Gastric Aspiration and Lung Transplantation

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Abstract

Lung transplantation has become a viable therapeutic option for patients with end-stage lung disease, however, despite improvements in surgical techniques and post-operative management long term survival is poor when compared to those of other solid organ transplants. The long term success is limited by the onset of obliterative bronchiolitis (OB) and its clinical correlate bronchiolitis obliterans syndrome (BOS). Obliterative bronchiolitis is a chronic pathology involving inflammation and airway fibrosis causing allograft dysfunction. It is thought to occur as a response to both immunological and non-immunological mechanisms, and there is increasing evidence to suggest that gastro-oesophageal reflux with subsequent aspiration is a contributing factor.

The aims of this project were to investigate whether gastric aspiration is occurring in lung transplant recipients and whether it can predispose a patient to the development of chronic rejection (OB/BOS). This was investigated using the gastric protease pepsin as a biomarker for gastric aspiration in the bronchoalveolar lavage (BAL) of lung transplant recipients. In addition, to further understand the link between aspiration and the development of OB/BOS the effects of pepsin on mucus and cytokine production from primary bronchial epithelial cells from lung transplant patients and goblet cells were investigated.

From cross-sectional analyses pepsin levels were found to be elevated in lung transplant recipients compared to normal and disease controls, with the highest levels been found in the acute rejection (grade ≥A2) group (normal: median, 1.1, range 0-2.3ng/ml vs. all transplant: median 8.3, range 0-51.7ng/ml, P =0.02). Further analysis involving a longitudinal cohort of patients also confirmed that pepsin was present in the BAL of lung transplant recipients. A cut off value for a 'high' pepsin level was prospectively determined using a separate and 'clinically well' stable transplant control group (75^{th} percentile pepsin level, 10.4ng/ml). These subjects were documented to be free from rejection, infection or any clinical problems commonly associated with transplantation. Patients with early elevated levels of BAL pepsin (i.e. above 10.4ng/ml

at 3 months post-transplant) were estimated to develop BOS at 3.0 times the rate of those with low early BAL pepsin. This is the first longitudinal study of BAL pepsin in lung transplant recipients which shows a trend for decreased survival in patients with early elevated BAL pepsin levels (60% BOS free in those with high early BAL pepsin vs. 80% BOS free in those with low early BAL pepsin at 3 years post-transplant). This shows a need for further investigations with increased patient numbers to reach statistical significance and confirm these results.

The effect of pepsin and gastric juice on bronchial epithelial and goblet cell cultures was also investigated. The viability of the cells was not affected with the addition of pepsin, however the addition of whole gastric juice did cause a significant reduction in epithelial cell viability. In addition, mucin production from goblet cells was significantly increased at 72h on addition of 50μg/ml pepsin at pH 7.4 (median values pH 7.4; 163.4μg/ml and pH 7.4 & pepsin; 448.9μg/ml, P=0.038) and at pH 7.0 (median values pH 7.0; 55.3μg/ml and pH 7.0 & pepsin; 327.2μg/ml, P=0.016). Previous investigations from our group have shown that interleukin-8 (IL-8) can stimulate the production of mucin from goblet cells *in vitro,* therefore media from epithelial and goblet cells stimulated with pepsin was measured for IL-8, however there was no significant increase in production from either cell type. This suggests that the increase in mucin production in cells stimulated with pepsin is not mediated through an IL-8 pathway, therefore other mechanisms should be investigated.

This thesis supports the hypothesis that gastric aspiration may be an important injury in lung transplantation and that pepsin is a potentially useful biomarker that may be associated with chronic allograft damage.

Publications and reviewed abstracts

Stovold, R., Forrest, I.A., Corris, P.A., Murphy, D.M., Smith, J.A., Decalmer, S., Johnson, G.E., Dark, J.H., Pearson, J.P. and Ward, C. (2007). "Pepsin, a Biomarker of Gastric Aspiration in Lung Allografts: A Putative Association with Rejection." American Journal of Respiratory & Critical Care Medicine **175**(12): 1298-1303.

Murphy, D.M., Ward, C., Forrest, I.A., Pritchard, G., Jones, D., Stovold, R., Fisher, A.J., Cawston, T.E., Lordan, J.L. and Corris, P.A. (2006). "The Phosphodiesterase Type IV Inhibitor Cilomilast Decreases Pro-inflammatory Cytokine Production From Primary Bronchial Epithelial Cells in Lung Transplantation Patients." The Journal of Heart & Lung Transplantation **25**(12): 1436-1440.

Decalmer, S., **Stovold, R.**, Jones, H., Pearson, J.P., Ward, C., Houghton, L. et al. Gastro-oesophageal Reflux, Micro-aspiration and Chronic Cough. *In submission.*

Stovold, R., Corris, P.A., Fisher, A.J., Lordan J., Pearson, J.P. and Ward, C. "Does pepsin in gastric juice aspirated into the lung allograft stimulate mucus hypersecretion". Presented at the 28th annual meetings and scientific sessions of the International society for heart and lung transplantation Boston, 2008.

Stovold, R., Forrest, I.A., Murphy, D.M., Johnson, G.E., Lordan J. Fisher, A.J., Corris, P.A., Pearson, J.P. and Ward, C. "Longitudinal assessment of pepsin levels in lung allografts suggests aspiration is an ongoing injury, which can occur early following lung transplantation." Presented at the British thoracic society winter meeting London 2008.

Stovold, R., Ward C., Corris P.A., Pearson, J.P. "Exposure to pepsin can modify mucus secretion from goblet cells."[abstract] Presented at the 15th United European Gastroenterology Week Pairs, 2007.

Stovold, R., Forrest, I.A., Murphy, D.M., Corris, P.A., Fisher, A.J., Dark, J.H., Pearson, J. P., Smith, J.A., Decalmer, S. and Ward, C. (2006). "Evidence of increased gastric aspiration in acute lung allograft rejection [abstract]." Thorax **61**: AS006.

Stovold, R., Ward, C., Smith, J.A., Decalmer, S., Dark, J.H., Corris, P.A. and Pearson, J.P. (2006). "A role for gastric reflux in lung allograft rejection [abstract]." Gut **55 supplement v**: AOP-G-266.

Stovold, R., Forrest, I., Murphy, D.M., Corris, P.A., Dark, J.H., Pearson, J.P., Smith, J.A., Decalmer, S. and Ward, C. (2006). "Evidence of Increased Gastric Aspiration in Acute Lung Allograft Rejection [abstract]." European Respiratory Journal **28**: A771.

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Abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
BEBM	Bronchial epithelial basal media
BEGM	Bronchial epithelial growth media
blt	Bilateral lung transplant
BOS	Bronchiolitis obliterans syndrome
BSA	Bovine serum albumin
CF	Cystic fibrosis
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CV	Coefficient of variance
Da	Daltons
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's modified eagles medium
DPX	Distrene-80 plasticizer xylene
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
ENT	Ear nose and throat
FCS	Foetal calf serum
FEV_1	Forced expiratory volume over 1 second
GI	Gastro-intestinal
GM-CSF	Granulocyte macrophage colony stimulating factor
GOR	Gastro-oesophageal reflux
GORD	Gastro-oesophageal reflux disease
H_2	Histamine 2
H_2RA	Histamine 2 receptor antagonist
HLA	Human leukocyte antigen
hlt	Heart-lung transplant
HRP	Horseradish peroxidase
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis

LBB	Lymphocytic bronchitis/bronchiolitis
LNF	Laparoscopic Nissen fundoplication
LOS	Lower oesophageal sphincter
LPR	Laryngeal-pharyngeal reflux
LPS	Lipopolysaccharide
MMF	Mycophenolate mofetil
MMP	Matrix-metallo protease
MUC	Mucin gene (human)
n/N	Number
NCCP	Non-cardiac chest pain
OB	Obliterative bronchiolitis
P	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PG	Pepsinogen
PPH	Primary pulmonary hypertension
PPI	Proton pump inhibitor
r^2	Goodness of fit of linear regression
RMPI	Acronym for Roswell Park Memorial Institute, where this culture media was developed
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
slt	Single lung transplant
TBB	Transbronchial biopsy
TBS	Tris buffered saline
TGF	Tumour growth factor
TLI	Total lymphoid irradiation
TMB	3,3',5,5'-Tetramethylbenzidine
TNBS	Trinitrobenzylsulphonic acid
TNF	Tumour necrosis factor
Tx	Transplant
v/v	Volume for volume
w/v	Weight for volume
w/w	Weight for weight

Chapter 1

Introduction

1.1 Lung transplantation

1.1.1History of and indications for lung transplantation

Lung transplantation has become a viable therapeutic option for a variety of end stage lung diseases, and since the first successful isolated unilateral lung transplant performed in 1983 by the Toronto Group (Toronto Lung Transplant Group 1986), the International Society for Heart and Lung Transplantation (ISHLT) has reported data on approximately 3200 heart-lung transplants and over 23000 lung transplants. The number of transplants performed each year peaked in 2005 with 2169 (Trulock et al. 2007). The number of single lung transplants has remained fairly constant since 1994; however, the number of bilateral transplants has been increasing each year, and exceeded the number of single transplants for the first time in 2002 (Trulock et al. 2005). This move towards bilateral transplants is likely to be due to better survival statistics when compared to unilateral transplantations for certain pre-transplant indications (Meyers et al. 1999; Hadjiliadis et al. 2002; Trulock et al. 2005).

The majority of lung transplants are carried out for the following conditions; chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), cystic fibrosis (CF) and α_1-anti-trypsin deficiency emphysema. Figures 1.1

and 1.2 show the distribution of pre-transplant indications for single and bilateral transplants for the period of January 1996 to June 2006.

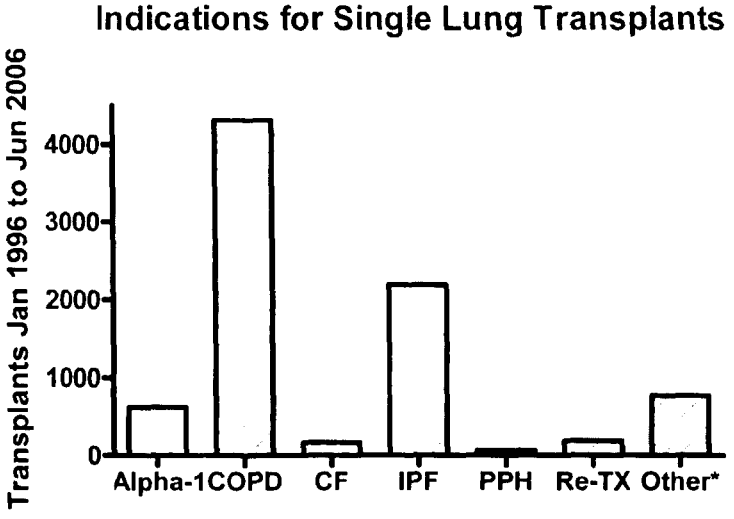


Figure 1.1. Main indications for single lung transplants over the period of January 1996 to June 2006. Alpha-1: anti-trypsin deficiency emphysema (8%), COPD: chronic obstructive pulmonary disease (52%), CF cystic fibrosis (2%), IPF: idiopathic pulmonary fibrosis (26%), PPH: primary pulmonary hypertension (1%) and Re-TX: re-transplantation (2%) *Others include: Sarcoidosis, Bronchiectasis, Congenital Heart Disease, lymphangioleiomyomatosis and OB (non-ReTx). This figure has been modified from Trulock et al 2007.

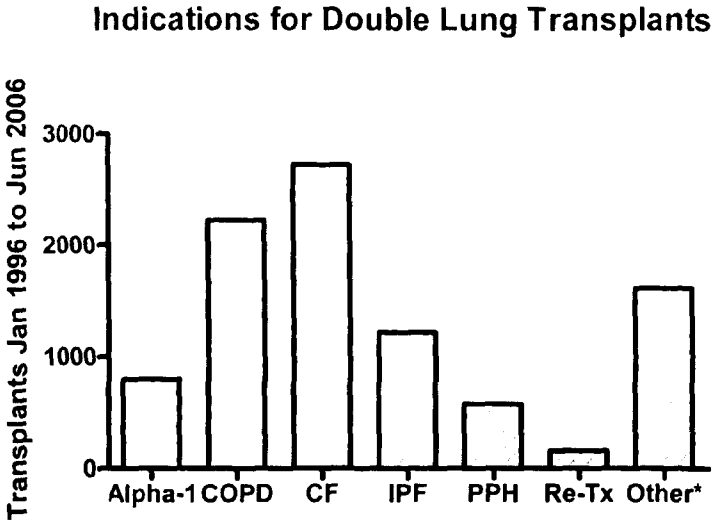


Figure 1.2. Main indications for bilateral lung transplants over the period of January 1996 to June 2006. Alpha-1: anti-trypsin deficiency emphysema (9%), COPD: chronic obstructive pulmonary disease (24%), CF: cystic fibrosis (29%), IPF: idiopathic pulmonary fibrosis (13%), PPH: primary pulmonary hypertension (6%) and Re-TX: re-transplantation (2%). *Others include: Sarcoidosis, Bronchiectasis, Congenital Heart Disease, lymphangioleiomyomatosis and OB (non-ReTx) This figure has been modified from Trulock et al 2007.

Experience gained over the last two decades shows that the 1 year survival rate has increased over this time period and figure 1.3 illustrates the survival rates for three different eras (1988-94, 1995-99 and 2000-06). The most recent era (2000-06) has the best 1 year survival rate at approximately 80%, followed by 74% for 1995-99 and finally 70% for 1988-94 (Trulock et al. 2007). This difference indicates improvements in surgical techniques, organ preservation and post operative management (Meyers et al. 1999; Moffatt et al. 2005), however, at around 5 years the difference in survival rate between the three eras becomes less marked, indicating that more research is required to further improve long term outcomes.

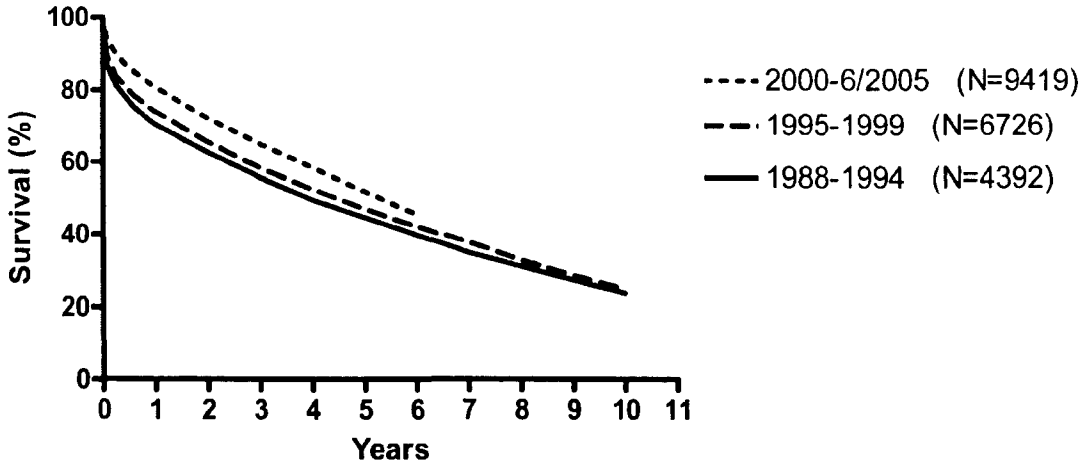


Figure 1.3 Survival rates by era for adult lung transplantations performed between January 1988 and June 2006. This figure has been modified from Trulock et al 2007.

1.1.2 Acute and chronic rejection in lung transplantation

Long term survival rates for lung transplant recipients are poor when compared to those of other solid organs. One of the major limitations to long term survival in lung transplantation is the onset of obliterative bronchiolitis (OB). OB is a chronic disease process involving the fibrosis of and cellular deposition in airways causing allograft dysfunction. The histological diagnosis of OB requires tissue obtained from either transbronchial biopsies (TBB) or from open lung biopsy and is graded according to internationally accepted criteria that was instituted by the Lung Rejection Study Group in 1990, and was revised in 1995 (Yousem et al. 1990; Yousem et al. 1996). In this revised criteria as well as grading OB it was put forward that the relative activity of the inflammatory infiltrate should be noted, and would be graded as either, a-Active or b-Inactive. The following definitions are taken from the revised criteria from Yousem et al (Yousem et al. 1996):

Active: In addition to the fibrosis, there are intra-bronchiolar and/or peri-bronchiolar submucosal and peri-bronchiolar mononuclear cell infiltrates usually associated with ongoing epithelial damage.

Inactive: Dense fibrous scarring without cellular infiltrates; this represents old cicatricial change in the small airways with a lack of significant submucosal and peri-bronchiolar inflammatory infiltrates.

Obliterative bronchiolitis is thought to represent an overall response to epithelial injury that results from multiple insults to the graft. The functional changes are thought to be due to submucosal scarring, which can lead to total obliteration of the airway (figure 1.4).

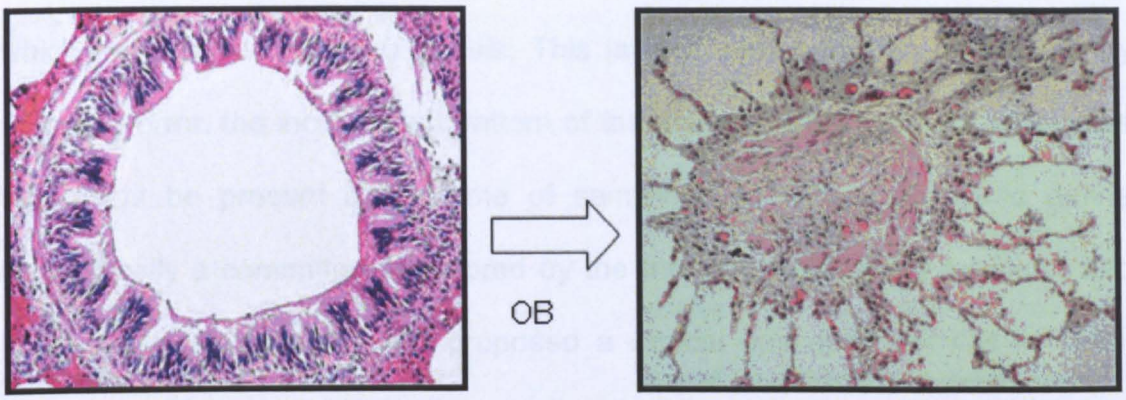


Figure 1.4: Transbronchial biopsies of a normal and a remodelled airway showing complete obliteration. These biopsies were taken and processed at the Freeman Hospital, Newcastle, UK.

Histological diagnosis is, however, invasive and not always sensitive enough which may lead to under-diagnosis. This lack of sensitivity can be caused by sampling error, the inconsistent pattern of the disorder, or any other pathologies that might be present at the time of sampling. As OB is difficult to define histologically a committee sponsored by the International Society for Heart and Lung Transplantation (ISHLT) proposed a clinical description of OB in 1993, termed bronchiolitis obliterans syndrome (BOS) (Cooper et al. 1993). BOS represents the functional manifestation of chronic rejection in lung allograft patients and is defined by a persistent decline in lung function measured by forced expiratory volume in 1 second (FEV_1). A baseline FEV_1 is measured (an average of the patient's two best post-transplant readings taken 3-6 weeks apart) and from this the status of BOS can be determined (table 1.1).

Status	FEV_1 Values (% of Post-transplant Baseline)
BOS-0	>80
BOS-1 (mild)	66-80
BOS-2 (moderate)	51-65
BOS-3 (severe)	≤50

Table 1.1. BOS classification using FEV_1 values (Cooper et al. 1993).

Although this classification system has gained universal acceptance some limitations have been noted. The original system defines BOS as a 20% reduction in FEV_1, however, this may miss early and small but potentially important changes in lung function, and therefore in the updated diagnostic criteria an extra *potential* BOS stage (BOS 0-p) was added to the system. This status is given with a decrease of 10-19% in baseline FEV_1 (Estenne et al. 2002).

Bronchiolitis obliterans syndrome is thought to be the functional manifestation of the pathophysiological changes that occur in OB. It is the most common chronic complication affecting the allograft itself. Figure 1.5 shows freedom from BOS in adult lung allograft recipients for follow-up between 1994 and 2006 taken from the international registry (Trulock et al. 2007). At 5 years post-transplant almost 50% of recipients have developed BOS.

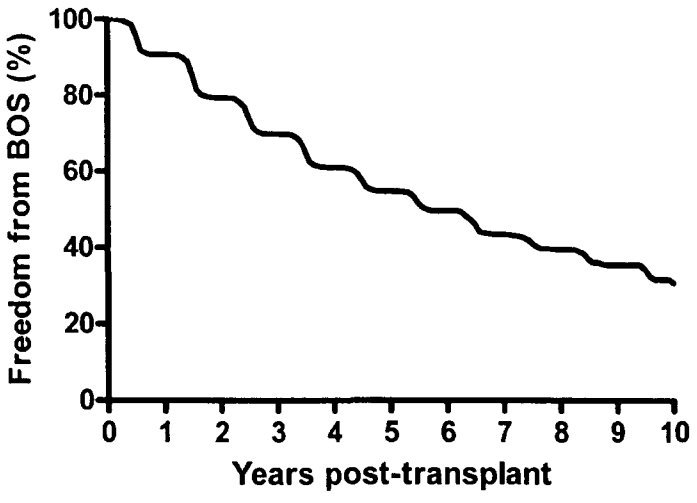


Figure 1.5 Freedom from bronchiolitis obliterans syndrome (BOS) in adult lung allograft recipients for follow-up between 1994 and 2006. This figure has been taken from Trulock et al 2007.

The fibroblastic scarring process in OB involves the respiratory bronchioles. Mononuclear cell infiltration of all layers of the bronchiolar wall in the active phase of OB can lead to the smooth muscle layers being destroyed by extension of the fibrous tissue into the peri-bronchiolar interstitium. As well as fibrosis of the airways there can be epithelial damage and even loss with lymphocytic infiltration. Extensive fibrosis connected with destruction of the smooth muscle may lead to extrinsic compression of the lumen in a constrictive form of bronchiolitis obliterans (Stewart 1994).

The origin of the fibroblasts responsible for this pathology is controversial, but these cells may originate from a variety of sources, including, *in situ* proliferation of resident fibroblasts or recruitment of circulating monocytes (Stewart 2004). There is also evidence to suggest that epithelial to mesenchymal transition (EMT) may be another source in this fibrotic response (Kalluri and Neilson 2003). This process involves epithelial cells becoming fibroblasts and can be described as a loss of epithelial markers and a gain of fibroblastic characteristics. An early marker of EMT is the expression of the human homologue of the fibroblast specific protein 1, S100A4. In addition, matrix-metallo proteases (MMP) 7, 2 and 9 are also associated with EMT and collagen type IV, a major component of the reticular basement membrane of airway epithelia, is a common substrate for these enzymes. Disruption of the reticular membrane by MMPs can cause damage, inflammation and further EMT, as epithelial cells rely on contact with the basement membrane to maintain their phenotype (Kalluri and Neilson 2003).

