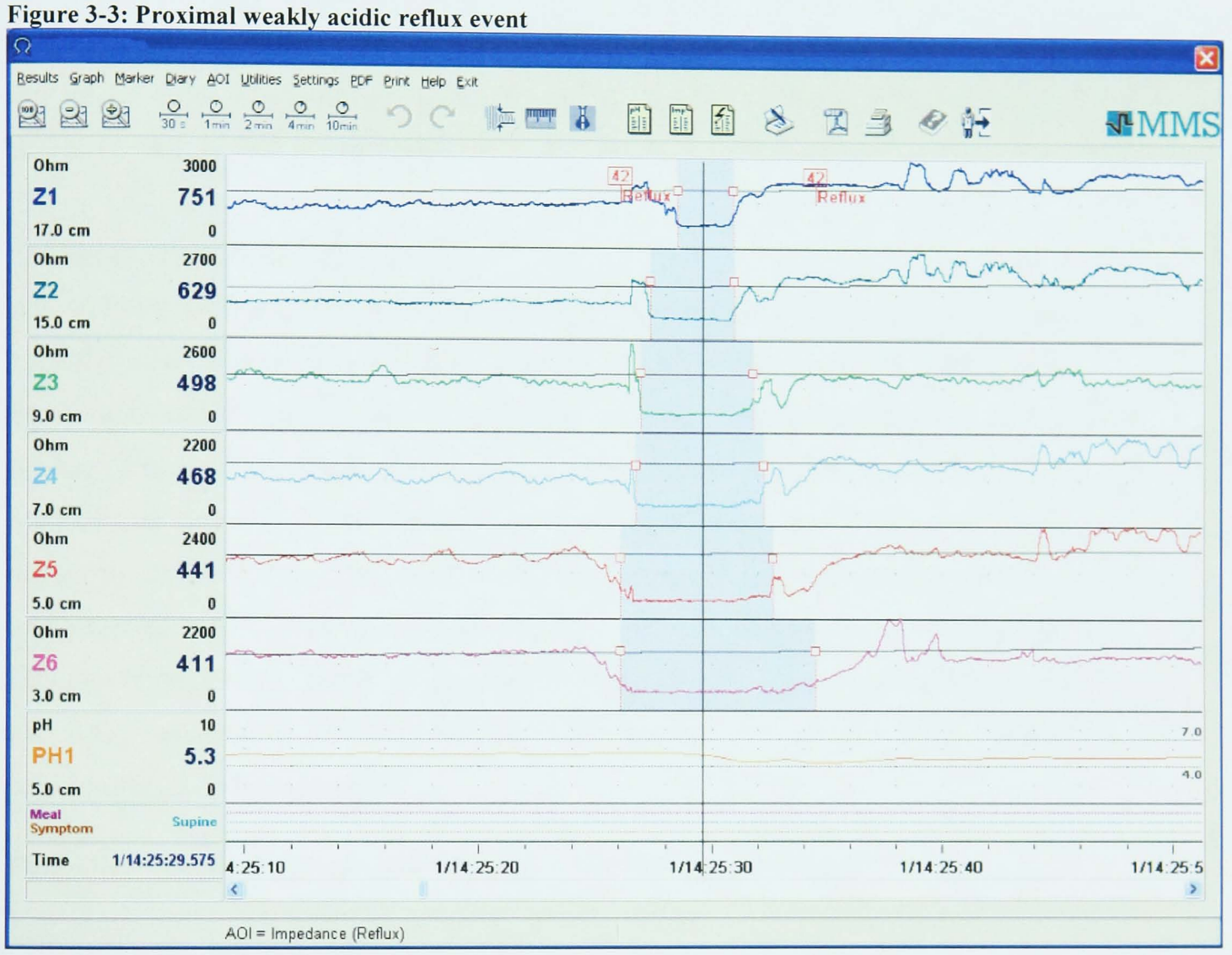
Aspiration

Pepsin was detected in 11/15 (73%) BALF samples- median 18ng/ml (range 0-43). 4/15 BALF samples had no pepsin. When compared to our normal controls (these were historical BALF samples collected from four healthy volunteers at bronchoscopy) median 5.5 (range 0-12.6ng/ml) the median from our current sample was higher. This was not statistically significant (p=0.1). Using 2 enzymatic bile salt assays no bile could be detected in 15/15 samples. Using a more sensitive tandem mass spectrometry with a lower limit of detect of 0.1μmol/L, we could detect conjugated bile salts in 2/15 of the lavage samples. One of these was just above the lower level of detection 0.2μmol/L. Four "normal" BALF samples showed no evidence of bile salts at a lower limit of detection.

Consequently we re-analysed the BALF samples after extraction which gave an increased minimum levels of detected of 0.001μmol/l. All 15 samples now showed detectable bile salts with the highest bile salt concentration present being 1.19μmol/l. The median value for bile salts in the 15 patients was 0.049μmol/l, which considering that normal serum levels range from 0-10μmol/l and taking into account the 180ml of

saline used to collect approximately 1ml of lung bathing fluid, then values up to 0.056µmol/l would be within the normal range. Only 2/15 patients had abnormal levels of bile salts in their BALF. Four normal controls were analysed with a median bile salt concentration of 0.009µmol/l (range 0.005-0.011µmol/l).

Figure 3-3: Proximal weakly acidic reflux event



Legend: This figure is from an actual study patient trace demonstrating an asymptomatic proximal weakly acidic reflux event. The sequential drop in impedance from channel Z6 to Z1 shows that the event reaches 17cm above the lower oesophageal sphincter. The pH (bottom trace) does not drop below 4, indicating that this is a weakly acidic event. The symptom button has not been pressed, suggesting this event was not noticed by the patient.

No correlation existed between RSI and distal reflux indices. A correlation existed between RSI and proximal reflux events (r=0.533, p=0.006). However the RSI failed to significantly predict or exclude proximal reflux in patients (Table 3-4).

Manometry to reflux indices

A statistically significant negative correlation existed between LOS pressure and total impedance reflux events (n=17) (p=0.03, r=-0.52) and LOS pressure and oesophageal acid exposure (p=0.02, r=-0.55) (n=17).

Evidence of GORD and aspiration

Interleukin 8 was detected in 15/15 samples. Median levels (1,057pg/ml (range 156-15,559)) were greater than reported normal controls (median 27.5pg/ml (range 8.7-84.6)) and stable lung transplant recipients (median 558pg/ml (range 36-1076)) (Zheng, Walters et al. 2000). No correlation existed between reflux indices/aspiration markers and BALF IL-8 levels or IL-8 and neutrophil levels (Table 3-6). Cell counts are shown in Table 3.6. There were increased percentages of neutrophils, eosinophils and macrophages but decreased lymphocytes when compared to stable controls (Zheng, Walters et al. 2000). A correlation existed between proximal reflux events and BALF neutrophils (n=13) (r=0.52, p=0.03) (Figure 3-4). No correlation existed between reflux indices and PFTs.

Figure 3-4: Correlation between proximal reflux events and neutrophil counts

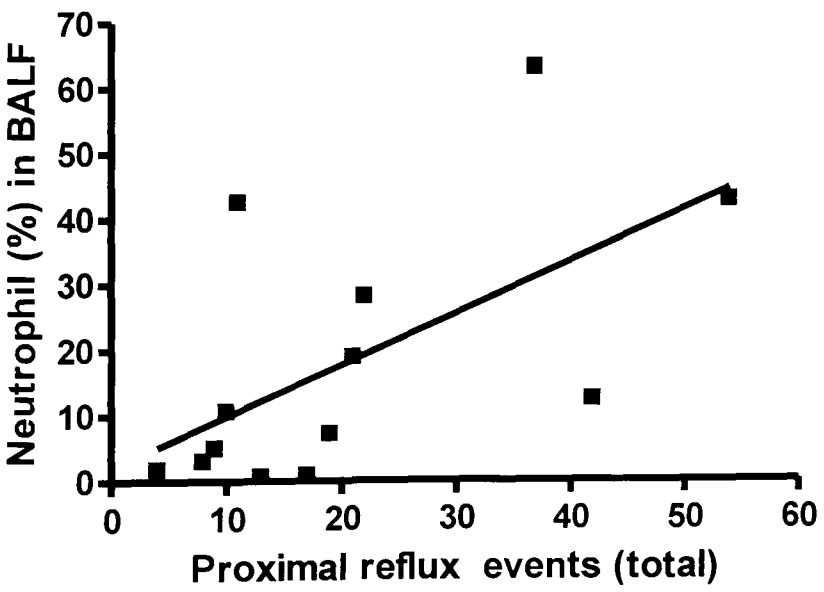


Table 3-6: The median total cell and neutrophil count and IL-8 count

	1 month	Normal values (Zheng. Walters et al. 2000)
Total BAL cell count (cellsx10^4/ml)	15.3 (1.04-68)	14 (12-16)
Neutrophils (%)	11 (0.6-63.2)	2.1 (1.6-2.6)
Lymphocytes (%)	5 (0-52)	20 (14-26)
Macrophages (%)	82.5 (20.2-97.8)	73 (66-80)
Eosinophils (%)	5 (0-52)	1.1 (0-2.2)
Interleukin 8	1057pg/ml (156-15559)	27.5pg/ml (8.7-84.6)

There was no significant difference in reflux indices in cystic fibrosis patients when compared to non cystic fibrosis patients, nor those with BALF colonisation when compared with non-colonised patients. Patients with or without A2 rejection had similar reflux indices.

3.5. Discussion

The main findings of this study are that aspiration is prevalent within the first month post-lung transplant. GORD as detected by pH-impedance was prevalent at one month post-transplant. Thirdly, there is a correlation between proximal reflux and BALF neutrophilia as shown in Figure 3-4.

The relationships learned from these findings are that GORD occurs frequently within the immediate post-lung transplant period and this is associated with elevated pepsin with the BALF, signifying aspiration. The correlation between proximal reflux and neutrophilia suggests that increased proximal reflux leads to increased aspiration causing allograft inflammation and damage. This adds more weight to the theory that patients with increased proximal reflux aspirate and injury their lungs.

Only one previous study has evaluated oesophageal physiology in the post-lung transplant population and shows oesophageal dysfunction to be common (Davis, Shankaran et al. 2010). There is a high prevalence of foregut motility problems in patients with end-stage lung disease (Cantu, Appel et al. 2004; D'Ovidio, Singer et al. 2005; Sweet, Herbella et al. 2006). This present study evaluated oesophageal manometry post-lung transplant and show almost half of the patients had oesophageal dysmotility which is in keeping with previous work. The high prevalence of oesophageal dysmotility may be related to the high prevalence of pre-transplant dysmotility, vagal damage or secondary to GORD and subsequent oesophageal injury.

The RSI has been shown to be useful in predicting LPR in the non-transplant population. In this study, the RSI correlated with reflux indices suggesting that the RSI score may be a surrogate marker of extra-oesophageal reflux. One difficulty with its use may be the fact that many of the symptoms- cough, hoarseness, and breathlessness- may be attributed to pulmonary pathology as well as extra-oesophageal reflux. This may suggest a need for further evaluation using other questionnaires specifically developed for the transplant population. In the lung transplant population, the RSI questionnaire could not predict nor exclude reflux or aspiration. Other methods for assessing reflux and microaspiration are required.

A previous study has shown increased prevalence and severity of GORD post lung transplantation with up to 75% of patients having demonstrable reflux on pH

monitoring (Young, Hadjiliadis et al. 2003). This post-transplant level of GORD is similar to our findings with pH-impedance. In other studies, using pH-impedance, almost 30% of patients had exclusively weakly-acidic reflux (Blondeau, V. Mertens et al. 2008; King, Iyer et al. 2009). These are similar to our results where 25% of patients had exclusively weakly-acidic reflux.

GORD is associated with worse pulmonary function tests in the post-transplant population (Hadjiliadis, Duane Davis et al. 2003; King, Iyer et al. 2009). One study showed a negative correlation between FEV_1 measurements and distal oesophageal acid exposure (Hadjiliadis, Duane Davis et al. 2003). This study had a longer follow up period(median 558days) and this may explain why no significant changes were detected in the current study. Proximal oesophageal reflux was associated with decreased lung function (Hadjiliadis, Duane Davis et al. 2003) and increased non-acid reflux, as detected by pH-impedance. This has been associated with increased levels of BOS (King, Iyer et al. 2009). The present study suggests that proximal reflux leads to lung injury via aspiration.

Bronchoalveolar lavage pepsin levels have been shown to be higher in the transplanted population suggesting gastric aspiration (Ward, Forrest et al. 2005; Blondeau, V. Mertens et al. 2008). The highest levels were present in patients with acute rejection (Stovold, Forrest et al. 2007). A recent study revealed a correlation between pepsin levels and BAL neutrophil levels- a marker of injury (Blondeau, V. Mertens et al. 2008). The present study shows pepsin to be an early marker of aspiration, detectable at one month. When compared to previously reported normal controls (stable lung transplant recipients), the median from the present study was higher, suggesting aspiration.

Other studies have discovered bile salts in the BALF and shown high levels to be associated with early onset BOS (D'Ovidio, Mura et al. 2005; Blondeau, V. Mertens et al. 2008). A major finding from this study is the rarity of bile salts in the BALF in the immediate post-transplant period. This rarity may have clinical indications. If biomarkers develop a role in the indications for surgery, pepsin may be a better early marker of aspiration and injury.

The elevated levels of the pro-inflammatory cytokine IL-8 suggests that there may be associated injury at this time point. Neutrophils contribute to chronic rejection and

elevated BALF neutrophilia has been associated with mortality (Zheng, Walters et al. 2000). The correlation in our study between proximal reflux events and BAL neutrophilia suggests that reflux is deleterious to the allografts.

There are several limitations to this study. These include the small numbers in our cohort. A large sample base would allow stronger conclusions to be made. The short-term follow up to this study and small numbers prevent it from proving a link between GORD and BOS which only develops after 6 months.

Most patients had pathological GORD at one month but the amount of acid suppression is unknown, as is the effect that this has on reflux. Factors influencing the efficacy of PPI therapy to suppress acid reflux include a lack of compliance, genetic variation, drug metabolism and *Helicobacter* pylori infection (Bredenoord and Smout 2008). In this study, the effects of these complex factors is unknown. It would have been interesting to assess those patients both on and off PPI to assess the differences PPI would make.

Further limitation lies with the analysis of biomarkers of aspiration. A greater volume of saline was used to carry out BAL than in previous studies (180ml versus 100ml). Secondly, the present results were assessed at a different time and it may take time for bile salts to accumulate in the lung. Assay variability is a problem and could further influence results. A consensus is required over how to measure biomarkers so studies can be compared (Robertson, Shenfine et al. 2009). This may explain the variation between bile salt levels reported in this study and in previous papers. However, this study suggests the assays used in previous studies are inaccurate.

4. *Longitudinal changes in gastro-oesophageal reflux and aspiration in the first six months post lung transplantation*

4.1. Abstract

Bac kground

Longitudinal reflux and aspiration data is lacking in lung transplantation. This study was undertaken to assess the changes over the first six months post transplant.

Aim

The aim of this chapter was to analyse the longitudinal changes in reflux and aspiration in the first six months post-transplant.

Methods

Within the first 6 months post-transplantation, patients completed a Reflux Symptom Index (RSI) questionnaire for symptoms of extra-oesophageal reflux and underwent objective assessment for reflux (manometry & pH/impedance). Protocol was to undergo testing at one, three and six months. Patients were assessed on maintenance PPI. BALF was assessed for pepsin and bile salts.

Results

Over the first six months there was an increase in reflux indices. Nine patients underwent assessment at one and six months. At one month 5/9 patients were positive for reflux at six months 8/9 were positive. Despite decreases in immunosuppression and normalising lung function there was a trend to increase in reflux parameters over the first six months post-transplant. Aspiration determined by pepsin in the BALF decreased over the first six months.

Conclusion

Reflux/aspiration is prevalent early post-operatively and there was an increase in reflux indices over the first six months. Some patients who were free from reflux at one month developed reflux at six months. This occurred despite decreases in immunosuppression and no deterioration in lung function. In several patients an increase in reflux parameters mirrored increased immunosuppression. Despite this, aspiration decreased over the first six months, suggesting an improvement in the defences against aspiration.

4.2. Introduction

The previous work of this thesis has shown that reflux is common post-transplant and that it is associated with elevated pepsin, a marker of aspiration and injury. This was also associated with evidence of allograft inflammation. These are prominent findings. It is important to assess whether this situation will improve, remain constant or deteriorate after this time point. If they remain similar or deteriorate, then they remain an issue for the allograft. If they improve, then this is unlikely to be a major problem. Longitudinal reflux data is currently lacking, and acceptable diagnostic tests are required (Sweet, Patti et al. 2009).

Our original intent was to assess patients at three time points, one month, three months and six months. This was universally unpopular and thus the data presented assesses patients at two time points- the first month and at six months post-transplant.

The aim of this chapter was to analyse the early changes in reflux and aspiration in the first six months post-transplant.

4.3. Methods

Ethical approval was obtained. Patients undergoing lung transplantation at the Freeman Hospital were studied in a longitudinal manner to test for the presence of reflux. Their lung allografts were under standard surveillance using bronchoscopy, bronchoalveolar lavage samples and pulmonary function tests. From 1st November 2007 to 1st November 2008 all newly transplanted lung recipients were approached to be recruited into the study.

Patients were assessed for GORD and aspiration at one and six months post lung transplantation, using a validated extra-oesophageal reflux questionnaire, pH/impedance measurements and BALF analysis. These assessments were performed around similar time periods as pulmonary function tests. Tests were performed with patients on their routine proton pump inhibitor. A detailed description of materials and methods can be found in chapter two.

4.4. Results

Participation was difficult as many patients did not tolerate multiple pH-impedance measurements. Twenty two patients refused any measurements, five patients underwent one pH-impedance test, eight patients underwent two pH-impedance tests and only four patients underwent three pH-impedance tests.

Seventeen patients underwent assessments of reflux at one month. Nine patients underwent repeat assessments of reflux at one and six months. No patient underwent changes in PPI therapy between longitudinal measurements. Their details are shown in Table 4-1 and Table 4-2.

Table 4-1: Demographics of patients who underwent repeat assessments of GORD

Patient	Age	Sex	Indication for transplant	Type of transplant	Proton pump inhibitor therapy
1	29	F	CF	SSLT	Lansoprazole 30mg bd
2	25	F	CF	SSLT	Lansoprazole 30mg bd
3	46	F	COPD	RSLT	Omeprazole 20mg bd
4	32	F	CF	SSLT	Lansoprazole 30mg od
5	42	M	COPD/asthma	SSLT	Lansoprazole 30mg od
6	46	M	Histiocytosis X	SSLT	Lansoprazole 30mg od
7	29	M	CF	SSLT	Lansoprazole 30mg od
8	49	F	COPD	SSLT	Lansoprazole 30mg od
9	46	M	CF	SSLT	Lansoprazole 30mg od

Table 4-1 Key: CF= cystic fibrosis, COPD= chronic obstructive pulmonary disease, SSLT= single sequential lung transplant, RSLT= right single lung transplant

Table 4-2: Longitudinal data on immunosuppression, lung function and GORD, from one to six months post-lung transplant

	Immunosuppression			Lung Function					
Pat	1 month	6 month	Sum	1 mo FEV1	6 mo FEV1	1 mo FVC	6mo FVC	1mo Ratio	6 mo Ratio
1	Aza 100mg Pred 10mg Tacro 4/4	Aza 100mg Pred 10mg Tacro 4/3	+/-	2.14	2.76	2.2	2.79	96	98.9
2	Aza 100mg Pred 10mg Tacro 5/4	Aza 100mg Pred 10mg Tacro 5/4	+/-	1.8	2.5	2.08	3.47	87	72
3	MMF 2160mg bd Pred 30mg Tacro 4/4	MMF 1080mg bd Pred 10mg Tacro 4/3	-	1.34	1.28	1.93	2.12	69	60.4
4	MMF 1080mg bd Pred 50mg Tacro 5/5	MMF 720mg bd Pred 10mg Tacro 4/3	-	0.94	2.39	1.32	2.98	71	80.2
5	Aza 100mg Pred 20mg CyA150/150	Aza 125mg Pred 15mg CyA175/150	+/-	3.7	4.43	4.49	4.5	82	98
6	Aza 150mg Pred 20mg CyA250/250	Aza 100mg Pred 10mg CyA150/150	-	2.45	3.17	2.6	3.83	94	83
7	Aza 125mg Pred 40mg CyA 275/275	Aza 25mg Pred 10mg CyA125/125	-	3.2	5.52	3.31	5.65	97	98
8	MMF 1500mg bd Pred 40mg CyA 150/125	MMF 1500mg bd Pred 10mg CyA 125/100	-	1.03	1.78	1.52	2.39	68	74.5
9	MMF 750mg bd Pred 20mg CyA 300/300	MMF 1500mg bd Pred 10mg Tacro1/1	+/-	2.59	3.78	3.14	4.05	82	93

Pat	Immunosuppression			pH/Impedance measurements									
	1 month	6 month	Sum	1mo Acid Exp	6 mo Acid Exp	1 mo Vol Exp	6 mo Vol Exp	1 mo Rfx	6 mo Rfx	1 mo Prox Rfx	6 mo Prox Rfx	1mo RSI	6mo RSI
1	Aza 100mg Pred 10mg Tacro 4/4	Aza 100mg Pred 10mg Tacro 4/3	+/-	13.3	8.4	1.64	2.13	105	108	25	27	20	13
2	Aza 100mg Pred 10mg Tacro 5/4	Aza 100mg Pred 10mg Tacro 5/4	+/-	10	17.2	0.69	2.22	35	116	7	78	2	15
3	MMF 2160mg bd Pred 30mg Tacro 4/4	MMF 1080mg bd Pred 10mg Tacro 4/3	-	6	0.7	1.91	2.31	93	79	17	32	7	8
4	MMF 1080mg bd Pred 50mg Tacro 5/5	MMF 720mg bd Pred 10mg Tacro 4/3	-	1.6	31.9	1.13	0.96	58	77	22	11	21	1
5	Aza 100mg Pred 20mg CyA150/150	Aza 125mg Pred 15mg CyA175/150	+/-	13.1	23.4	3.63	1.90	111	125	42	28	10	7
6	Aza 150mg Pred 20mg CyA250/250	Aza 100mg Pred 10mg CyA150/150	-	7.4	0.3	1.64	0.42	111	24	8	5	6	0
7	Aza 125mg Pred 40mg CyA 275/275	Aza 25mg Pred 10mg CyA125/125	-	4.5	13.5	1.02	0.83	69	64	34	24	0	0
8	MMF 1500mg bd Pred 40mg CyA 150/125	MMF 1500mg bd Pred 10mg CyA 125/100	-	1.4	1.6	1.08	1.26	38	42	11	14	7	4
9	MMF 750mg bd Pred 20mg CyA 300/300	MMF 1500mg bd Pred 10mg Tacro1/1	+/-	1.1	5.4	0.89	1.24	63	89	14	30	12	6

Table 4-2 Key: Pat=patient, Aza=azathioprine, Pred= prednisolone, Tacro= tacrolimus, CyA= cyclosporin A, MMF= mycophenolate mofetil, Sum= overall changes in immunosuppression, FEV1= forced expiratory volume in 1 second, FVC= forced vital capacity, Ratio= FEV1/FVC ratio, Acid Exp= 24 hour oesophageal acid exposure (%), Vol Exp= 24 hour oesophageal volume exposure (%), Rfx= total impedance reflux events/24 hours , Prox Rfx= impedance proximal reflux events, 17cm above the lower oesophageal sphincter, per 24 hours.

Immunosuppression

From one to six months, immunosuppression therapy remained similar in four patients and decreased in five patients. Of the four patients who had a similar level of immunosuppression: two patients had an increase in all reflux parameters, one patient had an increase in three parameters but a decrease in acid exposure, one patient had an increase in two reflux parameters and a decrease in two reflux parameters. Of the five patients with a decrease in immunosuppression: one patient had an increase in all four reflux parameters, one had an increase in three parameters and a decrease in one parameter, two patients had an increase in two parameters and a decrease in two parameters. One patient decreased all four reflux parameters (Table 4-2). This suggests that during this time point that if there is no change in immunosuppression, the reflux will tend to increase and if immunosuppression is decreased then the changes are variable.

Questionnaire

Two patients had positive RSI scores at one month, of these one had a positive RSI score at six months. Seven patients had a negative RSI score at one month. Of these seven, six had a negative RSI score at six months. Median RSI score was 7 (range 0-21) at one months and 6 (range 0-15) at six months.

pH/Impedance Results

Five patients had evidence of reflux at one month and eight patients had evidence of reflux at six months. At three months, all had acidic reflux. At six months 6 had acid reflux and 2 had exclusively weakly acidic reflux. At one month four had abnormal proximal reflux. This increased to six patients by six months.

Table 4-3: Key pH/impedance results at one and six months post-lung transplant

	1month median & ranges (n=9)	6months median & ranges (n=9)	**Normal values**
Acid exposure (%)	**6 (1.1-13.3)**	**8.4 (0.3-31.9)**	**<4.5**
Oesophageal volume exposure (%)	**1.13 (0.69-3.63)**	**1.26 (0.2-2.31)**	**0.4-1.2**
No of reflux events	**69 (35-111)**	**79 (24-125)**	**(25-58)**
-Acid reflux events	**28 (3-57)**	**18 (4-98)**	**(10-35)**
-Weakly acid reflux events	**47 (7-84)**	**44 (18-72)**	**(5-18)**
-Non acid reflux events	**0 (0-1)**	**0 (0-1)**	**(1-7)**
Bolus clearance time (secs)	**14 (8-22.5)**	**13 (8-21)**	**(8-13)**
Proximal reflux events	**17 (7-42)**	**27 (5-78)**	**(4-17)**

Median acid exposure was 6% at three months and increased to 8.4% at six months (Figure 4-1). No patients had a positive relationship between their symptoms of cough/acid in the throat and acid reflux episodes on SI, SSI and SAP at three months. Only one patient showed a positive relationship between their symptom of cough/acid in the throat and acid reflux episodes on SAP and SI at 6 months. None had a relationship on SSI at 6 months.

Median oesophageal volume exposure increased from 1.13% at three months to 1.26% by six months (Figure 4-2) and the median number of reflux events increased from 69 at three months to 79 events at 6 months (Figure 4-3). Median proximal reflux events increased from over this period (17 to 27) (Figure 4-4). BCT was abnormal in four patients at three months and five patients at six months (Table 4-2,Table 4-3). Three patients showed a positive relationship between their symptom of cough/acid in the throat and impedance reflux events on SI and SAP at three month, and only one on SSI. Only one patient showed a positive relationship between their symptom of cough/acid in the throat and impedance reflux episodes on SI, none on SSI and two were positive on SAP at 6 months.

Table 4-4 compares all patients assessed at one month and six months post-transplant. This also demonstrates that, in general, reflux indices are higher at six months post-transplant. This data is not longitudinal.