Ward et al have investigated the occurrence of EMT in lung transplantation using biopsies and primary bronchial epithelial cell cultures taken from lung transplant recipients. The group found epithelial expression of S100A4 in large airway biopsies and primary cultures, and also found that in the biopsies S100A4 expression was accompanied by staining for MMP-7, supporting the role of EMT in lung transplantation. In addition, the primary epithelial cells treated with TGF-β (a prototypical driver of EMT) demonstrated increased MMP dependant invasion of collagen coated filters (Ward et al. 2005).

Several risk factors for post-transplant OB/BOS have been identified from studies in individual centres, with one of the most important being acute rejection. A grading system for acute rejection was also included in the 1990 and revised 1995 criteria from the Lung Rejection Study Group (Yousem et al. 1990; Yousem et al. 1996). It states that there are 5 grades (0-4) of acute rejection, summarised in table 1.2.

Grade	Severity of Rejection
A0	No acute rejection; no mononuclear infiltration, haemorrhage or necrosis.
A1	Minimal acute rejection; scattered, infrequent perivascular mononuclear infiltrates not obvious at low magnification (40 X). Blood vessels are cuffed by small, round and transformed lymphocytes.
A2	Mild acute rejection; perivascular mononuclear infiltrates surrounding venuoles and arterioles are recognisable at low magnification. Presence of subendothelial mononuclear infiltrates, eosinophils and coexistent airway inflammation.
A3	Moderate acute rejection; readily recognisable venuole and arteriole cuffing by dense perivascular mononuclear cell infiltrates. Eosinophils and occasional neutrophils are also common. By definition, there is extension of the inflammatory cell infiltrate including alveolar macrophages into perivascular and peri-bronchiolar alveolar septae and air spaces.
A4	Severe acute rejection; diffuse perivascular, interstitial, and air space infiltrates of mononuclear cells and prominent alveolar pneumocyte damage. Grade A4 can be distinguished from post-transplant acute lung injury by the presence of perivascular and interstitial mononuclear cells, as they are not present in peri-operative lung injury.

Table 1.2 A summary of the acute rejection grading system defined by the Lung Rejection Study Group (Yousem et al. 1990; Yousem et al. 1996).

In addition to these definitions, airway inflammation also has a grading system. The report states that the airway inflammation (lymphocytic bronchitis/bronchiolitis) should be listed as a B category and have 5 grades each being defined histologically (0-4, 4 being most severe) and also a BX category, which represents an un-gradable biopsy, due to sampling errors, infection or other problems that may have occurred at the time of sampling.

1.1.3 Risk factors for lung transplant rejection

In a review by Scott et al identifying risk factors and therapeutic strategies for BOS, 13 of 15 centres studied reported a significant association with acute rejection (Scott et al. 2005). In the two centres that did not show a significant association, one involved only a small number of cases of BOS, and the other involved surveillance biopsies only, which may not be sensitive enough to pick up an association (Norgaard et al. 1998; Swanson et al. 2000). A group from the Papworth Hospital also observed that the number of acute rejection episodes occurring in the first six months post-transplantation had a significant effect on subsequent BOS, with those who experienced three or more episodes being more at risk than those only experiencing one or two (Sharples et al. 1996). These data show that acute rejection is the most common and consistent risk factor for OB/BOS.

Other immunological risk factors include lymphocytic bronchitis/bronchiolitis (i.e. airway inflammation) and human leukocyte antigen (HLA) mismatching. Hussain

et al performed a retrospective study of lung transplant biopsies from patients surviving at least 90 days to investigate whether lymphocytic bronchitis/bronchiolitis (LBB), independent of perivascular rejection, influenced the development of OB/BOS (Husain et al. 1999). They showed that at 180 and 365 days the B scores (defining airway inflammation/LBB) in BOS patients were more than twice that of patients with no BOS, indicating a possible association between LBB and BOS. Lymphocytic bronchiolitis is thought to be a specific and specialised manifestation of acute rejection.

The association between OB/BOS and HLA mismatching is not as clear as the association between acute rejection and OB. A large multi-centre study of the effect of HLA mismatching on the outcome of lung transplantation did not show a significant difference in total HLA mismatches in patients with OB compared to those without (Quantz et al. 2000). However some other studies from single centres have reported a significant association between HLA mismatching and OB (Kroshus et al. 1997; Sundaresan et al. 1998), making it difficult to determine whether or not it is an important risk factor.

Other non-immunological risk factors have been put forward, including cytomegalovirus infections, other respiratory infections (including respiratory syncytial virus (RSV), parainfluenza virus (PIV) and influenza A, donor characteristics (for example age, or whether the donor suffered traumatic brain injury), prolonged graft ischemia time and type of transplantation (i.e. single or bilateral) (Hohfield et al. 1996; Boehler et al. 1998; Husain et al. 1999; Bowdish et al. 2004; Khalifah et al. 2004).

Gastro-oesophageal reflux (GOR) has also been implicated as a non-immunological factor leading to lung allograft dysfunction (Palmer et al. 2000; Davis et al. 2003; Cantu et al. 2004).

1.2 Gastro-oesophageal reflux

Gastro-oesophageal reflux (GOR) is defined as the retrograde movement of gastric contents into the oesophagus and can cause symptoms such as heartburn. Chronic reflux (gastro-oesophageal reflux disease, GORD) can also cause metaplasia of the lower oesophagus, in which the squamous epithelium is replaced with a columnar epithelium (Barrett's oesophagus). Barrett's oesophagus has been associated with an increased risk of developing oesophageal carcinoma (Badreddine and Wang 2008).

In addition, when the reflux of gastric content advances beyond the oesophagus to the larynx and pharynx (laryngopharyngeal reflux, LPR) further symptoms and pathologies can develop, some resulting from the aspiration of gastric contents into the lungs (Farrokhi and Vaezi 2007; Galmiche et al. 2008).

GORD is considered a common problem. Using a questionnaire Locke et al investigated the prevalence of reflux in a population based study in the USA. They found, after adjusting for age and sex that approximately 40% suffered from at least one episode of heartburn, one of the key symptoms of GORD, over the year of the study. Furthermore, approximately 18% suffered from heartburn on a regular basis (at least one episode a week) (Locke et al. 1997). Sandler et al also

report that GORD is the fourth most prevalent gastrointestinal disease in the USA with approximately 19 million cases per year, and it is also the most expensive. The total annual direct cost in the USA was 9.3 billion dollars for 1998 (Sandler et al. 2002).

1.2.1 Diagnosis of gastro-oesophageal reflux

There are several methods for diagnosing GORD, as outlined in the updated guidelines from DeVault et al. Firstly, in patients who present with un-complicated symptoms such as heartburn that is relieved by antacids the guidelines state that it is appropriate to offer empirical therapy and also suggest that it is reasonable to assume a diagnosis of GORD in patients who respond to such therapy (DeVault and Castell 2005).

For patients who do not respond to empirical therapy, or who are suspected of having more complicated disease (for example Barrett's oesophagus) endoscopy can be performed. Endoscopy allows visualisation of the oesophageal mucosa and allows biopsies to be taken. However, while endoscopy may confirm mucosal injury suggestive of GORD it does not provide evidence that the symptoms are actually related to reflux. In addition, many patients may suffer from symptoms of GORD but lack oesophagitis (inflammation of the oesophagus), therefore would be less likely to have endoscopic findings.

In patients who have persistent symptoms without evidence of mucosal damage ambulatory pH testing can be performed. This is the current 'gold standard' for the measurement of gastro-oesophageal reflux. Ambulatory pH monitoring allows the role of acid reflux to be studied in patients without any endoscopic findings over an extended period of time. Catheter based pH monitoring systems require a flexible catheter and a data logger to record the pH measurements. Typically, the catheter is inserted through the nose and the pH sensor is placed in the distal oesophagus, approximately 5cm above the lower oesophageal sphincter (LOS). Additionally, a second sensor can be included between 15 and 20cm above the LOS (figure 1.6,) (Tutuian and Castell 2006). Normally ambulatory pH testing is performed over 24h, however there is data to suggest that 16h studies can provide accurate information and also improve patient tolerance (Dobhan and Castell 1992).

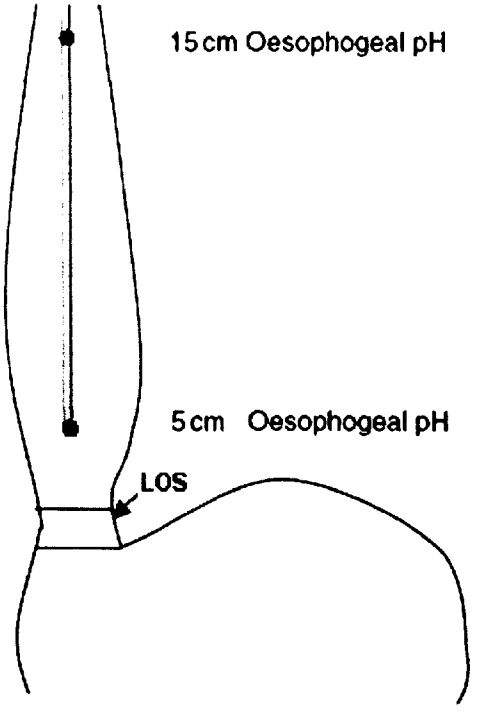


Figure 1.6 Ambulatory pH catheter placement, showing duel channel proximal and distal pH monitoring. LOS-lower oesophageal sphincter. This figure was modified from Tutuian and Castell 2006.

A positive result is one in which there is a sudden drop in intra-oesophageal pH to below pH 4.0. Once reflux has been established the number of and duration of episodes can help to quantify GOR.

Although ambulatory pH monitoring is considered the gold standard and is used by many clinicians there are certain limitations to this method. pH monitoring only identifies reflux events with a pH of 4.0 or below, therefore providing limited information on non-acidic reflux. As some patients are on acid suppression therapy but still have symptoms of GOR non-acid reflux may be important in some cases and should therefore be monitored.

This problem can be overcome through the use of combined multichannel intraluminal impedance and pH (MII-pH) monitoring. Impedance is a measure of the total opposition to current flow between adjacent electrodes. Pairs of electrodes are placed on a catheter inside the oesophagus and when liquid is present in-between two electrodes there is a rapid drop in impedance, as the ionic content of the liquid improves the electrical conductivity between the electrodes. Once the liquid has passed away from the electrodes the impedance will return to baseline. The presence of gas in the oesophagus is recognised by an increase in impedance as there are no electrical charges to close the circuit when the two electrodes are suspended in a gas.

With multichannel impedance (a series of electrodes placed along the catheter, (figure 1.7), the direction of movement can be determined; therefore swallowing (movement from proximal to distal) can be distinguished from reflux events (distal

to proximal movement). Combining multichannel impedance with conventional pH monitoring can provide a more comprehensive picture of what is happening in terms of reflux, for example the physical properties (i.e. whether the refluxate is a liquid or a gas), the chemical properties (i.e. acid or non-acid) and the direction of movement can be determined.

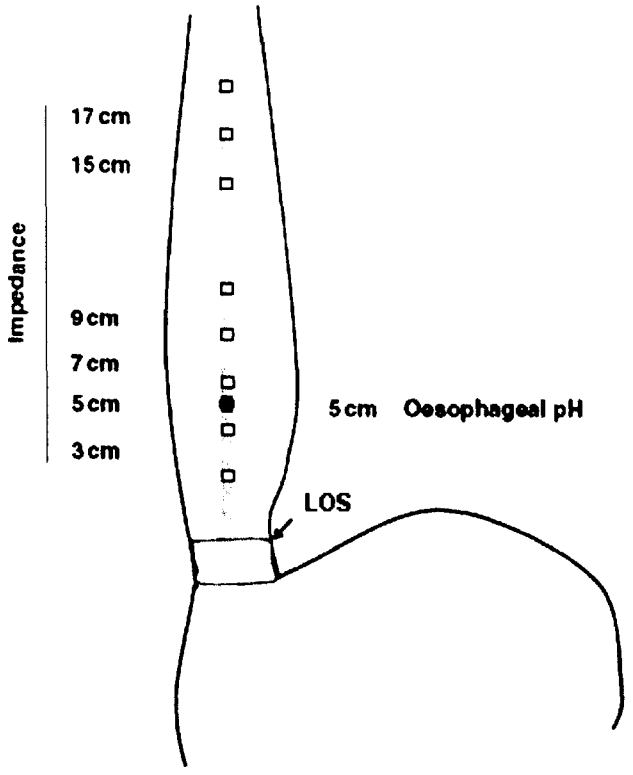


Figure 1.7 Combined multichannel intraluminal impedance and pH catheter. LOS-lower oesophageal sphincter. This figure was modified from Tutuian and Castell 2006.

1.2.2 Composition of gastric juice

Gastric juice is a fluid secreted by the gastric mucosa and the cells responsible for the secretions are arranged in structures known as gastric pits (figure 1.8). Pits are made up of several gastric glands and are distributed throughout the mucosa.

Gastric juice contains two major aggressors; hydrochloric acid and a group of proteolytic enzymes, pepsins. Hydrochloric acid is secreted by parietal cells located in the gastric pits and the pH of gastric juice is normally between 1 and 3. Gastric acid secretion can be stimulated by a variety of stimuli during both the cephalic and gastric phases of digestion. During the cephalic (head) phase the thought, taste, smell or sight of food, or swallowing can stimulate the release of acid through neural control, in which the vagus nerve stimulates acid secretion via muscarinic receptors and can also stimulate the release of gastrin (a hormone that increases gastric acid release) from G cells located in the antrum of the stomach.

During the gastric phase, like the cephalic phase, acid secretion can be stimulated by a wide range of stimuli, including distension of the stomach and also the presence of chemical constituents of food. This again causes an increase in gastrin levels which in turn causes histamine release from endocrine cells in the fundus. Histamine has a powerful effect on acid secretion as it binds to histamine-2 (H_2) receptors on parietal cells and stimulates them to produce acid. Acid production requires hydrogen ions that are secreted through a proton

pump (H^+K^+-ATPase) that is present on the membrane of an active parietal cell (also called the secretory canaliculus).

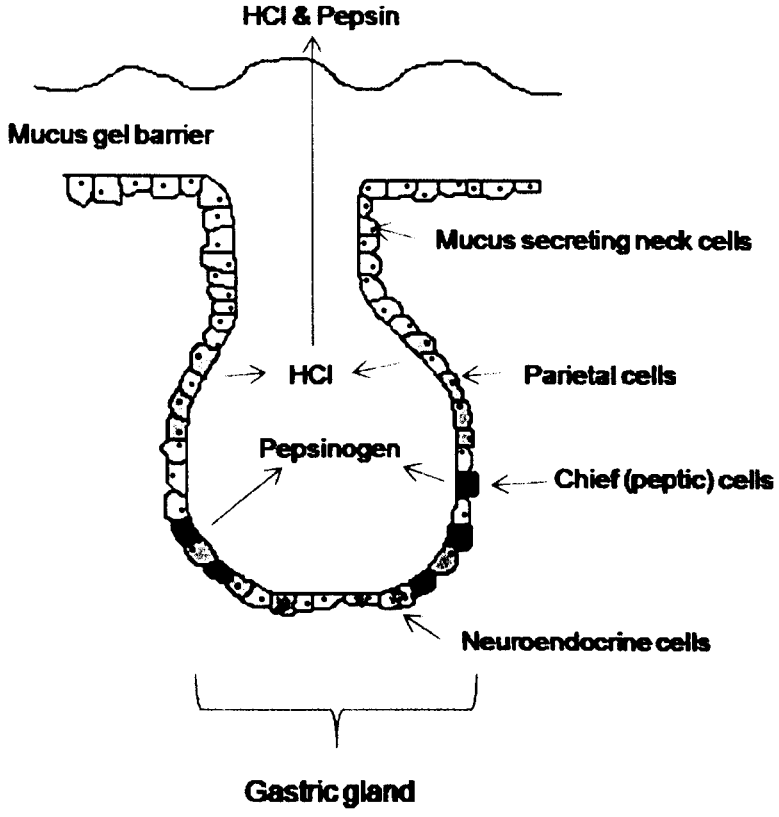


Figure 1.8 Structure of the gastric pits located in the mucosa of the corpus and fundus of the stomach.

In addition to hydrochloric acid gastric juice also contains proteolytic enzymes, or pepsins. The name pepsin was first described by Schwann in 1836 (Schwann 1836) and since then pepsin, or pepsin-like enzymes have been isolated from many species. Pepsins are endopeptidases that belong to the aspartate proteinase family and they have an active site consisting of a deep cleft containing two aspartic acid residues within hydrogen bonding distance of each other (Andreeva et al. 1977). They have a broad specificity; however they prefer peptide bonds between aromatic amino acids, particularly phenylalanine and tyrosine (Powers et al. 1977).

Pepsins are secreted in a precursor form, pepsinogen, by chief cells. Under acidic conditions pepsinogen is rapidly converted to pepsin and the reaction can be autocatalytic. Pepsins are irreversibly denatured under alkali conditions, whilst pepsinogens can be resistant to denaturation up to pH 10 (Samloff 1971).

Eight zones of proteolytic activity were identified by Etherington and Taylor in human gastric juice by agar gel electrophoresis (pepsins 1, 2, 3, 3a, 4, 5, 6 and 7) (Etherington and Taylor 1969). Pepsin 3 is the most abundant of the pepsins found in gastric juice followed by pepsin 5. Pepsins 1-4 correspond to pepsin A and pepsins 5 and 6 to pepsin C (also known as gastricsin). Pepsin 7 corresponds to a slow moving protease (SMP) described by Samloff and relates to pepsin B (Samloff 1971).

The different pepsins vary in their pH optima and relative proteolytic activity depending on substrate type, pH, temperature and solute and substrate

concentration. They are active at acidic pH with optimal activity at approximately pH 2.0 (Foltman 1981).

Two distinct groups of pepsinogens have been isolated, groups I and II and according to Samloff et al they give rise to seven pepsinogens (PG1-7) (Samloff 1971). Group I pepsinogens (PG1-5) produce pepsin A on activation and group II (PG6 & 7) produce pepsin C.

The conversion of pepsinogen to pepsin has been described in detail for porcine pepsinogen (James and Sielecki 1986). When activated by a decrease in pH to 5 or below pepsinogen loses two peptides which make up the pro-enzyme segment of 44 amino acids found at the N-terminal of the protein. At a pH above 5 electrostatic interactions between amino acids on the pro-enzyme segment and carboxyl groups on the enzyme stabilise the conformational shape of the protein. When the pH drops below 5 the carboxyl groups become protonated and the electrostatic interactions are broken, causing a conformational change which results in the hydrolysis of the peptide bond between leucine and isoleucine at positions 16 and 17, which takes place at the active site of the enzyme. This results in a pro-pepsin form and is further converted into active pepsin by other pro-pepsin or active pepsin molecules cleaving the peptide bond between leucine and isoleucine at positions 44 and 45 (figure 1.9) (Perlmann 1963; Dykes and Kay 1976).

It is also possible for the gastric juice to contain bile acids that have been refluxed back into the stomach from the duodenum. Kauer et al performed a

study in which they assessed the concentration of bile acids in the gastric refluxate of GORD patients and found that 86% showed at least trace amounts of bile during one or more collection of the gastric refluxate (Kauer et al. 1997).

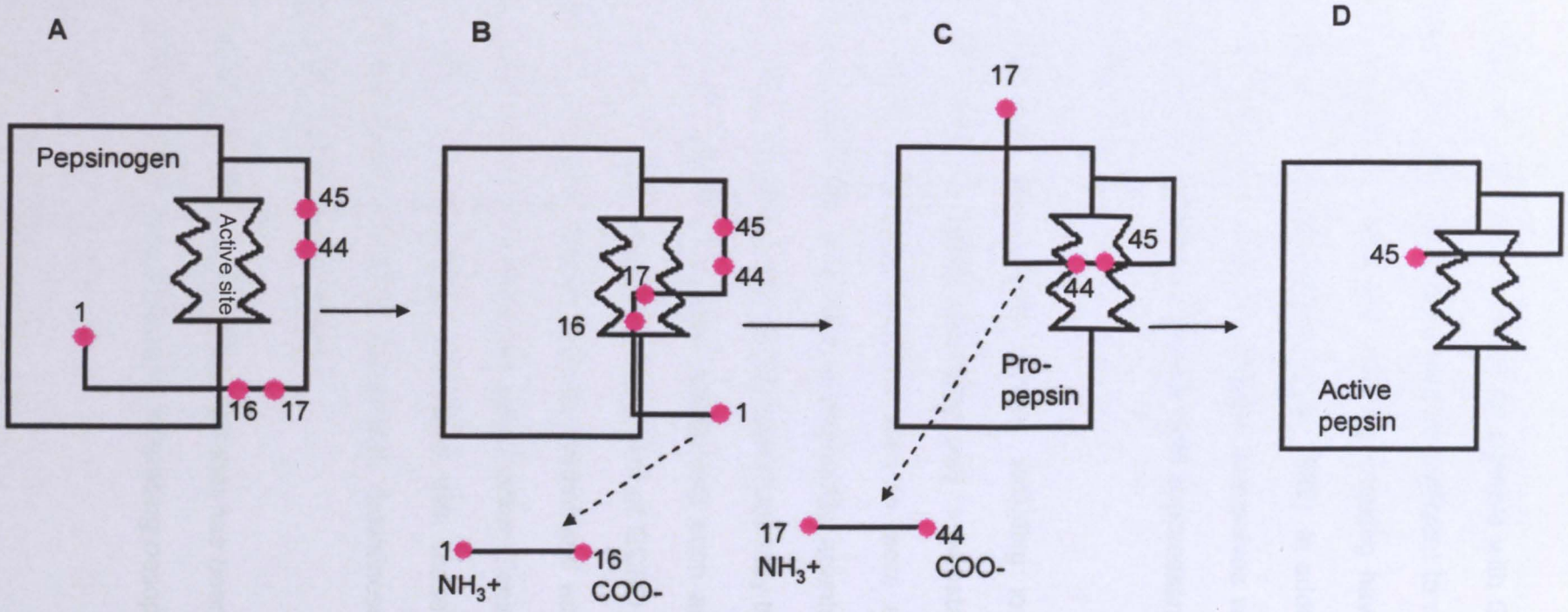


Figure 1.9. Pepsinogen activation. **A-**At pH levels above 5 electrostatic interactions between positively and negatively charged amino acids hold a cleft over the active site. **B-**At pH levels below 5 the negatively charged amino acids become protonated and lose their charge, breaking the interactions. This causes the cleft to fall into the active site of the enzyme, allowing part of the cleft to be cleaved (between amino acids 16 and 17). **C-** The active site is then partially freed, allowing more of the cleft (amino acids 17-44) to be cleaved off by either the enzyme itself or by other active pepsin molecules. **D-** This results in the active form of the enzyme. Model based on porcine pepsin (Perlmann 1963; Dykes and Kay 1976; James and Sielecki 1986).

1.2.3 Current treatments for gastro-oesophageal reflux disease

There are a variety of treatments available for patients with GORD. In patients with milder forms a lifestyle modification may be sufficient to reduce symptoms, for example, decreased fat intake and stopping smoking have been shown to reduce reflux (Becker et al. 1989; Waring et al. 1989). In addition, patients with milder GORD may choose to treat the problem themselves with antacids, anti-refluxants (e.g. alginates) or over-the-counter acid suppressants (e.g. histamine 2 receptor antagonists).

Defects in oesophageal and gastric motility, including lower oesophageal incompetence and delayed gastric emptying play a substantial role in the pathogenesis of GORD and may therefore lead to more severe symptoms requiring additional treatment. The use of promotility agents or those which decrease lower oesophageal sphincter (LOS) relaxations may therefore be useful in selective patients. Dopamine receptor antagonists such as metoclopramide and domperidone have been used in the treatment of GORD on the basis that dopamine stimulates the sympathetic nervous system and acts to reduce LOS and gastric tone and inhibit antro-duodenal coordination (Tonini 1996), however there are a number of side effects associated with these types of drugs, particularly metoclopramide, including depression, drowsiness and involuntary diskinetic movements (Ganzini et al. 1993).

Cisapride is a serotonin (5-HT_4) receptor agonist that has been shown to relieve symptoms of GORD by enhancing LOS tone, increasing oesophageal peristalsis

and also promoting gastric emptying (Tonini 1996). However as with the dopamine receptor antagonists there are side effects. In the case of cisapride the side effects are severe and can include fatal cardiac dysrhythmias which have therefore resulted in the product being withdrawn from the market (Chan-Tompkins and Babinchak 1996). The unwanted effects produced by these products demonstrate that more research is required and as a result of this promotility agents are not ideal monotherapy for patients with GORD, however they may be useful when used together with acid suppression therapy.

Acid suppression therapy can consist of histamine-2 receptor antagonists (H_2RAs) and/or proton pump inhibitors (PPIs). These therapeutic options have been developed over the last three decades and have led to a reduction of symptoms in the majority of patients.

The introduction of H_2RAs (including cimetidine, ranitidine, famotidine and nizatidine) made great advances in the ability of patients to control their symptoms of GORD, much more so than previously available options, such as antacids.

H_2RAs can relieve symptoms in approximately 60% of those treated, compared to 20% in placebo controls (DeVault and Castell 1995). All four of the H_2RAs are equivalent in efficacy at comparable doses and the best results are seen when they are taken twice daily. In addition, H_2RAs are more effective when given to a fasting patient, therefore are most effective when taken before bed on an empty stomach. Despite their effectiveness H_2RAs are rarely used as maintenance

therapy as patients can become tolerant (Hatlebakk and Berstad 1996) and they are actually much less effective than PPIs in controlling acid secretion (Katz and Tutuian 2001).

There are five available proton pump inhibitors; omeprazole, lansoprazole, rebeprazole, pantoprazole and more recently, esomeprazole. Esomeprazole is an optical isomer of omeprazole that has a slower metabolic clearance and therefore may provide more effective control over acid secretion (Lind et al. 2000).

PPIs are protonatable weak bases. The un-protonated form accumulates in the acidic space of the secretory canaliculus of the active parietal cell. Following accumulation these compounds undergo an acid-catalysed rearrangement which allows them to react with thiol groups on cysteine residues present on the alpha subunit of the proton pump (H^+K^+ -ATPase, figure 1.10) located on the apical membrane of the parietal cell, causing inhibition of acid production (Besancon et al. 1997). Acid production is restored when new H^+K^+-ATPase molecules are converted to their active form at the canalicular membrane. As mentioned previously, parietal cells are stimulated to produce acid by the sight, smell or taste of food and as PPIs can only inhibit active pumps on the surface of the parietal cell they are most effective when taken just before a meal is consumed.

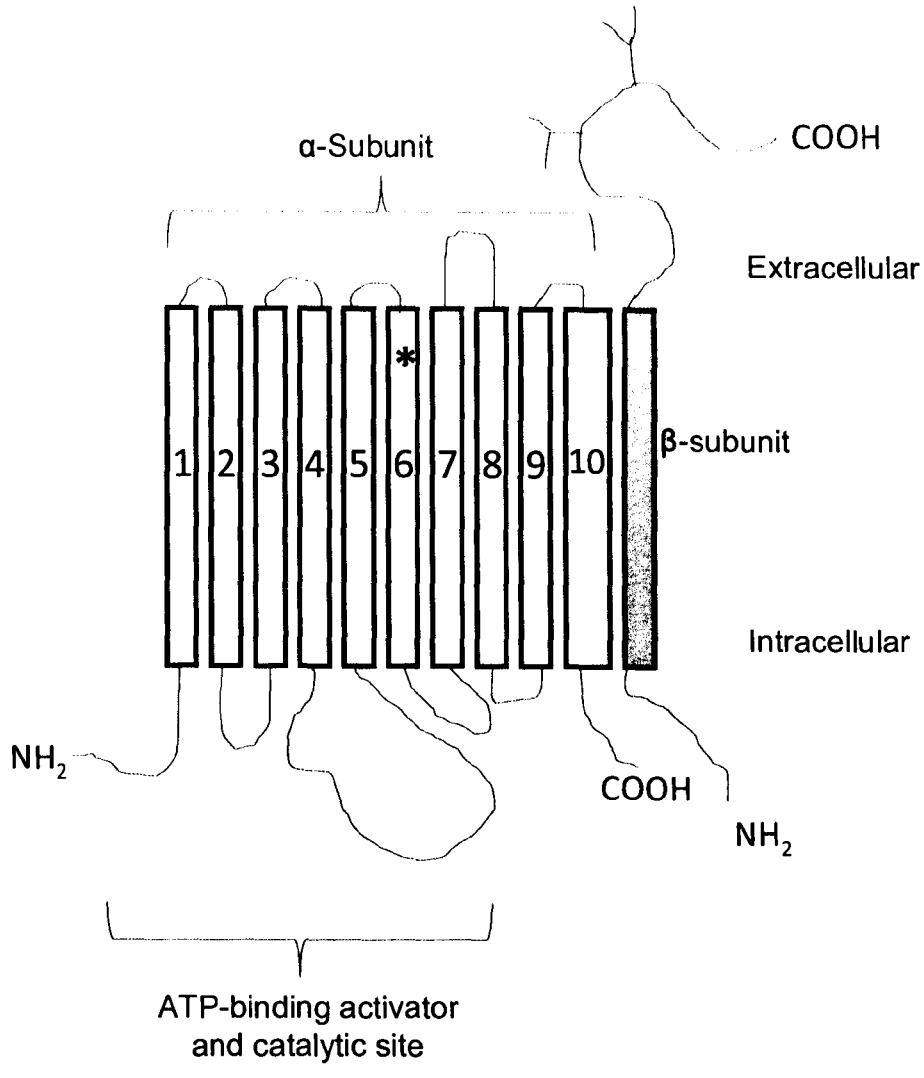


Figure 1.10 The proton pump (H^+K^+ -ATPase) of the parietal cell. The large mass of protein between transmembrane domains 4 and 5 contains ATP binding and phosphorylation sites involved in conformational transitions. * represents the site of a cysteine residue that is bound by proton pump inhibitors (e.g. omeprazole) (Pearson and Brownlee 2005).

Although PPIs are the most effective medical therapy for controlling the symptoms of GORD there are a number of patients who require higher doses and some in which symptoms are not resolved despite these elevated levels (DeVault and Castell 2005). In addition, PPIs can be expensive, especially for those that require maintenance/continuous therapy, therefore alternatives including surgery or endoscopic techniques may be required.

Such patients may choose to undergo endoscopic techniques, as these are less invasive than open surgery. There are three main types of endoscopic treatment; radiofrequency application to the LOS, techniques designed to reduce reflux using endoscopic suturing devices and also techniques that require injecting the LOS.

Radiofrequency application using the Stretta device (Curon Medical, USA) is designed to increase the reflux barrier of the LOS. It consists of a wire guided balloon tipped with a four needle catheter that can deliver radiofrequency energy to the gastro-oesophageal junction. When the balloon is inflated the four electrodes are placed into the oesophageal wall to make deep thermal lesions that result in fibrosis of the muscular sphincter (Schwartz and Smout 2007). One study has shown that radiofrequency application can reduce symptoms in 61% of patients compared to 30% in a sham procedure, however PPI use was not reduced compared to sham and no reduction was seen in oesophageal acid exposure time. This suggests that a reduction in oesophageal sensitivity may be responsible for the reduction in symptoms (Corley et al. 2003).

Better results have been seen with endoscopic suturing, where two or three plications (folds) are made at or below the gastro-oesophageal junction. One study has shown a significant improvement in the number of patients off PPIs after 3 months compared to sham and also in GORD symptoms. After 1 year 41% of patients experienced no or few symptoms, however 29% of patients were retreated within the year (Arts et al. 2005; Schwartz et al. 2007).

The last main endoscopic technique involves the injection of a biopolymer into the LOS, on the basis that it will be incorporated into the muscle to augment the sphincter, creating a barrier against reflux. Early results showed that symptoms were decreased compared to sham procedures, but oesophageal acid exposure was not reduced (Devière et al. 2005). In addition, one of the main biopolymers, Enteryx (Boston Scientific, USA), was recalled in 2005 after some serious adverse effects were reported, including renal failure, severe oesophageal stenosis and death.

Despite the effectiveness of other treatments for GORD patients may still choose to undergo anti-reflux surgery. The Society of American Gastro-Enterological Surgeons (SAGES) recommend that surgical therapy should be considered in those who have failed medical management, have complicated GORD (e.g. Barrett's), atypical symptoms (e.g. asthma) or choose to have surgery for lifestyle considerations (e.g. age, expense of medications etc.) (Society of American Gastrointestinal Endoscopic 1998).

The most common form of anti-reflux surgery is Nissen fundoplication, in which the fundus of the stomach is wrapped all the way around the distal oesophagus (figure 1.11). This increases the pressure in the lower oesophagus and consequently reduces the occurrence of reflux. Since Dellemagne first described the laparoscopic fundoplication in 1991 patient interest in anti-reflux surgery has increased (Dallemagne et al. 1991). In 1990 4.4 anti-reflux procedures were performed per 100 000 adults, however in 1997 this had more than doubled to 12.0 per 100 000 adults (Finlayson et al. 2003).

Laparoscopic Nissen fundoplication (LNF) has showed some promising results in the treatment of GORD. It can provide symptom relief in up to 83-93% of patients (Hunter et al. 1996; Lafullarde et al. 2001; Khajanchee et al. 2002) and normalisation of oesophageal acid exposure time in 80-91% (Hunter et al. 1996; Eubanks et al. 2000). In addition, LNF procedures have high patient satisfaction (96% satisfied (Bammer et al. 2001)) and show superior quality of life scores compared to those of patients treated with medical therapy (Fernando et al. 2002).

However, as with all surgeries there is a risk of intra-operative complications and also of developing post-operative symptoms. Pessaux et al investigated the morbidity of anti-reflux surgery in a review of 1470 patients operated on between 1992 and 1996 from 20 different hospital centres (Pessaux et al. 2002). 2.1% of patients experienced intra-operative complications, including bleeding from short gastric vessels, gastric and oesophageal perforation, splenic lesion and pneumothorax. Early post-operative complications were seen in 2.9%, with

asymptomatic pneumothorax and pulmonary embolism being the most common. 4.1% of patients experienced other post-operative side effects, the most common including gas bloat (36 patients), diarrhoea (10 patients) and epigastric pain (6 patients). In addition, 51.7% of patients experienced dysphagia (difficultly in swallowing) in the first 6 weeks; however this was reduced to 5.9% at 3 months.

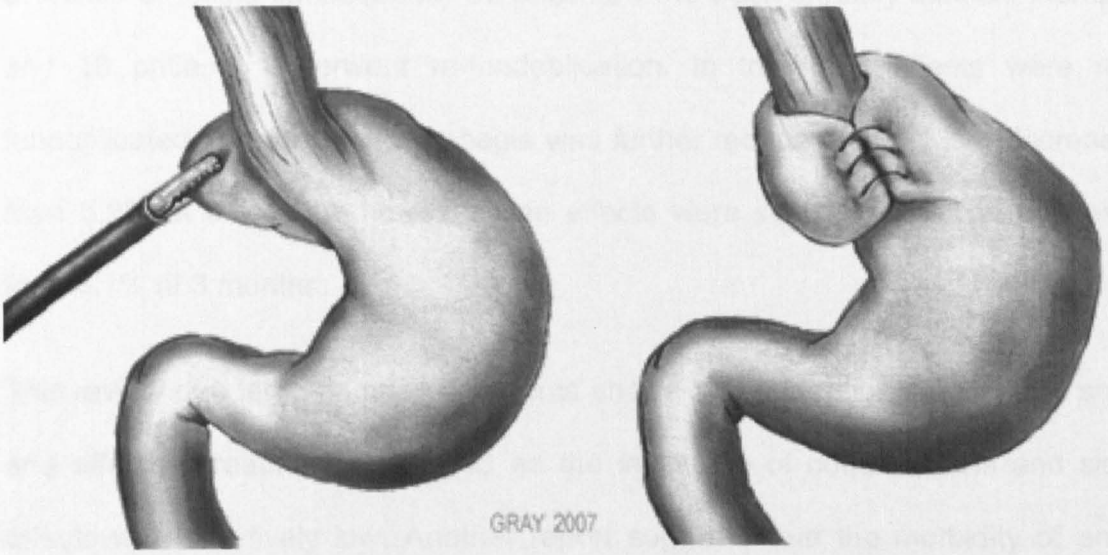


Figure 1.11 Nissen fundoplication, in which the fundus is wrapped around the distal oesophagus (Grey 2007).

At 3 years 78 patients (5.3%) had recurrence of symptoms and objective evidence of reflux, consequently 60 patients were back on daily medical therapy and 18 patients underwent re-fundoplication. In total 35 patients were re-fundoplicated. Incidence of dysphagia was further reduced to 0.35% a decrease from 5.9% at 3 months; however side effects were seen in 6.5%, an increase from 4.1% at 3 months.

This review of a large number of patients shows that anti-reflux surgery is a safe and effective treatment for GORD as the incidence of complications and side effects were relatively low. Another report suggests that the morbidity of anti-reflux surgery can be reduced by adequate patient selection and attention to technical detail during the operation, therefore high-volume, specialist centres are linked with better surgical outcomes (Stein et al. 1996).

1.2.4 Extra-oesophageal manifestations of gastro-oesophageal reflux

In addition to gastro-oesophageal reflux and its associated symptoms (for example heartburn, 'typical GORD') some patients may suffer from extra-oesophageal reflux, i.e. when the refluxate reaches the larynx/pharynx (laryngopharyngeal reflux, LPR). Symptoms of LPR can include laryngitis and chronic cough amongst others ('atypical reflux').

Symptoms of laryngitis can include hoarseness, sore throat, dysphagia and globus (the feeling of a lump in the throat). As many as 60% of chronic laryngitis

and difficult to treat sore throat cases are thought to be associated with acid reflux (Vaezi 2003). The most common mechanism for laryngeal damage caused by reflux is the direct contact of the refluxate with the laryngeal mucosa. Animal studies have shown that exposure of the laryngeal tissue to gastric contents results in inflammation, oedema, granularity and ulceration (Adhami et al. 2004). In addition, pepsin has been demonstrated in laryngeal biopsies (located in the interstitium and cytoplasm of laryngeal epithelial cells) in patients with ear, nose and throat (ENT) symptoms (Johnston et al. 2004).

Extra-oesophageal reflux is also the most common cause of non-cardiac chest pain (NCCP). NCCP is defined as recurrent episodes of angina-like chest pain in patients who have a normal cardiac work up (Wong and Fass 2004). Locke et al have shown that NCCP is more commonly reported in patients who experience regular heartburn (at least once a week) compared to those who suffer from irregular heartburn (less than once a week) and those who have no symptoms of GORD (37%, 30% and 7.9% respectively) (Locke et al. 1997). In addition, using 24h pH monitoring, Fass et al demonstrated that up to 60% of patients with NCCP had abnormal oesophageal acid exposure, suggesting a link between NCCP and GORD (Fass et al. 1998).

As well as extra-oesophageal symptoms GORD has been associated with respiratory complications such as chronic cough. In a review by Morice and Kastelik GORD was found to be responsible for up to 41% of chronic cough cases (Morice and Kastelik 2003). Reflux of gastric contents is thought to stimulate cough in two main ways; either indirectly, by stimulating an

oesophageal-bronchial cough reflex or, alternatively, reflux of gastric contents can directly irritate the upper respiratory tract, mainly the larynx and the lower respiratory tract by micro or macro-aspiration. Reflux of gastric contents into the distal oesophagus alone is enough to stimulate cough via the indirect oesophageal-bronchial mechanism (Ing et al. 1994; Irwin et al. 2006).

In the case of direct stimulation of the larynx/airways repeated macro-aspiration seems unlikely, as it can result in abscess formation and pneumonia, conditions that are not often associated with chronic cough. Micro-aspiration of refluxate may however play a role, and our group have previously measured pepsin levels in the BAL of a small number of chronic cough patients as a marker of gastric aspiration (Stovold et al. 2007). Pepsin levels were comparable to those of healthy volunteers, however this could be due to an over-sensitive cough reflex preventing the gastric contents reaching the lungs, therefore a logical next step would be to investigate pepsin in sputum samples from cough patients.

1.2.5 Lung transplantation and GOR

There is evidence to suggest that lung transplant recipients have a number of risk factors for GOR. Lung transplant surgery causes significant damage to the vagal innervation of the gastro-intestinal (GI) tract, which controls gastric motility. Involuntary gastric motility responses are directed to the stomach mainly via efferent vagal fibres. There are two types of efferent fibres that control the excitation or relaxation of gastric smooth muscle; low threshold cholinergic fibres that control contraction of gastric smooth muscle and high threshold non-

cholinergic, non-adrenergic nerve fibres, responsible for relaxing the body and fundus and mediate the efferent link of receptive relaxation and gastric accommodation reflexes. These active reflexes are necessary for the prevention of excessively high intragastric pressures, and are damaged by vagotomy (Stadaas 1975). Truncal vagotomy also results in an initial dis-coordination of antral phasic contractions, which results in delayed gastric emptying due to a decrease in force of contraction (Victor and Miller 1989).

Lung allograft patients are also on immunosuppressive therapy, a regimen that can include cyclosporine, which has been known to reduce gastric motility. In addition to these factors, cough reflexes and mucociliary clearance reflexes are attenuated and these would normally play a role in defence against aspiration (Veale et al. 1993).

Even though a high prevalence of GOR has been reported in lung transplant recipients it is not entirely clear as to whether it is due to vagotomy resulting from surgery, immunosuppressive therapy or if it is a pre-existing condition, as many lung diseases, especially idiopathic pulmonary fibrosis (IPF) and cystic fibrosis (CF) have already been associated with GOR (Feigelson et al. 1987; Tobin et al. 1998; Brodzicki et al. 2002; Raghu 2003).

A paper from Young et al describes a study they performed investigating the occurrence of GOR both pre and post lung transplantation (Young et al. 2003). They performed 24h pH testing, oesophageal manometry and gastric emptying studies on 23 adult patients both before and after transplantation. Pre-transplant

studies were performed at a median of 66 days prior to the transplant (range 1 to 443 days) and at a median of 100 days post-transplant (range 47 to 248 days).

Their investigation showed that 35% (8 of 23) of patients had an abnormal acid contact time before the transplant. This increased to 65% (15 of 23) after transplantation. All of the patients who experienced GOR before transplantation still had abnormal acid contact times after transplantation, indicating that pre-transplant GOR is predictive of post-transplant GOR. An additional 7 patients had completely normal pH studies prior to transplantation but acquired GOR after transplantation.

There are other references to GOR causing lung allograft dysfunction in the literature (Palmer et al. 2000; Benden et al. 2005). One to note is from the Duke University group. Their lung transplant program has performed over 450 lung transplantations between April 1992 and July 2003, and over that time period 202 patients have been evaluated for reflux postoperatively by pH probe. Originally, pH studies were not performed on patients at all, and then they were performed on symptomatic patients, and more recently pH studies are performed on all patients as part of their pre and postoperative evaluation. In their studies 63% (23 of 36) had abnormal pre-operative pH studies and 76% (127 of 167) had abnormal post-operative pH studies. The patients were split into 4 groups; no history of reflux, reflux with no fundoplicative surgery, reflux with early fundoplicative surgery (median 36 days, with a range of 0 to 87 days) and reflux with late fundoplicative surgery (median 447 days, with a range of 106 to 2999 days). A total of seventy six patients underwent post-transplant fundoplication.

At 1 year post-transplantation the patients who had reflux and early surgery showed the greatest freedom from BOS (100% free), followed by those with reflux and no surgery (92%), then those with no history of reflux (91%) and finally those with reflux and late surgery (90%, figure 1.12).

After 3 years, freedom from BOS in those with reflux and early surgery was significantly higher than all of the other groups (reflux with early surgery, 100%; no history of reflux, 62%; reflux and no surgery 60% and reflux with late surgery 47%). These results show that an improvement in the freedom from BOS can be seen with fundoplication, however, the surgery has to be performed early in the transplant experience, as this is not the case in patients with more advanced stages of BOS, shown by figure 1.12, at 3 years more than 50% of patients with reflux and late surgery had developed BOS.

The paper also makes a point of mentioning that for patients undergoing fundoplication, either early or late, there is no in-house or 30 day mortality, indicating that the surgery is safe and could delay the onset of BOS, and therefore could possibly increase long term survival rates (Cantu et al. 2004).

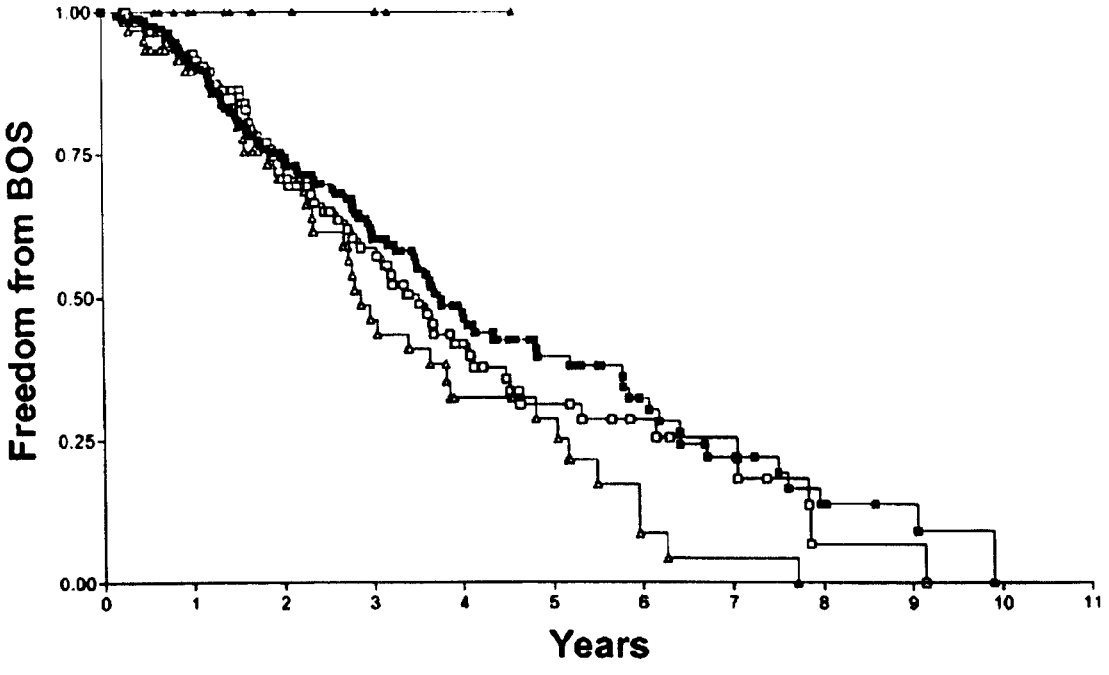


Figure 1.12 Freedom from BOS in the different groups. ▲-reflux early surgery; ■-no history of reflux; □- reflux no surgery and Δ- reflux late surgery. This graph was taken from the Chamberlain memorial paper, Cantu et al 2004.