Table 4-4: Key pH/impedance results at one and six months post-lung transplant (n=17 vs n=9)

	1month Median & ranges (n=17)	6months Median & ranges (n=9)	**Normal values**
Acid exposure (%)	**4.8 (1-79.9)**	**8.4 (0.3-31.9)**	**<4.5**
Oesophageal volume exposure (%)	**1.6 (0.71-5.48)**	**1.26 (0.2-2.31)**	**0.4-1.2**
No of reflux events	**72 (27-147)**	**79 (24-125)**	**(25-58)**
-Acid reflux events	**25 (2-90)**	**18 (4-98)**	**(10-35)**
-Weakly acid reflux events	**38 (5-140)**	**44 (18-72)**	**(5-18)**
-Non acid reflux events	**0 (0-3)**	**0 (0-1)**	**(1-7)**
Bolus clearance time (secs)	**15 (8-26.5)**	**13 (8-21)**	**(8-13)**
Proximal reflux events	**17 (4-54)**	**27 (5-78)**	**(4-17)**

Figure 4-1: Changes in oesophageal acid exposure (%) from one to six months post-lung transplant

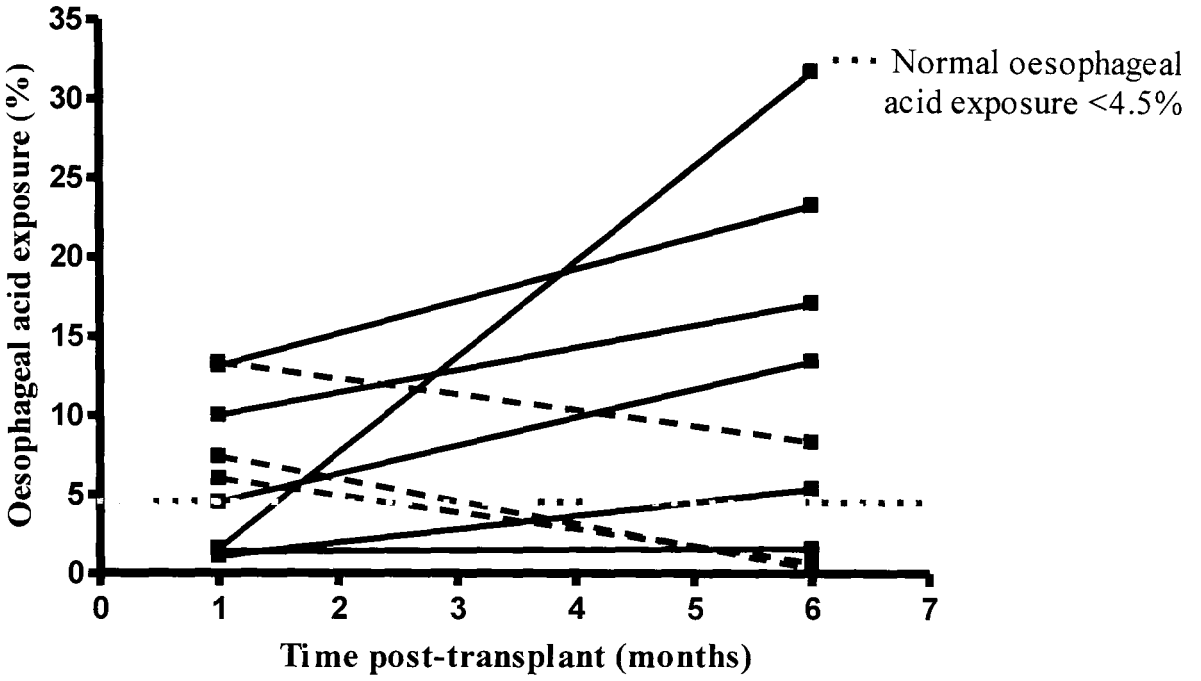


Figure 4-2: Changes in oesophageal volume exposure (%) from one to six months post-lung transplant

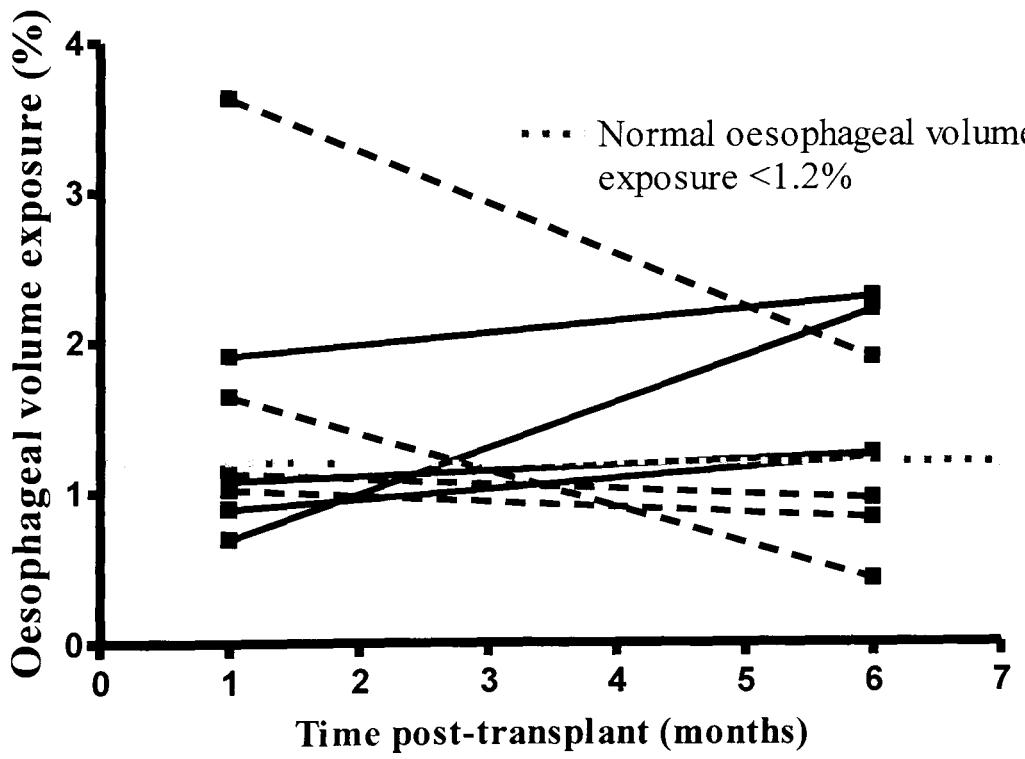


Figure 4-3: Changes in total reflux events from one to six months post-lung transplant

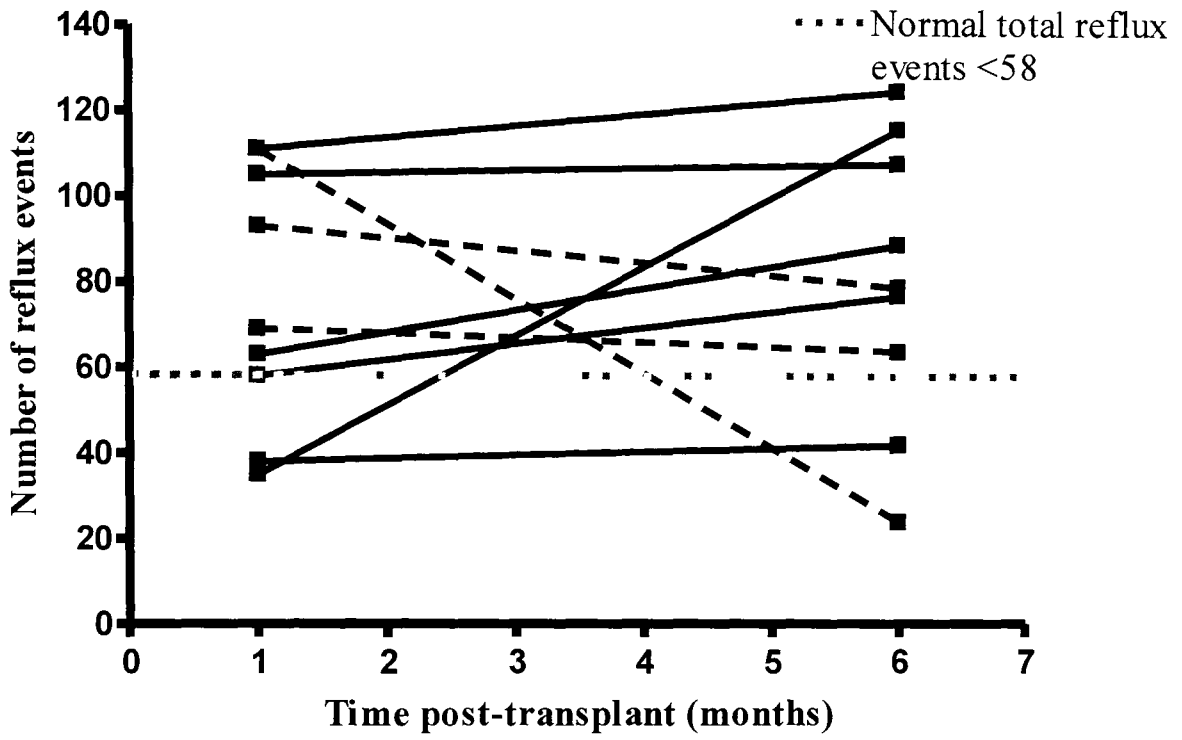
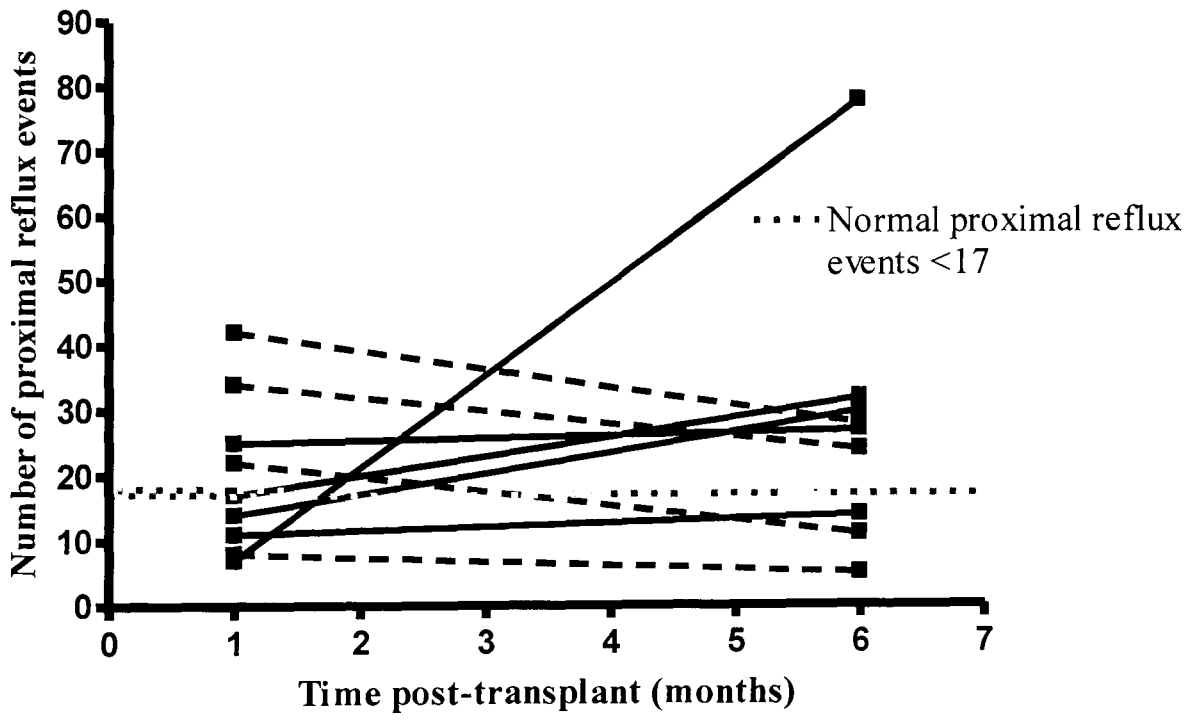


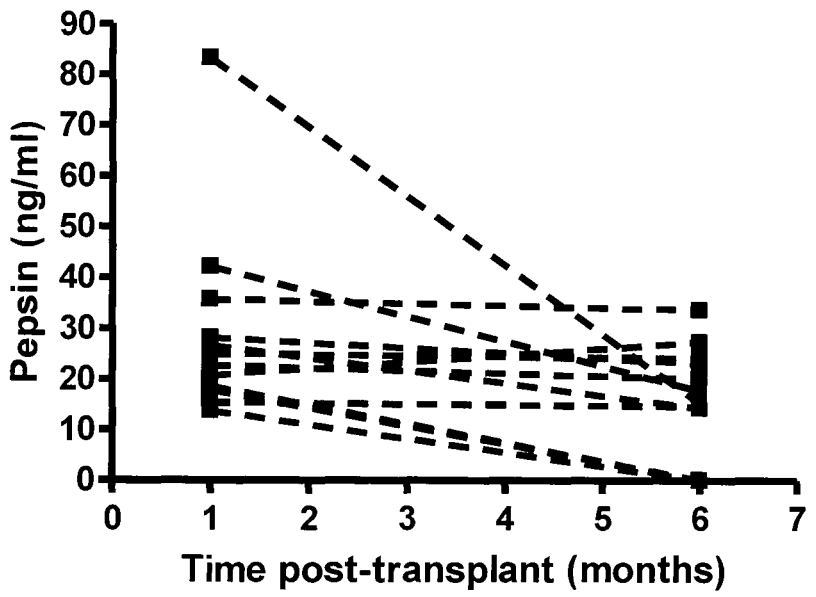
Figure 4-4: Changes in proximal reflux events from one to six months post-lung transplant



Biomarkers of aspiration

Only 13/17 had BALF available at one and six months. Median pepsin levels decreased from 23 ng/ml (range 14-83ng/ml) at one month to 15ng/ml (range 0-34ng/ml) at six months ($p<0.001$) (Figure 4-5). Median bile salt levels were 0μmol/l at three months and six months. At both time points only two patients had bile salt levels greater than 0.1μmol/l. There did not appear to be any trends between immunosuppression, reflux indices and biomarkers of aspiration in these patients (n=13) during these time points.

Figure 4-5: Pepsin levels at one and six months post-lung transplant



Statistics

There was no statistical significant differences in reflux indices from one to six months using Wilcoxon non-parametric paired t-tests. However there was a significant reduction of pepsin from one to six months (n=13) ($p<0.001$).

4.5. Discussion

The main findings of this study are that over the first six months the prevalence of reflux increases and aspiration, as denoted by BALF pepsin level, decreases.

The relationships learned from these findings are that despite decreasing immunosuppression and improvement in lung function, the prevalence of reflux increases. Surprisingly microaspiration improves with a decrease in BALF pepsin levels. A potential explanation of this finding is that over this time pulmonary defence mechanisms improve. This may be by allograft re-innervation, improved cough reflex (Duarte, Terminella et al. 2008) and muco-ciliary clearance which reduce the amount of aspiration the allografts encounter.

With impedance measurements, there is some intra- and inter-individual variability. Bredenoord et al evaluated 20 healthy volunteers, 2 weeks apart, and found that there was more variability between different subjects than within the same subjects at different times (Bredenoord, Weusten et al. 2005; Wise and Murray 2007). Reproducibility has not been assessed in the lung transplant population. Impedance monitoring has been shown to be well reproducible and at least as reproducible as pH monitoring (Bredenoord, Weusten et al. 2005; Wise and Murray 2007). This may suggest that these are real changes shown here during a period of dynamic anatomical, physiological and pharmacological changes for patients.

One study demonstrated an increase in the prevalence of reflux from 35% pre-transplantation to 65% post-transplantation (Young, Hadjiliadis et al. 2003). Previous studies, attempting multiple impedance measurements, have been unsuccessful due to patients refusing multiple measurements. (Blondeau, V. Mertens et al. 2008) Only one study exists comparing reflux at two different times post-transplant. This study comparing GORD in the first year post-transplant supports our observations (D'Ovidio, Mura et al. 2006). It shows an increase in the prevalence of GORD (16 out of 50 patients) at 3 months to (16 out of 30) at 12 months (D'Ovidio, Mura et al. 2006). Only twelve patients had multiple measurements and unfortunately changes for repeat measurements in the same individuals were not described.
The current study demonstrated an increase in the prevalence of GORD over the first six months post-transplant despite decreasing immunosuppression and improvement in lung function. Although reflux increased in the absence of augmented immunosuppression, if immunosuppression was kept stable then reflux indices tended to increase. If

immunosuppression decreased then changes in reflux were variable. This suggests that some changes in reflux may be related to immunosuppression but other factors may play a role.

This study tried to identify the optimum time to assess for reflux and its changes over a 6 month period. The current data does not identify an ideal time but repeat assessments of GORD may be an advisable component of post-transplant follow up. This series demonstrates that multiple catheter based assessments are unpopular. It highlights a need for identifying markers of GORD and aspiration which are specific and well tolerated (Robertson, Griffin et al. 2009).

Pepsin has been shown to be a marker of aspiration and of injury (Ward, Forrest et al. 2005; Stovold, Forrest et al. 2007). Little is known about the natural history of aspiration post-lung transplant and the variability of pepsin levels over time. Over the first six months, there was a statistically significant decrease of median BALF pepsin level. This is paradoxical as, over this time, GORD increased in prevalence. Several reasons may explain these findings. Given the strength of the p-value, biological variability is unlikely to explain this data. The most plausible explanation is that pulmonary defence mechanisms improve. Re-innervation of the allograft by the vagal nerve would improve sensation and secreto-motory function. Muco-ciliary clearance is shown to be reduced post-transplant (Veale, Glasper et al. 1993) but it is unknown if this improves with time. The cough reflex has been shown to improve over the first year post-transplant (Duarte, Terminella et al. 2008). Combined with decreasing post-operative pain and improved lung function, these factors may improve mechanical defences against aspiration.

The main weaknesses of this study were the low numbers, poor patient recruitment and compliance. Further larger studies should be performed to assess whether this paradox- a decrease of median BALF pepsin level occurring over the time period when GORD increases in prevalence- is maintained. Multiple impedance measurements were universally unpopular amongst patients. For future studies, a recommend maximum of two measurements per patient should be implemented.

5. *Analysis of Gastric Juice and Cell Stimulation Experiments*

5.1. Abstract

Introduction

There is a limited understanding of the pathophysiology of aspiration induced damage post lung transplant. Studies are needed to assess the contents and potential damaging components of gastric juice. It is also necessary to understand cellular mechanisms involved in injury from pathological levels of injurious agents.

Aims

The aim of this chapter was to assess the components of gastric juice and to perform cell culture experiments to increase our understanding of the potential pathophysiology of aspiration.

Methods

Gastric juice samples, from both transplant and predominantly non-transplant patients, were collected and analysed for pH, pepsin, bile, trypsin and bacteriology. Goblet cells were stimulated with porcine pepsin and primary bronchial epithelial cells (PBECs) were stimulated with porcine pepsin and gastric juice from both lung transplant and non-transplant patients. Viability, IL-8 and MUC 5AC production were assessed from goblet cells and PBECs.

Results

Gastric juice samples were collected from 65 patients (56 non-transplant patients and 9 lung transplant recipients). 28/65 patients were on PPI. Median pepsin levels were 380μg/ml (0-3892μg/ml), median bile salts levels were 50μmol/l (0-8000μmol/l), trypsin 5μg/ml (4-100μg/ml) and mean pH 3.7 (0.8-8.4) levels were established. Bacteria were present in 11/18 samples (1 of 2 samples analysed for microbiology from lung transplant recipients). Stimulation of HT29 MTX goblet cells with pepsin had no effect on IL-8 on cell viability but reduced MUC5AC production. Stimulation of PBECs with pepsin led to an increased IL-8 production, but did not affect cell viability. Stimulation of PBECs with diluted gastric juice led to a varied response in IL-8 production, but consistently resulted in cell death.

Conclusion

We suggest a novel pathophysiological mechanisms linking aspiration to infection: that gastric juice is a reservoir for bacteria may lead to allograft infection via the direct introduction of pathogens. Cell work suggests aspiration may down-regulate mucus, increase interleukin production and leads to cell death. However, any increase in IL-8 production is unlikely to arise from goblet cells. We propose a subsequent model of aspiration induced lung epithelial injury.

5.2. Introduction

There have been limitations to human and animal studies undertaken performed to look at the effects of chronic aspiration in lung transplant recipients (Robertson, Shenfine et al. 2009). Previous work has suggested that pepsin and bile are important biomarkers of aspiration. These biomarkers have not yet been fully validated. In our current study pepsin was elevated in the BALF of transplant recipients, and bile salts were rare, suggesting pepsin to be a more reliable biomarker of aspiration. To further assess the validity of biomarkers, gastric juice samples of lung transplant patients and normal controls were analysed: firstly to assess the gastric concentrations of pepsin and bile salts to determine whether reported BALF levels are feasible; secondly to obtain background data on the intra-gastric levels in both lung transplant recipients and non-transplant patients; thirdly to analysed gastric juice for other potential damaging compounds e.g. trypsin and bacteria and finally to guide cell culture experiments. It has been suggested that there is a link between aspiration and infection (Vos, Blondeau et al. 2008). This has previously been hypothesised as aspiration damaging the innate immunity in the lung, leading to a weakened response to infection (D'Ovidio, Mura et al. 2006). Another proposal is that although this may be one mechanism linking the two together, that the aspirate itself may contain pathogens- bacterial, viral and fungal (Robertson, Ward C et al. 2010).

Currently there is a limited understanding of the pathophysiology of aspiration induced damage at a cellular level. Cell culture experiments (PBECs and goblet cells) with pepsin and gastric juice are necessary to develop our understanding of this pathophysiological process. Mucus homeostasis is important in health and as a defence against infection. Therefore it is important to study mucus production as alteration in mucus homeostasis may be detrimental to allograft function and health.

The aim of this chapter was to assess the components of gastric juice and to perform cell culture experiments to increase our understanding of the potential pathophysiology of aspiration.

5.3. Methods

Methods are described in detail in Chapter 2. In summary gastric juice was collected at routine endoscopy from both transplant and non-transplant patients. Samples were analysed for pH, pepsin, bile salts, trypsin and microbiology.

Cellular experiments were performed using HT29-MTX goblet cells and primary bronchial epithelial cells. Cultured cells were stimulated with porcine pepsin and diluted gastric juice. Cells were assessed for their response to this challenge with regards to IL-8 production, MUC5AC production and cell viability.

5.4. Results

5.4.1. Gastric juice

Sixty five gastric juice samples were collected (56 from non-transplant patients and nine from lung transplant recipients). The mean age of all patients was 53.1years (Range 20-88years). 44 were female 18 were male. Three patients did not have their gender recorded. 28/65 were on PPI therapy. Three historical samples had been taken from patients after pentagastrin stimulation. There was a variety of pathology identified (Table 5-1). Several patients had more than one pathology present.

Table 5-1: Patient category identified at gastroscopy to collect gastric juice samples

Pathology	Number	Pathology	Number
Normal	10	Hiatus hernia	28
Gastric ulcer/erosion	7	Barrett's oesophagus	7
Gastritis	12	Oesophageal adenocarcinoma	2
Peptic ulcer	5	Oesophageal nodule	1
Oesophagitis	19	Pyloroplasty	1
Duodenal ulcer/duodenitis	7	Gastro-jejunostomy	1
Lung transplant	9	Gastric polyp	1

Mean pH was 3.74 (range 0.8-8.4). Median pepsin levels were 380µg/ml (range 0-3892). 77% 50/65 contained active pepsin. Median bile salt levels were 50µmol/l (range0-8000). 83% (54/65) contained bile salts. Only 11/65 (17%) patients had levels above 300µmol/l. Median trypsin levels were 5µg/ml (range 4-100). 100% (13/13) contained trypsin. A summary is shown in Table 5-2. 11/18 (61%) had bacteria- pathogens including *Pseudomonas* aeruginosa, *Klebsiella*, *Proteus*, and fungal pathogens (*candida).*

Table 5-2: Mean/median values of all gastric juice samples

pH (Mean)	3.74 (range 0.8-8.4)
Pepsin (median) *	380µg/ml (range 0-3892)
Bile (median)	50µmol/l (range 0-8000)
Trypsin (median) *	5µg/ml (range 4-100)
Bacteria	11/18

*(based on an activity assay)

5.4.2. *Comparison of gastric juice analyses from patients on and off PPI therapy*

Twenty eight patients had samples collected whilst on PPI therapy, 37 patients had samples collected with no PPI or anti-acid therapy. Those treated with PPI had a higher mean pH (5.02) than those without PPI therapy (2.7) ($p<0.0001$) (Figure 5-1). Patients without PPI therapy had a higher median pepsin level 572 µg/ml vs those on PPI therapy 107µg/ml ($p=0.049$) (Figure 5-2). 82% (31/38) of patients not taking a PPI as opposed to 68% (19/28) on PPI therapy had pepsin detected. Median bile salt levels were similar in both groups (70µmol/l vs 55µmol/l) ($p=0.97$) (Figure 5-3) as were median trypsin activity levels (9 µg/ml vs 5 µg/ml) ($p=0.29$). A summary is shown in Table 5-3. Bacteria were present in the gastric juice of 4/6 without PPI and 7/12 patients with PPI. This was not statistically significant on Fisher exact test ($p=1.0$).

Table 5-3: Demographics and values of those on PPI versus no PPI

	No PPI	PPI
Age	55.6 (20-81)	56 (24-88)
Sex Female Male Unrecorded	 25 9 3	 17 9
pH	2.7 (0.8-7.9)	5.0 (1.6-8.4) *
Pepsin	572µg/ml (0-3,772)	107µg/ml (0-3,892) *
Bile	70µmol/l (0-8,000)	55µmol/l (0-8,000)
Trypsin	9µg/ml (4-100)	5µg/ml (4-15)
Bacteria	4/6	7/12

Figure 5-1: pH of gastric juice on/off PPI

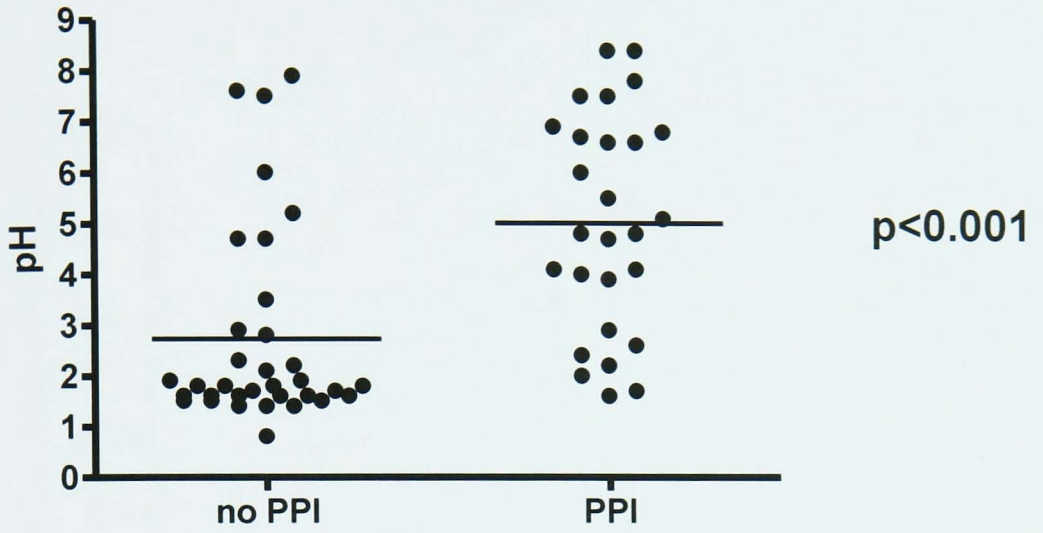


Figure 5-2: Pepsin levels of gastric juice on/off PPI

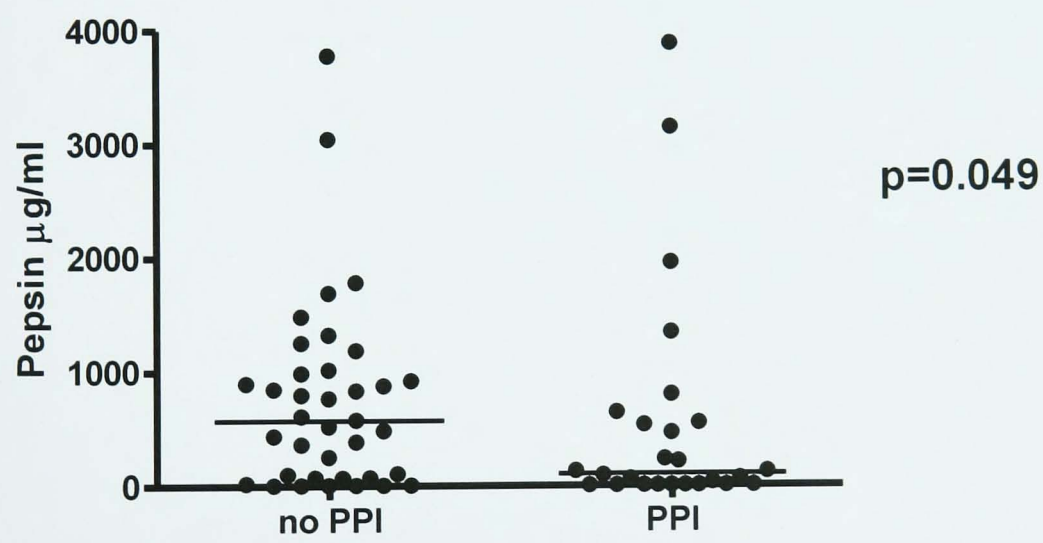
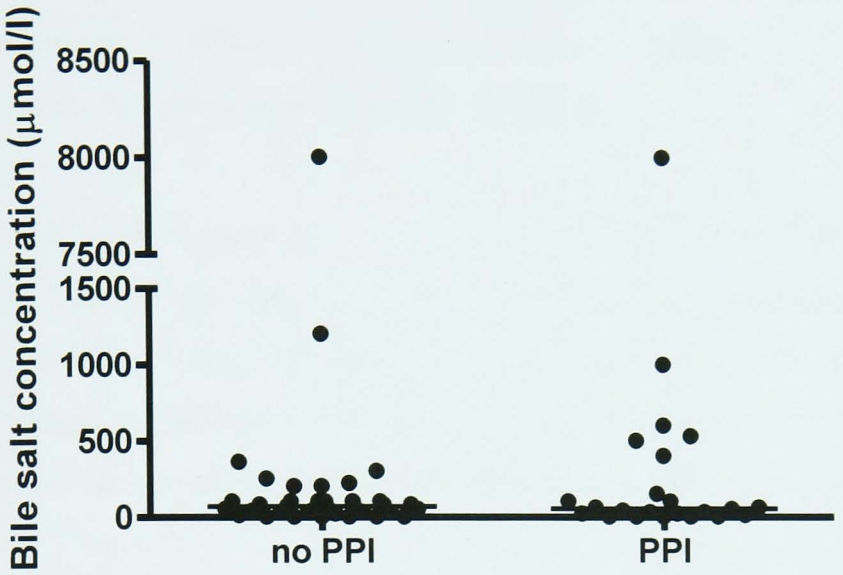


Figure 5-3: Bile salt concentration of gastric juice on/off PPI



5.4.3. *Gastric juice from lung transplant recipients*

Nine samples of gastric juice were obtained from lung transplant recipients (9 female) with a median age of 30years (Range 24-60years). 2 patients had stopped their PPI for gastroscopy the other seven remained on their PPI therapy.