This data is however, controversial. Cantu et al have identified certain limitations in their study, for example, they mention that the retrospective nature and non-random selection of patients introduces a bias. This becomes most relevant when comparing differences in group demographics, mainly age and diagnosis. They also point out that the patients in the early fundoplication group are the most recent, and therefore benefit from the experience gained throughout the program.

Although these studies give evidence of reflux in lung transplantation, and that fundoplicative surgery can improve the outcome of bronchiolitis obliterans syndrome, they do not give any evidence of aspiration of gastric content into the lung. More recent studies have focussed on measuring biomarkers of gastric aspiration in the bronchoalveolar lavage fluid (BAL) of transplant patients.

D'Ovidio et al have investigated the presence of bile salts in the BAL of their patients as a marker of duodenal-gastro-oesophageal reflux (D'Ovidio et al. 2005). BAL was analysed using a commercially available kit based on a spectrophotometric enzymatic assay and bile salts were detected in 66% of patients BAL (71 out of 107) and were significantly higher in patients with BOS (0p-3) compared to patients without BOS (1.6 and 0.3μmol/l respectively, P=0.002). In addition, patients with early onset BOS (within 12 months) had significantly higher BAL bile salts compared to patients with late onset (2.6 and 0.8μmol/l respectively, P=0.02). Patients with high levels of bile in their BAL (≥8μmol/ml) also had higher levels of interleukin-8 (IL-8) and higher neutrophil

counts than those with low levels (between 0 and 8μmol/l) or if no bile was detected. Interestingly, IL-8 and alveolar neutrophils have both previously been described as possible clinical markers of BOS development (DiGiovine et al. 1996; Riise et al. 1999; Zheng et al. 2006).

In addition Blondeau et al have investigated the presence of bile salts and pepsin in the BAL of transplant patients, along with performing pH-impedance measurements. They found that 46% of lung transplant patients had increased gastro-oesophageal reflux, defined as having one or more abnormal reflux indices (increased acid exposure, volume exposure or number of reflux events). All transplant patients had detectable levels of pepsin in their lavage, measured by ELISA, compared to 49% with detectable bile salts, measured using a kit based on an enzyme converting bile salts and thio-NAD to 3-keto steroids and thio-NADH. The rate of formation of thio-NADH is determined by measuring the change in absorbance and therefore allowing quantification (Bioquant, USA). Significantly more patients with BOS had detectable bile in their lavage compared to patients without BOS. This suggests that while pepsin may be a more sensitive marker of aspiration, bile acids may be a more specific marker for the development of BOS.

In their study a small number of patients had increased non-acidic reflux that would not have been diagnosed with pH monitoring alone. In addition, patients who were on proton pump inhibitor (PPI) therapy had significantly reduced oesophageal acid exposure and a reduced number of reflux events; however levels of pepsin and bile were comparable in both patients who were on PPIs and

those that were not. This suggests that while PPI treatment can reduce gastric acid secretion, it can not prevent gastric aspiration and therefore is unlikely to prove useful in the treatment of allograft damage resulting from such injury (Blondeau et al. 2008).

1.3 Current treatments for lung transplant rejection

1.3.1 Immunosupression

The modern era of transplantation arose from the discovery of the calcineurin inhibitor cyclosporine (Borel et al. 1976). Since then most lung transplant recipients have received a triple-immunosupression maintenance regimen consisting of a calcineurin inhibitor (most commonly cyclosporine or tacrolimus (Trulock et al. 2007), an antimetabolite (azathioprine or mycophenolate mofetil (MMF)) and corticosteroids.

The action of activated cytotoxic T cells via an indirect allo-recognition pathway is thought to play a major role in the injury to the airway epithelium in lung transplant rejection. Cyclosporine is a fungal polypeptide that forms complexes with intra-cytoplasmic proteins to inhibit calcineurin, a protein phosphatase that stimulates interleukin 2 (IL-2) production and is involved in T cell activation. Tacrolimus, also known as FK506, is a macrolide antibiotic and also inhibits the production of IL-2 via binding of FK506 binding protein-12 (Schreiber et al. 1991). Azathioprine, an antimetabolite, limits T and B cell proliferation through inhibiting

DNA, RNA and *de novo* purine synthesis. MMF also inhibits lymphocyte proliferation, but exhibits increased selectivity and decreased toxicity compared to azathioprine. MMF is a pro-drug of the active compound mycophenolic acid, an inhibitor of inosine monophosphate dehydrogenase, an enzyme involved in the *de novo* synthesis of guanosine monophosphate (Snell and Westall 2007). Most lung transplant programmes include a moderate dose of steroids in the immunosuppressive regimen comprising of intravenous methylprednisolone (0.5-1mg/kg/day) for several days followed by oral prednisolone (0.5mg/kg/day) (Knoop et al. 2004). Small studies have shown improved lung function with the use of steroids (Takao et al. 1995; Speich et al. 1997), however a larger study showed no advantage of inhaled corticosteroids in the treatment of acute or chronic rejection (Whitford et al. 2002). In addition there have been some reports of successful corticosteroid withdrawal years after transplantation (Knoop et al. 2004; Borro et al. 2005).

1.3.2 Total lymphoid irradiation

One of the major treatment strategies for managing chronic rejection (OB/BOS) is to intensify the patient's immunosuppressive therapy, with the intention of removing the alloimmune response to the graft. However in practice, a patient's lung function can continue to decline. Irradiation of the lymphoid tissue will act to interfere with the alloimmune response to the graft and should provide additional

immunosupression to that achieved through conventional drug based therapies (Tochner and Slavina 1988).

Fisher et al have investigated the safety and efficacy of total lymphoid irradiation (TLI) in lung transplant patients. 37 lung recipients were treated with TLI for progressive BOS, of which 27 completed at least 80% of the full course. Of the 10 who did not complete, 2 died early as a result of advanced BOS, 2 developed severe infections that lead to pneumonia requiring hospitalisation and the remaining 6 developed marrow suppression.

The mean (±SD) pre-TLI FEV_1 in the 27 patients that completed at least 80% of the treatment was 1.35L (±0.53) and the mean (±SD) post-TLI FEV_1 was 1.60L (±0.71), P=0.006. In addition, the effect of TLI on the progression of BOS was assessed and the rate of decline in FEV1 was 122.7ml/month pre-TLI vs. 25.1ml/month post TLI, P=0.0004 (Fisher et al. 2005). This study provides evidence that TLI is well tolerated and could provide additional immunosupression in an attempt to slow the rate of progression of BOS.

1.3.3 Azithromycin

Macrolide antibiotics have shown anti-inflammatory properties in some respiratory conditions, including asthma, cystic fibrosis and diffuse panbronchiolitis (Kudoh et al. 1998; Equi et al. 2002; Gotfried 2004). In addition to these lung disorders the macrolide azithromycin has been shown, in a number

of small studies, to have a positive effect on the management of BOS in lung transplant recipients.

The first study involved six lung transplant recipients with BOS grade 1 or greater that did not respond to augmentation of immunosupression. At the time of the last follow-up (up to 6 months after starting azithromycin therapy) five out of the six patients showed significant improvement in lung function (FEV_1) compared to baseline values at the start of the azithromycin therapy. The mean increase in absolute FEV_1 was 0.5L (range -0.18 to 1.36L), representing an average improvement in absolute FEV_1 of 46% (Gerhardt et al. 2003). Another study from Yates et al including data on 20 patients also shows a significant increase in FEV_1 with azithromycin therapy that is sustained beyond 3 months of follow up. Median improvement was 110ml (range -70 to 730ml) (Yates et al. 2005).

A recent study from Gottleib et al includes a larger number of patients (n=81) and a longer follow-up period (mean 1.3 ± 0.5 years). The mean pre-treatment FEV_1 in all patients was 1.46L (range 0.46 to 3.04L). A response to azithromycin was seen in 30% of patients at 6 months. The average increase in FEV_1 was 17% (range -4 to 43%) at 3 months and 16% (range 11 to 32%) at 6 months. Interestingly, analysis of BAL (available in 62 out of 81 patients) revealed a significantly higher proportion of neutrophils in responders compared to non-responders, showing a potential predictive value of BAL neutrophilia for treatment response in these patients (Gottlieb et al. 2008).

The mechanism of action of azithromycin is unknown, however it is of interest that macrolides are pro-motility agents and have been shown to decrease gastric emptying time (Sifrim et al. 1994; Arts et al. 2005).

1.3.4 Miscellaneous treatments

Murphy et al have investigated the effect of the phosphodiesterase type IV inhibitor cilomilast on the release of pro-inflammatory cytokines from cultures of bronchial epithelial cells taken from lung transplant patients. Previously, cilomilast has been shown to reduce inflammatory cells present in biopsies taken from COPD patients (Gamble et al. 2003). In addition, Murphy et al have shown that cilomilast can significantly reduce interleukin 8 (IL-8) and GMCSF (granulocyte-macrophage colony-stimulating factor) production from epithelial cell cultures (Murphy et al. 2006). IL-8 and GMCSF are associated with neutrophilic airway inflammation and airway remodelling seen in obliterative bronchiolitis (Hamilton and Anderson 2004; Belperio et al. 2005), suggesting that cilomilast may have effects relevant to the pathophysiology of chronic rejection seen in lung transplantation.

Re-transplantation is also an option for selected transplant patients with BOS, however with the limited number of donors available and an increasing number of potential recipients it remains controversial.

1.4 Airway mucus

1.4.1 Mucin structure and function

As well as graft rejection, mucus hypersecretion along with poor airway clearance and altered cough have been observed in lung transplant patients (Veale et al. 1993). Hypersecretion of mucus has also been observed in several respiratory diseases including COPD, CF and asthma (Callaghan-Rose and Voynow 2006).

Mucus is a mixture of water, ions, glycoproteins, proteins and lipids that coats epithelial surfaces (including the respiratory, gastrointestinal and reproductive tracts). In the airways one of the main functions of mucus is to protect the lungs by trapping foreign particles and pathogens and facilitate their removal by ciliary transport. As well as providing a physical barrier, mucus also contains a range of immunological factors with antibacterial, antiviral and antifungal properties (Rogan et al. 2006).

Mucus glycoproteins (mucins) are the major macromolecular constituent of mucus and they are expressed in two main forms; the secreted or gel forming mucins and the membrane bound mucins. Mucins are complex glycoproteins with a large molecular weight ranging in size from several hundred to several thousand kDa. They are characterised by their tandem repeat domain, which is composed of tandemly repeating amino acid residues, and is heavily glycosylated through *O*- or *N*-glycosyl linkages (figure 1.13). The more widely studied *O*-linkages are made between *N*-acetylgalactosamine and the hydroxyl

groups of serine and threonine residues, while *N*-linked carbohydrate chains are formed through bonds between *N*-acetylglucosamine and asparagine residues.

The predominant secreted mucins in the airway appear to be those coded for by the *MUC5AC* and *MUC5B* genes (Callaghan-Rose and Voynow 2006). MUC5AC is mainly produced by the goblet cells present in the surface epithelium and MUC5B by the secretory cells of the submucosal glands. However, Chen et al have shown that *MUC5B* can be expressed by surface epithelial cells as well as submucosal gland cells in airway tissue sections obtained from patients with COPD and asthma, whilst *MUC5AC* expression was restricted to the surface epithelial cells (Chen et al. 2001).

Normally, in humans goblet cells are present in the large airways and become less dense towards the lung periphery, with few or none being present in the small airways. This is also true of the submucosal glands, with no glands being present in the small non-cartilaginous airways. In chronic airway disease, the submucosal glands increase in size and the number of goblet cells also increase (goblet cell hyperplasia). Furthermore, goblet cells can appear in the small airways via metaplasia of non-goblet cells (Jeffery and Li 1997). The respiratory and terminal bronchioles can not be cleared by cough and since the mucociliary clearance is reduced compared to the large airways, excess mucus production can be a problem, possibly leading to occlusion of the small airways (Aikawa et al. 1992). This increase in the number of goblet cells and size of submucosal glands in airway disease is associated with an increase in the volume of mucus produced in the airways and can have significant clinical effects (Prescott et al. 1995; Vestbo et al. 1996).

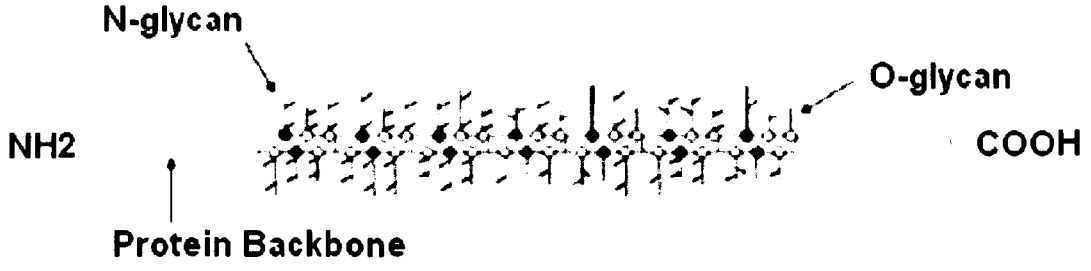


Figure 1.13 A schematic diagram of a secreted mucin. A MUC protein backbone usually consists of an N terminal domain, a C terminal domain and an area of high tandem repeats (shaded) that has O-and N-linked oligosaccharides (O-glycans, N-glycans respectively) attached. Mucins are classified by their MUC protein backbone, which is encoded by a *MUC* gene. This figure is modified from Callaghan-Rose and Voynow 2006.

1.4.2 Regulation of mucus secretion

The regulation of MUC genes has been previously investigated *in vitro* and a number of inflammatory cytokines have been shown to up-regulate MUC5AC expression, for example, TNF-α, IL-6 and IL-17 (Chen et al. 2003; Callaghan-Rose and Voynow 2006). Smirnova et al have also shown that IL-8 can stimulate MUC5AC secretion from goblet cells (Smirnova et al. 2002). In addition, the group has shown that bacterial LPS up-regulates both IL-8 and MUC5AC mRNA expression and secretion from goblet cells, suggesting that the up-regulative effect of LPS is partially mediated through an IL-8 dependant mechanism (Smirnova et al. 2003).

Other factors known to stimulate MUC5AC expression *in vitro* include prostaglandins, matrix matallo-proteinases (MMPs), neutrophil elastase, reactive oxygen species and also exogenous toxins, such as tobacco smoke and environmental pollutants (Voynow et al. 2006).

Such factors are thought to stimulate the up-regulation of mucins either via direct epithelial stimulation, or via leukocyte recruitment and activation (figure 1.14) (Jackson 2001). They act by binding to specific surface cell receptors, for example, P2Y2 and toll like receptors (Voynow et al. 2006). Additionally, the epidermal growth factor (EGF) receptor has been implicated in mucin gene regulation through a variety of stimuli (Takeyama et al. 1999). Binding of these receptors is then thought to activate mitogen-activated protein kinase (MAPK) pathways, which in turn activate the transcription factor, nuclear factor kappa B

(NFκB), which will regulate mucin gene expression (Callaghan-Rose and Voynow 2006). Other transcription factors can also mediate mucin gene expression, for example SP1 and AP-1 (Perrais et al. 2002; Gensch et al. 2004).

Few studies have been carried out investigating mucus hypersecretion in lung transplantation and therefore further work is required. It is possible that any pepsin present in the lung could contribute to the stimulation of epithelial/goblet cells causing them to synthesise/secrete mucin, as other diseases that are associated with mucus hypersecretion have also been linked to gastro-oesophageal reflux for example CF, COPD and asthma (Feigelson et al. 1987; Havemann et al. 2007; Kempainen et al. 2007).

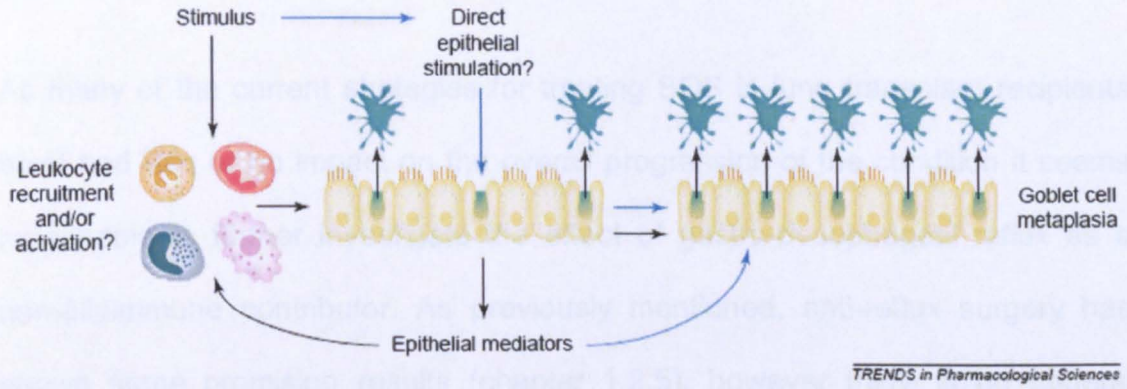


Figure 1.14 Stimulation of mucus production in goblet cells. Increased mucin production can be mediated through either direct stimulation of the epithelial cells or via leukocyte recruitment and activation. This figure is taken from Jackson 2001.

1.5 Outline of study

As many of the current strategies for treating BOS in lung transplant recipients have had little or no impact on the overall progression of the condition it seems reasonable to further investigate the effect of gastro-oesophageal reflux as a non-alloimmune contributor. As previously mentioned, anti-reflux surgery has shown some promising results (chapter 1.2.5), however there is no general consensus on when to perform surgery or which patients will benefit the most, therefore further investigations are required.

There are three overall aims of this study. The first is to investigate whether aspiration is occurring in lung transplant recipients by measuring levels of the gastric protease pepsin, as a biomarker of gastric aspiration, in the bronchoalveolar lavage (BAL) from transplant patients. The second is to measure pepsin levels in a longitudinal cohort to investigate the variation in levels over time and also if high levels of pepsin in the BAL can predispose or predict for chronic rejection (OB/BOS). Finally, to investigate the possible links between any pepsin present in the lung and damage caused to the epithelium using primary bronchial epithelial cell cultures taken from lung transplant patients.

Chapter 2

Materials and methods

2.1 Reagents

All reagents used in these experiments were obtained from Sigma UK, Fisher Scientific UK or BDH UK unless otherwise stated. All antibody concentrations were determined by the manufacturer, unless otherwise stated.

2.2 Collection of samples

Ethical approval was received from the relevant local research ethics committees for collection of all samples, including bronchoalveolar lavage and gastric juice samples.

2.2.1 Bronchoscopy and BAL collection

Bronchoscopy was performed in accordance with international guidelines (BTS Bronchoscopy Guidelines Committee 2001). Patients were pre-medicated with intravenous midazolam, and 4% lignocaine was applied topically to the nose, pharynx and larynx and below the vocal cords as required, up to a maximum of 8mg/kg body weight.

Bronchoalveolar lavage (BAL) was standardised to a 3 x 60ml procedure, during which O_2 saturation was routinely measured. The BAL sample was spilt and

assessed for clinical microbiology and differential cell counts on Giesma stained cytocentrifuge preparations. Cell free BAL supernatants were prepared by centrifuging the samples at room temperature and 1500rpm for 10 minutes (Centra-3, IEC). Aliquots were then snap frozen by immersion in liquid nitrogen and stored at -80 °C prior to experimentation.

2.2.2 Collection of gastric juice

Endoscopy was performed for standard indications. After xylocaine throat spray or up to 5mg midazolam endoscopy was performed using a fibre-optic endoscope. Gastric juice when present was aspirated out of the stomach and caught using a trapper (Pennine Healthcare, UK).

2.3 Preparation of standards

2.3.1 Purification of MUC5AC mucin standards

Media incubated with mucus secreting goblet cell line HT29-MTX (seeded at approximately 50000 cells/well) was collected until sufficient volume was acquired (approximately 500ml) and frozen at -20°C. The media was then defrosted, pooled and concentrated by vacuum dialysis followed by dialysis against deionised water to remove low molecular weight material. Once concentrated the media was adjusted to a density of 1.42g/ml with caesium

chloride and centrifuged at 4°C and 40000rpm for 48h (Centrikon T-1170).The resulting density gradient was separated into 8 equal fractions and their densities were measured. Each fraction was then exhaustively dialysed against 20 litres of deionised water for 3 days with 4-6 changes per day.

After dialysis the absorbance of each undiluted fraction was measured at 260 and 280nm on a spectrophotometer to determine the protein/nucleic acid concentration. A periodic acid-Schiff's (PAS) assay (Mantle and Allen 1978) was also performed on each fraction to determine the glycoprotein concentration. Mucin containing fractions were pooled and freeze-dried.

2.3.1.1 Periodic acid-Schiff's assay

This assay relies on 1,2-glycol groups being oxidised to aldehydes, leading to the production of a purple/magenta coloured compound that can be read on a spectrophotometer at 555nm (Mantle and Allen 1978).

A standard curve of 0-100µg was prepared using 1mg/ml papain digested pig gastric mucus and made up to a total volume of 1ml with deionised water. 100µl of periodic acid solution (0.1% periodic acid in 7% acetic acid) was added to 1ml of sample or standard and incubated at 37°C for 1h. After incubation 100µl of Schiff's solution (0.017g/ml sodium metabisulphate in Schiff's reagent, Biorad UK) pre-incubated for 1h at 37°C was added. Colour was developed for 30 minutes at room temperature. Samples and standards were then read at 555nm on a spectrophotometer.

2.3.2 Purification of human gastric juice

Gastric juice samples from the first 4 patients undergoing endoscopy after ethical approval was received were collected and pooled. Samples collected were of an abnormal pH (between pH 5.0 and 7.0) due to these patients taking proton pump inhibitors. The samples were adjusted to a density of 1.42g/ml with caesium chloride and centrifuged at 4°C and 40000rpm for 48h (Centrikon T-1170). The resulting density gradient was separated into 8 equal fractions and their densities were measured. Each fraction was then exhaustively dialysed against 20 litres of deionised water for 3 days with 4-6 changes per day.

After dialysis the absorbance of each undiluted fraction was measured at 260 and 280nm on a spectrophotometer to determine the protein/nucleic acid concentration. Protein containing fractions 1-3 were freeze dried. Fraction 1 contained a precipitate so was centrifuged for 5 minutes at 1500rpm (Centra-3, IEC) and pellet and supernatant were freeze-dried separately.

The individual freeze-dried fractions were then run on an SDS-PAGE gel to determine whether they contained pepsin (molecular weight approximately 35000).

2.3.2.1 SDS-PAGE

SDS-PAGE was performed using the Pharmacia PHAST gel system. The freeze dried fractions and 2 porcine pepsin controls were made up to a concentration of 2.5mg/ml in reducing buffer (0.0625M Tris buffer at pH 6.8 containing 2% (w/v)

SDS, 10% (v/v) glycerol, 0.001% bromophenol blue and 5% mercaptoethanol). Samples and controls were heated for 2 minutes at 100°C before 5µl was applied to the gel (PhastGel™ Gradient 4-15%). The gel was run (100V, 10mA, 1W and 4VH (volt hours)) until the tracking dye had reached the end of the gel. The gel was then stained overnight with a coomassie blue solution (0.05% coomassie blue, 25% (v/v) propan-2-ol and 10% (v/v) acetic acid in deionised water), followed by de-staining for 24h with a solution containing 25% (v/v) propan-2-ol and 10% (v/v) acetic acid in deionised water.

The gel was scanned on a densitometer (GS-800, Biorad, UK) and the relative mobility of the fractions were determined by calculating the distance moved on the gel from the point of application.

In addition, an ELISA was performed on each fraction to determine pepsin concentration (refer to methods 2.4.5).

2.4 Analytical methods

2.4.1 Determination of pepsin concentration by Slot/Blot enzyme linked immunosorbent assay (ELISA)

A nitrocellulose transfer membrane (Whatman, UK) with a pore size of 0.2µm was supported on blotting paper, wetted with deionised water and inserted into the Minifold II 72-well slot/blot apparatus (Whatman Schleicher & Schuell). Porcine pepsin standards and 100µl of each BAL sample were added to

individual wells in triplicate and the block attached to a vacuum source. Once the samples had been absorbed onto the membrane it was removed and incubated in phosphate buffered saline (PBS) with 2% bovine serum albumin (BSA) overnight at 4°C, or alternatively for 2h at room temperature. This was to prevent non-specific binding. After blocking, the membrane was incubated with a primary antibody for 1.5h (anti-pepsin, Biodesign International, USA) diluted to 1/2000 with 1% BSA in PBS. After incubation the membrane was washed with 2 changes of 0.5% Tween20 in PBS followed by 3 changes of PBS. The membrane was then incubated with a secondary antibody (horseradish peroxidase conjugated anti-sheep/goat, Sigma, UK) diluted to 1/10000 with 1% BSA in PBS for 1.5h at room temperature. After incubation, the washing was repeated and colour was developed using 0.05% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) with 0.03% H_2O_2 in PBS for 5 minutes. When the colour was sufficiently developed the membrane was rinsed with tap water and then left to dry over night before it was read on a Shimadzu scanning densitometer at 595nm. Negative controls were produced for determination of non-specific binding of antibody by omitting samples from the primary antibody incubation; in its place they were incubated in 1% BSA in PBS only. A schematic diagram of this method is shown in figure 2.1.

2.4.2 Determination of MUC5AC mucin concentration by Slot/blot ELISA

The slot/blot method using a nitrocellulose transfer membrane of pore size 0.45µm was repeated to determine MUC5AC mucin concentration (see schematic, figure 2.1). A monoclonal MUC5AC antibody (Vision Biosystems, UK) was used to detect MUC5AC mucin concentration and was diluted to 1/75 with 1% BSA in PBS, followed by a secondary antibody (horseradish peroxidase conjugated anti-mouse, Sigma UK) diluted to 1/5000 with 1% BSA in PBS. Again, negative controls were performed by omitting samples from the primary antibody step.

Mucin standards for this assay were purified from cell media collected from a human mucus secreting goblet cell line (HT29-MTX, refer to methods 2.3.1).

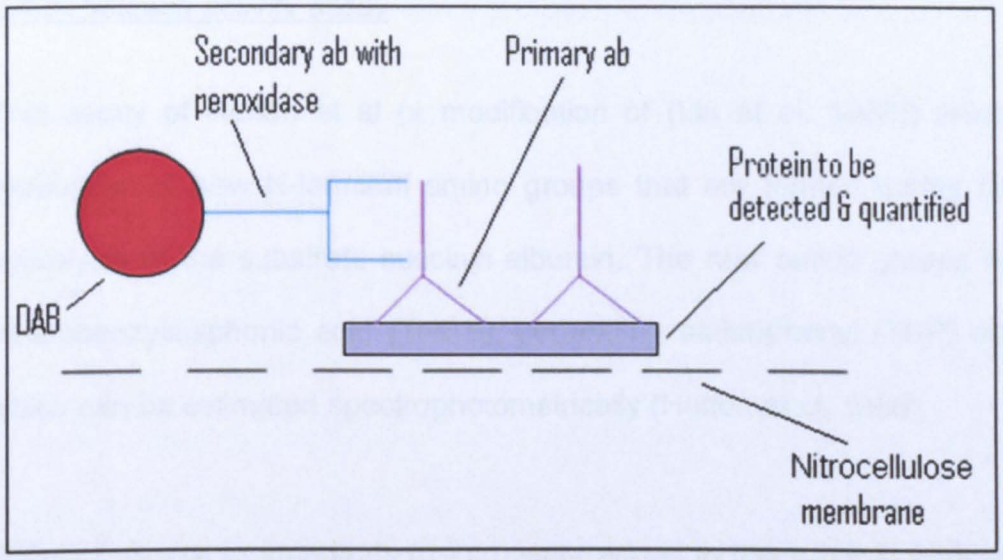


Figure 2.1: Schematic of the slot/blot ELISA assay.

2.4.3 Protease activity assay

This assay of Hutton et al (a modification of (Lin et al. 1969)) relies on the production of new N-terminal amino groups that are formed during proteolytic hydrolysis of the substrate succinyl albumin. The new amino groups react with trinitrobenzylsulphonic acid (TNBS), generating trinitrophenyl (TNP) derivatives which can be estimated spectrophotometrically (Hutton et al. 1986).

200µl of sample or standards (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, the tubes were then mixed, covered and incubated at 37°C for 1h. 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) the tubes were then mixed and placed in a water bath 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 1h. 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.

2.4.4 Determination of interleukin 8 concentration by sandwich ELISA

Interleukin 8 concentration was measured using a DuoSet ELISA kit (R&D Systems, USA):

A 96 well plate (Maxisorp, Nunc) was coated with a capture antibody diluted to a working concentration of 4.0µg/ml in PBS and left overnight at room temperature. After incubation the plate was washed with three changes of wash buffer (0.05% Tween20 in PBS) and dried by blotting with clean paper towels.

The plate was blocked for at least 1h with 1% BSA in PBS at room temperature and the wash step repeated. 100µl of samples (diluted 1 in 10) or standards (0-2ng/ml) made up in reagent diluent (0.1% BSA, 0.05% Tween20 in Tris-buffered saline (TBS), pH 7.2-7.4) were added and incubated for 2h at room temperature. Again the wash step was repeated followed by the addition of 100µl of the detection antibody (diluted to a working concentration of 20ng/ml with reagent diluent) and was left for 2h at room temperature. After washing, 100µl of streptavadin-HRP (diluted to 1/200 with reagent diluent) was added to each well and incubated for 20 minutes at room temperature. The wash step was repeated and 100µl of substrate solution (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)) was added to each well and colour was developed for approximately 20 minutes. The reaction was stopped with 100µl of 1% SDS and the plate was read on a standard plate reader at 405nm.

2.4.5 Determination of pepsin concentration by direct ELISA

The development of this assay for the measurement of pepsin concentration is dealt with in chapter 4.

100µl of sample (diluted to 1 in 5) or standards (0-10ng/ml) made up in PBS were coated onto a 96 well plate (Maxisorp, Nunc) and were incubated overnight at room temperature. The plate was then washed with three changes of wash buffer (0.05% Tween20 in PBS) and dried by blotting with clean paper towels.

The plate was blocked for at least 1h with 1% BSA in PBS at room temperature and the wash step repeated. After washing 100µl of the primary antibody (anti-pepsin, Biodesign International, USA) diluted to 1/2000 with reagent diluent (0.1% BSA, 0.05% Tween20 in Tris-buffered saline (TBS), pH 7.2-7.4) was added and incubated for 2h at room temperature.

Again the wash step was repeated followed by the addition of 100µl of the secondary antibody (HRP-conjugated anti sheep/goat, Sigma, UK) diluted to 1/10000 with reagent diluent and was left for 2h at room temperature. After washing, 100µl of substrate solution (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)) was added to each well and colour was developed for approximately 20 minutes at room temperature. The reaction was stopped with 100µl of 1% sodium dodecyl sulphate (SDS) and the plate was read on a standard plate reader at 405nm.

Negative controls were performed by omitting samples from the primary antibody step and incubating with reagent diluent only to determine the degree of non-specific binding of the secondary antibody.

2.5 Cell culture methods

2.5.1 Primary bronchial epithelial cell culture

Epithelial cells taken from bronchial brushings were obtained from lung transplant patients undergoing routine bronchoscopy and cultured according to the method described by Forrest et al (Forrest et al. 2005)(figure 2.2). Cells were used at passage 1-3 only:

Bronchial brushings were obtained from sub-segmented bronchi and were dispersed in 5 ml of sterile PBS and 5ml of RPMI and 10% foetal calf serum (FCS). The cells were centrifuged at room temperature and 1000 rpm for 5 minutes (Centra-3, IEC). The supernatant was removed and the cell pellet was re-suspended in 2ml of bronchial epithelial basal media (BEBM; Lonza, Switzerland) supplemented with bronchial epithelial growth media (BEGM) Singlequots (Lonza, Switzerland), penicillin and streptomycin. The cells were then transferred to a collagen coated T25 flask (Vitrogen 100; Cohesion, USA) and placed in an incubator at 37°C and 5% CO_2. Media was changed every 48h until the cells reached confluency.

Once confluent the cells were passaged using trypsin and neutralised with an equal volume of RPMI supplemented with 10% FCS. The cell suspension was centrifuged, supernatant removed and the pellet re-suspended with fresh media. The cells were then transferred to a T75 flask for expansion followed by a 24 well plate for experimentation. All flasks and plates are collagen coated prior to addition of the cells.

Cells were stained with haematoxylin & eosin and for cytokeratin to ensure an epithelial phenotype of the cells.

2.5.1.1 Haematoxylin & eosin staining

Cells were grown on 8 chamber slides until confluent. They were then fixed in 100% acetone for ten minutes at room temperature and allowed to air dry. The cells were stained with Harris Haematoxylin for 1 minute and then were washed in running tap water for 2-3 minutes. The slides were then differentiated in 0.1% acid alcohol and washed again with tap water until blue (slides were checked under the microscope). The slides were then counter-stained with Eosin Y for 2 minutes and washed in running tap water, then dehydrated with industrial methylated spirits (95% for 30 seconds, 2 x 99% for 30 seconds followed by 100% xylene). The slides were then mounted in DPX (Distrene-80, Plasticizer, Xylene).

2.5.1.2 Cytokeratin staining

Confluent cells grown in 8-chamber slides were stained for cytokeratin using the ImmPRESS™ detection system (Vector, USA) (figure 2.3).

Cells were fixed in 100% acetone for 10 minutes at room temperature and left to air dry. Sections on the slide were then ringed with a hydrophobic pen. Each section was treated with a Tris buffered saline (TBS)/Tween solution (TBS adjusted to pH 7.6 and 0.1% (v/v) Tween20) for 15 minutes. After removing the TBS/Tween solution slides were blocked with 2.5% normal horse serum for 30 minutes. Once blocked the slides were incubated with 100µl of the primary antibodies (a mixture of two anti-cytokeratin antibodies, raised against cytokeratin 6 & 8 and 5, 6, 8, 17 & 19, Dako, Denmark) diluted to 1/50 in 3% BSA/TBS/Tween, or the isotype control also diluted 1/50 in 3% BSA/TBS/Tween for 1h at room temperature. The slides were then washed with TBS for 5 minutes. Endogenous peroxidases were blocked with 0.3% H_2O_2 in methanol for 30 minutes, followed by washing with running tap water for at least 5 minutes, after which slides were rinsed with TBS.

Excess buffer was removed without drying out the cells on the slides. 100µl of ImmPRESS™ reagent containing the secondary antibody (horseradish peroxidase conjugated anti-mouse/rabbit) was then added for 30 minutes. After washing and removing any excess buffer the slides were incubated with DAB for 5-10 minutes, whilst being checked under the microscope for sufficient colour development. The sections were then washed with running tap water and

counter-stained with Harris Haematoxylin for 1 minute. Slides were washed gently under the tap until blue, and were then dehydrated with industrial methylated spirits (95% for 30 seconds, 2 x 99% for 30 seconds followed by 100% xylene). The slides were then mounted in DPX. (Distrene-80, Plasticizer, Xylene).

2.5.2 Goblet cell culture (HT29-MTX)

A human goblet cell line HT29-MTX, kindly provided by Dr Thecla Lesuffler (INSERM U178, France), was grown at 37°C and 10% CO_2 in a T75 flask in 12ml of Dulbecco's modified eagles medium (DMEM) supplemented with 10% FCS (figure 2.4). Confluent cells were passaged using trypsin and transferred to a 24 well plate for experimentation.

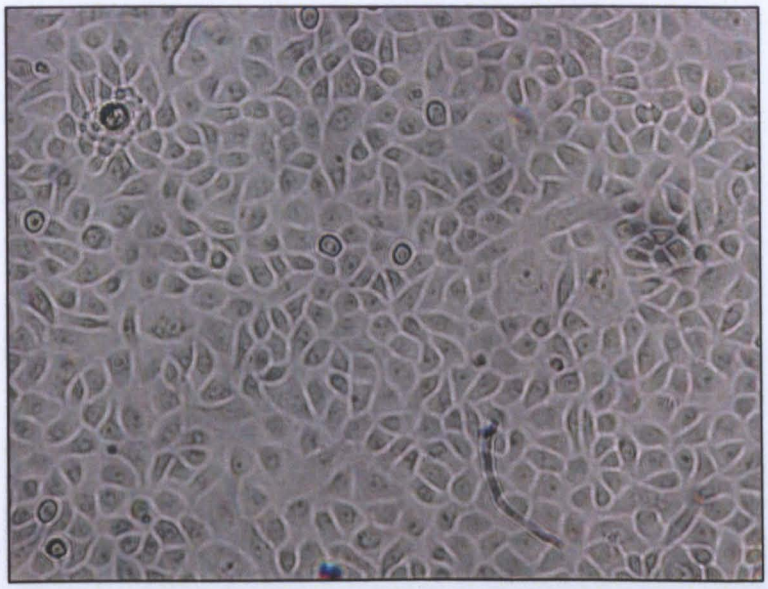


Figure 2.2 Human primary bronchial epithelial cells growing in culture under normal conditions (singlequot supplemented BEBM, Lonza, Switzerland, at 37°C and 5% CO_2, cells were seeded at approximately 50000/well).

Figure 2.3 Human primary bronchial epithelial cells stained for cytokeratin. Cells were grown in 8-chamber slides and stained for cytokeratin using antibodies against cytokeratin 6 & 8 and 5, 6, 8, 17 & 19.

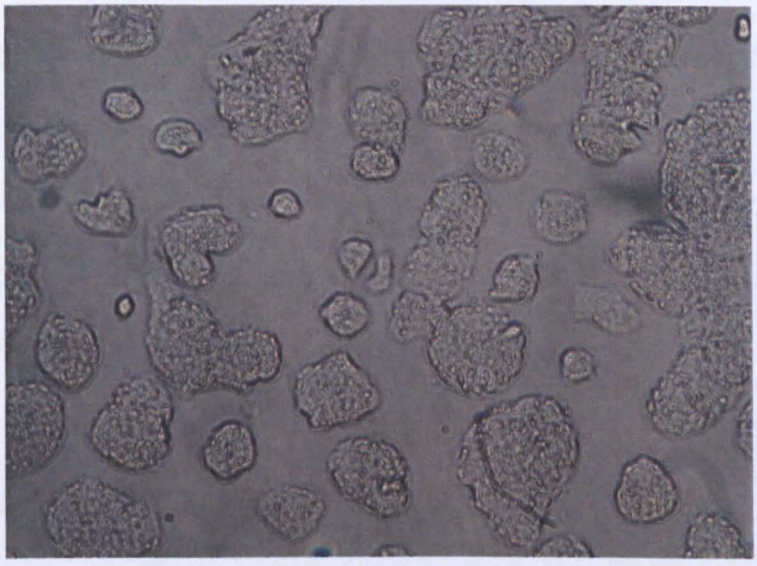


Figure 2.4 Human goblet cells (cell line HT29-MTX) growing in culture under normal conditions (FCS supplemented DMEM, Sigma UK, at 37°C and 10% CO_2; cells were seeded at approximately 50000/well).

2.5.3 Pepsin challenge on epithelial and goblet cells

Once the goblet cells and epithelial cells reached confluency in 24 well plates they were incubated with 500µl of media (DMEM, serum free, Sigma UK or BEBM, without singlequots, Lonza Switzerland respectively) at pH 7.4 or pH 7.0 (media adjusted with 1M HCl) and either with or without pepsin (porcine pepsin at a concentration of 10ng/ml, 50ng/ml or 50µg/ml). Media was collected at 24, 48 and 72h and frozen for future analysis of mucin and/or interleukin 8.

2.5.4 Gastric juice challenge on epithelial cells

Epithelial cells were also challenged with human gastric juice. Confluent cells in 24 well plates were incubated with 500µl of either neat gastric juice or gastric juice at one of the following dilutions; 1/4, 1/20, 1/100, 1/1000, 1/5000 or 1/10000 (diluted with BEBM without singlequots, Lonza, Switzerland). Controls were also performed by incubating the cells with media free from gastric juice or serum. The media was removed after 24h, after which a viability assay was performed.

2.5.5 Viability assay

Viability of both the goblet and epithelial cells was measured using the CellTiterblue assay (Promega, USA).

This assay relies on 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 that of resazurin, which is 605nm.

Challenge media was removed from the cells and stored at -20°C for further analysis. TiterBlue reagent 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 their standard conditions for 2-4h. Absorbance was then measured at 560nm on a spectrophotometer and values converted to a percentage of the normal control. 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 the media alone is responsible for the colour change.

Chapter 3

Cross-sectional analysis of pepsin present in BAL from lung transplant recipients

3.1 Introduction

Chronic rejection in the form of obliterative bronchiolitis (OB) and it's clinical correlate bronchiolitis obliterans syndrome (BOS) remains the biggest limitation to long term survival in lung transplantation. The pathophysiology is poorly understood, but the number and severity of acute rejection episodes are consistently linked with the development of OB. In addition non-alloimmune mechanisms are becoming increasingly recognised as risk factors for the development of post-operative OB and gastro-oesophageal reflux (GOR) with subsequent aspiration has been implicated, not only in the development of OB, but in other airway and lung diseases (Feigelson et al. 1987; Tobin et al. 1998; Palmer et al. 2000; Brodzicki et al. 2002; Raghu 2003).

As a result of this some centres are now assessing their patients for reflux, by pH monitoring and/or impedance both pre and post-operatively, and the Duke group in particular have taken a very aggressive approach and only feed their patients through a G-J tube until GOR-corrective surgery is performed (Cantu et al. 2004).

pH monitoring and impedance can diagnose reflux; however they do not tell us whether the gastric contents are reaching the lungs. Therefore a biomarker approach may be more accurate in detecting gastric aspiration. In 2005 Ward et al investigated whether the gastric protease pepsin could be found in the lavage of lung transplant patients (Ward et al. 2005). They showed, in a small number of patients, that pepsin was present in all of the lung allograft recipients. The study was however, small and not powered to look at possible associations between acute or chronic rejection and gastric aspiration.

I have therefore investigated whether pepsin, a marker of gastric aspiration, is present in the lungs of transplant recipients and also if high levels are associated with the presence of acute rejection and/or OB.

3.2 Patient demographics

Patient demographics are summarised in Table 3.1. Bronchoalveolar lavage (BAL) microbiology and transbronchial biopsy pathological rejection assessments are summarised only for the lung transplant recipients.

In the lung transplant recipients twelve patients had stable lung function with no evidence of clinically significant acute rejection (6 had A0, 6 A1) or BOS or clinical evidence of infection (stable lung transplant recipients). One patient in this group had asymptomatic infection with *Pseudomonas aeruginosa*. Twelve patients had biopsy proven acute rejection (A2 or greater) without BOS and twelve patients were diagnosed as having BOS.

3.2.1 BAL returns

Median BAL return was 85ml (range 35-115) in the allograft patients, 85ml (range 60-110) in the normal controls and 67.5ml (range 50-100) in the chronic cough disease control group, indicating technically satisfactory BAL procedures.