Median gastric juice pH was 3.5 (range 1.4-7.8). Median intragastric pepsin concentration was 391µg/ml (Range 0-3,892µg/l). Median intragastric bile acid concentration was 95µmol/l (range 0-2,200µmol/l). Three of eight patients had levels above 300µmol/L, which has been proposed as the lower limit of intragastric bile salts for detection of bile salts in the BALF. Only 2 samples were analysed for trypsin one had 5µg/ml the other 12µg/ml.

One of two patients had positive microbiological cultures. Pathogens grown included *Lactobacillus* and *Candida* species, as well as *Pseudomonas* aeruginosa. Two other patients had oesophageal candidiasis visible on OGD. There was no significant difference between pH (p=0.73), pepsin (p=0.88), bile salt levels (p=0.47) between transplant and non-transplant patients, although there was a significant difference in median age (p=0.0001). Results are shown in Table 5-4.

Table 5-4: Summary of analysis of lung transplant recipient gastric juice

	Lung transplant	Non-transplant
Age	30 (24-60)	59.4 (20-88)*
Sex Female Male Unrecorded	 9 0	 35 18 3
PPI No PPI	7 2	19 37
pH	3.5 (1.4-7.8)	3.7 (0.8-8.4)
Pepsin	391µg/ml (0-3,892)	380µg/ml (0-3,772)
Bile	95µmol/l (0-2,200)	60µmol/l (0-8,000)
Trypsin	8.5µg/ml (5-12) (n=2)	5µg/ml (4-100) (n=9)
Colonised	1/2	10/16

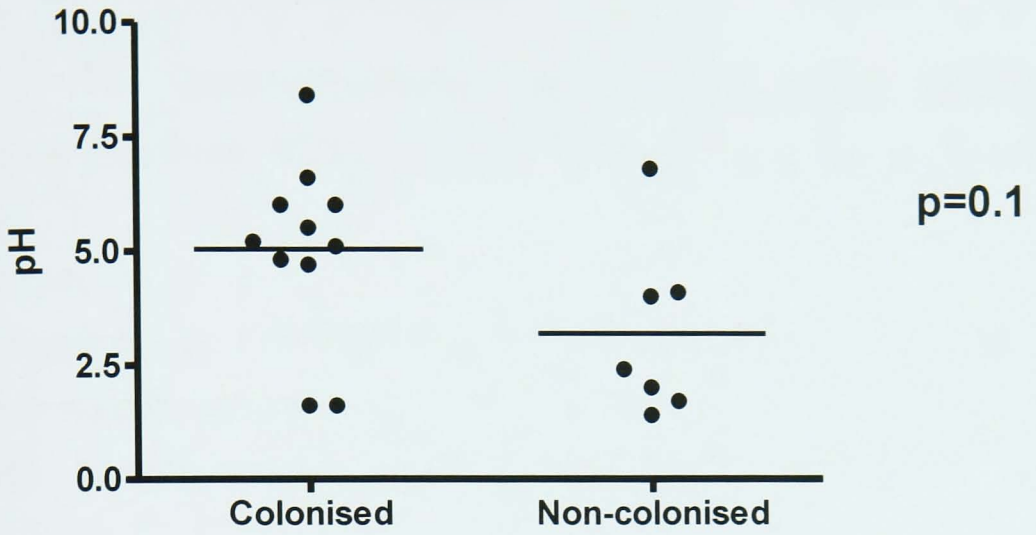
5.4.4. *Comparison of colonised versus non-colonised samples*

Eighteen samples were analysed for bacteriology (16 from non-transplant patients and two from transplant recipients). Eleven of eighteen (61%) patients were colonised. Pathogens detected included *Pseudomonas* aeruginosa, *Klebsiella*, *Proteus*, and fungal *Candida*. There was no significant difference in age (65 versus 59y) (p=0.53), sex (p=0.32), or PPI use (7/11 versus 5/7 (p=0.41) using Fisher exact test, in patients colonised or non-colonised. Median pH was higher in colonised samples pH 5.2 vs 2.4 (p=0.1). Of note 2 colonised samples had low pH 1.6, 1.7 (Figure 5-4). Median pepsin levels were lower in colonised samples 460 vs 798 (p=0.61). Bile salt and trypsin levels were similar in colonised versus non colonised samples (50 vs 50 p=0.59 and 6.5 vs 5 p=0.72 respectively) (Table 5-5). Analyses were performed using non-parametric t-tests.

Table 5-5: Summary of analysis of colonised gastric juice

	Colonised (n=11)	Non-colonised (n=7)
Age	65 (30-80)	59 (30-75)
Sex Female Male	 6 5	 6 1
PPI No PPI	7 4	5 7
pH	5.2 (1.6-8.4)	2.4 (1.4-6.8)
Pepsin	460 (0-3772)	798 (0-3892)
Bile	50 (0-2050)	50 (20-500)
Trypsin	6.5 (4-100)	5 (4-12)

Figure 5-4: pH of samples colonised/non-colonised



5.4.5. *Cell culture experiments*

5.4.6. *Stimulation of goblet cells (HT29-MTX) with porcine pepsin*

Results from HT-29 MTX cultured and exposed to pepsin are described below.

Viability

The stimulation of HT-29MTX goblet cells with pepsin over 48 hours did not lead to cell death at concentrations of 25 to 100µg/ml (Table 5-6) as assessed by TiterBlue Assay at 48 hours (repeated culture n=1, with repeated wells n=3, overall n=3).

Table 5-6: Viability at 48 hours of HT-29MTX goblet cells stimulated with porcine pepsin

	Viability at 48 hours
Control (n=3)	100%
25µg/ml porcine pepsin (n=3)	100%
50 µg/ml porcine pepsin (n=3)	100%
100 µg/ml porcine pepsin (n=3)	97%

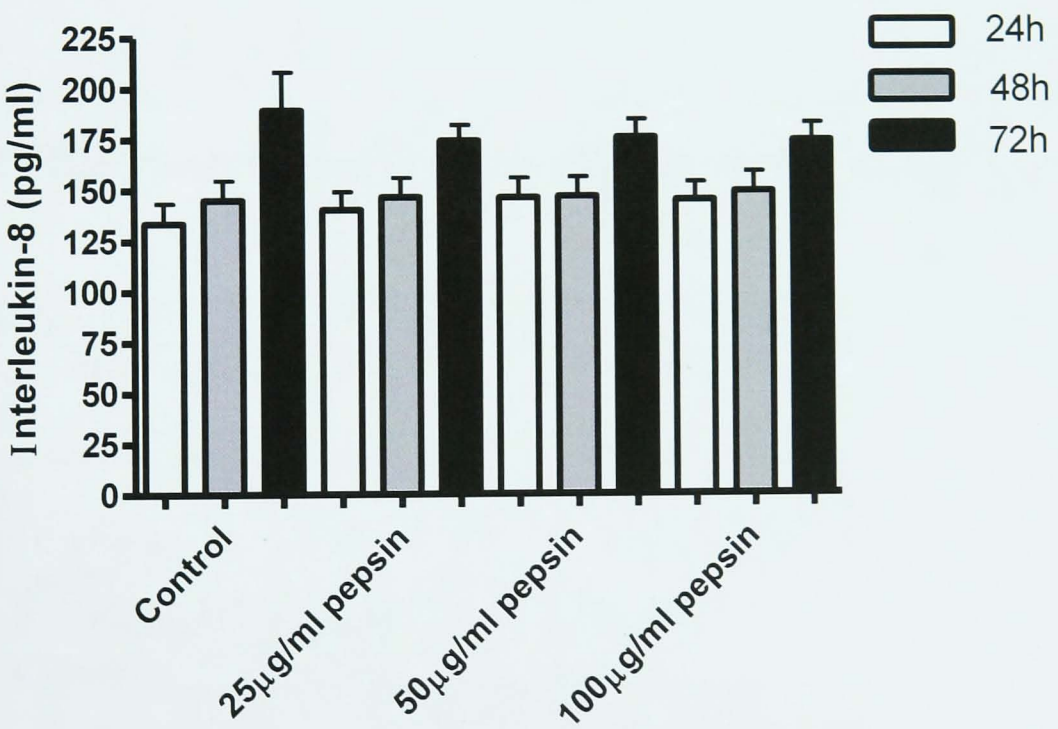
Interleukin-8

Stimulation of HT29-MTX goblet cells with porcine pepsin (concentration 25-100µg/ml) did not stimulate an increase in IL-8 production over a 72 hour period (repeated culture n=2, with repeated wells n=5, overall n=10). Levels are shown in Table 5-7 and Figure 5-5.

Table 5-7: Interleukin 8 concentration (on successive days) from goblet cells challenged with porcine pepsin

	IL-8 (pg/ml) at 24 hours	IL-8 (pg/ml) at 48 hours	IL-8 (pg/ml) at 72 hours
Control	136 (119-172)	144 (123-180)	186 (153-255)
25µg/ml porcine pepsin	139 (121-173)	145 (124-181)	175 (153-201)
50 µg/ml porcine pepsin	143 (125-182)	145 (123-182)	176 (149-204)
100 µg/ml porcine pepsin	144 (123-176)	147 (128-186)	172 (149-204)
P values on Kruskall Wallace analysis	P=0.19	P=0.83	P=0.85

Figure 5-5: Interleukin 8 concentration (on successive days) from goblet cells challenged with porcine pepsin



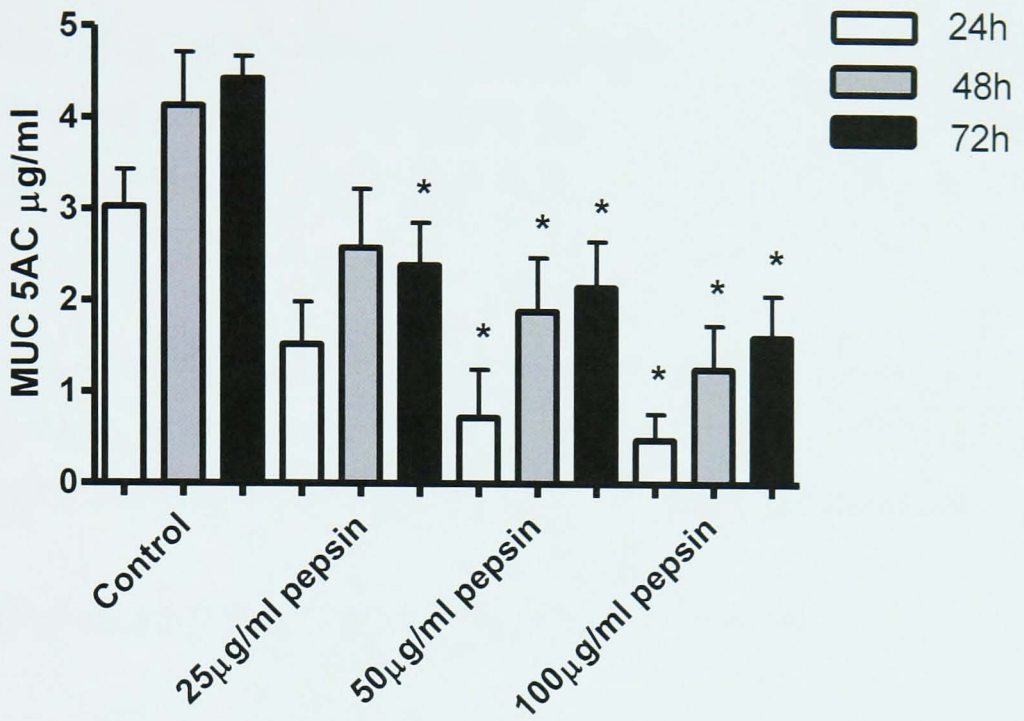
MUC5AC

Stimulation of HT-29MTX goblet cells with porcine pepsin (concentration 25-100µg/ml) down regulated MUC5AC concentrations at 24, 48 and 72 hours (repeated culture n=5, with repeated wells n=3; overall n=15). There was a dose dependent effect with a decreasing MUC5AC level as pepsin concentration increased. There was a statistically significant decrease in MUC5AC concentrations at 24 hours in samples challenged with 50µg/ml (p=0.03) and 100µg/ml (p=0.008) but not with 25µg/ml porcine pepsin when compared to controls. This was similar at 48 hours for 50µg/ml (p=0.03), 100 µg/ml (p=0.03), 25µg/ml (p=0.22) when compared to controls. By 72 hours there was a statistically significant decrease in MUC5AC concentrations in samples challenged with 25µg/ml (p= 0.016), 50µg/ml (p=0.016) and 100µg/ml (p=0.008) of porcine pepsin when compared to controls. There was no statistically significant difference between the three concentrations of pepsin (25,50,100µg/ml) at any time point (24h,48h,72h). Levels are shown in Table 5-8 and Figure 5-6.

Table 5-8: MUC5AC concentration (on successive days) after stimulate with porcine pepsin over 72 hours

	MUC 5AC (µg/ml) at 24 hours	MUC 5AC (µg/ml) at 48 hours	MUC 5AC (µg/ml) at 72 hours
Control (n=15)	3.03 (1.92-4.18)	4.13 (2.7-5.8)	4.43 (3.67-5.11)
25µg/ml porcine pepsin (n=15)	1.52 (0.65-3.08)	2.59 (1.36-4.87)	2.40 (1.27-4.04)*
50µg/ml porcine pepsin (n=15)	0.72 (0-2.81)*	1.89 (0.75-4.1)*	2.16 (1.2-4.01)*
100µg/ml porcine pepsin (n=15)	0.48 (0-1.56)*	1.26 (0.5-3.06)*	1.62 (0.9-3.39)*
P values on Kruskall Wallace analysis	P=0.01	P=0.039	P=0.011

Figure 5-6:MUC5AC mucin concentration (on successive days) after stimulation with porcine pepsin



5.4.7. *Primary bronchial epithelial cells*

5.4.8. *Stimulation of primary bronchial epithelial cells with porcine pepsin*

Results were analysed from PBECs cultured and exposed to concentrations of pepsin over 48 hours (repeated culture n=2, with repeated wells n=5, overall n=10).

Viability

Cell viability was assessed by TiterBlue Assay (repeated culture n=1, with repeated wells n=2, overall n=2). The stimulation of PBEC with porcine pepsin over 48 hours did not lead to cell death at concentrations of 25, 50 and 100µg/ml of pepsin (Table 5-9).

Table 5-9: Viability at 48 hours of PBECs stimulated with porcine pepsin

	Viability at 48 hours
Control	100%
25µg/ml porcine pepsin	100%
50 µg/ml porcine pepsin	99%
100 µg/ml porcine pepsin	98%

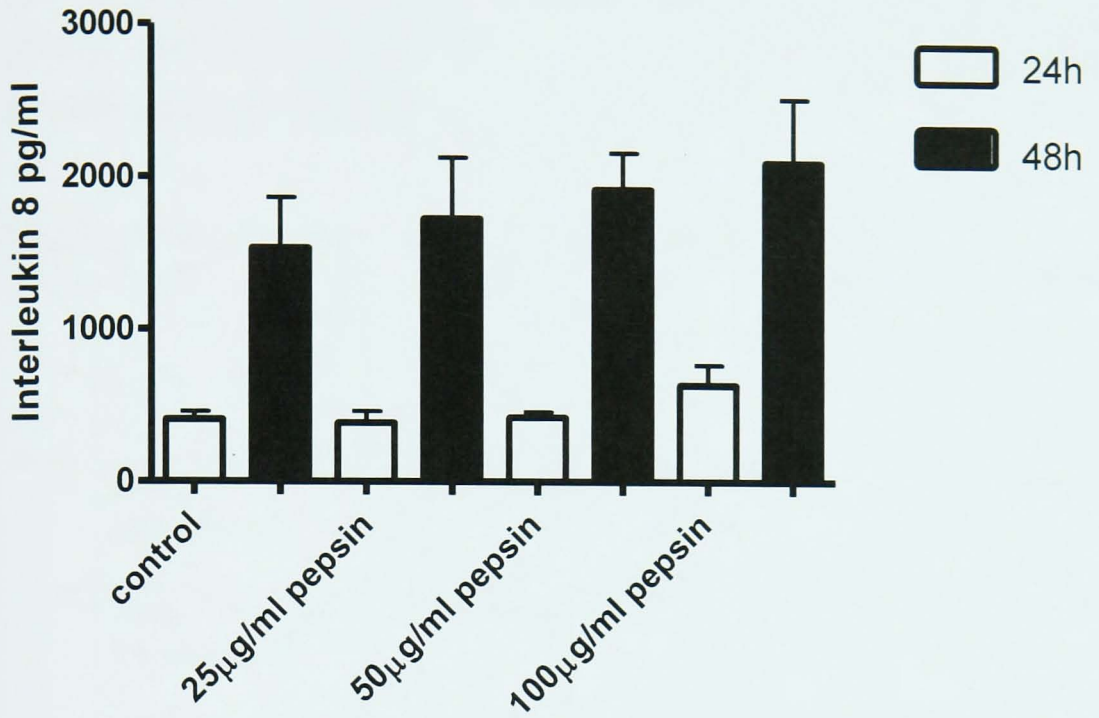
Interleukin-8

Stimulation of PBECs with pepsin (25, 50 and 100µg/ml), over a 48 hour period, did not lead to a significant increase in IL-8 production. Levels are shown in Table 5-10 and Figure 5-7.

Table 5-10: Interleukin 8 concentration (on successive days) from PBECs challenged with porcine pepsin

	IL-8 (pg/ml) at 24 hours	IL-8 (pg/ml) at 48 hours
Control (n=15)	408 (263-654)	1535 (648-2970)
25µg/ml Porcine Pepsin (n=15)	386 (249-755)	1728 (546-2986)
50 µg/ml Porcine Pepsin (n=15)	424 (305-574)	1925 (1273-2650)
100 µg/ml Porcine Pepsin (n=15)	638 (314-1158)	2103 (920-3750)
P values on Kruskall Wallace analysis	P=0.3	P=0.7

Figure 5-7: Interleukin 8 concentration (on successive days) from PBECs cells challenged with porcine pepsin



MUC5AC

Control samples of PBEC cells did not produce MUC5AC and stimulation of PBEC cells with pepsin did not result in the production of MUC5AC over 48 hours (repeated culture n=1, with repeated wells n=2, overall n=2).

5.4.9. *Stimulation of epithelial cells with gastric juice*

Cells, collected at bronchoscopy, were cultured from three different human lung transplant recipients. These were named Cell Culture A, B and C. These cell lines were stimulated with three different gastric juices (Table 5-11).

Table 5-11: Summary of gastric juice samples for PBEC stimulation

Sample	Patient	pH of original gastric juice sample	Pepsin	Bile	Trypsin	Pathogens
1	Lung transplant	1.6	1346µg/ml	530µmol/ml	5µg/ml	*Pseudomonas* Aeruginosa; Candida sp
2	Non-transplant	5.5	3153µg/ml	600µmol/ml	15µg/ml	*Acinetobacter* junii; Candida sp
3	Non-transplant	1.7	1319µg/ml	80µmol/ml	5µg/ml	Nil

Viability

Viability was not calculated for cell culture A. Viability for cell culture B, was calculated with Trypan Blue technique (repeated culture n=1, with repeated wells n=1, overall n=1). Viability for cell culture C was calculated with TiterBlue assay (repeated culture n=1, with repeated wells n=1, overall n=2). A fourth plate using cell culture B was assessed by both TiterBlue and Trypan Blue to compare the accuracy of these methods. Stimulation of PBECs with diluted gastric juice led to cell death. Gastric juice diluted to 1/1,000 concentration led to only 18-28% survival at 24 hours, 1/5,000 led to 32-55% survival and 1/10,000 led to 50-67% survival These results are shown in Table 5-12, Table 5-13, Figure 5-8 and Figure 5-9. Error bars are not shown due to the low numbers of viability assays performed.

Table 5-12: Viability of PBECs (cell culture B) stimulated with gastric juice

	Viability at 24 hours (sample 1)	Viability at 24 hours (sample 2)	Viability at 24 hours (sample 3)
Control	96%	98%	95%
1/1,000	18%	25%	20%
1/5,000	43%	49%	55%
1/10,000	56%	62%	66%

Figure 5-8: Viability of PBECs (cell culture B) stimulated with gastric juice

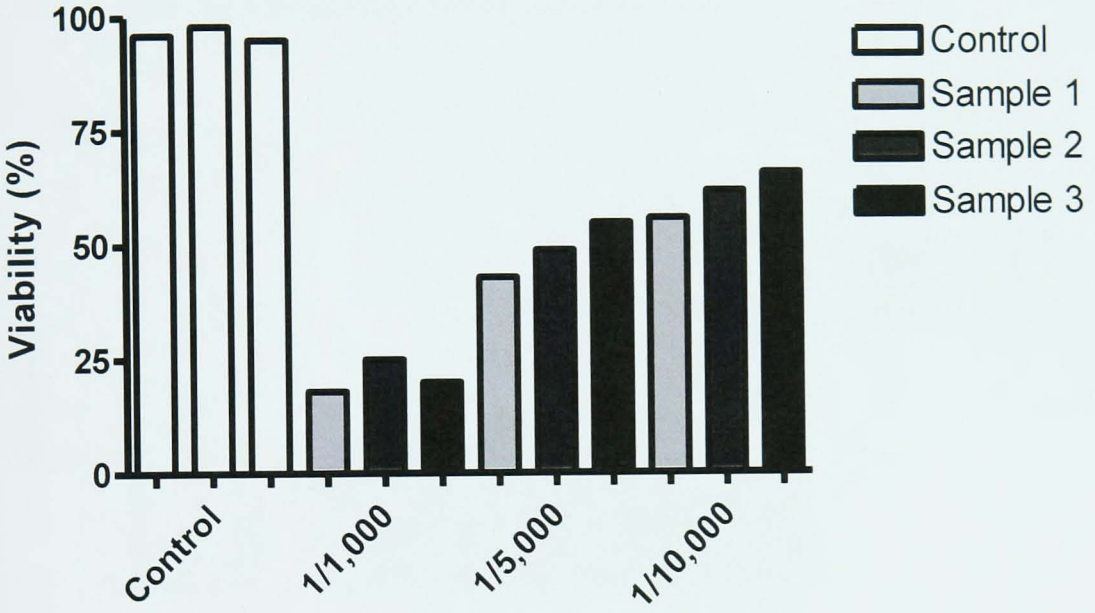
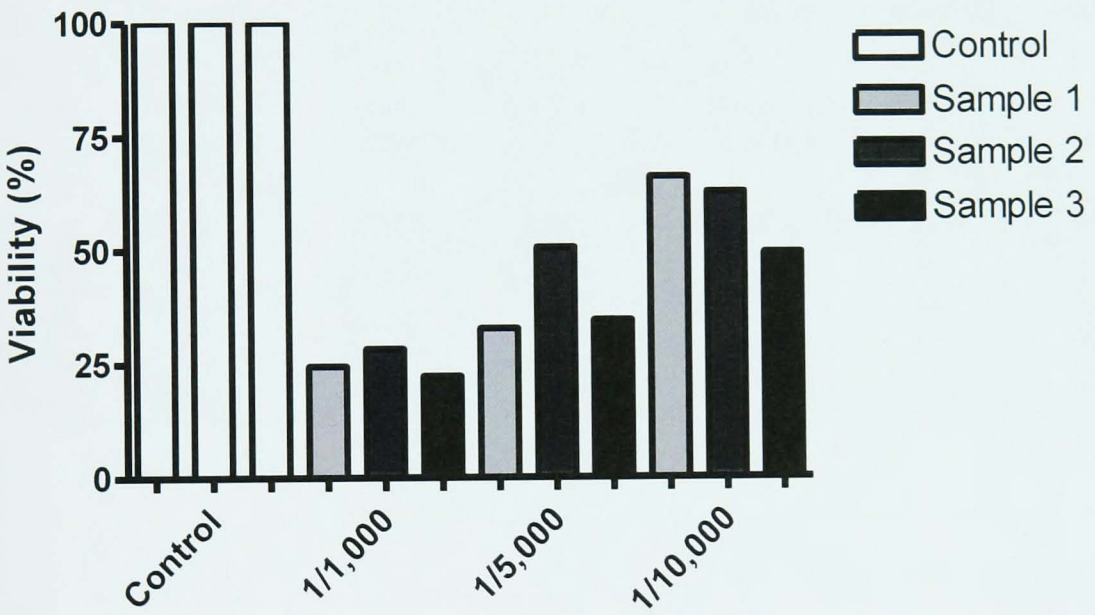


Table 5-13: Viability of PBECs (cell line C) stimulated with gastric juice

	Viability at 24 hours (sample 1)	Viability at 24 hours (sample 2)	Viability at 24 hours (sample 3)
Control	100%	100%	100%
1/1,000	24%	28%	22%
1/5,000	33%	51%	35%
1/10,000	66%	63%	50%

Figure 5-9: Viability of PBECs (cell culture C) stimulated with gastric juice



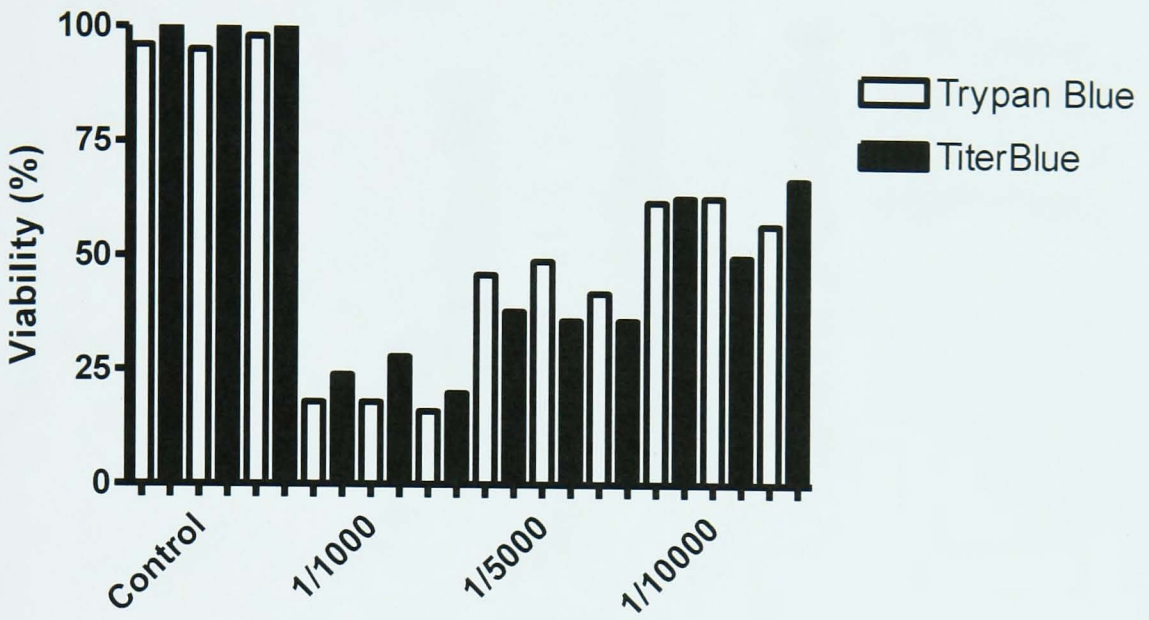
5.4.1. *Comparison of TiterBlue and Trypan Blue viability assays*

Viability for one plate was calculated with both TiterBlue (repeated culture n=1. with repeated wells n=2, overall n=2) and Trypan blue (repeated culture n=1, with repeated wells n=1, overall n=1) techniques to assess for any differences in the results (Table 5-14,Figure 5-10). There was no significant difference on Bland-Altman analysis for the two different methods of assessment.