Subject no.	Group	age	sex	Diagnosis	Op.	Time post Tx (weeks)	Biopsy	BOS Score	Micro-biology	Pepsin ng/ml	ant-acid therapy
1	a	42	f	ipf	blt	4	A3B2	*	neg	8.5	N
2	a	35	f	pph	hlt	4	A2B2	*	neg	31.2	R
3	a	50	m	emp	slt	1	A2B2	*	neg	26.5	Oi
4	a	47	f	emp	slt	26	A2B0	0	neg	51.7	Oi
5	a	41	m	cf	blt	1	A3B2	*	neg	5.4	R
6	a	20	f	cf	blt	1	A2B2	*	neg	10	Oii
7	a	36	f	pph	hlt	24	A2B0	0	neg	22.3	Oi
8	a	38	f	ob	blt	4	A2B1	*	neg	9.1	N
9	a	25	f	pph	hlt	4	A3B2	*	neg	10.2	Oi
10	a	56	f	emp	slt	12	A2B2	0	neg	10.7	R
11	a	51	f	emp	blt	4	A2B1	*	neg	11.7	L
12	a	25	f	cf	blt	12	A2Bx	0	neg	20.6	Oi
13	s	61	f	lam	slt	24	A0B0	0	neg	8.1	Oi
14	s	43	f	lam	blt	52	A1B0	0	neg	4.2	L
15	s	35	m	sar	slt	24	A1B1	0	neg	1.4	R
16	s	55	f	ipf	slt	4	A0B1	*	neg	2.5	L
17	s	37	m	emp	blt	26	A1B1	0	neg	0	L'
18	s	48	f	ob	blt	1	A1B0	*	neg	2.9	L
19	s	40	m	ipf	slt	52	A0Bx	0	neg	10.2	L
20	s	49	m	emp	blt	12	A1Bx	0	pa	9.9	L
21	s	59	f	emp	slt	52	A0B0	0	neg	6.4	L
22	s	39	m	emp	blt	24	A0B0	0	neg	7.4	R
23	s	39	m	cf	blt	52	A1B1	0	neg	12.6	L
24	s	22	f	cf	blt	12	A0Bx	0	neg	0	N
25	b	24	m	cf	blt	80	A0B1	2	pa	12.8	R
26	b	54	m	ipf	slt	230	AuBx	1	neg	19.1	R
27	b	21	m	cf	blt	60	A1Bx	3	pa	3.5	N
28	b	20	f	cf	blt	54	A0B1	3	pa	7.7	Oi
29	b	51	f	ipf	slt	280	AuB1	3	neg	9.3	R
30	b	16	f	cf	blt	112	A1Bx	3	neg	0	Oii
31	b	53	m	emp	blt	140	A0Bx	2	neg	1.3	L
32	b	28	m	cf	blt	34	A1Bx	2	pa	5.8	L
33	b	27	f	cf	blt	180	A1B1	3	neg	15.6	N
34	b	57	f	emp	slt	28	A0B0	1	asp	7.3	Oi
35	b	17	m	cf	blt	26	A0Bx	2	neg	1.3	N
36	b	29	m	cf	hlt	160	AuBx	2	pa	0	Oi

Table 3.1. Patient demographics, a- acute rejection (grade A2 or higher), s – stable, b-BOS. ipf - idiopathic pulmonary fibrosis, lam - lymphangioleiomyomatosis, cf-cystic fibrosis, sar-sarcoidosis, emp-emphysema, pph-primary pulmonary hypertension, ob- obliterative bronchiolitis. slt-single lung transplant, blt- bilateral lung transplant, hlt-heart lung transplant. neg -negative, pa- *Pseudomonas aeruginosa*, asp-*Aspergillus*. Au-denotes biopsies that are technically un-gradable for acute rejection. * -patients un-gradable for BOS as their biopsies were taken within 12 weeks of transplantation. N-no acid suppression, R-ranitidine 150mg twice daily, Oi- omeprazole 20mg once daily, Oii- omeprazole 20mg twice daily, L- lansoprazole 30mg once daily, L'- lansoprazole 15mg once daily.

3.3 Analysis of BAL Pepsin

3.3.1 Statistical analysis of BAL pepsin data

The statistical analysis of data contained in this chapter was performed with the help of a statistician. The median allograft pepsin levels between groups were compared using the Kruskal Wallis test (non parametric one-way analysis of variance) with a *post-hoc* Mann Whitney test (two tailed throughout). P-values for groups of pair-wise comparisons detailed in 3.3.3 were adjusted using the Bonferroni method. Unadjusted P-values are reported, because Bonferroni corrections made no difference to the statistical significance using $P<0.05$ as a cut-off.

Possible confounding variables:

1. Time post transplantation.

A rank based Spearmans (non parametric) correlation was used to evaluate a possible relationship between time post transplantation and BAL pepsin levels.

2. A model including demographic variables.

A model was examined to evaluate possibly confounding, variables for BAL pepsin levels which included group, sex, age, operation type and antacid status. Time post transplantation could not be assessed in this model because it was impossible to dissociate this from group status e.g. BOS patients are further post transplantation than other groups. Pair-wise comparisons were performed

between the means of the groups and adjusted for all other variables in the model, with a Bonferroni correction.

3.3.2 Comparison of lung transplant recipients and control subjects

Pepsin levels were analysed in the lavage of 4 normal controls, 17 chronic cough disease controls and 36 transplant patients. Pepsin levels from all transplant patients (median 8.3, range 0-51.7ng/ml) were higher than both the control groups (normal: median, 1.1, range 0-2.3ng/ml, chronic cough: median 0, range 0-2.6ng/ml) (all transplant group vs. normal group $P = 0.02$, vs. Chronic cough group $P < 0.0001$) (Figure 3.1).

Moreover, BAL pepsin levels were significantly raised in lung transplant recipients without BOS compared to the control groups, suggesting that gastric aspiration is present in lung transplant patients without any significant airflow limitation (Figure 3.2).

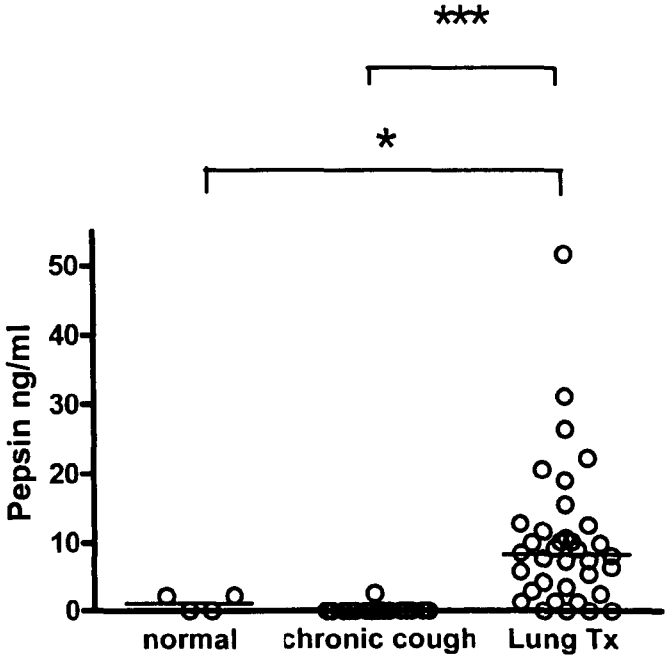


Figure 3.1. BAL Pepsin levels (ng/ml) of all transplant recipients vs. normal and chronic cough control groups. Lines represent the median values. Lung TX-lung transplant. Normal n=4, c cough n=17 and lung tx n=36.

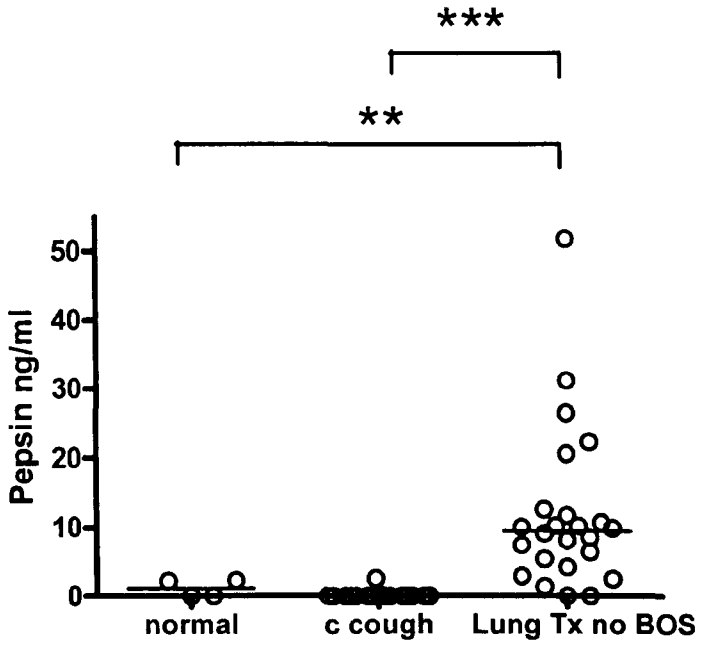


Figure 3.2. BAL Pepsin levels (ng/ml) of transplant recipients without BOS vs. normal and chronic cough control groups. Lines represent the median values. Lung TX-lung transplant, BOS-bronchiolitis obliterans syndrome. Normal n=4, c cough n=17 and lung tx no BOS n=24

3.3.3 Comparison of lung transplant recipients with stable lung function, acute rejection and BOS

BAL pepsin levels were elevated in stable lung transplant recipients, subjects with acute rejection and subjects with BOS. The highest levels were found in recipients with A2 or greater acute rejection (Figure 3.3), with no significant difference between stable patients (A0-1) and BOS patients. The statistical significance of these results, with a Bonferroni correction for multiple comparisons, remained the same after adjustment for age, gender, operation type and whether the patients were treated with antacid therapy.

When compared to normal and chronic cough controls (both GORD and no-GORD) BAL pepsin levels were significantly raised in the A2 or greater acute rejection group (vs. normal $P= 0.004$ and vs. both chronic cough groups $P<0.001$).

Pepsin levels were not significantly different in patients treated with a maintenance dose of acid suppression therapy (median 8.7, range 0-51.7ng/ml) compared to patients who were untreated (median 6.0, range 0-15.6ng/ml, $P = 0.3$).

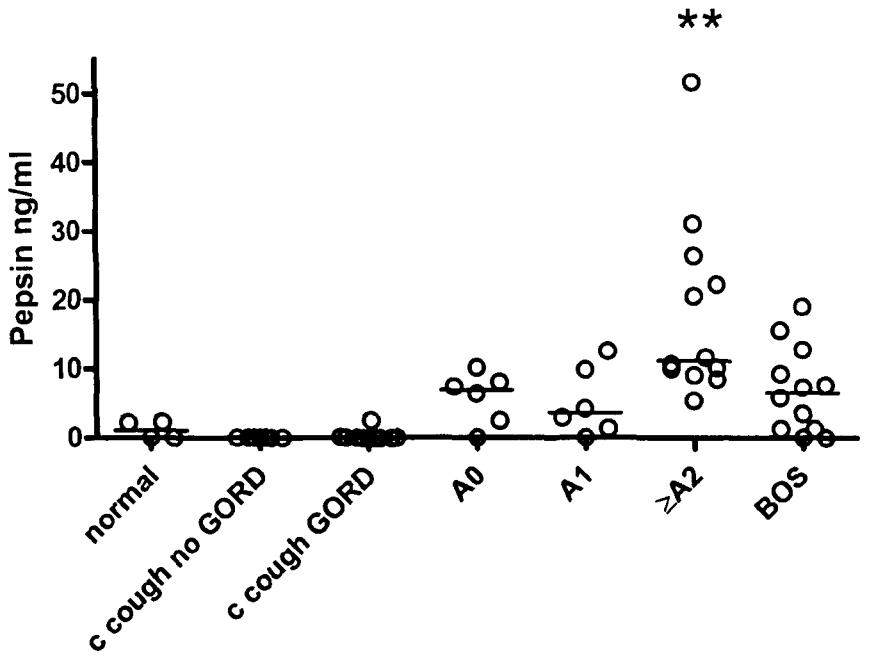


Figure 3.3. Values of pepsin (ng/ml) recovered from BAL. Lines represent the median values. c. cough-Chronic Cough, GORD-gastro-oesophageal reflux disease. A0 and A1 are clinically stable allograft recipients with grade A0 or A1 acute vascular rejection. The ≥A2 group are subjects with A2 or greater acute rejection. BOS - allograft recipients diagnosed with Bronchiolitis Obliterans Syndrome. Normal n=4, c cough no GORD n=7, GORD n=10, A0 n=6, A1 n=6, ≥A2 n=12 and BOS n=12

3.3.4 Airway inflammation

Grades of airway inflammation were highest in the clinically significant acute rejection group (≥A2) (median B scores; 0 in A0, 1 in A1 and 2 in ≥A2. P=0.02 A0 vs. ≥A2) (figure 3.4 and table 3.1), showing a possible association between gastric reflux, inflammation and acute rejection.

3.3.5 Potential confounding variables

1. Time post transplantation.

There was no relationship between time post transplant in all subjects and BAL pepsin level (r=0.144 P=0.4).

2. A model including demographic variables.

A model was examined to evaluate possibly confounding variables for BAL pepsin levels which included group, sex, age, operation type and antacid status. The adequacy of the model fit was examined and found to be reasonable. With a Bonferroni correction the conclusions reached in the basic analysis above were confirmed (i.e. A2 statistically significantly higher pepsin levels on average than the stable (P=0.006) or BOS (P=0.03) patient groups, with no significant difference between stable and BOS patient groups (P=0.9).

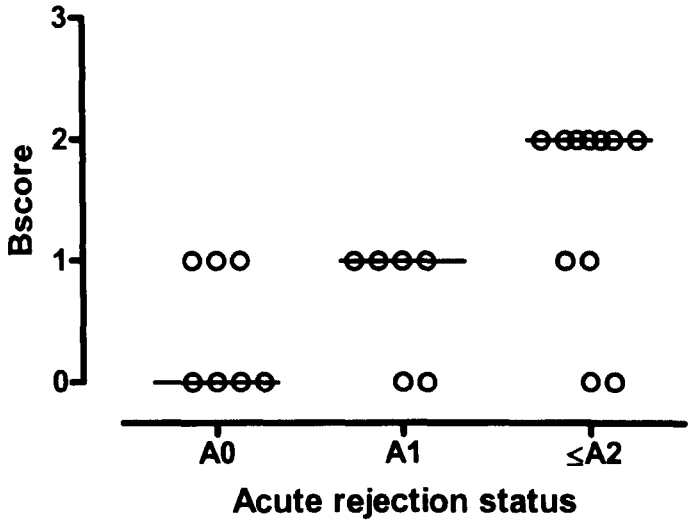


Figure 3.4. Acute rejection status vs. inflammation (B score). Lines represent median values. A0 n=7, A1 n=6 and ≥A2 n=11.

3.4 Discussion

Ward et al have previously shown for the first time that pepsin, a marker of gastric aspiration, can be found in the airways of lung allograft recipients (Ward et al. 2005). This has now been confirmed in a larger group of patients, with more control data. It has also been shown that levels of pepsin were highest in lung allograft recipients with histologically verified A2 or greater acute rejection. Furthermore these patients have the highest grades of airway inflammation, suggesting a possible link between aspiration, acute rejection and inflammation.

Alloimmune mechanisms have been the traditional focus of research and therapeutic intervention, however this study adds to the growing evidence that supports the role of non-alloimmune mechanisms as risk factors for the development of chronic lung rejection. Accumulating clinical evidence suggests that gastro-oesophageal reflux (GOR) with sequential aspiration may be an important source of injury and this is underlined by a recent paediatric lung transplantation study from Great Ormond Street, which showed that all patients had evidence of GOR post transplant, except one individual who had received a previous fundoplication for confirmed gastro-oesophageal reflux disease (GORD) (Benden et al. 2005). Additionally, Benden et al reported that all subjects with acute rejection had moderate to severe GORD and that no episodes of acute cellular rejection occurred post-Nissen fundoplication (Benden et al. 2005). The implication from this, as well as the finding of higher levels of pepsin in adult patients with A2 or greater acute rejection, is that non alloimmune injury may contribute to a pathology previously attributed solely to alloimmunity.

For this study acute rejection was defined as A2 or greater as this is the trigger to alter clinical management at the Freeman hospital. However, there has been debate on the importance of minimal acute rejection (A1) as a risk factor for BOS (Hopkins et al. 2004; Khalifah et al. 2005). This study indicates that in A1 rejection pepsin levels were not raised. This could support the concept that aspiration injury is additive to alloimmunity, and where present, results in higher grades of injury. This is speculative however, and the suggestion of a possible association between acute rejection and gastric aspiration requires further studies which may in particular better define the role of A1 rejection.

As pepsin levels are elevated in all transplant recipients compared to controls, it would appear that aspiration may be an ongoing source of injury. It has previously been shown that other non-alloimmune injuries, such as ischemia-reperfusion may further pre-dispose the transplanted lung to alloimmune injury (Serrick et al. 1997) and this study supports the importance of both alloimmune and non-alloimmune damage to overall injury in lung transplantation.

The present findings and previous work indicate that a biomarker approach to studying gastric aspiration in lung allografts is informative and practicable. One such reason why biomarker approaches may be important is that calcineurin inhibitors and other drugs which may be augmented to treat presumed alloimmune rejection have adverse effects on gastric motility and may in fact pre-dispose patients to aspiration.

The lack of any pepsin in the BAL samples from chronic cough patients, even when 10 patients out of 17 were diagnosed with GORD can be explained by the fact that a diagnosis of GORD does not mean that patients are refluxing out of the oesophagus and hence aspirating. Even if the refluxate reaches the upper airway it is almost certainly cleared by a hyper-active cough reflex (Niimi and Chung 2004). In contrast, lung allograft recipients are regarded as being especially vulnerable to aspiration since they have gastroparesis, impaired cough and an impaired mucociliary clearance (Higenbottam et al. 1989; Laube et al. 2007).

Clinical research in human patients is often limited to studies of association therefore; the use of animal models can provide important information. Work in a rat model of lung transplantation from the Duke group has shown that lung allografts challenged with gastric juice demonstrated severe grade 4 acute rejection with significant monocyte infiltration, fibrosis, and lung destruction (Hartwig et al. 2006). Aspiration was also associated with increases in CD8+ T cells and this 'proof of principle' study indicates that aspiration may indeed lead to pathological changes previously attributed to T cell, alloimmune based mechanisms (Takehisa et al. 2002; Boehler and Estenne 2003).

This study does not seem to show an association between patients with BOS and higher levels of pepsin in the lavage. A potential explanation for this is that the study is cross sectional and subjects with BOS tend to be sampled at a later time point compared with the other groups, which is important as gastroparesis specifically associated with peri-operative vagal damage and early post

transplant events would be less marked. Further insights into this would be provided through longitudinal assessments of gastric aspiration and its relationship with allograft dysfunction.

There is also increasing evidence to suggest that duodeno-gastro-oesophageal reflux may be involved in chronic lung rejection, as bile acids have been measured in the lavage of lung transplant recipients (Palmer 2006; Blondeau et al. 2008). Unlike pepsin, bile acids seem to show some association with the development of BOS. In one study from the Toronto group patients with bile acids present in their lavage had a lower freedom from BOS (D'Ovidio et al. 2005). In a second study they also showed that higher levels of bile acids in the lavage fluid was associated with impaired lung allograft innate immunity, demonstrated by reduced surfactant collectins and altered phospholipids (D'Ovidio et al. 2006).

This study has shown that patients who are being treated with maintenance proton pump inhibitors (PPI) still show evidence of gastric aspiration. On initial consideration this may be unexpected, however, approximately 12-20% of GORD patients are resistant to acid suppression therapy (Ahlawat et al. 2005) and, in addition, it is important to recognise that proton pump inhibitors do not prevent reflux *per se*., but rather act to reduce acidic reflux. Mildly acidic, neutral or alkaline reflux, which will still contain pepsin is not the target of PPI therapy but may still be an important source of aspiration injury, which requires a biomarker approach to monitor more accurately. It is therefore understandable that these

allograft patients will continue to reflux and aspirate when on maintenance acid suppression therapy.

Consequently, alternative treatments should be considered. There is evidence from the Duke group to suggest that early fundoplicative surgery can provide a survival benefit. At 3 years post-transplantation patients that had been diagnosed with reflux and had an early fundoplication were 100% free from BOS, compared to 62% in patients with no history of reflux, 60% in those with a history of reflux and a late fundoplication and 47% in those with a history of reflux and no surgery (Cantu et al. 2004). Previously the Duke group did not perform pH studies on their patients. Now they assess all patients as part of their pre and post-operative evaluation and often fundoplicate within the initial hospitalisation for transplantation.

The general consensus is that fundoplication is a safe and effective treatment of GORD, however, there are some risks and putting patients that have already undergone a major surgery through another operation may not always be the answer. Assessing which patients will derive clinical benefit of GOR corrective surgery is therefore important to minimise unnecessary complications.

In this study 24 hour oesophageal pH measurements were not taken. As mentioned previously such measurements would not tell us whether gastric contents have been aspirated into the lung, however, the patient is more likely to be aspirating if they are refluxing on a regular basis so future studies may benefit from combining traditional methods of monitoring reflux (i.e. 24-hour pH

monitoring or 24h pH with impedance) with a more novel approach i.e. measuring biomarkers to get a more complete picture of what is happening in these patients.

Chapter 4

Development of a plate ELISA for the measurement of pepsin

4.1 Introduction

Gastro-oesophageal reflux with subsequent aspiration is associated with many lung diseases as well as lung transplant rejection (Feigelson et al. 1987; Tobin et al. 1998; Brodzicki et al. 2002; Raghu 2003). Measuring biomarkers is becoming a useful tool in the diagnosis of aspiration. Previous investigations in our laboratory have shown that pepsin is present in the bronchoalveolar lavage (BAL) of transplant patients using a slot/blot ELISA (Ward et al. 2005; Stovold et al. 2007); however the manufacturer of the nitrocellulose membranes required for the slot/blot ELISA changed, and as a result the membranes no longer work within the assay. In addition, the slot/blot can only process a relatively small number of samples at a time. It was therefore decided to change the format of the assay to allow a higher through-put of samples.

The aim was to develop a new ELISA assay that can detect the gastric enzyme pepsin in BAL. As well as having a high through-put of samples the assay should have a sensitivity range that covers levels of pepsin expected within bronchoalveolar lavage, for example, BAL found positive for pepsin using the

slot/blot had levels ranging from 1.3 to 51.7ng/ml, therefore the new assay should also be able to detect pepsin levels in the range of 1ng/ml.

The assay should be reproducible to allow comparisons between patient's samples and also between different patients. This can be measured by comparing standard curves from different assays and also including an internal standard in the assay. The assay should be relatively simple and as well as analysing more samples should not take too long to run.

The assay should be specific for pepsin, therefore antibodies should not cross react with any other proteins likely to be present in the lavage, for example certain serum proteins (for example albumin or γ-globulins) and should also measure pepsin in the presence of molecules that may interfere with antibody binding, for example mucins. The slot/blot ELISA was initially used as mucus can be a problem in plate ELISAs.

Currently, there are other centres world-wide using pepsin as a marker of aspiration; however, there is a wide variation in levels being reported. For example, Blondeau et al report levels over 1000ng/ml in BAL using an ELISA based method, whilst Metheney et al are measuring pepsin in tracheal aspirates using a gel based immunoassay and give either a positive or negative result with no quantitation and also measuring pepsin in tracheal aspirates in children, Farhath et al use a proteolytic assay and report levels over 2000ng/mg of protein (Metheny et al. 2006; Blondeau et al. 2008; Farhath et al. 2008). In addition, there are some groups who use bile salts as a marker of duodenal/gastric

aspiration instead of or as well as pepsin, as there is some evidence that bile salts correlate with BOS development (D'Ovidio et al. 2005; Blondeau et al. 2008).

4.2 Development of a sandwich ELISA for the determination of pepsin concentration

To improve through-put of samples the assay was changed from a membrane based, to a 96-well plate ELISA. Sandwich ELISAs generally demonstrate good sensitivity so this was the preferred format to begin with. To my knowledge there were no commercial human pepsin antibodies available at the time (December 2006), so a human pepsinogen antibody was acquired for use as the capture antibody (anti-human pepsinogen I, Europa Bioproducts, UK), along with the porcine pepsin antibody used in the previous assay as the detection (anti-porcine pepsin, Biodesign International, USA). Pepsinogen is the inactive form of pepsin and loses only 44 amino acids to become pepsin below pH 5 (chapter 1.2.2, figure 1.9), therefore the pepsinogen antibody should also recognise pepsin in our assay, unless it reacts with the 44 amino acids that are lost in the conversion process.