Table 5-14: Viability of PBECs (cell culture B) stimulated with gastric juice assessed by Trypan Blue and TiterBlue assays

	Viability at 24 hours (sample 1) Trypan Blue	Viability at 24 hours (sample 1) TiterBlue	Viability at 24 hours (sample 2) Trypan Blue	Viability at 24 hours (sample 2) TiterBlue	Viability at 24 hours (sample 3) Trypan Blue	Viability at 24 hours (sample 3) TiterBlue
Control	96%	100%	95%	100%	98%	100%
1/1,000	18%	24%	18%	28%	16%	20%
1/5,000	46%	38%	49%	36%	42%	36%
1/10,000	62%	63%	63%	50%	57%	67%

Figure 5-10: Viability of PBECs (cell culture B) stimulated with gastric juice assessed by Trypan Blue and TiterBlue assays



Interleukin-8

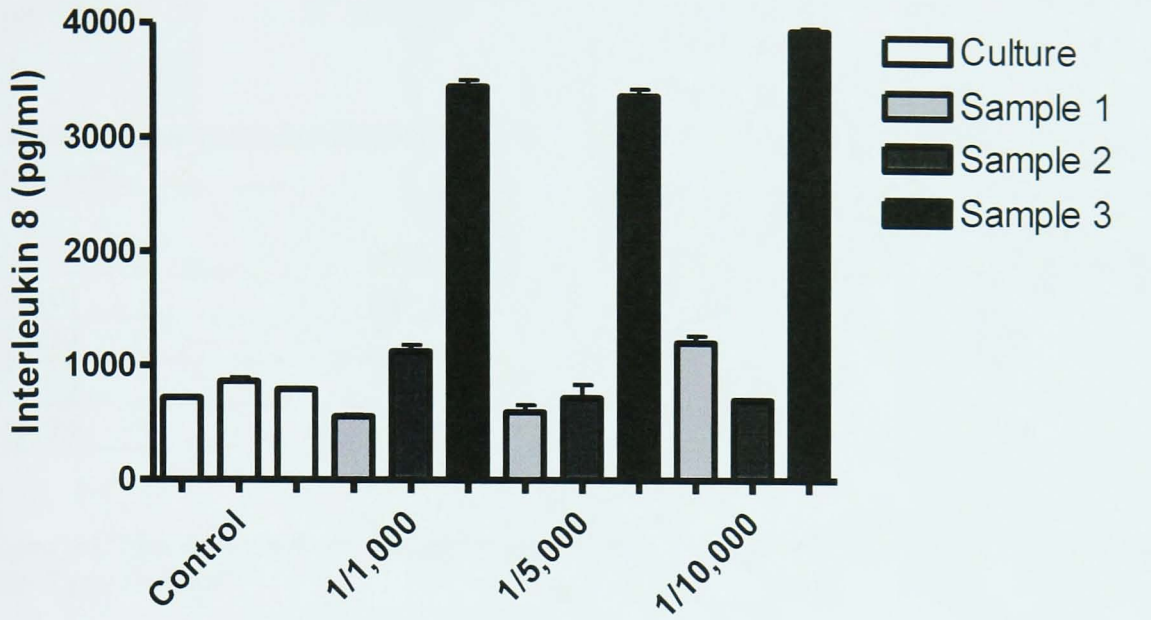
Stimulation of PBECs with diluted gastric juice (1/1,000 to 1/10,000) over a 24 hour period had a variable effect on IL-8 production (repeated culture n=1, with repeated wells n=7, overall n=7 from each experiment).

In cell culture A, stimulation with sample 3 of the gastric juice led to an increase in IL-8 production, whereas samples 1,2 did not have a major effect. Levels are shown in Table 5-15 and Figure 5-11.

Table 5-15: Interleukin 8 production from PBECs (cell culture A) challenged with gastric juice

	IL-8 (pg/ml) at 24 hours (sample 1)	IL-8 (pg/ml) at 24 hours (sample 2)	IL-8 (pg/ml) at 24 hours (sample 3)
Control	719	863	791
1/1,000	570	1128	3451
1/5,000	599	723	3377
1/10,000	1214	709	3941

Figure 5-11: Interleukin 8 concentration (on successive days) from PBECs (cell culture A) challenged with diluted gastric juice

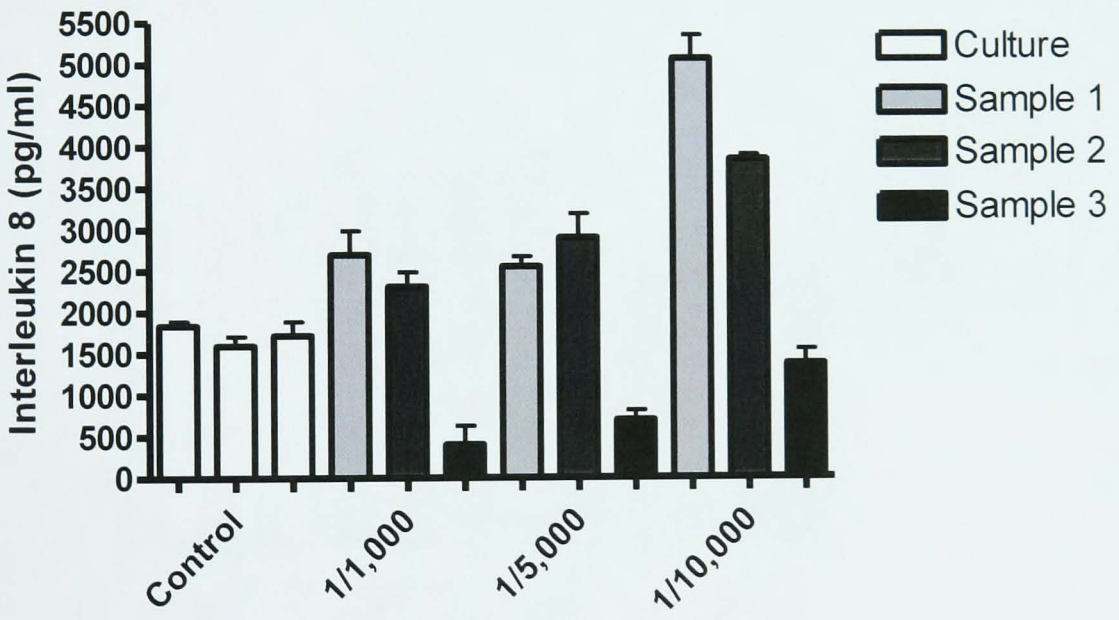


In cell culture B samples one and two led to an increase of IL-8, sample three led to a decrease in IL-8 (Table 5-16,
Figure 5-12) (repeated culture n=1, with repeated wells n=7, overall n=7 from each experiment).

Table 5-16: Interleukin 8 concentration (on successive days) from PBECs (cell culture B) challenged with gastric juice

	IL-8 (pg/ml) at 24 hours (sample 1)	IL-8 (pg/ml) at 24 hours (sample 2)	IL-8 (pg/ml) at 24 hours (sample 3)
Control	1839	1598	1719
1/1,000	2697	2314	404
1/5,000	2558	2905	699
1/10,000	5070	3851	1380

Figure 5-12: Interleukin 8 concentration (on successive days) from PBECs (cell culture B) challenged with diluted gastric juice



In cell culture C, stimulation with gastric juice led to a decreased IL-8 production (Table 5-17,

Figure 5-13) (repeated culture n=1, with repeated wells n=7, overall n=7 from each experiment). This was not statistically significant.

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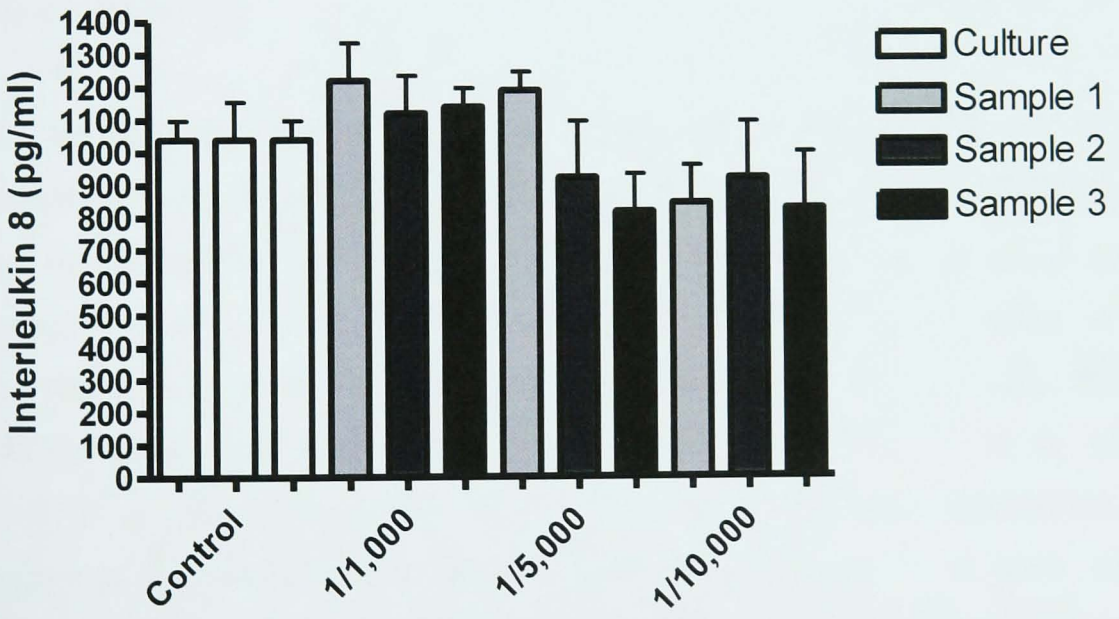
Table 5-17: Interleukin 8 concentration (on successive days) from PBECs (cell culture C) challenged with gastric juice

	IL-8 (pg/ml) at 24 hours (sample 1)	IL-8 (pg/ml) at 24 hours (sample 2)	IL-8 (pg/ml) at 24 hours (sample 3)
Control	1039	1039	1039
1/1,000	1221	1120	1139
1/5,000	1189	923	818
1/10,000	844	923	828

Figure 5-13 Interleukin 8 concentration (on successive days) from PBECs (cell culture C) challenged with diluted gastric juice



5.5. Discussion

The main findings of this chapter are:- bacterial contamination is present in gastric juice: exposure of the HT29-MTX cell line to pepsin led to a decrease of mucin production, but did not result in IL-8 release or reduced cell viability; exposure of primary bronchial epithelial cells to diluted gastric juice led to cell death.

The experiments reported have shown gastric juice to be heavily contaminated with organisms. The number of contaminated samples was greater than expected. This may be important for lung transplant recipients as gastric juice may act as a reservoir for allograft infection. Subsequent aspiration of gastric juice could directly introduce infection into the lung allograft. The reduction in mucus in response to stimulation of goblet cells with pepsin suggests that aspiration may degrade the protective mucus barrier lining respiratory epithelium. The significant cell death encountered after stimulation of primary bronchial epithelial cells by diluted gastric juice suggests microaspiration may be an important injury to lung allografts.

An important function of gastric juice is to inactivate and destroy micro-organisms (Martinsen, Bergh et al. 2005). The low pH and digestive enzymes of gastric juice provide a poor environment for bacterial growth and are often bactericidal (Gotley, Morgan et al. 1990; Verdu, Viani et al. 1994; Martinsen, Bergh et al. 2005). Some bacteria have developed an acid tolerance response and can survive in acidic environments (Martinsen, Bergh et al. 2005). Gastric juice is normally strongly acidic with a pH of 1-3 due to hydrochloric acid secretion (Verdu, Viani et al. 1994). PPI therapy increases intra-gastric pH and may predispose the gastric juice to bacterial colonisation (Verdu, Viani et al. 1994; Martinsen, Bergh et al. 2005). A pH of 4-7 has no bactericidal effect (Martinsen, Bergh et al. 2005; Zhu, Hart et al. 2006). One study showed PPI therapy led to increased gastric pH and bacterial overgrowth. Mean bacterial counts increased from 0.47 to 5.13 $x10^6$ cfu/ml, whilst mean pH increased from 2.51 to 5.79 (Goddard and Spiller 1996). Previous studies have suggested a link between PPI therapy and pneumonia in critical care patients (Tryba and Cook 1995) and in patients in the community (Herzig, Howell et al. 2009). Acid inhibition alters the gastric flora and if this is aspirated, it may then lead to pneumonia (Vakil 2009). The current study showed bacteria, including *Pseudomonas* aeruginosa, in gastric juice of both lung transplant recipients and non-transplant patients. Of interest, this was a biofilm forming species capable of allograft colonisation and refractory to conventional antibiotics (Robertson, Griffin et al.

2009). These bacteria are likely to have entered the gastric environment via the oropharynx from swallowed sputum or saliva. The oropharyngeal flora may have been altered by immunosuppressive therapy. The importance of this intra-gastric bacterial colonisation is that gastric juice may act as a reservoir of infection and if reflux/aspiration occurs pathogens may be introduced and re-introduced into allografts (Botha, Archer et al. 2008; Vos R, Vanaudenaerde BM et al. 2008). This risk is not altered by the original source of these bacteria.

The concentration of pepsin in gastric juice has been reported as 100-600μg/ml (Wallace 1989; Gotley, Morgan et al. 1991; Balan, Jones et al. 1996). The present study's intragastric results are comparable with published levels. PPI treatment significantly lowered active pepsin concentrations, showing pepsin to be more active at a lower pH. The intragastric bile salt levels detected in lung transplant recipients were similar to normal controls. Levels of bile salts in BALF have been reported from 0-32μmol/l (D'Ovidio, Mura et al. 2005; Blondeau, Mertens et al. 2009). It is hard to equate the BALF levels reported with the described gastric levels, knowing the subsequent dilutions. Aspirates will be diluted by oesophageal, oropharyngeal and bronchial secretions. There will be a further dilution of 100-200 times through the BAL lavage fluid volume. High levels of bile salts(>300μmol/l) will be detected, levels lower than this will be undetectable. Only 17% (11/65) had bile salt levels >300μmol/l. Trypsin is a protease secreted by the pancreas into the duodenum. In one study, trypsin was found in 17 of 365 gastric juice aspirates (Gotley, Morgan et al. 1991). The present activity assay shows trypsin was present in gastric juice aspirates but at levels a hundred fold less than pepsin. Thus it will be a less useful biomarker of aspiration.

Alterations in mucus homeostasis may be problematic in lung transplant recipients (Veale, Glasper et al. 1993). Little has been published on down-regulation of mucus production which could lead to drying of the epithelial surface. Aspiration may down-regulate mucus production and homeostasis leading to epithelial injury, damage and increased infection. This study has shown mucus secretion by a goblet cell line to be down-regulated by pepsin.
Stimulation of goblet cells with porcine pepsin did not lead to an increase in IL-8 production nor affect cell viability. There was a down-regulation of MUC5AC production and this may reduce the protective effects of MUC5AC on the respiratory epithelia. This may lead to cell injury and facilitate infection and colonisation. The reduction of MUC5AC may be a result of reduced production or as a result of MUC5AC degradation by pepsin. However, experiments

were performed at pH7.4 and pepsin has no activity at this pH. Further experimental work should be undertaken to reveal the mechanisms of these results.

It has been shown that exposure of a porcine transplant lung to gastric juice increases indirect alloimmunity (Meltzer, Weiss et al. 2008), Cell death, induced by aspiration, leads to inflammation, scarring and fibrosis and the release of MHC peptides which could activate the indirect immune system. The current experiments show that exposure of epithelial cells to dilute gastric juice leads to cell death. This may explain the link between aspiration and the indirect alloimmune response (Meltzer, Weiss et al. 2008). Interleukin 8 is a marker of injury and is produced by many cells in response to injury. The biological mechanisms of BOS may involve elevated IL-8 (D'Ovidio, Mura et al. 2005). Stimulation of PBECs with pepsin did not affect IL-8 production significantly and did not lead to cell death. No measurable MUC5AC was produced by the PBEC in a control situation or after stimulation with pepsin. This is to be expected as MUC5AC is predominantly produced by differentiated goblet cells. It suggests, that in this submerged culture, the cells are undifferentiated and that there were few if any differentiated goblet cells present in these cultures.

Chapter 3 has shown an association between proximal reflux, aspiration and BALF IL-8 levels. If allograft epithelial cells are releasing IL-8 then other stimuli in gastric juice other than pepsin are responsible.

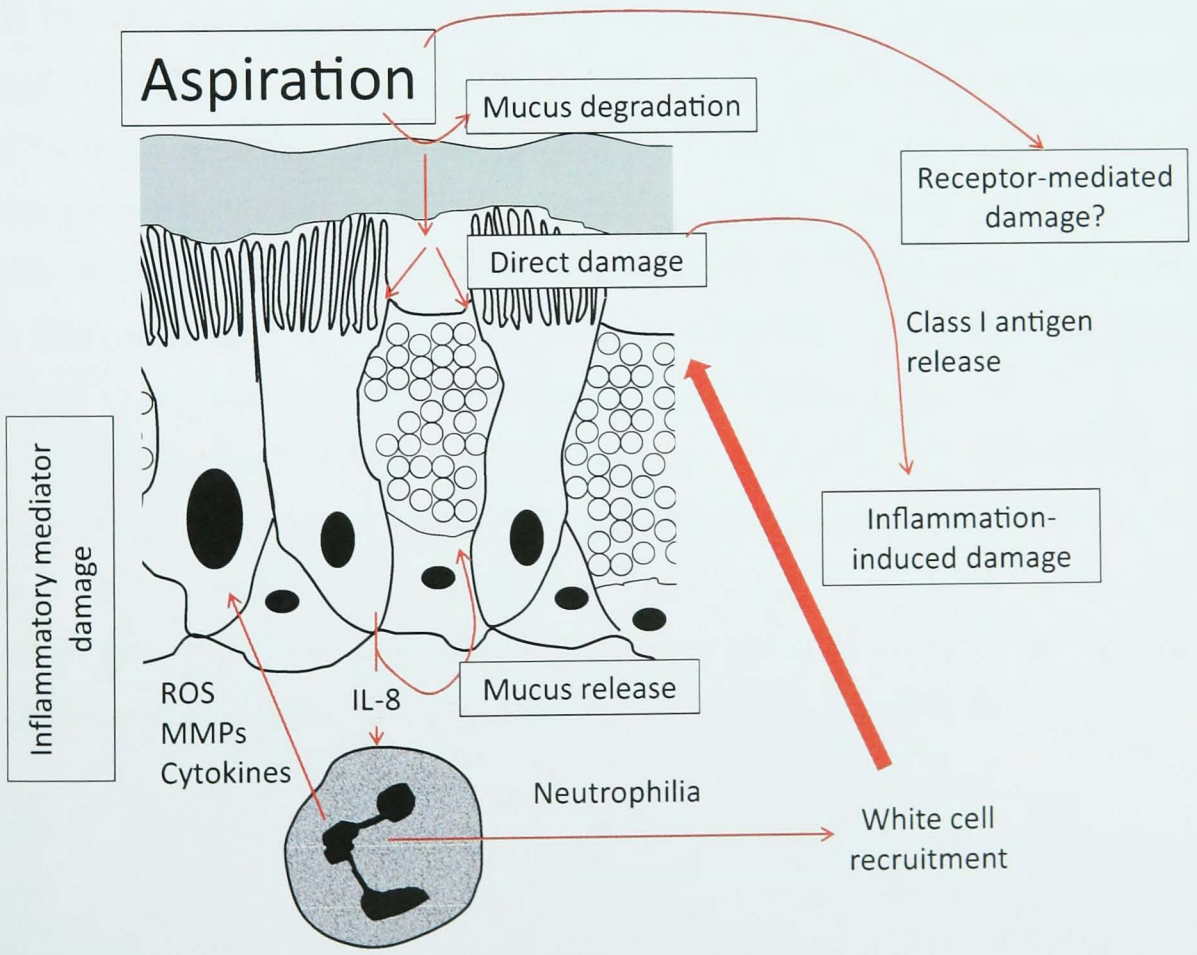
The exposure of epithelial cells to diluted gastric juice resulted in cell death. IL-8 production was variable which may be partly due to the cell death. Several samples had elevated IL-8 suggesting injury. Although there was a varied response in IL-8 production it must be noted that samples underwent significant cell death, most notably at the 1/1,000 concentration (up to 84%). If this is corrected for, i.e. allowing for the decrease in cell population, then there is a general increase in IL-8 production, up to seventeen times control levels. This suggests that although these samples have fewer cells, they have increased IL-8 production. However, this deduction must be interpreted with caution as it is unknown whether dying cells increase IL-8 production and whether or not the process of cell death releases stores of IL-8 from inside cells. Of interest in cell culture A, the gastric juice which triggered the greatest IL-8 production was the sample which tested negative for microbiology (Sample 3).

Cell death was much greater at a 1/1,000 dilution (80%) when compared to a 1/10,000 dilution. This suggests that even after significant dilution, aspiration still has the potential to be injurious.

Model of injury

This study hypothesises a model of injury (Fig 5.14). Aspiration induces damage through a variety of causes of injury:- acid, pepsin, bile and trypsin. Aspiration introduces pathogens into the lung leading to infection and colonisation. The first protective layer in the epithelium is the mucus layer. Respiratory epithelial mucus secretion may be down-regulated by pepsin, exposing the epithelium to injury and direct cellular damage. This leads to inflammation induced damage, cell death and cytokine release from epithelial cells. IL-8 release triggers several responses including mucus production and neutrophilia. The latter leads to damage through reactive oxidative species cytokines and matrix metalloproteinases production. This results in epithelial to mesenchymal transition which converts epithelial cells to fibroblasts and subsequently may lead to obliterative bronchiolitis. The cell death leads to inflammation and the MHC I molecules shed into circulation could trigger an indirect immune response (Meltzer, Weiss et al. 2008).

Figure 5-14: Hypothesised model of aspiration induced damage in lung allograft



(Pearson 2009) (Artwork by IA Brownlee)

The main weakness of this study was the relatively small numbers involved. Gastric juice samples were time consuming to collect and analyse. Cells were time consuming to grow. Our samples of gastric juice were collected after fasting. Night-time and post-prandial levels remain unknown. The sample population was heterogeneous with significant variance in demographics, pathology and individual PPI use. These variables may affect the results of this study and reduce the applicability to individual populations. Using the endoscope raises the possibility of contamination of gastric juice samples. Current methods for sterilisation of endoscopes have been shown to kill all bacteria (Cronmiller, Nelson et al. 1999; Allison, Bradley CR et al. 2008). The risks of contamination were minimal but oropharyngeal contamination remains a possibility.

To analyse pepsin, we used an activity assay. If the pepsin has been exposed to a pH >7 then it will be irreversibly denatured and will not be detected by this assay. These samples require further analysis using an ELISA.

The goblet cell line (HT29-MTX) was derived from a colorectal cancer cell line and thus may be an inaccurate model. It was used as respiratory goblet cells are difficult to isolate and culture. This cell line has some similar properties to respiratory goblet cells and expresses MUC5AC and MUC5B. Thus, it is an acceptable model. Due to cell line death and problems with cell culture, we did not stimulate the goblet cell line with gastric juice. The effects of stimulating this cell line with gastric juice and pepsin cannot be compared.

The PBECS used were undifferentiated. Differentiated cell cultures are more resilient to injuries (Parker, Sarlang et al. 2010) but are more difficult to culture. Results from in vitro experiments cannot always be extrapolated to an in vivo environment.

Cell death was assessed by Trypan blue which relies on cell counts and could be open to human error. An experiment comparing this with the assay based TiterBlue model, revealed that there was no significant difference between the two methods and a simple human observation method did not consistently over or under read cell viability. This simple, quick test could be used as an indicator of cell death to direct future experiments.

6. *Effects of anti-reflux surgery on reflux symptoms and quality of life in lung transplant recipients*

6.1. Abstract

Introduction

Gastro-oesophageal reflux disease (GORD) has been suggested to be a risk factor for BOS post-lung transplant. Anti-reflux surgery has been performed in some patients and may be associated with improved lung function and survival. Little has been published on the effects of this on symptoms and quality of life of laparoscopic fundoplication in adult lung transplant recipients.

Aim

The aim of this study was to evaluate the effects of anti-reflux surgery on reflux symptoms and quality of life in lung transplant recipients.

Methods

Between 1st June 2006 and 1st October 2009, all lung transplant recipients undergoing anti-reflux surgery were studied. Patients were operated on for symptomatic GORD or for GORD with decreased lung function. Quality of life was assessed before and after surgery using Gastrointestinal Quality of Life Index (GIQLI), DeMeester and Reflux Symptom Index (RSI) questionnaires. Body Mass Index and pulmonary function were followed up from transplant to the current date.

Results

Nine patients (3 male/6 female) with a median age of 41 years (range 24-57years) were operated on during this period. Laparoscopic Nissen fundoplication was the procedure of choice. There was no peri-operative mortality and no major complications occurred. Median hospital stay was 2 days (range 2-4 days). 7/8 patients were satisfied with the results of surgery 6 weeks post-operatively and 4/5 at six months. There was an improvement in median RSI, DeMeester and GIQLI scores at six weeks and this was maintained at six months. Median BMI decreased from 22.5 (range 18.5-29) pre-fundoplication to 21.1 (Range 17.6-29.4) at six months post-fundoplication ($p=0.0012$). Median FEV_1 was 2.35L pre-operatively and 2.68L at latest follow up (median 174 days post-fundoplication (range 68-1082days)).

Conclusion

Fundoplication was associated with an improvement in reflux symptoms and overall quality of life in this population.

6.2. Introduction

The earlier chapters of this thesis have focused on the deleterious effects and high prevalence of GORD post lung transplant (D'Ovidio and Keshavjee 2006; Robertson, Griffin et al. 2009). Anti-reflux surgery may be associated in this population with an increased survival and improved lung function (Davis, Lau et al. 2003; Cantu, Appel et al. 2004).

In routine patient populations fundoplication has been shown to improve symptoms and quality of life. Little evidence exists to support a benefit of this therapy on symptoms and quality of life in lung transplant recipients. For this study three validated questionnaires were used- (DeMeester, GIQLI and RSI questionnaires).

The DeMeester reflux regurgitation questionnaire is a validated straightforward tool (DeMeester, Wang CI et al. 1980). It is based on a score of 0-3 for symptoms of reflux, regurgitation and dysphagia. The higher the score, the worse the symptoms are.

The RSI (Figure 2.1) is a 9 item questionnaire which assesses both oesophageal and extra-oesophageal reflux symptoms. It is easily administered and highly reproducible. The higher the score, the worse the symptoms are. A RSI score of greater than 13, is abnormal (Belafsky, Postma et al. 2002).

The GiQLI was chosen as it is a straightforward quality of life questionnaire which addresses both global symptoms of well-being and also gastrointestinal focused questions (Kirk 1986; Eypasch, Williams et al. 1995). It allows us to look at the effects of fundoplication on quality of life without too much focus on the transplant process. The questionnaire is made up of 36 questions, 17 physical (8 related to upper gastrointestinal symptoms) and 19 social. Each question is scored from 0-4. The higher the GIQLI score the greater the quality of life.

There have been reports of weight loss after anti-reflux surgery in both non-transplant (Neumayer, Ciovica et al. 2005) and the transplant community (Burton, Button et al. 2009). This study also assessed Body Mass Index (BMI) pre and post-operatively.

The aim of this study was to evaluate the effects of anti-reflux surgery on reflux symptoms and quality of life in lung transplant recipients.

6.3. Methods

Between 1st June 2006 and 1st December 2009, all lung transplant recipients undergoing anti-reflux surgery at the Northern Oesophago-Gastric Unit were studied. Surgery was considered for patients with symptomatic reflux alone, refractory to PPI therapy, or for reflux associated with deteriorating lung function. All lung transplant recipients, in our unit, are routinely prescribed prophylactic PPI therapy to prevent steroid induced ulceration.

Reflux status was assessed by oesophageal manometry, pH-impedance (Ohmega, MMS System, Utrecht, The Netherlands) and endoscopy. Patients underwent a thorough pre-operative assessment to ensure fitness for surgery. Patients were followed up clinically with emphasis on lung function, satisfaction and quality of life and BMI. Patient satisfaction was assessed by directly questioning of patients. Lung function was assessed in accordance with American Thoracic Society/European Respiratory Society guidelines. The RSI, DeMeester reflux questionnaire and GIQLI questionnaires were completed pre-operatively, 6 weeks and 6 months post-operatively. Patients were asked about satisfaction at 6 weeks and 6 months post-operatively.
Statistical analysis was carried out using non-parametric paired t-tests (Wilcoxon) with Graphpad Prism software (San Diego, CA, USA).

Laparoscopic Nissen fundoplication was performed. Access to the abdominal cavity was via 4 ports and an epigastric stab incision for the Nathanson retractor to retract the liver. Initially the oesophageal hiatus was dissected to mobilise the oesophagus. The posterior vagus was preserved and a window was created behind the oesophago-gastric junction. The posterior crura were repaired to tighten the hiatus, and a loose 360° wrap was tailored with 3 sutures. One further suture was used to anchor the wrap to the oesophagus and right crus. Percutaneous endoscopic gastrostomy (PEG) fistulae were repaired when present. They were divided with an Endostapler. The PEG wound was then excised and the deficit in the abdominal wall and skin were closed. Local anaesthesia was inserted into the peritoneal cavity and infiltrated in the wounds.

6.4. Results

6.4.1. Demographics

Nine patients (6 women, 3 men) with a median age of 41 years (range 24-57years) underwent fundoplication. Indications for lung transplant were cystic fibrosis 5, COPD/asthma 1, pulmonary fibrosis 2, Pulmonary fibrosis/asthma 1. Eight underwent single sequential lung transplant, 1 had a right single lung transplant (Table 6-1). Indications for fundoplication were heartburn (n=5) or heartburn and extra-oesophageal symptoms (n=4). Symptoms occurred despite PPI therapy. PPI used included lansoprazole 30mg od (n=1), 30mg bd (n=4) (one of these patients also took ranitidine 150mg nocte), rabeprazole 20mg od (n=2) and esomeprazole 40mg bd (n=1). Median pre-operative BMI was 22.7 (range 18.5-29).

Table 6-1: Patient demographics

Age	
-Median	41 years
-Range	24-57 years
Sex	
-Male	3
-Female	6
Underlying pathology	
-Cystic fibrosis	5
-COPD/asthma	1
-Pulmonary fibrosis	2
-Pulmonary fibrosis/asthma	1
Transplant	
-SSLT	8
-RSLT	1
-LSLT	0
-HLT	0

Oesophageal physiology

All patients underwent oesophageal physiology measurements. All (9/9) underwent oesophageal manometry (n=9), one of 9 had pH monitoring (n=1) whilst 8/9 had combined pH-impedance (n=8). Results of these tests are shown in Table 6-2. One patient who underwent surgery had a normal DeMeester score and acid exposure on PPI therapy. The decision was made to operate as they had symptomatic reflux, oesophagitis and abnormal volume exposure on impedance measurements.

Table 6-2: Results of pre-fundoplication investigations

Oesophageal physiology	Median	Range
Lower oesophageal sphincter pressure length Mean distal peristaltic amplitude	 13 2.5 47.9	 9.3-26 1.5-3.5 75.4-165.9
Peristalsis normal abnormal	 7 2 (NSD, SOC)*	 n/a n/a
Reflux indices Acid exposure DeMeester score Oesophageal volume exposure Total reflux events Proximal reflux events	 17.2 61.1 1.58 68 19	 1.6-33.1 7.5-115.2 0.5-3.84 21-125 3-78
FEV_1 (% predicted)	87.8	33.4-139.5

*NSD= Nonspecific dysmotility, SOC= Simultaneous oesophageal contractions

6.4.2. *Morbidity and mortality*

There were no deaths or serious post-operative complications. One patient developed minor post-operative dysphagia which increased their post-operative stay by 2 days. Barium swallow revealed no significant hold-up and symptoms subsequently settled.

6.4.3. *Overall satisfaction*

Overall 8/9 patients were satisfied at 6 weeks and 7/8 patients were satisfied at 6 months. At six weeks one patient was unsatisfied due to dysphagia. At six months one patient was unsatisfied due to pain at the site of her PEG fistula and abdominal bloating.

6.4.4. *Quality of life*

Overall there was a statistically significant improvement in symptoms and quality of life scores over the first six months post-fundoplication (Table 6-3).

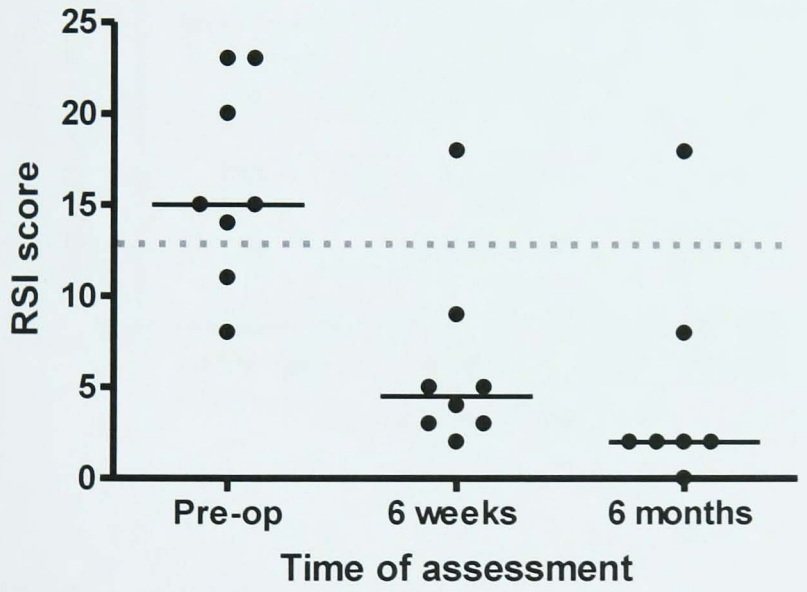
Table 6-3: Median (and range) quality of life questionnaire scores pre & post-fundoplication

	Pre-operative	Six weeks	Six months
GIQLI	106 (65-132)	118 (63-133)	128 (75-142)
DeMeester	4 (1-6)	1 (0-4)	1 (0-2)
RSI	15 (8-23)	3.5 (2-18)	2 (0-18)

6.4.5. *Reflux symptom index questionnaire*

Pre-fundoplication RSI was positive on 6/8 patients and this decreased to 1/8 being positive for RSI by six weeks and 1/7 being positive at six months. The median RSI improved from 15 (range 8-23) pre-operatively (n=8) to 3.5 (range 2-18) at six weeks post-fundoplication (n=8) (p=0.008) and 2 (range 0-18) at six months (n=7) (p=0.016) (Figure 6-1). There was no statistical difference between RSI scores at six months and six weeks (p=0.44). The improvement in RSI score was through an amelioration of both heartburn and extra-oesophageal symptoms.

Figure 6-1: Graph of RSI score over the first six months post fundoplication



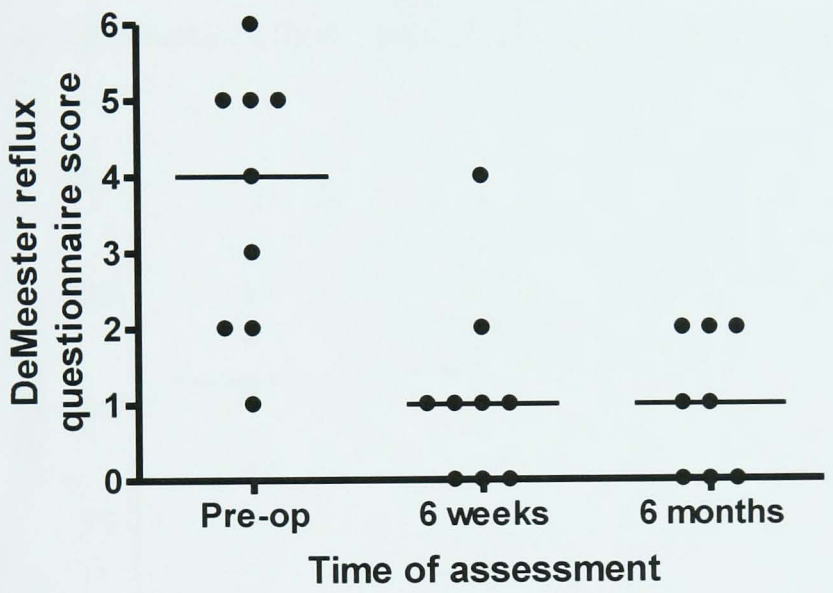
Legend: Dotted line is placed at RSI=13, the cut off between normal and abnormal scores

6.4.6. *DeMeester reflux questionnaire*

There was an improvement in median DeMeester reflux questionnaire score from 4 (range 1-6) pre-operatively (n=9) to 1 (range 0-4) at six weeks (n=9) and 1 (range 0-2) by six months (n=8) (

Figure 6-2). There was a statistical significance between pre-operative scores and six weeks (p=0.039) and pre-operative scores and six months (p=0.023). There was no significant difference between scores at six weeks and six months. (p=0.63).

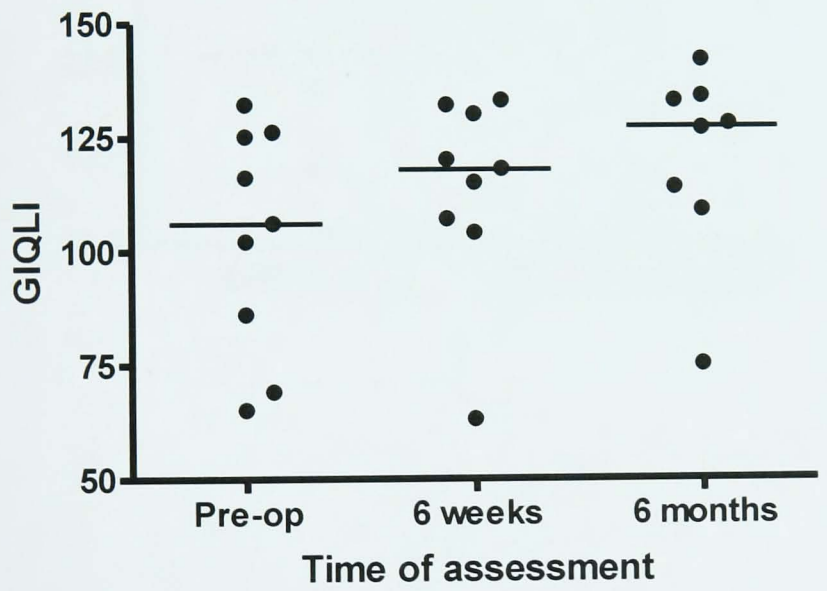
Figure 6-2: Graph of DeMeester reflux questionnaire score over the first six months post fundoplication



6.4.7. *Gastrointestinal quality of life index*

There was a statistically significant improvement in median GIQLI score from 106 (range 65-132) pre-operatively (n=9) to 118 (range 63-133) at six weeks (n=9). This was 128 (range 109-134) by six months (n=8) (Figure 6-3). There was a significant difference between GIQLI scores pre-operatively and at six weeks (p=0.001) and six months (p=0.023). There was also a statistically significant improvement from six weeks to six months (p=0.003). The improvements were in both physical and social categories. Seven points of the overall median improvement of 22 points were in social functioning, but the predominant improvement was via amelioration of physical symptoms.

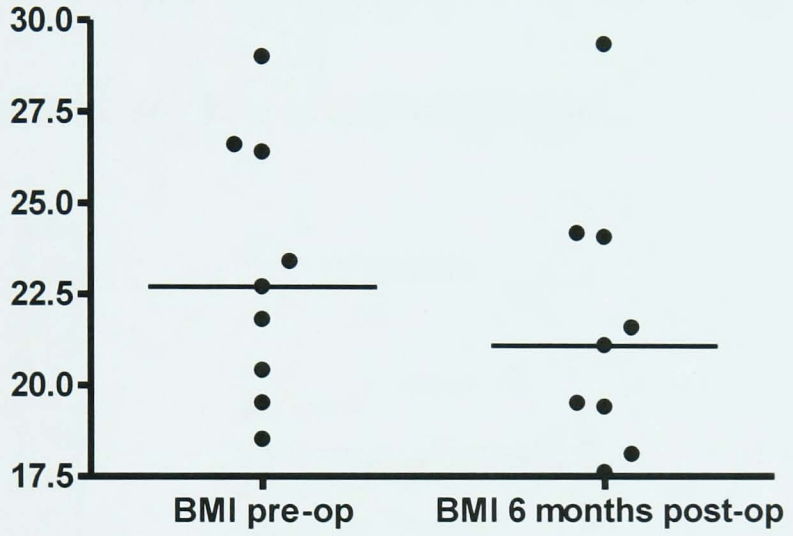
Figure 6-3: Graph of GIQLI score over the first six months post fundoplication



6.4.8. *Body mass index*

Median BMI decreased from 22.7 (range 18.5-29) pre-fundoplication to 21.1 (Range 17.6-29.4) at six months post-fundoplication (p=0.001) (Figure 6-4). Four patients kept a steady weight and five patients had a decrease in weight post-fundoplication.

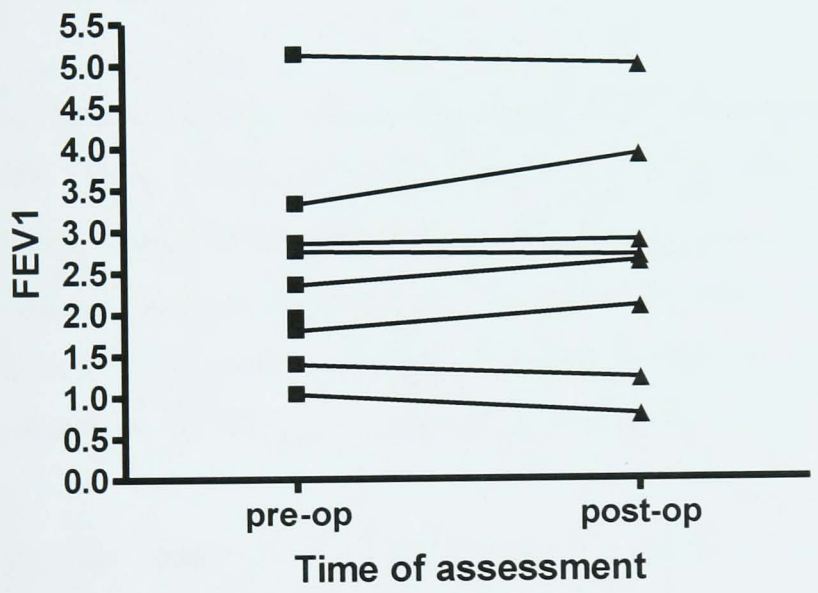
Figure 6-4: Graph of BMI score over the first six months post fundoplication



6.4.9. *Lung function*

Patients were followed up for a median of 174 days post-fundoplication (range 68-1082days). Median FEV_1 was similar pre-fundoplication 2.35L (range 1.03-5.12L) and post-fundoplication 2.68L (0.79-5.03L) (Figure 6-5). This was not statistically significant (p=0.38). Pre-fundoplication, five patients had no evidence of BOS, whilst the remaining four patients had BOSp (n=1), BOS1 (n=1), BOS 2 (n=1) and BOS 3 (n=1). One patient had a worsening BOS score from BOS 2 to 3 during follow up. The patient with BOSp had a reversal of this to BOS 0. All other patients remained stable.

Figure 6-5: Graph of PFTs pre and post fundoplication



6.5. Discussion

The main findings of this study was that in lung transplant recipients, anti-reflux surgery improves both reflux and extra-oesophageal reflux symptoms; there was an improvement in quality of life after surgery; in the first six months post-fundoplication BMI decreased.

Anti-reflux surgery improves both reflux and extra-oesophageal reflux symptoms. The subsequent improvement in quality of life was derived predominantly from an improvement in physical symptoms but also an improvement in social functioning. This suggests that fundoplication is of benefit to lung transplant recipients. The improvements in extra-oesophageal reflux symptoms suggest that these patients suffer from laryngopharyngeal reflux. The decreased BMI within the first six months post-fundoplication is of unknown significance.

The Duke University Transplant Group have published several papers(Lau, Palmer et al. 2002; Davis, Lau et al. 2003; Cantu, Appel et al. 2004; O'Halloran, Reynolds et al. 2004; Balsara, Cantu et al. 2008), with results suggesting that anti-reflux surgery may lead to increased survival and improved lung function post-transplantation (Cantu, Appel et al. 2004). The limitations and flaws of their studies are described previously. No conclusions can be drawn on the effects on lung function from this study.

Anti-reflux surgery in the lung transplant population has been shown to be safe (O'Halloran, Reynolds et al. 2004). Only one post-fundoplication death has been reported.(Burton, Button et al. 2009). This study reports no mortality or major morbidity to date. Post-operative stay was longer than for non-transplant patients. This may be partially due to the fact that transplant patients travel greater distances for surgery and can remain in hospital due to logistical reasons.

Fundoplication is associated with symptomatic improvement in the non-transplant population (Korolija, Sauerland et al. 2004; Yano, Sherif et al. 2009). It is recommended that questionnaires are completed between 1-3 months and then at one year post-operatively (Korolija, Sauerland et al. 2004). The present study assessed patients at six weeks and six months to obtain quality of life data at both short and medium term follow up. Only one study has previously assessed the effects of fundoplication on reflux symptoms in lung transplant recipients (Burton, Button et al. 2009). This paper did not use validated questionnaires and the results are therefore of limited value. The current study has demonstrated an improvement

in reflux and extra-oesophageal reflux symptoms using validated questionnaires, the DeMeester reflux questionnaire and the RSI.

Fundoplication has been shown to improve quality of life in the non-transplant population (Korolija, Sauerland et al. 2004; Yano, Sherif et al. 2009). In one previous transplant study, three quarters of patients had an improvement in quality of life scores. 88% rated the results of their surgery as excellent or good(Burton, Button et al. 2009). However, this used non-validated methods of assessment. The current study has shown that patients are generally satisfied with their procedure and there is an improvement in quality of life. The GIQLI questionnaire was used as it is validated and recommended for the assessment of anti-reflux surgery by the European Association for Endoscopic Surgery (Korolija, Sauerland et al. 2004).

It is well known that BMI decreased post-fundoplication, due to early satiety. This normally stabilises within the first six months. One previous study has shown this in the lung transplant population. The present study concurs with these results and shows a decrease in median BMI from 22.5kg/m^2 to 21.1kg/m^2 in the first six months post-fundoplication. The clinical significance of this is unknown, as the current study does not demonstrate whether this weight stabilises or undergoes further deterioration by one year. This requires further follow up.

This study has several limitations. Firstly, the numbers involved are small. The patients had a variety of indications for surgery, making the patient sample diverse. Secondly, fundoplication was performed at different times after transplant and no patients were operated on within 90 days, the suggested optimum time for therapy. No control group was present to compare symptoms or lung function and the study wasn't randomised. The overall follow up is limited and thus reduces the conclusions that can be drawn from this study.

7. Summary

7.1. Summary

Background

-Chronic microaspiration, secondary to extra-oesophageal reflux, may contribute to bronchiolitis obliterans syndrome post-lung transplant.

-Up to 75% of lung transplant patients have demonstrable gastro-oesophageal reflux disease on pH monitoring.

-Elevated biomarkers, pepsin and bile salts, have been documented in the broncho-alveolar lavage fluid post-transplant, suggesting microaspiration. Elevated pepsin is associated with acute rejection, and elevated bile salts have been linked to BOS.

-Early anti-reflux surgery may lead to increased survival and improved lung function, through preventing microaspiration and allograft injury.

-Little has been published on this topic and the current data is limited and flawed.

Results

Chapter 3

-GORD occurs frequently within the immediate post-lung transplant period.

-This is associated with elevated pepsin in the BALF, signifying aspiration.

-A correlation exists between proximal reflux and neutrophilia suggesting that increased proximal reflux leads to aspiration. This leads to allograft inflammation and damage.

-Bile salts are a less prevalent biomarker of aspiration.

Chapter 4

Despite decreasing immunosuppression and improvement in lung function, the prevalence of GORD increases over the first six months post-lung transplant.

-Microaspiration improves as suggested by a decrease in BALF pepsin levels.

-A potential explanation of this finding is that over this time point pulmonary defence mechanisms recover. This may occur through vagal re-innervation of the allograft, improved cough reflex and muco-ciliary clearance. These factors may reduce the amount of aspiration the allografts encounter.

Chapter 5

-Gastric juice may be colonised by pathogenic organisms.

-This may be due to the raised pH created by PPI therapy.

-This may be important for lung transplant recipients, as gastric juice may act as a reservoir for bacteria. Subsequent aspiration of gastric juice could directly introduce infection into the lung allograft.

-MUC5AC levels were reduced in response to stimulation of goblet cells with pepsin. This suggests that aspiration may degrade the protective mucus barrier lining respiratory epithelium.

-Cell death was encountered after stimulation of primary bronchial epithelial cells to diluted gastric juice. This suggests microaspiration may be an important cause of injury to lung allografts. This cell death could release MHC peptides from allograft epithelial cells which could activate the indirect immune system. This may explain the link between aspiration and the indirect alloimmune response.

Chapter 6

-Anti-reflux surgery is safe in selected lung transplant recipients.

-Anti-reflux surgery improves both reflux and extra-oesophageal reflux symptoms in lung transplant recipients.

-The subsequent improvement in quality of life was derived predominantly from an improvement in physical symptoms but also an improvement in social functioning.

-BMI decreases over the first six months post-fundoplication. This is of unknown clinical significance.

7.2. Future Work

More work is required to increase our understanding of microaspiration in these patients at a clinical and cellular level.

Clinical

-A larger study of reflux and aspiration should be performed with long-term follow up, to establish whether early GORD and aspiration is associated with BOS.

- A larger study of changes in GORD and aspiration over the first six months should be undertaken to assess whether the paradox of improving aspiration, despite worsening GORD is maintained.

-BALF pepsin levels should be collected and analysed at 1 year to establish whether aspiration further improves or is maintained at this time point.

Laboratory

-More gastric juice samples should be collected from homogeneous populations of patients.

-Gastric juice samples should be collected at differing times from the same patients to assess variability. Samples should be collected from patients both on and off PPI therapy to assess the effects of PPI on colonisation.

-In lung transplant recipients gastric juice microbiology should be compared with BALF microbiology to assess whether the same bacteria are present in both samples.

-Goblet cells should be stimulated with diluted gastric juice and also other individual agents which could be injurious (e.g. bile salts and trypsin) to assess the individual effects of these.

-Experiments should be repeated using a differentiated epithelial cell culture.

-Epithelial cells should be stimulated with individual components of gastric juice which could be injurious (e.g. bile salts and trypsin), to assess which component causes cell death and elevated IL-8.

Surgery

-Many unanswered questions remain about the role of laparoscopic fundoplication in lung transplant recipients. Does surgery improve lung function and survival? When is the optimal time for intervention? What are the optimum selection criteria for surgery?

-Individual units researching this topic should collaborate and undertake a large multi-centred randomised controlled trial of laparoscopic fundoplication in lung transplant recipients to answer the above questions.

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Appendices

Appendix 1: Special Trustees Grant

2008: £20,000: Research Grant from the Joint Research Scientific Executive Scientific Committee of the Newcastle Healthcare Charity (RVI/NGH) & Newcastle Upon Tyne Hospitals NHS Charity (FH) for: **The role of oesophageal impedance measurement and markers of aspiration in the detection of extra-oesophageal reflux disease and in the development of allograft dysfunction in human lung transplant recipients.** Written and submitted by myself.

Newcastle University

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Our Ref: PC/ML

20th March 2008

Professor S M Griffin
Professor of Gastrointestinal Surgery
Northern Oesophago-Gastric Unit
Royal Victoria Infirmary

Dear Professor S M Griffin

THE ROLE OF OESOPHAGEAL IMPEDANCE MEASUREMENT AND MARKERS OF ASPIRATION IN THE DETCTION OF EXTRA-OESOPHAGEAL REFLUX DISEASE AND IN THE DEVELOPMENT OF ALLOGRAFT DYSFUNCTION IN HUMAN LUNG TRANSPLANT RECIPIENTS

The Newcastle Healthcare Charity (the Trustees), on the recommendation of the Joint Research Executive Scientific Committee, are prepared to make a grant in respect of the above project subject to the terms set out below.

TOTAL AMOUNT:	£20,000
Comprising	
Salary Costs	£0
Equipment/Other	£0
Consumables	£20,000
Total:	£20,000
Duration:	12 months
Starting Date:	1st April 2008 (or as soon as possible thereafter)

Nothing in this offer constitutes a contract of employment and there shall not be charged to the project any costs falling to the employer other than arises directly from the pursuit of the research (specifically, sick pay and maternity leave costs are excluded); nor do the Newcastle Healthcare Charity accept any responsibility for claims which might arise from the conduct of the project directly or indirectly. It shall be the responsibility of the researcher and his/her employer to ensure that the project work is of an adequate quality, that the project is conducted in accordance with the protocol submitted to the JRE Scientific Committee (or as amended by it) and with all reasonable care.

Where appropriate, every endeavour will be made by the researcher and his/her employer to disseminate the results of the project. **The grant holder shall be obliged to submit a report (*circa* 1,000 words), no later than six months after the cessation of funding. No further application from the grant holder will be considered if a final report is outstanding.**

Any intellectual property arising from the Trustee-funded work should be commercially exploited when appropriate for the benefit of the Trustees and the institution. The Trustees waive any claim to ownership of intellectual property or data arising from the commercial exploitation of Trustees-funded research on the condition that grant holders and their administrative authorities agree to keep the Trustees fully informed of the development of any patentable property and to include the Newcastle upon Tyne Hospitals NHS Foundation Trust as an equitable partner (reasonably related to the Trustees' proportion of support) in any revenue-sharing agreements that may result from this.

It is essential that research is conducted to the highest ethical and scientific standards and all staff involved in the research should have read the Department of Health document 'Research Governance Framework for Health and Social Care' which defines the principles of good research. Researchers should fully understand the implications of research governance and make certain that their work meets its requirements.

Work cannot commence unless the completed Agreement has been returned to me. Please also enclose a copy of the approval letter from the relevant human (or animal) Ethics Committee, where appropriate.

Please indicate by signing and returning the attached copy of this letter whether you are willing to accept the grant offered on the terms stated above. It is also necessary to obtain the signed agreement of a finance officer who will be responsible for the administration of the grant.

Kind regards.

Yours sincerely,

PROFESSOR P F CHINNERY, PhD MRCPath FRCP
Chairman, Joint Research Executive Scientific Committee
On behalf of the Newcastle Healthcare Charity and
Newcastle upon Tyne Hospitals NHS Charity

The above offer of a grant is accepted upon the terms stated.

Principal Investigator

Date

Finance Officer for Trust/University
(delete one)
Date

Appendix 2: European Society For Organ Transplantation Fellowship

2008: £35,000: Fellowship from the European Society for Organ Transplantation-Clinical Research Grant for:

The role of oesophageal impedance measurement and markers of aspiration in the detection of extra-oesophageal reflux disease and in the development of allograft dysfunction in human lung transplant recipients. Written and submitted by myself. Awarded to myself directly.

European Society for Organ Transplantation

Dr. A.G.N. Robertson
Dept. of Cardiopulmonary Transplantation, Cardiothoracic Centre, Freeman Hospital, Newcastle upon Tyne, NE7 7RN, United Kingdom
andrewgnrobertson@doctors.net.uk

Groningen, May 20th, 2008

Reference: 2666
JCRG 08.006

Dear Dr. Robertson,

During the last ESOT Council Meeting all applicants for the ESOT Junior Clinical Research Grant were presented and discussed. We are delighted to inform you that you have been awarded the ESOT Junior Clinical Research Grant. The grant consists of a total amount of
€ 35.000.

It is very important, that you will inform the ESOT Treasurer Prof.Dr. H.J. Schlitt, Department of Surgery of the University of Regensburg, Germany, phone + 49 941 9446801, about the bank information of your institution. For your convenience, you will find attached a form which has to be filled out and returned to Professor Schlitt.

According to the new regulations we require that Grant Awardees will become a member of ESOT. In case you accept the grant, we kindly ask you to transform your ESOT Temporary Membership into a Full Membership asap.

A report about your research project in relation to the ESOT Junior Clinical Research Grant is required within 2 months after completion; failure to report will prohibit the recipient from applying for ESOT grants in the future. In case of a published article, the ESOT Junior Clinical Research Grant should be mentioned in the acknowledgements. All grant recipients will be mentioned during the next ESOT Congress in Paris in 2009.

Please, inform us about your plans and current time schedule concerning the fulfilment of your proposal.

On behalf of the ESOT Council,
yours sincerely,

Rutger J. Ploeg,
Secretary General of ESOT

Cc Professor H.J. Schlitt, Treasurer of ESOT,
hans.schlitt@klinik.uni-regensburg.de
Cc - Professor J.H. Dark, Consultant Cardiothoracic Surgeon,
j.h.dark@ncl.ac.uk
- Professor P.A. Corris, Professor of Thoracic Medicine,
Dept. of Cardiopulmonary Transplantation, Cardiothoracic Centre, Freeman Hospital, Newcastle upon Tyne, NE7 7RN, United Kingdom
paul.corris@ncl.ac.uk

Correspondence ESOT
c/o Prof.Dr. R.J. Ploeg, Department of Surgery, University Medical Center Groningen UMCG, PO Box 30.001, 9700 RB Groningen, The Netherlands
Telephone *31.50.3614430, Telefax *31.50.3611745, E-mail j.t.uildriks@chir.umcg.nl, Website http://www.esot.org

Appendix 3: OESO Award

2008: OESO 9th World Congress: Research Grant Award.