The pepsinogen antibody did not come with any suggested dilutions for use from the manufacturer; therefore a range from 1/10 to 1/2000 was investigated using standard curves with porcine pepsin (porcine pepsin A, Sigma, UK). However, no pepsin was detected, suggesting that the human pepsinogen antibody does not bind porcine pepsin. Consequently the porcine pepsin was replaced with human pepsinogen (human pepsinogen I, Sigma, UK) as the standard. Different concentrations of human pepsinogen were tried with increasing antibody concentrations; however there was still no detection.

Previous investigations have shown that the pepsin antibody from Biodesign will bind porcine pepsinogen; however, it is much less sensitive. At 1µg/ml of protein there is a ten-fold difference in antibody binding between pepsin and pepsinogen (Tasker 2003). Therefore the pepsinogen was incubated with hydrochloric acid at pH 2.0 for 1h at room temperature in an attempt to convert it to pepsin, hopefully allowing more efficient binding of the antibodies.

Using activated human pepsinogen as the standard gave a general increase in absorbance with increased concentration, showing evidence of some antibody binding, however the increase was very small with a poor r^2 value (slope=0.0009, r^2=0.6526 figure 4.1). Consequently the pepsinogen antibody was replaced with the anti-porcine pepsin to act as both the capture and detection antibody (recommended dilutions were 1/2000 and 1/10000 respectively). The detection antibody was conjugated to horseradish peroxidase to allow substrate binding. Standard curves were run with porcine pepsin, human pepsinogen and activated human pepsinogen. The porcine pepsin standard curve (0-10ng/ml) was acceptable (r^2=0.9976, slope=0.042 figure 4.2) and was therefore chosen for use in future assays.

4.2.1 Determination of antibody concentrations

To ensure nothing in the bronchoalveolar lavage would interfere in the assay spiking experiments were performed by adding a known amount of porcine pepsin to lavage samples that contained little or no pepsin and measuring the

recovery. Three samples were chosen and pepsin was added to give a final concentration of 5, 10, 15, 20 and 40ng/ml. Values recovered from the BAL can be seen in table 4.1.

Percentage recovery was lowest in the samples spiked with 40ng/ml pepsin (47-54%) and was best at 10ng/ml (74-95%). To try to improve recovery the capture antibody concentration was increased to 1/1000 and 1/500. Spiking experiments were repeated using one sample made up to give a final concentration of 10, 20 or 40ng/ml (table 4.2).

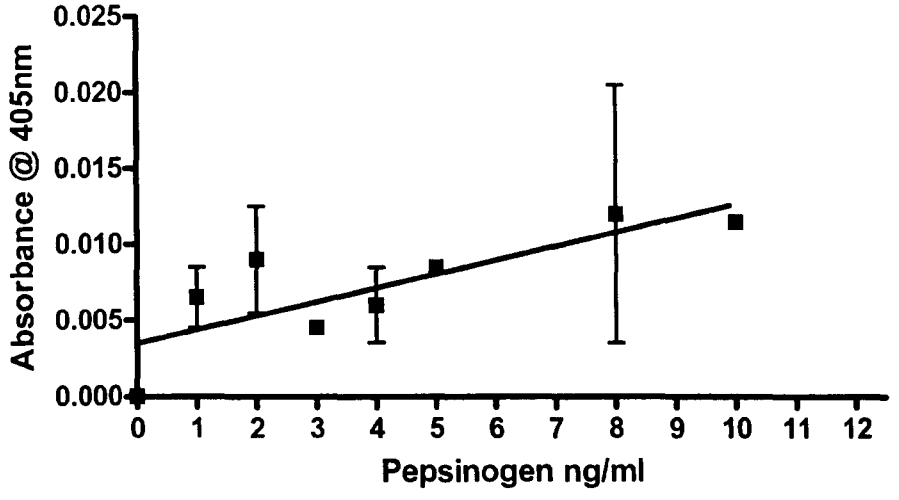


Figure 4.1 Standard curve with SEM from a sandwich ELISA with anti-human pepsinogen as the capture antibody (1/100), anti-pepsin as the detection antibody (1/100) and activated human pepsinogen as the standard. r^2=0.6526, slope=0.0009. Data is representative of 2 experiments.

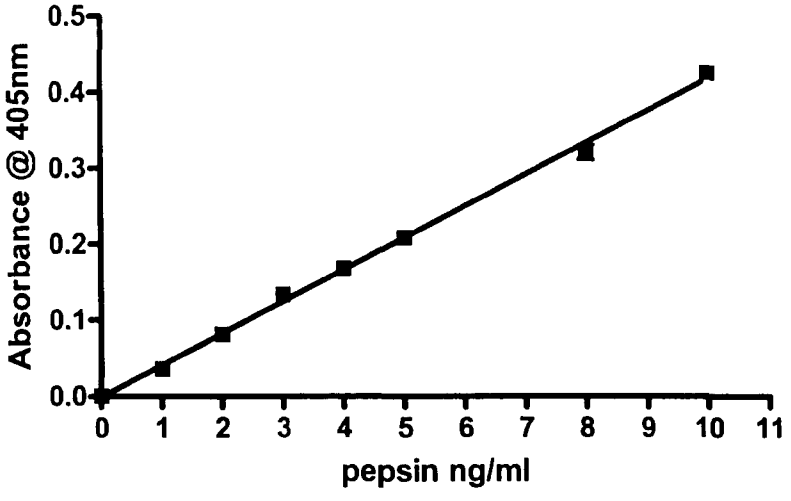


Figure 4.2 Standard curve with SEM from a sandwich ELISA using anti-porcine pepsin as both the capture (1/2000) and detection antibody (peroxidase conjugated, 1/10000) with porcine pepsin as the standard. r^2=0.9976, slope=0.042. Data is representative of 6 experiments.

Pepsin ng/ml → / Sample ↓	Neat BAL	5	10	15	20	40
A	0.2	3.1 62%	9.5 95%	11.6 77%	15.8 79%	21.6 54%
B	0.9	3.9 78%	8.4 84%	8.6 57%	13.8 69%	18.7 47%
C	0.1	3.3 66%	7.4 74%	11.3 75%	14.6 73%	20.0 50%

Table 4.1 Values of pepsin in ng/ml and percentage recovery from bronchoalveolar lavage spiked with porcine pepsin. Neat values (pepsin recovered from BAL with no added pepsin) have been subtracted from the spiked samples. The capture antibody was diluted to 1/2000 and the detection 1/10000. Data is representative of 3 experiments.

Capture antibody dilution →	1/2000 (Taken from table above)	1/1000	1/500
Neat BAL	0.2	0	0
10ng/ml	9.5 95%	6.9 69%	6.1 61%
20ng/ml	15.8 79%	15.3 77%	15.0 75%
40ng/ml	21.6 54%	26.6 67%	31.5 79%

Table 4.2 Values of pepsin in ng/ml and percentage recovery from bronchoalveolar lavage spiked with porcine pepsin. Neat values have been subtracted from the spiked samples. Differences in levels recovered from neat BAL are due to interassay variation. Detection antibody was diluted to1/10000. Data is representative of 3 experiments.

The standard curves (0-20ng/ml) for the different antibody dilutions (1/2000, 1/1000 and 1/500) were also compared (r^2=0.9902, 0.9935 and 0.9921 respectively). As 1/1000 gave a slightly better r^2 value and would use less antibody than 1/500, this dilution was used for further assays.

Spiking experiments were performed in a new BAL sample (made up to either 20 or 50ng/ml pepsin) with the new capture antibody concentration (1/1000) and with dilutions (1/2, 1/4 or 1/8 for 20ng/ml and 1/4 for 50ng/ml) to ensure the levels fit within or near to the top end of the standard curve (0-10ng/ml). Samples that were previously run using the slot/blot technique were also analysed to compare the assays (tables 4.3.1 and 4.3.2).

4.2.2 Removing interference within the sandwich ELISA

Pepsin recovery was poor in the spiked BAL at 50ng/ml (68% for neat and 38% when diluted 1/4, table 4.3.1) and also in the previously measured lavage. Levels were much lower than expected in samples 2, 3 and 4 (13%, 26% and 19% of expected based on the slot/blot ELISA respectively, table 4.3.2), suggesting that there was some interference within the assay, preventing the pepsin antibody binding the pepsin in the lavage. In an attempt to overcome this interference wells were saturated with capture antibody in the hope that there would still be enough available to capture any pepsin in the BAL despite the interference. Capture antibody was diluted 1/100 and the samples previously measured using the slot blot were repeated (table 4.3.2). Samples 1, 2 and 4 all measured higher levels of pepsin than when using the antibody at a dilution of 1/1000 (table 4.3.2).

However, levels were still not as high as previously measured in samples 2, 3 and 4 (29%, 14% and 41% of expected levels respectively) and the recovery in sample 1 was 263%.

Spiking experiments were also performed for 1, 5, 10 or 50ng/ml and recovery was still varied despite the increase in capture antibody concentration (range 80-136%, table 4.3.3), suggesting further investigations were required.

In an attempt to remove any small molecules that may interfere in the assay BAL samples were spiked with 5, 10 or 50ng/ml pepsin and then split into two. Half of the sample was dialysed against phosphate buffered saline (PBS) overnight at 4°C. Both dialysed and non-dialysed samples were analysed using the ELISA and compared.

There was an improvement in pepsin recovery from BAL spiked with 10ng/ml porcine pepsin after dialysing the samples (table 4.4); however recovery was over 200% for 5ng/ml and was not improved for 50ng/ml. This wide variation in levels recovered suggests that small molecules within the BAL were not causing the interference. To attempt to remove any larger molecules that may cause interference spiked BAL samples (5, 10 and 50ng/ml) were centrifuged for 10 minutes at 14000 rpm and room temperature (Eppendorf microcentrifuge, Germany). The recovery of pepsin in the centrifuged samples was improved for samples spiked with 5ng/ml (92% vs. 192% in non-centrifuged samples) however spiking with other concentrations was not consistent enough even to allow for a

correction factor (recovery ranged from 45% to 165%, table 4.4); therefore further investigations were still requited.

The standard curve used to determine the pepsin concentration is linear between 0 and 10ng/ml and is presumed to remain linear above 10ng/ml. However if this was not the case any extrapolated values above 10ng/ml could be incorrect. Therefore a standard curve using porcine pepsin of between 0 and 100ng/ml was carried out to ensure that it remained linear above 10ng/ml (figure 4.3). The standard curve was linear up to 100ng/ml; therefore, the difference in recovery for more concentrated samples can not be explained by non-linearity.

Sample	Recovered pepsin ng/ml/ %
Neat BAL	0.6
20ng/ml	15.4 77%
20ng/ml diluted 1/2	15.5 78%
20ng/ml diluted 1/4	18.7 94%
20ng/ml diluted 1/8	16.4 82%
50ng/ml	34.2 68%
50ng/ml diluted 1/4	19.0 38%

Table 4.3.1 Values of pepsin in ng/ml and percentage recovery from bronchoalveolar lavage spiked with porcine pepsin. Neat values have been subtracted from the spiked samples. Capture antibody was diluted to 1/1000 and detection to1/10000. Data is representative of 3 experiments.

Previously measured samples	Slot/blot results	Sandwich ELISA (1/1000 capture)	Sandwich ELISA (1/100 capture)
1	6.4	5.1 80%	16.8 263%
2	51.7	6.5 13%	14.8 29%
3	8.1	2.1 26%	1.1 14%
4	10	1.9 19%	4.1 41%

Table 4.3.2 Values of pepsin ng/ml and percentage recovery of expected based on the slot/blot from BAL using a slot/blot and a sandwich ELISA. Capture antibody dilutions are stated in the table and detection was diluted to 1/10000. Data is representative of 2 experiments for the slot/blot and 3 experiments for the sandwich ELISAs.

Sample	Recovered pepsin ng/ml and %
Neat BAL	0
1ng/ml	0.8 80%
5ng/ml	5.4 108%
10ng/ml	11.3 113%
50ng/ml	68.0 136%
50ng/ml diluted 1/10	46.2 92%

Figure 4.3.3 Values of pepsin in ng/ml and percentage recovery from bronchoalveolar lavage spiked with porcine pepsin. Capture antibody was diluted to 1/100 and detection to1/10000. Data is representative of 3 experiments.

Sample	Spiked BAL (no dialysis or centrifugation)	Dialysed	Centrifuged
BAL spiked with 5ng/ml	9.6 192%	14.6 292%	4.6 92%
BAL spiked with 10ng/ml	6.6 66%	9.0 90%	15.4 154%
BAL spiked with 50ng/ml	34.1 68%	31.9 64%	82.5 165%
BAL spiked with 5ng/ml Diluted 1/10	9.7 194%	2.2 44%	3.4 68%
BAL spiked with 10ng/ml Diluted 1/10	12.8 128%	2.2 22%	4.5 45%
BAL spiked with 50ng/ml Diluted 1/10	26.4 53%	19.6 39%	38.2 76%

Table 4.4 Values of pepsin ng/ml and % recovery from spiked BAL, spiked BAL that has been dialysed against PBS overnight at 4°C and spiked BAL that has been centrifuged at 14000rpm for 10 minutes. Capture antibody was diluted to 1/100 and detection 1/10000. Data shows the average of 2 experiments.

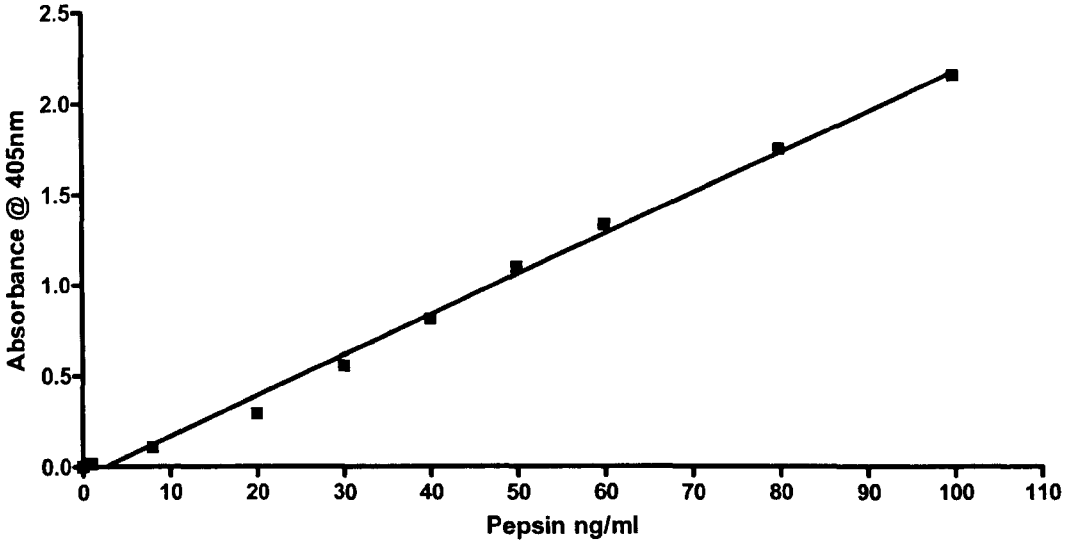


Figure 4.3 Standard curve with SEM from a sandwich ELISA using anti-porcine pepsin as both the capture (1/100) and detection antibody (peroxidase conjugated, 1/10000) with porcine pepsin as the standard. r^2=0.9952, slope=0.0222. Data is representative of 2 experiments.

With the dialysis and centrifugation steps failing to eliminate the variability in the assay, a literature search using Medline was performed to identify other possible interfering factors. One search documented the presence of human anti-animal antibodies as an interfering factor in immunoassays (Kricka 1999). The most common human anti-animal antibody is human anti-mouse, however human anti-rabbit, -goat, -sheep, -cow,-pig, -rat, and -horse have been identified and they can cause both false positive and false negative results within immunoassays (figure 4.4).

The Biodesign pepsin antibody is raised in goat; therefore if the BAL samples contained any human anti-goat it is possible that they could bind to the pepsin antibody and prevent it from capturing or detecting any pepsin present in the sample. The suggested solution to prevent this type of interference is to incubate the sample with a blocking agent containing IgG from the animal, in this case goat. This seemed like a sensible step to take, as whatever is causing the interference, whether it be human anti-goat or not, it could bind to goat IgG instead of the anti-pepsin, allowing more antibody to bind pepsin within the sample, reducing the possibility of a false negative result.

Spiking experiments giving a final concentration of 1, 5, 10 or 50ng/ml were repeated, this time adding 50ng/ml goat IgG to the reagent buffer used to dilute samples and make up the standard curves. The average recovery was 100.2% (range 61-126%, excluding recovery for 1 ng/ml as it is an outlier and also the lowest level of detection, table 4.5). Although the recovery was not 100% in all

samples it was improved and the variation was reduced, therefore the goat IgG was included in future assays.

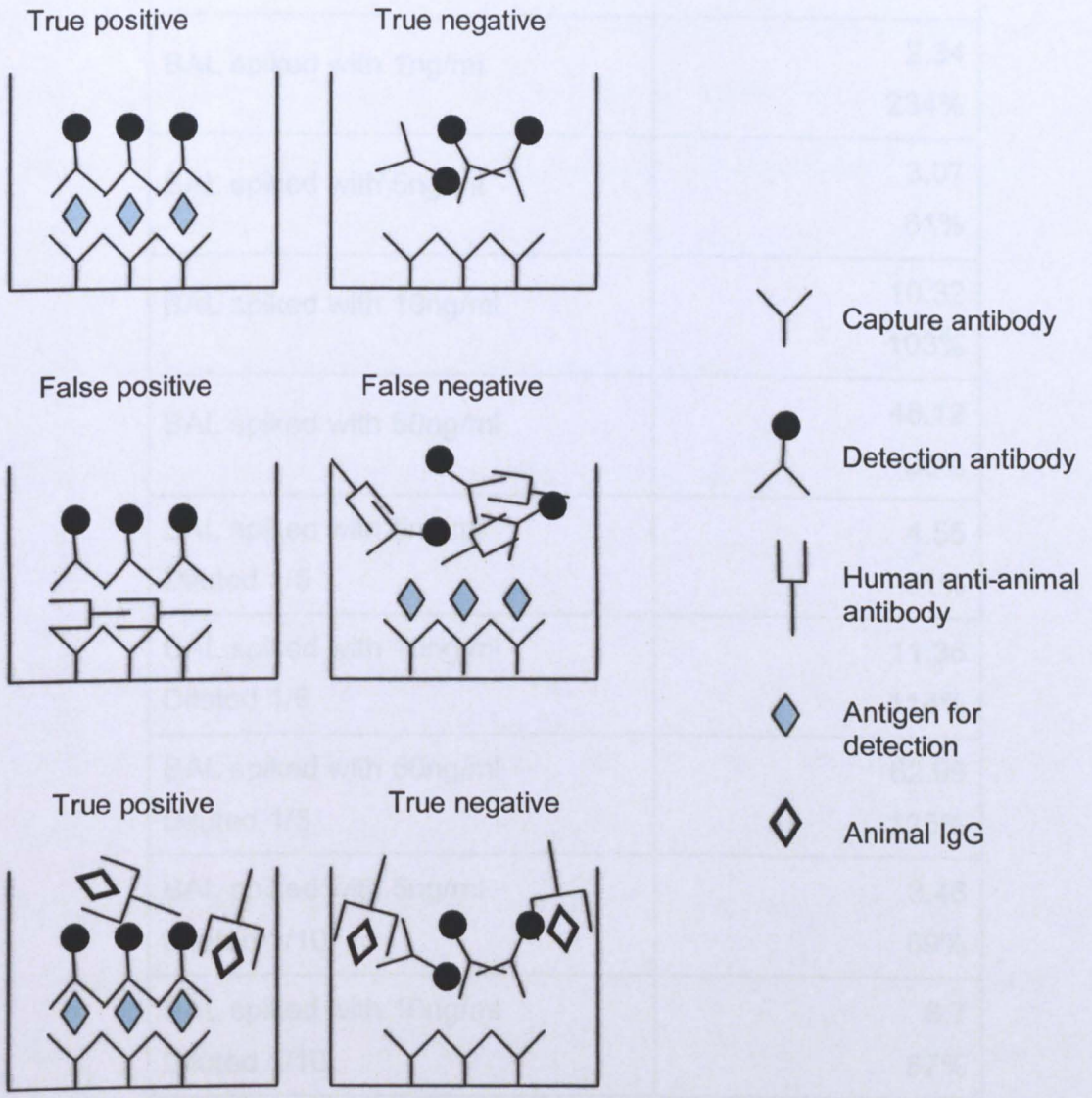


Figure 4.4 A schematic representation of how human anti-animal antibodies can cause interference within immunoassays and how including animal IgG can reduce this interference.

Spiked sample ↓	Recovered pepsin ng/ml and %
BAL spiked with 1ng/ml	2.34 234%
BAL spiked with 5ng/ml	3.07 61%
BAL spiked with 10ng/ml	10.32 103%
BAL spiked with 50ng/ml	48.12 96%
BAL spiked with 5ng/ml Diluted 1/5	4.55 91%
BAL spiked with 10ng/ml Diluted 1/5	11.36 114%
BAL spiked with 50ng/ml Diluted 1/5	62.99 126%
BAL spiked with 5ng/ml Diluted 1/10	3.46 69%
BAL spiked with 10ng/ml Diluted 1/10	8.7 87%
BAL spiked with 50ng/ml Diluted 1/10	47.07 94%

Table 4.5 Values of pepsin in ng/ml and percentage recovery from bronchoalveolar lavage spiked with porcine pepsin. Capture antibody was diluted to 1/100 (with buffer containing 50ng/ml goat IgG) and detection to1/10000. Data shows the average of 3 experiments.

To summarise, the sandwich ELISA was performed using a porcine pepsin standard with a porcine pepsin antibody (Biodesign International, USA) as both the capture (1/100) and detection (HRP-conjugated, 1/10000). Samples were run neat and with a 1/5 dilution to ensure they fit within the standard curve and samples and standards were diluted in buffer containing 50ng/ml goat IgG.

4.3 Production of a human gastric protein standard

After ethical approval was granted it was possible to collect gastric juice samples from patients undergoing endoscopy. These samples could then be used as controls to further validate the assay, as up until this point only porcine pepsin had been used in the spiking experiments since human pepsin is not commercially available. Four samples were collected and analysed with the sandwich ELISA. The basal level of pepsin in the gastric juice is approximately 200µg/ml in normal subjects (Pearson et al. 1986), however, this may be an over estimation as some of the patients used to collect gastric juice from were on proton pump inhibitors, therefore a range of dilutions were performed up to 1/40000 (to give approximately 5ng/ml) to ensure the samples fit within the standard curve and did not saturate the plate (table 4.6).

There was a very large variation in levels measured over the different dilutions. This could be a result of either errors being multiplied up when accounting for the dilution, i.e. an error of only 0.5ng/ml becomes 20000ng/ml when multiplied up by 40000, or when diluting the sample it is possible that any interfering factor is also

being diluted out allowing the antibodies to bind more pepsin, or it could be a combination of both.