9th World Congress

World Organization for Specialized Studies on Diseases of the Esophagus

oeso the esophagus

Presents

The OESO Third Place Award of Excellence to

A. Robertson

For the Protocol entitled

The role of oesophageal impedance measurement and markers of aspiration in the detection of extra-oesophageal reflux disease and in the development of allograft dysfunction in human lung transplant recipients

Presented to the OESO Research Committee
and Conference Attendees on April 6, 2008

Stephen J. Sontag,
Jury President

Henry D. Appelman,
President of OESO

Robert Giuli,
Executive Director of OESO

Appendix 7: Trevor Clay Memorial Grant from the British Lung Foundation

24 November 2008

BRITISH LUNG FOUNDATION

Dr Chris Ward
Institute of Cellular Medicine
4th Floor William Leech Building
Medical School
Framlington Place
Newcastle-upon-Tyne NE2 4HH

Dear Dr Ward

Award of a British Lung Foundation Grant

I am pleased to confirm the award of a British Lung Foundation research grant as detailed below.

The grant is subject to our Grant Regulations and Conditions, dated November 2007, as enclosed. Any changes to these will be advised and it is your responsibility and that of the Host Institution to take appropriate action to comply with these changes.

Grant Holder(s):
i) Principal Grant Holder: Dr Chris Ward

ii) Co-Grant Holder(s): Professor Paul Corris, Professor S M Griffin, Professor J Pearson & Dr Andrew Robertson

Title of Research: The role of oesophageal impedance measurement and markers of aspiration in the detection of extra-oesophageal reflux disease in human lung transplant recipients.

Type of Award:	Duration:	Amount awarded:
Trevor Clay Memorial Grant	12 months	£5,600

Appendix 3: NIHR-CRN Registration

This project has been accepted for and included in the National Institute of Health Research UK-Clinical Research Network (NIHR CRN ID:6486) with myself as study co-ordinator.

NIHR CRN
NIHR Clinical Research Network
Coordinating Centre

NHS
National Institute for
Health Research

NIHR Clinical Research Network
Coordinating Centre
Fairbairn House
71-75 Clarendon Road
Leeds LS2 9PH

Tel: 0113 343 2314
Fax: 0113 343 2300
Email: info@ukcrn.org.uk
www.crncc.nihr.ac.uk

20th February 2009

Professor S M Griffin
Northern Oesophago-Gastric Unit
Royal Victoria Infirmary
Newcastle upon Tyne
NE1 4LP

Dear Professor Griffin

Re The role of oesophageal impedance measurement in detection of gastro-oesophageal reflux disease in human lung transplant (NIHR CRN ID: 6486)

Thank you for completing the minimum dataset for the above study. I can confirm that the study is eligible for, and has therefore been included on, the National Institute for Health Research (NIHR) Clinical Research Portfolio. The record for this study can be viewed on the Portfolio Database at http://www.ukcrn.org.uk/index/clinical/portfolio_new.html.

Benefit of inclusion in the NIHR Portfolio

Inclusion in the NIHR Portfolio of studies **ensures your study can access NHS service support and research infrastructure support in England** (i.e. support to help with study promotion, approval, identification of eligible patients, recruitment, and follow up etc). This support is now flowing through the Comprehensive Clinical Research Network to the 25 Comprehensive Local Research Networks (CLRNs) across England. Funding allocations to the CLRNs include an activity-based component driven by the data which are held on the UKCRN Portfolio Database and it is therefore essential that your study record is kept up-to-date. Please contact us as soon as possible via email (portfolio@ukcrn.org.uk) if any changes are required.

Collecting your accrual data

In order to ensure that your study remains on the NIHR Portfolio and receives appropriate support through the relevant Comprehensive Local Research Network(s), the UKCRN Coordinating Centre must collect accrual data for the above study from April 2008 and then each month on an ongoing basis.

If you haven't already had the opportunity to send this data to us, we would be grateful if you could do so as soon as possible. Accrual data should be supplied via

UKCRN Accrual Upload System and we will be contacting you in the near future to talk you through this process. Further information and data templates for uploading accrual data can be found on the UKCRN website at: http://www.ukcrn.org.uk/index/clinical/portfolio_new/P_accrual.html. Please contact us (accrual@ukcrn.org.uk) if you have any queries about the process.

We would also encourage you to provide data on accrual prior to April 2008 in order to contribute to the CCRN "baseline" and to provide information on the overall level of recruitment into this study. This can be submitted in a simplified format, simply stating the total number of patients recruited prior to April 2008.

Additional and new studies

Please note that some new studies funded by NIHR Partners (as defined in the Eligibility Criteria) might need to undergo a further adoption process prior to inclusion onto the Portfolio (e.g. if individual studies are part of a programme grant). All new "non-automatic" studies (those funded by non-UK governments, e.g. EU, NIH, and industry-supported, non-industry sponsored - IITs) will also need to undergo a full adoption process.

UKCRN is keen to ensure that all studies which are eligible for inclusion into the NIHR Portfolio are identified so that they can be supported through the Comprehensive Clinical Research Network. If you are aware of any other potentially eligible studies which are recruiting or actively following up patients from April 2008, and which have not yet been confirmed as being on the Portfolio, we would be very grateful if you would let us know. Further details are available at http://www.ukcrn.org.uk/index/clinical/portfolio_new.html.

Thank you for your support in this exercise which will be critical to the successful development of the national Comprehensive Clinical Research Network. Our aim is to ensure the provision of high quality infrastructure to support clinical research in the NHS and support the delivery of your study.

Please do not hesitate to contact me if you require any further information.

Best wishes

Dr Sam Taylor
Portfolio Lead
NIHR Clinical Research Coordinating Centre (NIHR CRN CC)
Fairbairn House
71-75 Clarendon Road
Leeds
LS2 9PH

Tel: 0113 343 0403
Fax: 0113 343 2300
Email: s.taylor@ukcrn.org.uk
www.crncc.nihr.ac.uk

APPENDIX 3: Ethical Approval Submission, Ethical & Trust R&D Approval

All studies except clinical trials of investigational medicinal products

REC Ref	07/H0908/70
Short title of Study	Oesophageal impedance in human lung transplant recipients
Chief Investigator	Professor S Michael Griffin
Sponsor	Newcastle Upon Tyne NHS Foundation Trust

Please complete this checklist and send it with your application

- Send **ONE** copy of each document (except where stated)
- ALL accompanying documents must bear version numbers and dates (except where stated)
- When collating please do NOT staple documents as they will need to be photocopied

Document	Enclosed?	Date	Version	Office use
Covering letter on headed paper	• Yes ○ No			
NHS REC Application Form, Parts A&B	Mandatory	04/10/2007	2	
Site-Specific Information Form (for SSA)	○ Yes • No			
Research protocol or project proposal (6 copies)	Mandatory	23/08/2007		
Summary C.V. for Chief Investigator (CI) – Prof Griffin	Mandatory	23/8/2007		
Summary C.V. for supervisor (student research) – Prof Griffin	• Yes ○ No	23/08/2007		
Research participant information sheet PIS Impedance	• Yes ○ No	04/10/2007	2	
Research participant information sheet PIS Gastric Juice	• Yes ○ No	04/10/2007	2	
Research participant consent form Impedance	• Yes ○ No	04/10/2007	2	
Research participant consent form Gastric Juice	• Yes ○ No	04/10/2007	2	
Letters of invitation to participants	○ Yes ○ No			
GP/Consultant information sheets or letters	○ Yes ○ No			
Statement of indemnity arrangements	○ Yes ○ No			
Letter from sponsor	○ Yes ○ No			
Letter from statistician	○ Yes ○ No			
Letter from funder	○ Yes ○ No			
Referees' or other scientific critique report	○ Yes ○ No			
Summary, synopsis or diagram (flowchart) of protocol in non-technical language	○ Yes ○ No			
Interview schedules or topic guides for participants	○ Yes ○ No			
Validated questionnaire	○ Yes ○ No			
Non-validated questionnaire	○ Yes ○ No			
Copies of advertisement material for research participants, e.g. posters, newspaper adverts	○ Yes ○ No			

website. For video or audio cassettes, please also provide the printed script.			

An application form specific to your project will be created from the answers you give to the following questions

1. Is your project an audit or service evaluation?

○ Yes • No

2. Select one research category from the list below:

○ Clinical trials of investigational medicinal products
• Clinical investigations or other studies of medical devices
○ Other clinical trial or clinical investigation
○ Research administering questionnaires interviews for quantitative analysis, or using mixed quantitative qualitative methodology
○ Research involving qualitative methods only
○ Research limited to working with human tissue samples and/or data
○ Research tissue bank

If your work does not fit any of these categories, select the option below:

○ Other research

2a. Select one category from the list below:

• Is this a clinical investigation of a medical device?
○ Is this a performance evaluation of an in vitro diagnostic device?
○ Is this a drug-device combination of both an investigational medical device and an investigational medicinal product?
○ Is this a post-market surveillance study of a CE-marked product?

2b. Please answer the following questions:

a) Does the study involve the use of any ionising radiation? ○ Yes • No
b) Will you be taking new human tissue samples? • Yes ○ No
c) Will you be using existing human tissue samples? ○ Yes • No

3. Is your research confined to one site?

• Yes ○ No

4. Does your research involve work with prisoners?

○ Yes • No

5. Do you plan to include in this research adults unable to consent for themselves through physical or mental incapacity?

Yes • No

6. Is the study, or any part of the study, being undertaken as an educational project?

- Yes ○ No

6a. Is the project being undertaken in part fulfilment of a PhD or other doctorate?

- Yes ○ No

NHS Research Ethics Committee NHS
Application form for a clinical investigation of a medical device

This form should be completed by the Chief Investigator, after reading the guidance notes. See glossary for clarification of different terms in the application form.

Short title and version number: (maximum 70 characters – this will be inserted as header on all forms)
Oesophageal impedance in human lung transplant recipients

Name of NHS Research Ethics Committee to which application for ethical review is being made:
County Durham & Tees Valley 2 REC

Project reference number from above REC: 07/H0908/70
Submission date: 04/10/2007

A1 Title of the research

Full title: The role of oesophageal impedance measurement in detection of gastro-oesophageal reflux disease in human lung transplant recipients

Key words: Lung Transplant; reflux; impedance

A2. Chief Investigator

Title:	Professor
Forename/Initials:	S Michael
Surname:	Griffin
Post:	Professor of Gastrointestinal Surgery
Qualifications:	MD, FRCS
Organisation	
Work Address:	Northern Oesophagogastric Unit, Royal Victoria Infirmary
	Queen Victoria Rd
	Newcastle upon Tyne
Post Code:	NE1 4LP
E-mail:	Michael.Griffin@nuth.nhs.uk
Telephone:	+44(0)191 282 0234
Fax:	+44(0)191 282 0237
Mobile:	

A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application

A3. Proposed study dates and duration

Start date: 0[illegible] 2007
End date: 0[illegible] 2010
Duration: Years: 3 Months: 0

A4. Primary purpose of the research: *(Tick as appropriate)*

- ☐ Commercial product development and/or licensing
- ☐ Publicly funded trial or scientific investigation
- ☑ Educational qualification
- ☐ Establishing a database/data storage facility
- ☐ Other

Question(s) 5 disabled.

A6. Does this research require site-specific assessment (SSA)? (Advice can be found in the guidance notes on this topic.)

○ Yes • No

If No please justify

If Yes an application for SSA should be made for each research site on the Site-Specific Information Form and submitted to the relevant local Research Ethics Committee. Do not apply for SSA at sites other than the lead site until the main application has been booked for review and validated by the main Research Ethics Committee.

Management approval to proceed with the research will be required from the R&D office for each NHS care organisation in which research procedures are undertaken. This applies whether or not the research is exempt from SSA. R&D applications in England, Wales and Scotland should be made using the Site-Specific Information Form.

A7. **What is the principal research question objective?** *(Must be in language comprehensible to a lay person.)*

The aim of this study is to evaluate study how frequently gastro-oesophageal reflux disease (GORD) occurs after lung transplant and its role in the development of chronic lung dysfunction, which is progressive loss of lung function after lung transplantation. Many patients after lung transplant suffer from gastro-oesophageal reflux disease (GORD), which is when the stomach contents and acid from the stomach leak up into the gullet (oesophagus). This may cause heartburn and other symptoms. It not fully known whether this reflux disease is related to the decay of lung function.

A8. **What are the secondary research questions objectives?** *(If applicable, must be in language comprehensible to a lay person.)*

The secondary research objectives are to assess the effects of fundoplication surgery on reflux and on lung function. Fundoplication is an operation performed to tighten the lower oesophagus and prevent reflux leaking up from the stomach.

A9. **What is the scientific justification for the research? What is the background? Why is this an area of importance?** *(Must be in language comprehensible to a lay person.)*

BACKGROUND
Lung transplantation has been performed since 1963. Compared to other transplanted organs survival is poor. Only 40% of patients are alive 5 years after their transplant. This is commonly due to chronic lung dysfunction after the transplant. (Davis, 2003).

SCIENTIFIC JUSTIFICATION
It is thought that chronic aspiration of stomach contents may contribute to chronic lung dysfunction (progressive loss of lung function). This is a fairly recent concept and was first described in 1990. There is a high incidence of GORD after lung transplant. This is related to various factors including damage to the nerve supplying the stomach during surgery, anti-rejection medication, and the high incidence of GORD before surgery.
One of the proposed mechanisms for increased reflux in post transplant patients is delayed emptying of the stomach. An increase in the volume of the stomach is known to cause reflux into the gullet. If the nerves are damaged at the time of transplant this would promote delayed stomach emptying and may be a significant contributing factors to reflux after surgery.
Post-transplant there are impaired lung defence mechanisms - cough and clearance of mucous and spit. Mucous clearance has been shown to be less than 15% of normal after a transplant. These factors predispose the new lungs to damage.

Post-operatively, patients are put on anti-acid therapy. This reduces symptomatic heartburn, and the acid levels in reflux, but not refluxed material itself, which can still damage the lungs. Impedance is a small device that can be placed in the gullet to measure reflux whether it is acid or not. It is an exciting new technology which is more accurate than current acid detection studies (Wise, 2007). These non acid reflux events may contribute to the development of chronic lung dysfunction. The use of this technology enables us to study reflux in a "real life situation", unlike previous studies where anti-acid therapy has been discontinued artificially, for acid monitoring studies (Davis, 2003). The older technique will miss episodes of non-acid reflux.

There is existing research into aspiration of stomach contents in lung transplant patients and subsequent lung dysfunction, worldwide and also from this unit. There have been some high profile publications from this centre (Stovold, 2007, Ward, 2005). Our project will bring state of the art objective measurements of reflux and will compliment existing work on measurements of aspiration from our laboratory.

To accurately quantitate the levels of pepsin being aspirated into the lungs, the ELISA used must be calibrated with human pepsin. The only source of human pepsin is gastric juice. Therefore taking gastric juice is an essential part of this study.

IMPORTANCE
This is a very important topic. Two key papers published in 2003 by Davis and in 2007 by Stovold, suggest

the following: (a) that anti-reflux surgery may lead to increased survival and improved lung function after transplant, by preventing lung damage through reflux and (b) that gastric refluxate is reaching the transplanted lung and is harmful to lung function.
However this is unclear and the relationship between reflux disease and chronic lung dysfunction needs to be defined. Although there are suggestions that fundoplication surgery has improved lung function and survival the studies have been small and retrospective. Further research is needed.

References

- R. Stovold, I.A. Forrest, P.A. Corris, D.M. Murphy, J.A. Smith, S. Decalmer, G.E. Johnson, J.H. Dark, J.P. Pearson and C. Ward. Pepsin a biomarker of gastric aspiration in lung allografts. Am. J. Respir. Crit. Care Med 2007; Vol 175 1298-1303.

- Wise JL, Murray JA. Utilising multichannel intraluminal impedance for diagnosing GERD: a review. Diseases of the Esophagus 2007. Vol 20:83--88.

- Ward C, Forrest IA, Brownlee IA, Johnson GE, Murphy DM, Pearson JP, Dark JH, Corris PA. Pepsin pre activity in bronchoalveolar lavage fluid is suggestive of gastric aspiration in lung allografts. Thorax 2005; Vol 60(10):872-4.

-Davis RD, Lau CL, Eubanks S, Messier RH, Hadjiliadis D, Steele MP, Palmer SM. Improved Lung Allograft Function after fundoplication in patients with gastroesophageal reflux disease undergoing lung transplantation. Journal of Thoracic & Cardiovascular Surgery 2003. Vol 125(3):533-542.

A10-1. Give a full summary of the purpose, design and methodology of the planned research, including a brief explanation of the theoretical framework that informs it. It should be clear exactly what will happen to the research participant, how many times and in what order.

This section must be completed in language comprehensible to the lay person. It must also be self-standing as it will be replicated in any applications for site specific assessment on the Site-Specific Information Form. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.

Purpose & Theory
Largely asymptomatic stomach reflux is present in most patients after lung transplantation. Subsequent aspiration of stomach contents into the lung can be detected using appropriate biomarkers and reduces lung function. Fundoplicative surgery reduces reflux disease and biological markers of aspiration, with the consequence of improving survival and lung function.

Our aims are:
-To measure pH/impedance in a study of lung transplant recipients, to objectively assess reflux disease.
-To see if impedance can replace pH monitoring in reflux patients.
-To measure patient symptoms of reflux disease, using a specific questionnaire.
-To compare objective assessment of reflux disease (impedance) with patient experience of symptoms (questionnaire).
-To compare objective and clinical assessments of reflux and symptoms with markers of aspiration in the fluid removed from the lungs (pepsin, bile salts) and clinical and pathological changes in lung function.
-To evaluate the effect of fundoplication surgery on the above.

Patients undergoing lung transplantation at the Freeman Hospital will be studied to test for the presence of reflux. Their lungs will be under surveillance using bronchoscopy (a test to look inside the lungs), fluid samples will be taken from the lungs and lung function tests will be performed. This is already routine practice.
Over an 18 month period a group of 40 new lung transplant patients will have reflux and acid levels measured in the gullet using a small probe passed through the nose, at 1, 3 and 6 months post transplant. This will be performed immediately before the bronchoscopy and a fluid sample on each occasion. Patients will be approached to be recruited into the study by Dr Robertson, during their post-operative stay, once they are beginning to recover from surgery and returning to health. Overall there will be no extra visits, but the patient's one month, 3 month and 6 month visit will last for 2 days as opposed to one day. During this time patient's will receive free accommodation in the available Transplant accommodation at the Freeman Hospital.

The extra procedure performed is called an impedance test. Each patient will undergo this procedure 3 times at 1 month, 3 month and 6 month post-transplant. Impedance is a new test (10 years old) similar to a standard pH catheter. It consists of a thin walled tube (2mm in diameter) which will be placed through the nostril into the gullet to look for reflux for a distance of approximately 45cm. The tube consists of a series of small rings which detect changes of resistance between these rings. Liquids have low resistance gases have a high resistance. This device is able to detect changes in resistance ad various points along the tube. This enables this device to distinguish between swallows and reflux events, determine the composition of the reflux event (gas/liquid) and the level of reflux. Impedance devices have been in use for over 10 years and the devices used in the study have been used in the UK for 3 years in both clinical and research settings. Impedance devices are used routinely throughout the UK and worldwide. UK centres include Glasgow Royal Infirmary, Univeristy College London Hospitals, Nottingham, Manchester (paediatrics) and Plymouth. We also use this device clinically at the Northern Oesophago-Gastric Cancer Unit in the Royal Victoria Infirmary. The device used is CE marked in line with European standards and is manufactured to comply with the European Medical Devices Directive (93/42 EEC) and therefore does not require MHRA approval. There is a completed Pre Purchase Questionnaire (PPQ) from Ardmore Healthcare Ltd that confirms this compliance. The device itself, has been operationally check by the electronic department on receipt and has been placed on the Newcastle Upon Tyne Hospitals Trust asset register. (Trust Safety Number: Safety Information for Impedance- 155951)

There will no dietary restrictions during this study and patients will be encouraged to try to have a 'normal' a diet as possible to allow a 'real life' assessment of their reflux.
The role of reflux disease in the development of chronic lung dysfunction after lung transplant is controversial. Although there is a growing body of evidence to suggest a link, this has not been definitively proven.
Similar tests for reflux disease are currently routine practice at several lung transplant centres (Duke's Centre, North Carolina, USA; St Louis, Missouri, USA; Great Ormond St, London). They are also routinely done for patients at the RVI suffering from reflux. Instead of an old-fashioned probe (24 hr pH) a newer more accurate probe will be used (impedance). This will be positioned with the help of a slightly larger tube (manometry) placed into the gullet through the nose. This larger tube will be in place for about 10-20 minutes. Patients will also be asked to fill in a questionnaire to see if they have symptoms.
Lung fluid samples will be analysed as routine practice and also as comparable with current research.

The degree of reflux detected (how often, how severe, and whether it is acid or not) will be compared with molecular measures of reflux. The detection of pepsin (a protein made in the stomach) and bile salts (from the liver and small intestine) in the lung fluid and the presence of cells of inflammation in the lung fluid sample will be used to assess the relevance of the detected reflux episodes.
Patients with significant reflux at the 3 month assessment will be offered anti-reflux surgery as part of their clinical management at the Royal Victoria Infirmary. All patients will have a 6 month impedance performed. Those patients who decide to have surgery will be included in the follow -up.

Lung Surveillance
Routine lung surveillance will be performed by the respiratory physicians specialising in pulmonary transplant. This will be undertaken using bronchoscopy. At bronchoscopy, there are normally fluid samples and biopsies taken. Evidence of rejection from biopsies, assessment of lung fluid for infection, an analysis of inflammation cells will be used to assess the status of the graft. These are routine measurements.
Some extra bronchoalveolar lavage samples will be taken and analysed to look for the presence of pepsin, bile acids, (evidence of stomach contents entering the lungs) and production of an inflammatory molecule called interleukin (IL)-8. This means the bronchoscopy will last about 5 minutes longer than normal.
Pulmonary Function Tests will be studied as part of routine follow up, including FEV1, FVC, FEF25-75 spirometry with expiratory flow volume loops.

Results will be studied to see if there is a link between severe reflux of stomach contents and lung dysfunction.

Those with significant reflux at 3 months will be offered the opportunity to undergo surgery to stop them refluxing at the Royal Victoria Infirmary to try to prevent the development of bronchiolitis obliterans and thus improve survival. This is also routine practice at several lung transplant centres worldwide, but is a controversial issue. Currently apart from Great Ormond St, no other UK lung transplant unit is performing this procedure. However there is some evidence to suggest that this improves patient survival.

Results will then be analysed to see if there is a link between reflux, lung fluid samples and lung dysfunction.

The measures of pepsin (a protein found in the stomach) causing lung damage have been shown to be important (Stovold). Much research has been performed in general on pepsin and also on its role of in lung disease by Professor Pearson. Pepsin levels are measured using a standard kit called an ELISA. The

ELISAs are designed to check for pepsin derived from the stomach of pigs, not humans. It would therefore be of scientific interest to assess these detection kits using human pepsin. This would be obtained from samples from routine washouts of patients stomachs at scheduled endoscopies of non-transplant patients. The patient's management would not be altered in any way as the endoscopy is performed for clinical reasons and the washouts of the stomach are to enable the surgeon to look at the inside of the stomach during the procedure. The fluid is normally discarded after the procedure. It could be used to extract human pepsin and then our assay could be evaluated. This collection of human pepsin would be used to see if our assays are accurate in detecting human samples and thus are scientifically accurate.

A10–2. In which parts of the research have patients, members of the public or service users been involved?

- ☐ As user-researchers
- ☐ As members of a research project group
- ☐ As advisor to a project
- ☐ As members of a departmental or other wider research strategy group
- ☑ None of the above

Please provide brief details if applicable:

A10–3. Could the research lead to the development of a new product/process or the generation of intellectual property?

○ Yes ○ No • Not sure

A11. Will any intervention or procedure, which would normally be considered a part of routine care, be withheld from the research participants?

○ Yes • No

A12. Give details of any clinical intervention(s) or procedure(s) to be received by research participants over and above those which would normally be considered a part of routine clinical care. *(These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material.)*

Additional Intervention	Average number per participant		Average time taken (mins/hours/days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.
	Routine Care	Research		
Other		3	3x 1day	Manometry and Impedance testing will be performed in the oesophageal laboratory of the Northern Oesophagogastric Unit of the Royal Victoria Infirmary by the clinical research fellows. Namely Dr Andrew Robertson a clinical research fellow and Specialty Registrar (ST1) in General Surgery, who has completed a foundation programme and has experience in inserting nasogastric tubes. He is being trained in manometry, pH studies and impedance by Mr Sultan, a Specialty Registrar (ST3) in General Surgery

A13. Give details of any non-clinical research-related intervention(s) or procedure(s). *(These include interviews, non-clinical observations and use of questionnaires.)*

Additional intervention	Average number per participant	Average time taken (mins/hours/days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.
Other Questionnaire	3	1-2 hour	Patients will be asked to fill in a straight forward questionnaire looking at symptoms of reflux disease (i.e. heartburn, cough, discomfort whilst eating)

A14. Will individual or group interviews/questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews group discussions, or use of screening tests for drugs)?

○ Yes • No

The Information Sheet should make it clear under what circumstances action may be taken.

A15. What is the expected total duration of participation in the study for each participant?

6 months

A16. What are the potential adverse effects, risks or hazards for research participants either from giving or withholding medications, devices, ionising radiation, or from other interventions (including non-clinical)?

Manometry and impedance are low risk procedures. Many patients undergo manometry and pH studies (an old-fashioned measurement similar to impedance) without experiencing any complications. The main risk is of discomfort to the nose, throat or gullet.

A17. What is the potential for pain, discomfort, distress, inconvenience or changes to lifestyle for research participants?

The main potential for distress to participants is from the manometry test, which lasts about 20 minutes. This may cause discomfort to the nose, throat or gullet. Impedance causes less discomfort as it is a smaller tube.

A18. What is the potential for benefit to research participants?

Evidence has shown that if severe reflux is detected and treated with anti-reflux surgery, then patients have improved lung function and survival. Early lung dysfunction has been reversed through surgery. Studies have shown surgery to have no mortality and a small amount of complications. Several anti-reflux operations have been successfully performed here on lung transplant patients.

A19. What is the potential for adverse effects, risks or hazards, pain, discomfort, distress, or inconvenience to the researchers themselves? *(if any)*

None. This is a low risk investigation.

A20. How will potential participants in the study be (i) identified, (ii) approached and (iii) recruited? *Give details for cases and controls separately if appropriate.*

40 patients who have undergone lung transplant at the Freeman will be approached and recruited into this study, after informed consent is obtained ; to obtain 30 completed patients.

Routine endoscopy patients at the Royal Victoria Infirmary, Newcastle General will be approached randomly and will be recruited to donate gastric juice.

A21. Where research participants will be recruited via advertisement, give specific details.

☑ Not Applicable

If applicable, enclose a copy of the advertisement/radio script/website/video for television (with a version number and date).

A22. What are the principal inclusion criteria? *(Please justify)*

Patient who have had a recent Lung transplant at the Freeman Hospital.

Non-transplant patients undergoing routine endoscopy

A23. What are the principal exclusion criteria? *(Please justify)*

Patient who have not undergone a recent lung transplant

A24. Will the participants be from any of the following groups? *Tick as appropriate*

- ☐ Children under 16
- ☐ Adults with learning disabilities
- ☐ Adults who are unconscious or very severely ill
- ☐ Adults who have a terminal illness
- ☐ Adults in emergency situations
- ☐ Adults with mental illness (particularly if detained under Mental Health Legislation)
- ☐ Adults with dementia
- ☐ Prisoners
- ☐ Young Offenders
- ☐ Adults in Scotland who are unable to consent for themselves
- ☐ Healthy Volunteers
- ☐ Those who could be considered to have a particularly dependent relationship with the investigator, e.g. those in care homes, medical students
- ☑ Other vulnerable groups

Justify their inclusion.

This study is designed to look at reflux in the lung transplant population and its role in the development of chronic allograft dysfunction. It is therefore necessary to include these patients in the study. There may be potential health benefits for participants of the study

- ☑ No participants from any of the above groups

A25 Will any research participants be recruited who are involved in existing research or have recently been involved in any research prior to recruitment?

• Yes ○ No ○ Not Known

If Yes, give details and justify their inclusion. If Not Known, what steps will you take to find out?

There is an ongoing study of chronic lung dysfunction after lung transplant at the Freeman Hospital which has full ethical approval. This study seeks to add on one minor clinical investigation and combine this with routine management to gain an understanding of reflux disease and lung dysfunction.

A26. Will informed consent be obtained from the research participants?

• Yes ○ No

If Yes, give details of who will take consent and how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material

If participants are to be recruited from any of the potentially vulnerable groups listed in A24, give details of extra steps taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative

If consent is not to be obtained, please explain why not

Informed Consent will be taken by Dr Andrew Robertson, Clinical Research Fellow with the aid of the enclosed information sheets. Patients will be encouraged to discuss participation with all members of the transplant team.

Copies of the written information and all other explanatory material should accompany this application.

A27. Will a signed record of consent be obtained?

• Yes ○ No

If Yes, attach a copy of the information sheet to be used, with a version number and date.

A28. How long will the participant have to decide whether to take part in the research?

Several weeks to 1 month

A29. What arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters etc.)*

All of our patients need to understand and retain explanations from a transplant perspective to be able to receive a lung transplant. Translators and interpreters will be used to inform patients who cannot understand English.

A30. What arrangements are in place to ensure participants receive any information that becomes available during the course of the research that may be relevant to their continued participation?

Patient will have regular contact with the transplant team.

A30–1. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? *Tick one option only.*

- ...e participant would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be ...ined. Any identifiable data or tissue would be anonymised or disposed of.
- ...he participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be ...etained and used in the study.
- The participant would continue to be included in the study.
- Not applicable – informed consent will not be sought from any participants in this research.

Further details

A31. Does this study have or require approval of the Patient Information Advisory Group (PIAG) or other bodies with a similar remit? *see the guidance notes)*

○ Yes • No

A32a. Will the research participants' General Practitioner (and or any other health professional responsible for their care) be informed that they are taking part in the study?

• Yes ○ No

If Yes, enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A32b. Will permission be sought from the research participants to inform their GP or other health professional before this is done?

• Yes ○ No

If No to either question, explain why not

It should be made clear in the patient information sheet if the research participant's GP/health professional will be informed.

A33. Will individual research participants receive any payments for taking part in this research?

○ Yes • No

A34. Will individual research participants receive *reimbursement of expenses* or any other *incentives or benefits* for taking part in this research?

• Yes ○ No

If Yes, indicate how much and on what basis this has been decided

Patients will not be foreseen to have to make extra journeys to Newcastle as the impedance measurements will coincide with their clinic visits. Accommodation will be provided free of charge. An allowance will be made for meals during their extra day stay in Newcastle. Patients will be informed that if any unforeseen expenses arise related to participation in the study, full reimbursement will be given.

A35. Insurance/indemnity to meet potential legal liabilities

Note: References in this question to NHS indemnity schemes include equivalent schemes provided by Health and Personal Social Services (HPSS) in Northern Ireland.

A35-1 What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the *sponsor(s)* for harm to participants arising from the *management of the research*?

Note: Where an NHS organisation has agreed to act as the sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, describe the arrangements and provide evidence.

- NHS indemnity scheme will apply

Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents

A35-2 What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the *sponsor(s) or employer(s)* for harm to participants arising from the *design of the research*?

Note: Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), describe the arrangements and provide evidence.

- NHS indemnity scheme will apply to all protocol authors

○ Other insurance or indemnity arrangements will apply (give details below)

Please enclose a copy of relevant documents.

A35-3. What arrangements will be made for insurance and or indemnity to meet the potential legal liability of *investigators collaborators* and, where applicable, *Site Management Organisations*, arising from harm to participants in the *conduct of the research*?

Note: Where the participants are NHS patients, indemnity is provided through NHS schemes or through professional indemnity. Indicate if this applies to the whole of the study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, describe the arrangements which will be made at these sites and provide evidence.

- All participants will be recruited at NHS sites and NHS indemnity scheme or professional indemnity will apply

○ Research includes non-NHS sites (give details of insurance indemnity arrangements for these sites below)

Please enclose a copy of relevant documents.

A36. Has the sponsor(s) made arrangements for payment of compensation in the event of harm to the research participants where no legal liability arises?

○ Yes • No

If Yes, give details of the compensation policy

Please enclose a copy of relevant documents.

A37. How is it intended the results of the study will be reported and disseminated? *Tick as appropriate*

- [x] Peer reviewed scientific journals
- [ ] Internal report
- [x] Conference presentation
- [x] Other publication
- [ ] Submission to regulatory authorities
- [ ] Access to raw data and right to publish freely by all investigators in study or by independent Steering Committee on behalf of all investigators
- [ ] Written feedback to research participants
- [ ] Presentation to participants or relevant community groups
- [ ] Other: none e.g. Cochrane Review, University Library

A38. How will the results of research be made available to research participants and communities from which they are drawn?

The results of research will be made available through presentations and publications.

A39. Will the research involve any of the following activities at any stage (including identification of potential research participants)? *Tick as appropriate*

- [x] Examination of medical records by those outside the NHS, or within the NHS by those who would not normally have access
- [ ] Electronic transfer by magnetic or optical media, e-mail or computer networks
- [ ] Sharing of data with other organisations
- [ ] Export of data outside the European Union
- [ ] Use of personal addresses, postcodes, faxes, e-mails or telephone numbers
- [ ] Publication of direct quotations from respondents
- [ ] Publication of data that might allow identification of individuals
- [ ] Use of audio/visual recording devices
- [x] Storage of personal data on any of the following:
  - [ ] Manual files including X-rays
  - [x] NHS computers
  - [ ] Home or other personal computers
  - [ ] University computers
  - [ ] Private company computers
  - [ ] Laptop computers

Further details

Relevant sections of patients' medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant.

A40. What measures have been put in place to ensure confidentiality of personal data? Give details of whether any encryption or other anonymisation procedures have been used and at what stage:

The Coldicott Principals will be adhered to.

A41. Where will the analysis of the data from the study take place and by whom will it be undertaken?

Analysis of the data from the study will be undertaken by the research team from the Newcastle Upon Tyne Hospital Trust and the University of Newcastle.

A42. Who will have control of and act as the custodian for the data generated by the study?

Professor Corris & Professor Griffin

A43. Who will have access to research participants' or potential research participants' health records or other personal information? *Where access is by individuals outside the normal clinical team, justify and say whether consent will be sought.*

The Clinical Team, the research team, personnel from regulatory authorities or from the sponsor (i.e. the Trust). Patients will be informed of this and consent will be sought.

A44. For how long will data from the study be stored?

7 Years 0 Months

Give details of where they will be stored, who will have access and the custodial arrangements for the data

Lung Transplant Patient Details will be stored in the Department of Respiratory Medicine Freeman Hospital Newcastle under the guardianship of Professor Corris. Details of the impedance measurements will be stored in the Northern Oesophago-Gastric Unit, Royal Victoria Infirmary under the guardianship of Professor Griffin.

A45-1. How has the scientific quality of the research been assessed? *(Tick as appropriate)*

- ☐ Independent external review
- ☐ Review within a company
- ☐ Review within a multi-centre research group
- ☐ Review within the Chief Investigator's institution or host organisation
- ☑ Review within the research team
- ☑ Review by educational supervisor
- ☐ Other

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review.

This potential project has undergone review by the research team, educational supervisor and the University of Newcastle. All involved have deemed this to be an important area of clinical research with potential benefit for patients. This project has been accepted for a MD thesis pending ethical approval.

A45-2. How have the statistical aspects of the research been reviewed? *(Tick as appropriate)*

- ☐ Review by independent statistician commissioned by funder or sponsor
- ☐ Other review by independent statistician
- ☐ Review by company statistician
- ☐ Review by a statistician within the Chief Investigator's institution
- ☐ Review by a statistician within the research team or multi-centre group
- ☑ Review by educational supervisor
- ☐ Other review by individual with relevant statistical expertise

In all cases give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution concerned.

	Title	Forename/Initials	Surname
	Professor	Paul	Corris

Department: Department of Respiratory Medicine
Institution: Freeman Hospital
Work Address: High Heaton
Newcastle upon Tyne

Postcode: NE7 7DN
Telephone: 0191 212 7462
Fax:
Mobile:
E-mail: Paul.Corris@ncl.ac.uk

Please enclose a copy of any available comments or reports from a statistician.

Question(s) 46-47 disabled.

A48. What is the primary outcome measure for the study?

The primary outcome measures for the study are impedance measurements, the presence of reflux markers in the lung fluid and lung function results

A49. What are the secondary outcome measures? (if any)

The secondary outcome measures for the study are lung function and patient survival

A50. How many participants will be recruited?
If there is more than one group, state how many participants will be recruited in each group. For international studies, say how many participants will be recruited in the UK and in total.

Up to 40 to get 30 completed evaluated patients

A51. How was the number of participants decided upon?

This is based on number of patients transplanted per year.

If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.

A52. Will participants be allocated to groups at random?

○ Yes • No

A53. Describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which

the data will be evaluated to meet the study objectives.

The results will be analysed by the research team and a statistician will be involved

A54. Where will the research take place? *(Tick as appropriate)*

- [x] UK
- [ ] Other states in European Union
- [ ] Other countries in European Economic Area
- [ ] Other

If Other, give details:

A55. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK, the European Union or the European Economic Area?

Yes • No

A56. In how many and what type of host organisations (NHS or other) in the UK is it intended the proposed study will take place?

Indicate the type of organisation by ticking the box and give approximate numbers if known

	Number of organisations
[x] Acute teaching NHS Trusts	1
[ ] Acute NHS Trusts	
[ ] NHS Primary Care Trusts or Local Health Boards in Wales	
[ ] NHS Trusts providing mental healthcare	
[ ] NHS Health Boards in Scotland	
[ ] HPSS Trusts in Northern Ireland	
[ ] GP Practices	
[ ] NHS Care Trusts	
[ ] Social care organisations	
[ ] Prisons	
[ ] Independent hospitals	
[x] Educational establishments	
[ ] Independent research units	
[ ] Other (give details)	

Other:

A57. What arrangements are in place for monitoring and auditing the conduct of the research?

Monitoring will be performed by the Study Sponsor i.e. the Trust.

A57a. Will a data monitoring committee be convened?

Yes • No

If Yes, details of membership of the data monitoring committee (DMC), its standard operating procedures and summaries of reports of interim analyses to the DMC must be forwarded to the NHS Research Ethics Committee which gives a favourable opinion of the study

What are the criteria for electively stopping the trial or other research prematurely?

n/a

A58. Has external funding for the research been secured?

◯ Yes • No

If No, what arrangements are being made to cover any costs of the research? If no external funding is being sought please say so:

This project will have minimal costs as there is free patient accommodation for transplant patients at the Freeman Hospital. The main costs will be for the person collecting the data and the impedances catheters. Grants are currently being sought to help cover these costs.

A59. Has the funder of the research agreed to act as sponsor as set out in the Research Governance Framework?

• Yes ◯ No

Has the employer of the Chief Investigator agreed to act as sponsor of the research?

• Yes ◯ No

Lead sponsor *(must be completed in all cases)*

Name of organisation which will act as the lead sponsor for the research:

Newcastle Upon Tyne NHS Foundation Trust

Status:

• NHS or HPSS care organisation ◯ Academic ◯ Pharmaceutical industry ◯ Medical device industry ◯ Other

If Other, please specify:

Address: R&D Clinical Research Facility 4th Floor Leazes Wing
Royal Victoria Infirmary
Newcastle upon Tyne

Post Code: NE1 4LP

Telephone:

Fax:

Mobile:

E-mail:

Sponsor's UK contact point for correspondence with the main REC *(must be completed in all cases)*

Title: Ms	Forename/Initials: Amanda Surname: Tonge
Work Address:	Clinical Research Facility, 4th Floor Leazes Wing Royal Victoria Infirmary Newcastle upon Tyne
Post Code:	NE1 4LP
Telephone:	0191 282 5959
Fax:	
Mobile:	
E-mail:	Amanda.Tonge@nuth.nhs.uk

Co-sponsors

Are there any co-sponsors for this research?

○ Yes • No

A60. Has any responsibility for the research been delegated to a subcontractor?

○ Yes • No

A61. Will individual *researchers* receive any personal payment over and above normal salary for undertaking this research?

○ Yes • No

A62. Will individual *researchers* receive any other benefits or incentives for taking part in this research?

○ Yes • No

A63. Will the host organisation or the researcher's department(s) or institution(s) receive any payment or benefits in excess of the costs of undertaking the research?

○ Yes • No

A64. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share-holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes • No

A65. Research reference numbers: *(give any relevant references for your study)*

Applicant's organisation's own reference number, e.g. R&D (if available):
Sponsor's protocol number:
Funder's reference number: n/a
International Standard Randomised Controlled Trial Number (ISRCTN): n/a
Project website: n/a

A66. Other key investigators/collaborators *(all grant co-applicants or protocol co-authors should be listed)*

Title: Professor Forename/Initials: Paul Surname: Corris

Post: Professor of Thoracic Medicine
Qualifications:
Organisation: Department of Respiratory Medicine, Freeman Hospital
Work Address: Freeman Hospital
High Heaton
Newcastle upon Tyne
Postcode: NE7 7DN
Telephone: +44 (0)191 21 27462
Fax:
Mobile:
E-mail: Paul.Corris@ncl.ac.uk

Title: Professor Forename/Initials: John Surname: Dark

Post: Professor of Cardiothoracic Surgery
Qualifications:
Organisation: Regional Cardiothoracic Centre
Work Address: The Freeman Hospital
Freeman Road
High Heaton, Newcastle upon Tyne
Postcode: NE7 7DN
Telephone: 0191 223 1450
Fax: 0191 233 1152
Mobile:
E-mail: J.H.Dark@ncl.ac.uk

Title: Dr Forename/Initials: Andrew G N Surname: Robertson

Post: Clinical Research Fellow
Qualifications:
Organisation: Northern Oesophago-Gastric Cancer Unit
Work Address: Royal Victoria Infirmary,
Queen Victoria Rd.,
Newcastle upon Tyne
Postcode: NE1 4LP
Telephone: 0774 385 6466
Fax:
Mobile:
E-mail: andrewgnrobertson@doctors.org.uk

Title: Dr	Forename/Initials: Andrew	Surname: Fisher	
Post:	Clinical Senior Lecturer in Respiratory Medicine		
Qualifications:			
Organisation:	Department of Respiratory Medicine, Freeman Hospital		
Work Address:	Freeman Hospital High Heaton Newcastle upon Tyne		
Postcode:	NE7 7DN		
Telephone:	01912137693		
Fax:			
Mobile:			
E-mail:	a.j.fisher@ncl.ac.uk		

Title: Professor	Forename/Initials: Jeff	Surname: Pearson	
Post:	Professor of Molecular Physiology		
Qualifications:			
Organisation:	Institute for Cell and Molecular Biosciences		
Work Address:	The Medical School University of Newcastle upon Tyne Framlington Place, Newcastle		
Postcode:	NE2 4HH		
Telephone:	+44 (0) 191 222 699		
Fax:	+44 (0) 191 222 742		
Mobile:			
E-mail:	J.P.Pearson@ncl.ac.uk		

Title: Dr	Forename/Initials: Christopher	Surname: Ward	
Post:	Lecturer in Respiratory medicine		
Qualifications:			
Organisation:	The Medical School		
Work Address:	University of Newcastle upon Tyne Framlington Place, Newcastle upon Tyne		
Postcode:	NE2 4HH		
Telephone:			
Fax:			
Mobile:			
E-mail:	chris.ward@ncl.ac.uk		

A67. What arrangements are being made for continued provision of the intervention for participants, if appropriate, once the research has finished? *May apply to any clinical intervention, including a drug, medical device, mental health intervention, complementary therapy, physiotherapy, dietary manipulation, lifestyle change, etc*

The Northern Oesophago-Gastric Unit has facilities to study reflux in its oesophageal laboratory and has the facilities to perform anti-reflux surgery

A68. **What are the main ethical issues with the research?**

Summarise the main issues from the participant's point of view, and say how you propose to address them

The main issue with this research is the addition of a minor monitoring procedure to the routine management and the use of human samples in the laboratory. However analysis of these samples has previously received ethical approval. Similar reflux studies are performed in other units as part of routine clinical practice

Indicate any issues on which you would welcome advice from the ethics committee

Question(s) not disabled

A70. Give details of the educational course or degree for which this research is being undertaken:

Name of student:

Dr Andrew G N Robertson BSc(Hons), MBChB(Hons)

Name and level of course/degree:

Doctorate of Medicine

Name of educational establishment:

University of Newcastle Upon Tyne

Name and contact details of educational supervisor:

Professor SM Griffin,
Professor of Gastrointestinal Surgery,
Northern Oesophago-Gastric Unit,
Royal Victoria Infirmary
Newcastle
NE1 4LP
Email: michael.griffin@nuth.nhs.uk
Telephone: 0191 282 0234

A71. Declaration of educational supervisor

I have read and approved both the research proposal and this application for the ethical review. I am satisfied that the scientific content of the research is satisfactory for an educational qualification at this level. I undertake to fulfil the responsibilities of a supervisor as set out in the Research Governance Framework for Health and Social Care.

Signature: .....

Print Name: Professor SM Griffin

Date: 16/08/2007 (dd/mm/yyyy)

A one page summary of the supervisor's CV should be submitted with the application

1. Give details of the medical device(s) to be used in the study

Device description	Impedance Catheter & Ambulatory Recording Device
Manufacturer	Ardmore Healthcare Limited/Medical Measurement Systems
Use	Detection of gastro-oesophageal reflux disease
Length of time since device came into use	3 years

* Does the device have a CE mark? • Yes ○ No

** For all products with CE mark please attach instructions for use*

2. Does the study involve the use of a *new* medical device or *new* implantable material or the use of an existing product outside the terms of its CE market intended purpose?

○ Yes • No

In addition to the instructions for use, the following details should be provided where applicable:

- Description of new device, materials, method of use or operation and a summary of the intended purpose
- Composition of any new implantable materials, including summary of biocompatibility findings from studies to date
- If already CE marked, a summary of any proposed changes to the CE marked intended purpose

Impedance is a new test (10 years old), similar to a standard pH catheter. It consists of a thin walled tube (2mm in diameter) which will be placed through the nostril into the gullet to look for reflux for a distance of approximately 45cm. This tube is connected to a small hand-held box which records the information. The tube consists of a series of small rings which detect changes of resistance between these rings. Liquids have low resistance gases have a high resistance. This device is able to detect changes in resistance ad various points along the tube. This enables this device to distinguish between swallows and reflux events, determine the composition of the reflux event (gas/liquid) and the level of reflux. Impedance devices have been in use for over 10 years and the devices used in the study have been used in the UK for 3 years in both clinical and research settings. Impedance devices are used routinely throughout the UK and worldwide. UK centres include Glasgow Royal Infirmary, University College London Hospitals, Nottingham, Manchester (paediatrics) and Plymouth. We also use this device clinically at the Northern Oesophago-Gastric Cancer Unit in the Royal Victoria Infirmary. The device used is CE marked in line with European standards and is manufactured to comply with the European Medical Devices Directive (93/42/EEC) and therefore does not require MHRA approval. There is a completed Pre Purchase Questionnaire (PPQ) from Ardmore Healthcare Ltd that confirms this compliance. The device itself has been operationally check by the electronic department on receipt and has been placed on the Newcastle upon Tyne Hospitals Trust asset register (Trust Safety Number: Safety Information for Impedance - 155951)

3. For electrical devices give summarised details of acceptance and safety testing

Safety testing has been performed by Jeff Stephenson, Electronic Services Officer at the Royal Victoria Infirmary. The device is CE marked in line with European standards and is manufactured to comply with the European Medical Devices Directive (93/42/EEC). A Pre Purchase Questionnaire (PPQ) from Ardmore Healthcare Ltd has been completed to confirm this compliance.

With regard to the device itself, it has been operationally check by the electronics department on receipt and has been placed on the Trust asset register.
Trust Safety Number: Safety Information for Impedance - 155951

4 **Is a medical device or other commercial company arranging this trial?**

○ Yes • No

a) Is this trial a clinical investigation requiring notification to the MHRA? ○ Yes • No

b) Does the company have a Notice of No Objection from the MHRA? • Yes ○ No

c) Has MHRA approval been applied for but not yet received? ○ Yes • No

Note: An application can be made prior to receipt of a valid Notice of No Objection from MHRA. The Notice will be issued subject to the sponsor subsequently receiving a favourable opinion. There is no requirement for a valid Notice of No Objection to be provided to relevant ethics committee before the research can be given a favourable opinion.

5. Have any of the medical devices been transferred from one organisation (legal entity) to another for the purpose of this trial?

○ Yes • No

Give details.

6 In cases of equipment or medical devices, what arrangements have been made with the manufacturer to provide indemnity?

The Manufacturer has insurance to provide indemnity.

Enclose a copy of the relevant correspondence, with a version number and date

1. **What types of human tissue or other biological material will be included in the study?**

Lung Fluid, Gastric Juice

2. **Who will collect the samples?**

Lung fluid will be collected at routine bronchoscopy by one of the respiratory physicians. Gastric juice will be collected after routine endoscopy by one of the surgeons.

3. **Will the samples be:** *(Tick as appropriate)*

☑ Obtained primarily for research purposes?
☑ Surplus tissue left over from tissue taken in the course of normal clinical care for diagnostic or therapeutic purposes?

4. **Will informed consent be obtained from donors for use of the samples:**

In this research?

• Yes ○ No

In future research?

○ Yes • No

5. **Will the samples be stored:**

In fully anonymised form? *(link to donor broken)*

○ Yes • No

In coded (anonymised for n? *(linked to donor but donor not identifiable to researchers)*

• Yes ○ No

If Yes, say who will have access to the code and personal information about the donor

Lung fluid samples are stored as part of another project which has full ethical approval. Access will be through Professor Corris as part of an ongoing study (NRES approved) into chronic lung dysfunction. The storage of these samples is not directly related to this research.

In a form in which the donor could be identifiable to researchers?

• Yes ○ No

If Yes, please justify

This storage is part of another research project and has full ethical approval. It is not directly linked to this proposal. The link between patients and samples allows a correlation between the clinical picture and laboratory studies. Otherwise this information is anonymous and protected.

6. **What types of test or analysis will be carried out on the samples?**

Samples will be analysed to look for evidence of stomach contents and markers of inflammation in lung fluid (pepsin, bile acid levels). Gastric samples will be used to test our laboratory measurements of pepsin, a stomach protein.

7. Will the research involve the analysis of human DNA in the samples?

○ Yes • No

8. Is it possible that the research could produce findings of clinical significance for individuals? *(NB, include relatives as well as donors)*

• Yes ○ No

9. If so, will arrangements be made to notify the individuals concerned?

• Yes ○ No ○ Not applicable

If No please justify. If Yes, say what arrangements will be made and give details of the support or counselling service

Patients are in regular contact with the transplant team and are very well supported.

10. Give details of where the samples will be stored, who will have access and the custodial arrangements.

Samples will be stored in the Freeman Hospital, Lung Transplant Human Tissue Bank. Access and custodial arrangements are through Professor Corris. This is not directly related to this project.

11. What will happen to the samples at the end of the research?

○ Destruction

• Transfer to research tissue bank
(If the bank is in England, Wales or Northern Ireland a licence from the Human Tissue Authority will be required to store the tissue for possible further research.)

○ Storage by research team pending ethical approval for use in another project
(Unless the researcher holds a licence from the Human Tissue Authority, a further application for ethical review should be submitted before the end of this project.)

○ Storage by research team as part of a new research tissue bank
(The bank will require a licence from the Human Tissue Authority. A separate application for ethical review of the tissue bank may also be submitted.)

○ Not yet known

Please give further details of the proposed arrangements:

Samples will be stored in the Freeman Hospital, Lung Transplant Human Tissue Bank in line with previous ethical approval. This storage is not related to this current research project. Lung fluid samples analysed as part of this research project will be destroyed after analysis. Gastric fluid samples will also be destroyed.

Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.
3. If the research is approved I undertake to adhere to the study protocol, the terms of the full application on which the main REC has given a favourable opinion and any conditions set out by the main REC in giving its favourable opinion.
4. I undertake to seek an ethical opinion from the main REC before implementing substantial amendments to the protocol or to the terms of the full application on which the main REC has given a favourable opinion.
5. I undertake to submit annual progress reports setting out the progress of the research.
6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register where necessary with the appropriate Data Protection Officer.
7. I understand that research records/data may be subject to inspection for audit purposes if required in future.
8. I understand that personal data about me as a researcher in this application will be held by the relevant RECs and their operational managers and that this will be managed according to the principles established in the Data Protection Act.
9. I understand that the information contained in this application, any supporting documentation and all correspondence with NHS Research Ethics Committees or their operational managers relating to the application

   - Will be held by the main REC until at least 3 years after the end of the study
   - May be disclosed to the operational managers of the appointing body for the REC in order to check that the application has been processed correctly or to investigate any complaint
   - May be seen by auditors appointed by the National Research Ethics Service to undertake accreditation of the REC
   - Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply

Optional - please tick as appropriate

☑ I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

Signature: .....

Print Name: Professor S Michael Griffin

Date: 04/10/2007 (dd/mm/yyyy)

Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co-sponsors by a representative of the sponsor nominated to take the lead for the REC application

I confirm that: *(tick as appropriate)*

- ☑ This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.
- ☑ An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.*
- ☑ Any necessary indemnity or insurance arrangements, as described in question A35, will be in place before this research starts.
- ☑ Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.
- ☑ Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.
- ☑ The duties of sponsors set out in the NHS Research Governance Framework for Health and Social Care will be undertaken in relation to this research.**

* Not applicable to student research (except doctoral research)
** Not applicable to research outside the scope of the Research Governance Framework.

Signature:

Print Name: Amanda Tortice

Post: Research Operations Manager

Organisation: Newcastle Upon Tyne Hospitals NHS Trust

Date: 04/10/2007 (dd/mm/yyyy)

County Durham & Tees Valley 2 Research Ethics Committee
Professorial Unit of Surgery
University Hospital of North Tees
Piperknowle Road
Stockton-on-Tees
TS19 8PE

Telephone: 01642 624164
Facsimile: 01642 624164

10 October 2007

Professor S Michael Griffin
Professor of Gastrointestinal Surgery
Northern Oesophagogastric Unit, Royal Victoria Infirmary
Queen Victoria Rd
Newcastle upon Tyne
NE1 4LP

Dear Professor Griffin

Full title of study: **The role of oesophageal impedance measurement in detection of gastro-oesophageal reflux disease in human lung transplant recipients**
REC reference number: **07/H0908/70**

Thank you for your letter of 04 October 2007, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Committee held on 08 October 2007. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

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

Ethical review of research sites

The Committee has designated this study as exempt from site-specific assessment (SSA. There is no requirement for [other] Local Research Ethics Committees to be informed or for site-specific assessment to be carried out at each site.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application	5.4	17 August 2007
Investigator CV		
Protocol	1	23 August 2007
Participant Information Sheet: Collection of gastric fluid samples	2	04 October 2007
Participant Information Sheet	2	04 October 2007
Participant Consent Form	2	04 October 2007
Participant Consent Form: Collection of gastric fluid samples	2	04 October 2007
Response to Request for Further Information		04 October 2007
Table of events		
revised pages of application form		

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Feedback on the application process

Now that you have completed the application process you are invited to give your view of the service you received from the National Research Ethics Service. If you wish to make your views known please use the feedback form available on the NRES website at:

https://www.nresform.org.uk/AppForm/Modules/Feedback/EthicalReview.aspx

We value your views and comments and will use them to inform the operational process and further improve our service.

07/H0908/70	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Kate Williams
Deputy Chair

Email: leigh.morgan@nth.nhs.uk

Enclosures: *Standard approval conditions*

Copy to: Ms Amanda Tortice, R & D Department, 4th Floor. Leazes Wing. Royal Victoria Infirmary, Newcastle upon Tyne

The Newcastle upon Tyne Hospitals NHS

NHS Tr[illegible]

Royal Victoria Infirm[illegible]
Queen Victoria Roa[illegible]
Newcastle upon Ty[illegible]
NE[illegible]

LRF/11A/196

2/11/07

Dr Andrew GN Robertson
Clinical research Fellow
Northern Oesophago-Gastric Cancer Unit
RVI

Dear Dr Robertson

Trust Approval for R&D Project: 4368

Title of Project:	**The role of oesophageal impedance measurement in detection of gastro-oesophageal reflux disease in human lung transplant recipients**
Principal Investigator	**Dr A GN Robertson**
Funder (proposed):	**Northern Oesophago-Gastric Unit**
Sponsor (proposed):	**The Newcastle upon Tyne Hospitals NHS Foundation Trust**

The Trust grants approval for the above project, dependent upon:

(i) you, as Principal Investigator, agreeing to comply with the Department of Health's Research Governance Framework for Health and Social Care, and understanding their responsibilities and duties (a copy of guidelines prepared by the Trust R&D Office are enclosed)

(ii) you, as Principal Investigator, ensuring compliance of the project with all other legislation and guidelines including Caldicott Guardian approvals and compliance with the Data Protection Act 1998, Health and Safety at Work Act 1974, any requirements of the MHRA (*eg* CTA, EudraCT registration), and any other relevant UK/European guidelines or legislation (*eg* reporting of suspected adverse incidents).

Sponsorship

The Newcastle upon Tyne Hospitals NHS Foundation Trust will act as Sponsor for this project, under the Department of Health's guidelines for research in health and social care.

In addition, the Trust has a Research Governance Implementation Plan, agreed with the Department of Health, in order to fully comply with Research Governance and fulfil the responsibility of a Sponsor.

As the Trust is acting as Sponsor for the research and where some of the research is taking place outside of Newcastle upon Tyne, then all costs must be met for research governance audit visits to those sites. It is the responsibility of the PI to provide confirmation to the Trust of who will pay these costs. Audit is required under the Research Governance Framework for Health and Social Care. (Please note that the Trust randomly audits 10% of all its active research annually.)

You must notify the R&D Office if any changes to the protocol, etc. are agreed with the Ethics Committee or if there are any associated changes in cost relating from such alterations. It is imperative that the R&D Office retains a *complete* and up-to-date set of all such material.

It is also the Principle Investigator's responsibility to ensure that all staff involved have Honorary Contracts, where necessary, issued prior to commencing the research. Please be aware that Honorary Contracts will not be issued without a favourable ethical opinion and funding.

In addition, unless otherwise agreed with the Trust, the research will be covered for negligence under the CNST (Clinical Negligence Scheme for Trusts), however cover for no-fault harm is the responsibility of the Principal Investigator to arrange if required.

Please also note that for any NHS employee who generates Intellectual Property *in the normal course of their duties*, it is recognised that the Intellectual Property Rights remain with the employer and not the employee.

Yours sincerely,

I R Fenwick CBE
Chief Executive

Enc

cc Mrs C Hughes, Finance Department, Room 203, Cheviot Court, Freeman Hospital
Dr AN Branson, Cancer services, Newcastle General Hospital

Appendix 7: Patient Information Sheets & Questionnaires

The Newcastle upon Tyne Hospitals
NHS Trust

Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Patient Information Sheet and Consent Form

Study Code:

Patient Initials: **Subject Number:**

Study Title: **The role of oesophageal impedance measurement in detection of gastro-oesophageal reflux disease in human lung transplant recipients.**

Name of Researchers: Professor Paul Corris, Professor Michael Griffin, Professor John Dark, Dr Jim Lordan, Dr Andrew Fisher and Dr Andrew Robertson

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

Ask us if there is anything that is not clear or if you would like more information.

Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the background and purpose of the study?

Rejection is a major problem for lung transplant recipients. This can occur at any time after a lung transplant; some patients develop chronic rejection soon after the transplant while others may go many years without it developing. The earliest sign of the start of this rejection is a drop in lung function, which can be measured with a simple blowing test. This early drop in lung function is often termed Bronchiolitis Obliterans Syndrome (BOS). All of the causes of BOS are as yet, not fully understood. One possible cause is now thought to be backflow of stomach contents into the lungs (known as reflux disease).

This backflow is most likely to be a low-grade, which means that patients will not always notice this. It is enough, however, to cause the airways to become inflamed and, if left untreated, to cause scarring.

It has been shown, in several studies here and in other lung transplant units, that contents from the stomach can flow backwards up the gullet and reach the transplanted lung. This is bad for lung function. It has also been shown that anti-reflux surgery, which is surgery to prevent this backflow, may lead to increased survival and improved lung function after transplant, by preventing lung damage.

We are now conducting a study that will, hopefully, answer the question as to how important this backflow is in causing rejection in patients who have had a lung transplant. If this is the case then surgery could be performed to prevent backflow and perhaps rejection.

This study will involve placing a thin tube through your nose into the gullet to measure this backflow. This tube is called an impedance catheter. The tube will be inserted into the gullet for 24 hours to measure the amount and severity of backflow you are experiencing. All patients in the study will have this test performed to determine whether they are suffering from backflow and how severe this problem is for them.

Why have I been chosen?

You have been asked to consider taking part in this study because you have had a recent lung transplant.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you decide not to take part in this study your management will be routine and your treatment will not be changed in any way.

What will happen to me if I take part?

Following the discussion and consent for the study, when you will be given the opportunity to ask questions:

- You will be asked to fill in a simple questionnaire to see if you are experiencing symptoms of backflow (e.g. heartburn)
- The impedance catheter will be placed in your gullet for 24 hours before your routine bronchoscopy to assess for the presence of backflow. To insert the impedance catheter another 10 minute study is performed on your gullet with a slightly large tube to work out where to place the catheter.

These impedance measurements would be performed the day before your routine bronchoscopies at 1, 3 and 6 months. If you were found to have severe backflow or early signs of rejection at 3 months you would be offered the opportunity to undergo anti-reflux surgery to prevent this backflow.

What do I have to do?

It is important that you attend your study visits – these will be the day before your routine bronchoscopies which are performed to check up on your new lungs.

We would ask you to keep a note of any adverse events that may happen and note if you are prescribed any new medication during the study.

What is the technology that is being tested?

An impedance catheter is a new technology that assesses reflux disease. It is made up of small monitors along a fine tube that can detect the changes in electrical resistance present in liquids and gases. Thus it can detect the presence of gas and liquid in your gullet and whether you are swallowing this gas/liquid or whether it is travelling in the wrong direction.

With your consent, your family doctor will be informed that you are taking part in this study. Your participation in this study will be written in your hospital notes so that all hospital staff will know that you are in the study.

What are the side effects of any treatment received when taking part?

No new treatment is being given during this study therefore there are no side effects.

What are the side effects of any study procedures?

The possible side effects of the manometry and impedance catheters are discomfort to the nose, throat or gullet. These are normally related to the manometry test which only lasts 10-20 minutes.

What are the other possible disadvantages and risks of taking part?

There are no foreseen disadvantages or risks of taking part.

What are the possible benefits of taking part?

We cannot promise the study will help you but if you are found to have severe reflux disease or worsening lung function, then you will be offered surgery. This has been shown in several studies to improve lung function and survival. The information we get might help improve the treatment of other lung transplant patients and people with chronic rejection.

What happens when the research study stops?
At the end of the study you will continue to be monitored in the transplant clinic.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Contact 0191 2231148). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure
Complaints can be sent to:
The Complaints Officer, Freeman Hospital, Newcastle. NE7 7DN

Will my taking part in the study be kept confidential?

By agreeing to take part in this study you are consenting to the study staff collecting personal data about you, including the following:

- Your date of birth
- Your sex
- Your race or ethnic origin
- Details of your medical condition e.g. reason for transplant, transplant date etc

The data will be collected and entered onto a secure database. Access to this database will be password protected and only available to your doctors and the research staff. All data stored on the computer will be coded, your name will not appear – you will be given a unique study number under which all data and test results will be entered.

Your data, and the data from all the patients taking part in this study, will be analysed to see whether the presence and severity of backflow of stomach contents has an effect on your lung function and on the markers of inflammation that are being looked at from the samples taken during your bronchoscopy.

Your medical records may also be looked at by representatives of regulatory authorities and by authorised people from the Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

All the information about your participation in this study will be kept confidential.

If you agree, we will notify your GP that you are taking part in the study. Participation in the study will also be noted in your hospital records so that anyone who treats you will be aware that you are taking part in the study.

What will happen if I don't want to carry on with the study?

Participation in any research study is completely voluntary and you can decide to withdraw from the study at any time. You may decide that you don't want to have the impedance measurements performed. If you do withdraw from the study any information collected may still be used.
Withdrawing from the study will not affect the level of care that you get from your doctors.

What if something goes wrong?

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against Newcastle upon Tyne Hospitals NHS Trust. The normal National Health Service complaints mechanisms will still be available to you.

What will happen to the results of the research study?

The results of this study will be published in a medical paper but your identity will not be revealed. This study is expected to go on for two years so any publication may not take place until 2009.
Your transplant doctor will be able to tell you the severity of the backflow of stomach contents during the study. If this is abnormal you will be able to discuss the treatment options available to you with your consultant. You will be able to find out the overall results of the study, if you wish to know them, once the study is completely finished.

Who is organising and funding the research?

This study has been funded by the Newcastle upon Tyne Hospitals NHS Trust and will be overseen by the Newcastle upon Tyne Hospitals NHS Trust.

Who has reviewed the study?

This study has been reviewed by the County Durham & Tees Valley 2 Research Ethics Committee.

Contact Details:

For further information about the study you can speak to one of the consultants,

Prof Corris, Prof Griffin, Dr Fisher or Dr Lordan.

Alternatively you can speak to the Research Fellow:

Andrew Robertson Tel: 0191 2820240

In case of an emergency you can contact the transplant registrar on call.

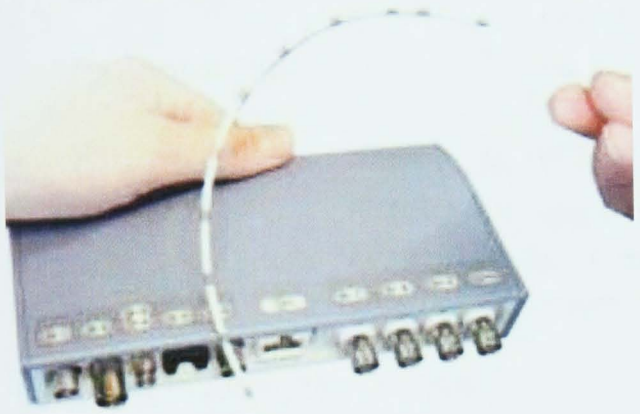
Thank you for taking the time to read this information sheet.

If you decide you would like to take part in this study, you will be given a copy of this information sheet and a signed consent from to keep.

TABLE OF EVENTS

	Visit 1 Screening	Visit 2	Visit 3	Visit 4
	Week1-4	1 month	3 months	6 months
Study Discussed / Information sheet given	X			
Informed Consent	X			
Medical History	X			
Routine Flow Volume & Bronchoscopy		X	X	X
Heartburn questionnaire		X	X	X
Impedance		X	X	X

Diagram of Impedance catheter which is a few millimetres in diameter.



The Newcastle upon Tyne Hospitals NHS
NHS Trust

Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Patient Consent Form

Study Code:

Patient Initials: **Subject Number:**

Study Title: **The role of oesophageal impedance measurement in detection of gastro-oesophageal reflux disease in human lung transplant recipients**

Lead Investigator: Andrew Robertson (Clinical Research Fellow)
Name of Researchers: Prof Corris, Griffin, Dr Fisher and Lordan

Please initial in the box

1. I confirm that I have read and understand the information sheet dated. 4th October 2007 (version 2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to my GP being informed of my participation in the study. ☐
5. I agree to take part in the above study ☐

Name of Patient	Signature	Date
Name of Person taking consent	Signature	Date

When completed, 1 for patient; 1 for researcher site file; 1(original) to be kept in medical notes.

The Newcastle upon Tyne Hospitals
NHS Trust

Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Patient Information Sheet and Consent Form

Study Code:

Patient Initials: **Subject Number:**

Study Title: **Collection of Gastric Fluid Samples for analysis and to assess its damaging effects on the human lung**

Name of Researchers: Professor Paul Corris, Professor Michael Griffin, Professor J Pearson, Dr Andrew Robertson

You are being asked to allow your doctor to keep fluid samples removed from the stomach at endoscopy. Before you decide if you are willing to take part, it is important for you to understand why the fluid samples are being collected, what this involves, how the information gathered will be used, and the possible benefits, risks and discomforts associated with the procedures. Therefore please take time to read the following information carefully and discuss it with others if you wish.

Ask us if there is anything that is not clear or if you would like more information. Take as much time as you want to decide whether or not you wish to take part.

Thank you for reading this.

What is the background and purpose of the study?

Your doctor will be one of the investigating doctors in a study of gastric juice and its role in damaging the human lung.

Why have I been chosen?

You have been asked to consider taking part in this study because you are scheduled for an endoscopy. You are being asked to allow your doctor to take your gastric secretions and send these to a laboratory in Newcastle. Your stomach's juice will be analysed and assessed for its role in damaging the human lung.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

If you decide not to take part in this study you will not be disadvantaged and your medical treatment and care will not be changed in any way.

What will happen to me if I take part?

You are already scheduled to undergo an endoscopy. During the procedure any fluid of the stomach is sucked out to allow your doctor to look at the lining of the stomach. Rather than being placed in a clinical waste bin, some of this fluid would be kept for analysis at the laboratory and used in experiments. This retaining of samples does not affect the endoscopy being performed by your doctor.

What are the possible side effects, risks and discomforts associated with this?

There are no anticipated side effects, risks or discomforts over and above the risks of endoscopy, which will be explained to you.

What are the possible benefits of taking part?
This study will not directly help you but will be of use to the scientific community.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (Contact 0191 2829697). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure

Complaints can be sent to:
The Complaints Officer, Freeman Hospital, Newcastle. NE7 7DN

How will my personal data be used?
The samples will be collected anonymously. No personal information will be collected for this research process.

What if something goes wrong?

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation

against Newcastle upon Tyne Hospitals NHS Trust. The normal National Health Service complaints mechanisms will still be available to you.

Who has reviewed the study?
This study has been reviewed by the County Durham & Tees Valley 2 Research Ethics Committee

Contact Details:

For further information about the study you can speak to one of the consultants,

Prof Griffin

Alternatively you can speak to the Clinical Research Fellow:

Andrew Robertson Tel: 0191 2829697

Thank you for taking the time to read this information sheet.

If you decide you would like to take part in this study, you will be given a copy of this information sheet and a signed consent from to keep.

The Newcastle upon Tyne Hospitals
NHS Trust

Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Patient Consent Form

Study Code:

Patient Initials: **Subject Number:**

Study Title: **Collection of Gastric Fluid Samples for analysis and to assess its damaging effects on the human lung**

Lead Investigator: Andrew Robertson (Clinical Research Fellow)
Name of Researchers: Prof Corris, Prof Griffin and Prof Pearson

Please initial in the box

1. I confirm that I have read and understand the information sheet dated. 27th April 2009 (version 4) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I agree to take part in the above study ☐

Name of Patient	Signature	Date
Name of Person taking consent	Signature	Date

When completed, 1 for patient; 1 for researcher site file; 1(original) to be kept in medical notes.

Extra-Oesophageal Reflux Study
Reflux Symptom Index Questionnaire Response Form

Patient Initials: _____ Screening Number: _____

Date: __ / __ / ____

Within the **last Month** how did the following problems affect you	0 = No Problem					5 = Severe Problem
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
Excess throat or postnasal drip	0	1	2	3	4	5
Difficulty swallowing food, liquids or pills	0	1	2	3	4	5
Coughing after you eat or after lying down	0	1	2	3	4	5
Breathing difficulties or choking episodes	0	1	2	3	4	5
Troublesome or annoying cough	0	1	2	3	4	5
Sensation of something sticking in your throat or a lump in your throat	0	1	2	3	4	5
Heartburn, chest pain, indigestion or stomach acid coming up	0	1	2	3	4	5
	RSI					

DeMeester Reflux Questionnaire

1) In the last 2 weeks have you suffered from heartburn (i.e. a burning sensation in the chest)?

grade 0, no symptoms	grade 1, occasional episodes	grade 2, reason for medical visit	grade 3, interference with daily activities

2) In the last 2 weeks have you suffered from regurgitation (acid or stomach contents coming up into your throat, mouth or lungs)?

grade 0, no regurgitation	grade 1, occasional episodes	grade 2, predictable on position of straining	grade 3, episodes of pulmonary aspiration, nocturnal cough or recurrent pneumonia

3) In the last 2 weeks have you suffered from dysphagia (difficulty swallowing or food getting stuck)?

grade 0, no dysphagia	grade 1, occasional episodes	grade 2, require liquid-to-clear diet	grade 3, episodes of esophageal obstruction

Overall are you satisfied with your operation?

Y/N

Comments:

The Gastrointestinal Quality of Life Index (GIQLI)

1. How often during the past 2 weeks have you had pain in the abdomen?

all of the time	most of the time	some of the time	a little of the time	never

2. How often during the past 2 weeks have you had a feeling of fullness in the upper abdomen?

all of the time	most of the time	some of the time	a little of the time	never

3. How often during the past 2 weeks have you had bloating (sensation of too much gas in the abdomen)?

all of the time	most of the time	some of the time	a little of the time	never

4. How often during the past 2 weeks have you been troubled by excessive passage of gas through the anus?

all of the time	most of the time	some of the time	a little of the time	never

5. How often during the past 2 weeks have you been troubled by strong burping or belching?

all of the time	most of the time	some of the time	a little of the time	never

6. How often during the past 2 weeks have you been troubled by gurgling noises from the abdomen?

all of the time	most of the time	some of the time	a little of the time	never

7. How often during the past 2 weeks have you been troubled by frequent bowel movements?

all of the time	most of the time	some of the time	a little of the time	never

8. How often during the past 2 weeks have you found eating to be a pleasure?

never	a little of the time	some of the time	most of the time	all of the time

9. Because of your illness, to what extent have you restricted the kinds of food you eat?

very much	much	somewhat	a little	not at all

10. During the past 2 weeks, how well have you been able to cope with everyday stresses?

extremely poorly	poorly	moderately	well	extremely well

11. How often during the past 2 weeks have you been sad about being ill?

all of the time	most of the time	some of the time	a little of the time	never

12. How often during the past 2 weeks have you been nervous or anxious about your illness?

all of the time	most of the time	some of the time	a little of the time	never

13. How often during the past 2 weeks have you been happy with life in general?

never	a little of the time	some of the time	most of the time	all of the time

14. How often during the past 2 weeks have you been frustrated about your illness?

all of the time	most of the time	some of the time	a little of the time	never

15. How often during the past 12 weeks have you been tired or fatigued?

all of the time	most of the time	some of the time	a little of the time	never

16. How often during the past 2 weeks have you felt unwell?

all of the time	most of the time	some of the time	a little of the time	never

17. Over the past week, have you woken up in the night?

every night	5-6 nights	3-4 nights	1-2 nights	never

18. Since becoming ill, have you been troubled by changes in your appearance?

a great deal	a moderate amount	somewhat	a little bit	not at all

19. Because of your illness, how much physical strength have you lost?

a great deal	a moderate amount	some	a little bit	none

20. Because of your illness, to what extent have you lost your endurance?

a great deal	a moderate amount	somewhat	a little bit	not at all

21. Because of your illnes, to what extent do you feel unfit?

extremely unfit	moderately unfit	somewhat unfit	a little unfit	fit

22. During the past 2 weeks, how often have you been able to complete your normal daily activities (school, work, household)?

never	a little of the time	some of the time	most of the time	all of the time

23. During the past 2 weeks, how often have you been able to take part in your usual patterns of leisure or recreational activities?

never	a little of the time	some of the time	most of the time	all of the time

24. During the past 2 weeks, how much have you been troubled by the medical treatment of your illness?

very much	much	somewhat	a little	not at all

25. To what extent have your personal relations with people close to you (family or friends) worsened because of your illness?

very much	much	somewhat	a little	not at all

26. To what extent has your sexual life been impaired (harmed) because of your illness?

very much	much	somewhat	a little	not at all

27. How often during the past 2 weeks, have you been troubled by fluid or food coming up into your mouth (regurgitation)?

all of the time	most of the time	some of the time	a little of the time	never

28. How often during the past 2 weeks have you felt uncomfortable because of your slow speed of eating?

all of the time	most of the time	some of the time	a little of the time	never

29. How often during the past 2 weeks have you had trouble swallowing your food?

all of the time	most of the time	some of the time	a little of the time	never

30. How often during the past 2 weeks have you been troubled by urgent bowel movements?

all of the time	most of the time	some of the time	a little of the time	never

31. How often during the past 2 weeks have you been troubled by diarrhoea?

all of the time	most of the time	some of the time	a little of the time	never

32. How often during the past 2 weeks have you been troubled by constipation?

all of the time	most of the time	some of the time	a little of the time	never

33. How often during the past 2 weeks have you been troubled by nausea?

all of the time	most of the time	some of the time	a little of the time	never

34. How often during the past 2 weeks have you been troubled by blood in the stool?

all of the time	most of the time	some of the time	a little of the time	never

35. How often during the past 2 weeks have you been troubled by heartburn?

all of the time	most of the time	some of the time	a little of the time	never

36. How often during the past 2 weeks have you been troubled by uncontrolled stools?

all of the time	most of the time	some of the time	a little of the time	never

Calculation of the score:

most desirable option: 4 points

least desirable option: 0 points

GIQLI score: sum of the points

Appendix 8: JAG Accreditation in Endoscopy

JAG

Certificate of Competency in Endoscopy

...on behalf of the Joi... ...visory Gr... ...stro...

I declare that Dr Andrew Robertson
has been formally assessed as competent to a standard commensurate with independent specialist practice in the under-noted modalities of endoscopy. Training leading to this certification was conducted in accordance with the current guidelines for training in endoscopy published by the JAG on GI Endoscopy.

MODALITIES	DATE
Diagnostic Upper GI Endoscopy	

Mr K Wynne
Name of Training Supervisor

Royal Victoria Infirmary
Training Unit Name

1/NTH/002
Unit Registration number

UGI/09/027(d)
Serial No:

Signature of JAG Chairman

22 May 2009
Date

JAG Stamp

JAG
...ISTERED

Joint Advisory Group on Gastrointest... ...
JCHMT, 6 St Andrews Place, Regent's Park, London NW1 4LB. Tel: (020) 79... ...74. Fax: (020) 7486 4160

Appendix 9: Breakdown of RSI scores at 1 month

Study No	RSI >13	Hoarseness	Clearing your throat	Excess throat or postnasal drip	Difficulty swallowing food, liquids or pills	Coughing after you eat or after lying down	Breathing difficulties or choking episodes	Troublesome or annoying cough	Sensation of something sticking in your throat or a lump in your throat	Heartburn, chest pain, indigestion or stomach acid coming up	RSI	Prox Rfx Events
1	N	0	0	0	3	0	0	2	1	0	6	8
2	Y	4	3	2	4	1	0	4	0	3	21	22
3	Y	2	2	0	4	1	0	2	2	4	17	10
4	N	0	1	4	0	0	0	1	0	1	7	17
5	N	0	0	2	2	0	0	0	2	0	6	8
6	N	0	0	3	0	0	0	0	0	1	4	4
7	Y	0	4	0	0	5	3	4	0	3	19	17
8	N	1	3	1	0	1	0	0	1	3	10	21
9	N	0	0	0	0	0	0	0	0	0	0	34
10	Y	3	3	3	4	4	4	3	4	4	32	37
11	N	2	0	1	1	1	0	3	0	2	10	42
12	N	0	0	0	0	0	1	1	1	1	4	19
13	Y	1	2	0	2	0	2	3	3	4	17	37
14	N	3	0	2	0	0	0	0	0	5	10	11
15	N	0	2	0	0	1	0	2	0	2	7	10
16	N	3	3	1	1	0	0	0	0	0	12	13
17	N	0	2	0	3	0	0	0	2	0	7	51

Appendix 10: Results of Gastric Juice Analysis

GJ Sample	Diagnosis	Medication	Age	Sex	pH	Bile Acid (uM)	Pepsin ug/ml	Trypsin (ug/ml)	Micro
SP	Normal	Nil	20	f	2.3	300	3039	n/a	
PU1	peptic ulcer	pentagastrin				0	0	n/a	
PU2	peptic ulcer	pentagastrin				0	65.4	n/a	
PU3	peptic ulcer	pentagastrin				0	916	n/a	
LTx1	LT	PPI	41	f	7.8	30	0	n/a	
LTX2	LT	PPI	24	f	n/a	1000	n/a	n/a	
LTx3	LT	PPI	29	f	4.8	30	0	n/a	
LTx4	LT	PPI	25	f	2.2	0	548	n/a	
CR1	oesophageal adenocarcinoma	Nil	61	m	1.9	100	380	n/a	
CR2	Gastritis	PPI	67	f	7.5	8000	61.9	n/a	
CR3	Barrett's/Gastric ulcer	PPI	62	f	3.9	0	640	n/a	
CR4	Barrett's	PPI	88	m	8.4	0	0	n/a	
CR5	Nad	Nil	81	m	2.2	100	1684	n/a	
CR6	oesophagitis, gastritis, duodenitis	Nil	37	m	1.8	250	358	n/a	
CR7	oesophagitis, gastritis,	Nil	73	m	2.9	30	0	n/a	
CR8	gasttric ulcer	PPI	71	m	4.7	n/a	n/a	n/a	
CR9	ulcer, oesophagitis, gastro-jejunostomy	PPI	82	f	6.7	10	0	n/a	
CR10	gastric erosion	PPI	51	f	1.7	100	123	n/a	
CR11	Normal	Nil	48	f	4.7	8000	1778	n/a	
CR12	Barrett's	PPI	84	m	6.9	0	210	n/a	
CR13	Oesophagitis	Nil	46	f	0.8	0	95.2	n/a	
CR14	Barrett's, duodenitis peptic ulcer	Nil	75	f	1.9	1200	68.2	n/a	
CR15	Oesophagitis	Nil	57	m	1.5	200	893	n/a	
CR16	metastatic carcinoma	PPI	70	m	6.6	100	0	n/a	
CR17	Normal	Nil	45	m	2.8	0	0	n/a	
LTx5	LT	PPI	35	f	2.9	150	233.2	n/a	
CR18	Normal	Nil	38	f	3.5	20	829	n/a	
CR19	gastric polyp	Nil	50	f	1.6	100	1010	n/a	
CR20	oesophagitis Grade 4, gastritis, duodenitis	Nil	53	m	1.7	100	606	n/a	
CR21	gastric ulcer	PPI	53	f	2.6	50	27.5	n/a	
CR22	Oesophagitis	Nil	75	f	1.6	200	67.4	n/a	
CR23	Oesophagitis	Nil	64	m	7.5	100	19.8	n/a	
CR24	Duodenitis	Nil	59	f	7.9	70	0	n/a	
CR25	healed gastric ulcer	PPI	47	f	4.1	60	1957	n/a	
CR26	gastric ulcer	Nil	67	f	1.6	30	873	n/a	
CR27	oesophagitis, duodenal ulcer	Nil	29	f	1.8	100	515	n/a	
CR28	mild antral gastritis	Nil	49	f	1.6	30	981	n/a	
CR29	oesophagitis grade 2	Nil	68	f	1.4	50	432	n/a	

CR30	oesophageal nodule	Nil	69	f	7.6	220	0	n/a	
CR31	oesophagitis Grade A	Nil	34	f	1.5	50	1478	n/a	
CR32	Normal	Nil	31	f	2.1	360	479	n/a	
LTx6	Normal	Nil	37	f	1.4	40	572	n/a	
LTx7	oesophagitis grade A, HH, bile in oesophagus	PPI	60	f	7.5	2200	0	n/a	
CR33	oesophagitis Grade A, HH	nil	42	f	1.5	50	791	n/a	
CR34	duodenitis, nodule	nil	48	f	1.6	80	1247	n/a	
CR35	oesophagitis, gastritis	nil	81	f	1.8	0	100	n/a	
CR36	oesophagitis Grade B, gastritis	nil	63	f	1.8	50	249	n/a	
CR37	oesophagitis Grade A,	PPI	75	f	2.4	20	798	n/a	N
CR38	Oesophagitis and pyloroplasty	PPI	56	m	6.6	400	0	n/a	Y
CR39	Barrett's oesophagus, HH	PPI	65	f	4.8	40	127	n/a	Y
CR40	HH	PPI	59	f	2	20	90	5	N
CR41	Duodenitis, HH, oesophagitis	nil	45	f	1.4	50	840	10	N
CR42	Gastritis, HH	PPI	42	f	5.5	600	3153	15	Y
CR43	Oesophagitis, HH	PPI	58	f	4.1	500	533	4	N
CR44	normal	nil	80	m	4.7	10	0	8	Y
CR45	gastric ulcer	PPI	50	m	8.4	0	57	5	Y
CR46	gastritis, HH	nil	78	f	1.6	80	761	4	Y
CR47	Barrett's oesophagus	PPI	73	m	5.1	60	460	4	Y
CR48	Barrett's oesophagus	PPI	62	m	6.8	n/a	0	n/a	N
CR49	gastritis	PPI	78	m	6	n/a	0	n/a	Y
CR50	normal	nil	55	f	5.2	2050	3772	100	Y
CR51	duodenal ulcer	nil	68	f	6	30	1181	20	Y
CR52	gastritis	nil	65	f	1.7	80	1319	5	N
LTx8	HH	PPI	30	f	1.6	530	1346	5	Y
LTx9	normal	PPI	30	f	4	n/a	3892	12	N