Dilution of gastric juice ↓	Sample 1	Sample 2	Sample 3	Sample 4
Neat juice	0	1.56	24.38	0
1/4	0	2.4	111.98	0.44
1/20	35.03	4.52	12.57	33.01
1/100	226.9	3.88	29.78	221.14
1/1000	1261.78	269.01	556.77	1506.38
1/10000	6862.63	3121.75	9020.84	12042.32
1/40000	20544.28	14213.55	29752.61	28601.57

Table 4.6 Values of pepsin in ng/ml measured in 4 samples of gastric juice over a range of dilutions using the sandwich ELISA (capture antibody diluted 1/100 and detection diluted 1/10000). Samples have been multiplied by the dilution factor. These values are from a single experiment.

As detailed previously, human pepsin is not commercially available so the gastric juice was purified to produce a human pepsin standard.

Pooled samples 1-4 were adjusted to a density of 1.42g/ml with caesium chloride and fractionated by ultra-centrifugation at 4°C and 40000rpm for 48h (Centrikon T-1170). The resulting 8 fractions were separated and weighed to ensure a density gradient was present (1.33-1.58g/ml, table 4.7). Each fraction was then exhaustively dialysed against deionised water for 3 days with 4-6 changes per day.

After dialysis the absorbance of each fraction was measured at 260 and 280nm on a spectrophotometer to determine the location of protein (i.e. pepsin containing fractions table 4.7). Protein containing fractions 1-3 were freeze dried. As fraction 1 contained a precipitate it was centrifuged for 5 minutes at room temperature and 1500rpm (Centra-3, IEC) prior to freeze-drying. The pellet and supernatant were freeze-dried separately.

The individual freeze-dried fractions were run on an SDS-PAGE gel using the Pharmacia PHAST gel system to determine whether they contained pepsin. The freeze dried fractions and two porcine pepsin controls were made up to a concentration of 2.5mg/ml in a reducing buffer. 5µl of each fraction/control was loaded onto the gel (chapter 2.3.2.1). Gels were stained for protein using the coomassie blue method and were then scanned on a densitometer (GS-800, Biorad, UK). The relative mobility of each of the fractions was determined by calculating the distance moved into the gel from the point of application (table

4.8). Fractions 1 (supernatant), 2 and 3 contained just one major band that moved a similar distance to the porcine pepsin; therefore it can be presumed that they contain a protein with the same molecular weight (approximately 35KDa).

Fraction	Density g/ml	Optical Density	
		260nm	280nm
1	1.33	2.522	Off scale
2	1.35	1.046	1.304
3	1.39	0.640	0.720
4	1.40	0.401	0.414
5	1.42	0.512	0.503
6	1.46	0.402	0.448
7	1.49	0.434	0.455
8	1.58	0.516	0.456

Table 4.7 Density in g/ml and optical density at 260 and 280nm of each fraction of the pooled gastric juice samples. Fraction 1 contained a dark green precipitate resulting in the absorbance being off the top of the scale at 280nm.

Sample	Relative mobility	Protein concentration compared to control average (mg/ml)
Control 1	0.59	
Control 2	0.58	
Fraction 1 (pellet)	0.77	8.89
Fraction 1 (supernatant)	0.60	0.14
Fraction 2	0.59	1.15
Fraction 3	0.60	0.04

Table 4.8 Relative mobility (the distance moved from loading divided by the distance moved by the tracking dye) for each protein containing fraction obtained from purifying human gastric juice. Protein concentration compared to the average of the controls has also been calculated using the optical densities of each fraction. 5µl of a 2.5mg/ml solution of the freeze-dried fraction was used to run the gel.

Staining the gel with coomassie blue allowed a semi-quantitative value for the protein concentration of each fraction (compared to the average of the porcine pepsin controls) to be calculated using the optical densities (table 4.8). Each fraction was made up to a concentration of 2.5mg/ml; however this was not reflected in the levels calculated by the protein assay (lower concentrations were seen in fractions 1 (supernatant), 2 and 3). This suggested that the freeze-dried fractions 1 (supernatant), 2 and 3 contained other non-protein components. Fraction 1 (pellet) had a high relative mobility compared to the porcine pepsin and also contained 8.89mg/ml protein, which was not plausible since only 2.5mg/ml was originally added, suggesting the fraction contained contaminants and was consequently discarded.

4.3.1 Determination of protein concentration of the purified gastric juice by ELISA

The remainder of the fractions were assayed for pepsin using the sandwich ELISA. The fractions were made up to a concentration of 1mg/ml pepsin, assuming that all the protein in the freeze-dried fraction was pepsin (i.e. fraction 2 contained 46% protein; therefore to make a 1mg/ml pepsin solution 460µl of reagent buffer should be added to 1mg of fraction 2). Fractions 1 and 3 came back negative and fraction 2 only contained 0.48ng/ml, suggesting that either the purification process had not been successful or that the ELISA was not recognising human pepsin.

To further validate the assay purified human pepsin (pepsin 3A, ion-exchange HPLC purified, donated by Technostics, UK) was used to run human pepsin standard curves using the sandwich ELISA (0-50ng/ml, figure 4.5). There was an increase in absorption with increasing pepsin concentration, however the increase was very small (slope=0.0004 compared to 0.0346 for porcine pepsin) and therefore could not be used.

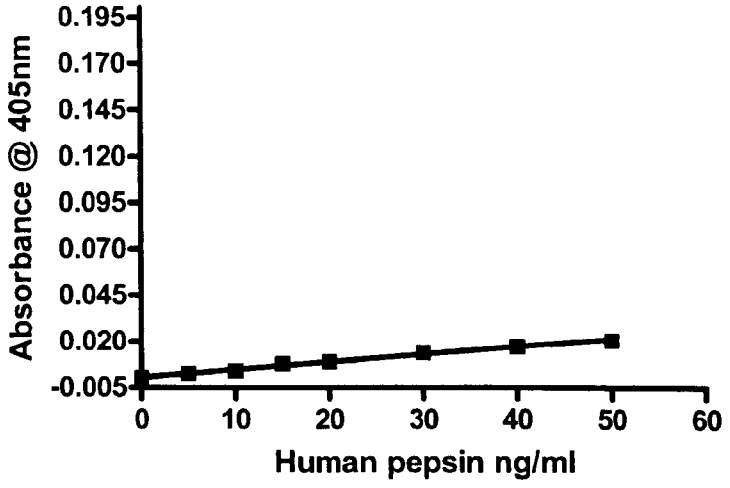


Figure 4.5 Standard curve with SEM from a sandwich ELISA with anti-porcine pepsin as both the capture (diluted to 1/100) and detection (1/10000) with human pepsin as the standard (Technostics, UK). r^2 =0.9924, slope=0.0004. This data is representative of 2 experiments.

4.4 Development of a direct ELISA for the determination of pepsin concentration

Based on the absorbance measured with 50ng/ml pepsin the antibody appears to be approximately 50 times less sensitive to human pepsin (Technostics, UK) than porcine pepsin (Sigma, UK) in the sandwich format (absorbance for 50ng/ml pepsin was 0.021 and 1.099 respectively when the substrate was read at 405nm). It is possible that the lack of sensitivity is related to the number of epitopes that are available for antibody binding, and if so, it would appear that there are more on porcine pepsin, allowing increased sensitivity. As the slot/blot ELISA appeared to demonstrate a better sensitivity using a single antibody it was decided to return to this direct format in a 96 well plate.

Human and porcine pepsin standards (Technostics and Sigma, UK respectively) of between 0-50ng/ml were coated onto a plate overnight at room temperature and were then incubated with the pepsin antibody (Biodesign International, USA) diluted to 1/2000 according to manufacturer's suggestion, followed by a secondary, horseradish peroxidase conjugated, antibody (anti sheep/goat, Sigma UK) diluted 1/10000. Once the substrate had been added and colour allowed to develop the plate was read at 405nm on a standard plate reader. Both human and porcine pepsin showed an increase in absorbance with increasing pepsin concentration (r^2 =0.9838, slope=0.0104 and r^2 =0.9871, slope=0.0190 respectively, figure 4.6). However, the porcine standards gave twice the absorbance of the human standards, demonstrating that the antibody is twice as sensitive to porcine pepsin as human pepsin.

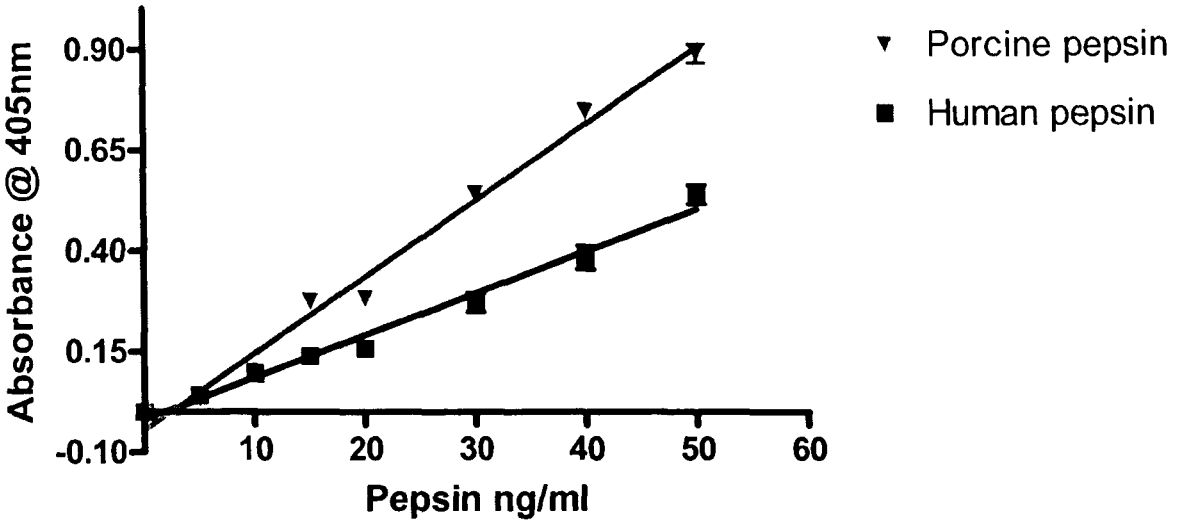


Figure 4.6 Porcine and Human pepsin standard curves with SEM from a direct ELISA using anti-pepsin (1/2000) followed by anti sheep/goat (peroxidase conjugated 1/10000). Porcine pepsin: r^2=0.9877, slope = 0.019, human pepsin: r^2=0.9858, slope = 0.010. The graph demonstrates that the Biodesign antibody is twice as sensitive to porcine pepsin than human pepsin (absorbencies at 20, 30 and 40ng/ml are 0.281 vs. 0.164, 0.543 vs. 0.271 and 0.748 vs. 0.383 respectively). Data is representative of 3 experiments.

4.4.1 Differences in antibody reactivity with human and porcine pepsin

To further understand the relationship between porcine and human pepsin antibody binding spiking experiments were performed by making BAL up to a concentration of 5, 20 or 50ng/ml using either human or porcine pepsin (Technostics UK and Sigma UK respectively). Levels were measured using both human and porcine standard curves (table 4.9).

When analysing BAL spiked with human pepsin, recovery using the human pepsin standard curve is approximately twice that of when using the porcine pepsin standard curve (average percentage recovery is 14.7% and 6% respectively). Again, this data suggests that the antibody is approximately twice as sensitive to porcine pepsin as human pepsin. Using this information commercially available porcine pepsin can be used as a standard throughout the assay; however a correction factor of 2 is required when analysing human samples.

Human and porcine pepsin recovery from the BAL is poor when using either the human or porcine pepsin standard curve to analyse the samples (maximum recovery is 16% in BAL spiked with human pepsin using the human standard curve and 30% in BAL spiked with porcine pepsin using the porcine standard curve). As well as demonstrating that the recovery is poor this data also suggests that the recovery of human pepsin from BAL is worse than porcine pepsin even when using a human pepsin standard, or that the human pepsin standard is not quite as pure as the porcine pepsin standard.

Sample	Recovered pepsin using human pepsin standard curve ng/ml	Recovered pepsin using porcine pepsin standard curve ng/ml
Neat BAL	3.4	1.5
BAL spiked with 5ng/ml porcine pepsin	2.7 54%	1.2 24%
BAL spiked with 20ng/ml porcine pepsin	11.7 59%	4.9 25%
BAL spiked with 50ng/ml porcine pepsin	36.7 73%	15.2 30%
BAL spiked with 5ng/ml human pepsin	0.8 16%	0.3 6%
BAL spiked with 20ng/ml human pepsin	2.7 14%	1.2 6%
BAL spiked with 50ng/ml human pepsin	7.2 14%	3.1 6%

Table 4.9 Values of pepsin (ng/ml) and percentage recovery from BAL spiked with human or porcine pepsin (Technostics, UK and Sigma, UK respectively) using either human or porcine standard curves. Neat values have been subtracted from the spiked samples. Data shows the average of 2 experiments.

4.4.2 Improving recovery of pepsin in BAL samples

To investigate how to improve recovery of pepsin in the BAL spiking experiments were performed by adding a known concentration of fraction 2 of the purified gastric juice (henceforth termed 'purified gastric protein') to phosphate buffered saline (PBS) and BAL to compare recovery rates, since PBS should contain no contaminating factors. Recovery of pepsin (using the purified gastric protein) in the BAL was poor (table 4.10) so BAL was diluted before a known concentration of purified gastric protein was added to investigate whether the interfering factor could be diluted out. A solution of purified gastric protein (1.6mg/ml purified gastric protein in deionised water) was used to spike PBS, BAL and diluted BAL (1/5 with PBS). Recovery compared to levels measured in PBS was on average 33% in neat BAL and 112% in diluted BAL (table 4.11), suggesting that the recovery can be improved by diluting BAL 1/5. Differences in levels measured with each run suggests that the pepsin was not homogenous throughout the purified gastric protein, however the purified protein can still be used to investigate recovery if the same solution is used to spike the PBS, neat BAL and diluted BAL. These results also suggest that not all of the protein in the purified gastric protein is pepsin, even though only one band was present on the SDS-PAGE gel when the gastric juice was purified. This suggests that the other protein components have a large molecular weight and therefore stayed at the origin, or have a low molecular weight and have run off the end of the gel.

Spiking experiments were repeated to ensure reproducibility using an alternative substrate (tetramethylbenzidine (TMB), R&D systems). Again, a solution of

purified gastric protein (1mg/ml purified gastric protein in deionised water) was used to spike PBS, neat BAL and BAL diluted 1/5 in PBS. Interestingly recovery was higher in neat BAL than PBS and diluted BAL with the alternative substrate, however compared to PBS average recovery was 91% in diluted BAL and 148% in neat BAL which again demonstrates that diluting the BAL gives a more accurate measurement of pepsin (table 4.12).

Previous investigations have shown that the pepsin antibody shows a small amount of non-specific reactivity with the serum proteins albumin and γ-globulins, however antibody binding of only 3.5% and 0.9% of that for 1μg of porcine pepsin was observed for 200μg of γ-globulins and albumin respectively (Tasker 2003).

To assess the reproducibility of the assay the coefficient of variation (CV) was calculated using an internal standard by dividing the standard deviation by the mean. The mean Intra-assay CV was 6.1% with a mean inter-assay CV of 9.7%.

In conclusion, human pepsin can be measured with a direct 96-well plate ELISA using a porcine pepsin antibody (Biodesign International, USA) and a porcine pepsin standard. However a correction factor of 2 must be included to allow for human/porcine differences in antibody binding and also, bronchoalveolar lavage (BAL) should be diluted 1/5 to remove any factors that may interfere with the detection of human pepsin. Negative controls should also be performed for all samples to eliminate any background readings by omitting samples from the primary antibody incubation.

Concentration of purified gastric protein	Recovered pepsin from PBS ng/ml	Recovered pepsin from BAL ng/ml
250ng/ml	12.6	0.6
500ng/ml	35.0	0.6
1000ng/ml	64.0	0.8

Table 4.10 Values of pepsin recovered from purified gastric protein diluted in PBS and BAL. Levels have been multiplied by 2 to correct for human/porcine differences in antibody binding. Data is representative of 3 experiments.

Sample	Recovered pepsin ng/ml
1.6mg/ml solution of purified gastric protein diluted 1/10 in PBS	684.1
1.6mg/ml solution of purified gastric protein diluted 1/10 in neat BAL	224.3 (33% of pepsin recovered from PBS)
1.6mg/ml solution of purified gastric protein diluted 1/10 in BAL diluted 1/5 with deionised water	765.4 (112% of pepsin recovered from PBS)

Table 4.11 Values of pepsin in ng/ml recovered from PBS, neat BAL and diluted BAL spiked with purified gastric protein. Values have been multiplied up by the dilution factor and have also been corrected for differences in human/porcine antibody binding. Data shows the average of 2 experiments.

Sample ↓	pepsin recovered ng/ml and % of PBS recovery
PBS	1408.1
Neat BAL	2083.7 148%
BAL diluted 1/5 (in deionised water)	1285.5 91%

Table 4.12 Values of pepsin in ng/ml recovered from PBS, neat BAL and diluted BAL spiked with purified gastric protein. Values have been multiplied up by the dilution factor and have also been corrected for differences in human/porcine antibody binding. Data shows the average of 5 experiments.

4.5 Discussion

Gastric aspiration is well documented in many lung diseases as well as lung transplant rejection (Feigelson et al. 1987; Tobin et al. 1998; Brodzicki et al. 2002; Raghu 2003). Measuring biomarkers is becoming increasingly important in identifying which patients are aspirating gastric content into the lungs and are therefore most likely to benefit from anti-reflux treatment. Previously in our laboratory, pepsin levels were measured in the bronchoalveolar lavage (BAL) of transplant patients using a slot/blot ELISA (Ward et al. 2005; Stovold et al. 2007). This assay was however limited in the number of samples it could process at a time, therefore, a new assay was developed based on a 96-well plate format.

Through a series of investigations I have shown that the new plate ELISA fulfils the necessary requirements of the assay set out before development began. These investigations have shown that with a correction factor, the assay, based on a porcine pepsin antibody can measure human pepsin using porcine pepsin as a standard. The assay has a lower limit of detection of 1ng/ml showing it is sensitive enough to measure levels of pepsin previously determined using the slot/blot ELISA. Finally, the inter-assay coefficient of variance was 9.7% indicating that the assay is reproducible.

Despite sandwich ELISAs generally demonstrating good sensitivity this was not the case for pepsin and therefore a direct format was chosen instead. This lack of pepsin detection using the human pepsinogen and the porcine pepsin antibodies as a pair could be explained by a number of things; firstly, the capture antibody

(anti human pepsinogen) may not be capable of binding human or porcine pepsin, as it may recognise part of the 44 amino acid chain that is lost when pepsinogen is converted to pepsin. This could be investigated using pepstatin, as this is part of the 44 amino acid sequence that is lost during pepsinogen conversion. Secondly, it is possible that the capture antibody can bind human and porcine pepsin, but when the capture antibody is bound the epitope for the detection antibody (anti porcine pepsin) is obstructed, or, finally it is also possible that the capture antibody can recognise human pepsin but only poorly. This may be the case as when activated human pepsinogen was used as the standard there was an increase in absorbance with an increase in concentration, however it was a very small increase, suggesting poor antibody binding (figure 4.1).

The lack of sensitivity of the sandwich ELISA when using the porcine pepsin antibody as both the capture and detection could be explained by porcine pepsin having two epitopes available for the antibody to recognise and human pepsin having only one. If human pepsin only contained one epitope it could be captured but not detected. This would also explain the two fold difference in antibody reactivity between porcine and human pepsin when using the direct format, as there are twice the number of epitopes available on porcine pepsin there is twice the antibody binding and therefore twice the detection (figures 4.6 and 4.7).

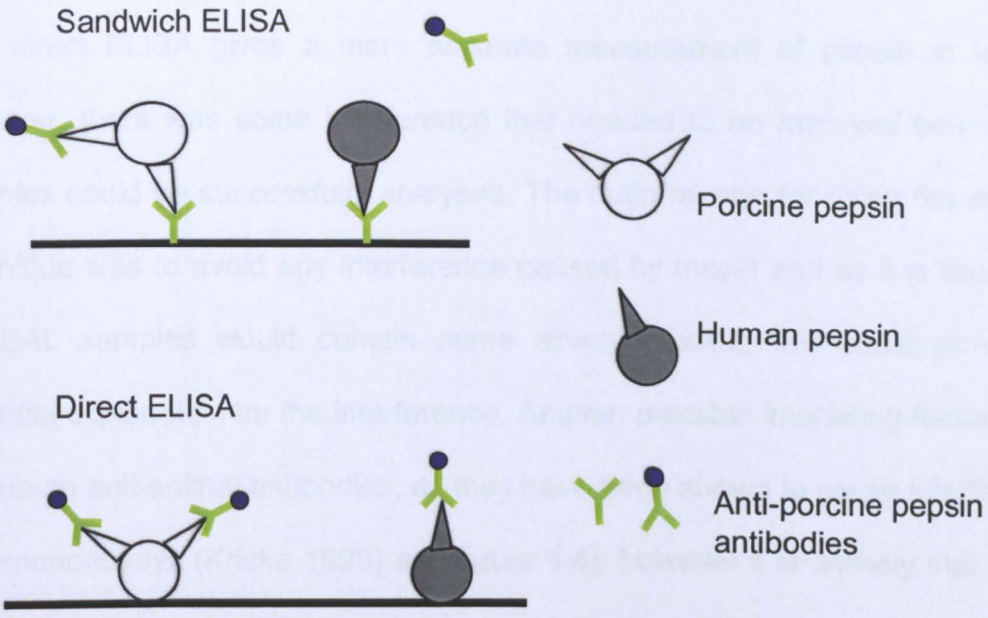


Figure 4.7 A proposed model of porcine pepsin antibody reactivity with porcine and human pepsin in a sandwich and a direct ELISA format.

The direct ELISA gives a more accurate measurement of pepsin in lavage, however, there was some interference that needed to be removed before BAL samples could be successfully analysed. The main reason for using the slot/blot technique was to avoid any interference caused by mucin and as it is likely that the BAL samples would contain some airway mucins; this could provide a potential explanation for the interference. Another possible interfering factor could be human anti-animal antibodies, as they have been shown to cause interference in immunoassays (Kricka 1999) and figure 4.4); however it is unlikely that all the patient's samples would contain these antibodies.

The thorough validation of this assay is important as although measuring biomarkers of aspiration is becoming increasingly popular across lung transplant centres world-wide the literature is still relatively small and the levels of pepsin being reported are reasonably varied. Blondeau et al are reporting levels over 1000ng/ml using a similar direct ELISA. They are using a porcine pepsin antibody as the detection followed by a secondary antibody (goat immunoglobulin G) labelled with horseradish peroxidase and tetramethylbenzidine (TMB) as the substrate (Blondeau et al. 2008). There is no mention of negative controls in their protocols, suggesting they do not perform them. This could help to explain why the group document high levels of pepsin, as any non-specific binding is not investigated and would therefore not be subtracted from the final concentration of pepsin in the BAL sample. In addition, there is also no mention of the BAL being diluted before it is analysed, my investigations have shown that neat BAL can give an over estimation of pepsin when using TMB as a substrate (table 4.12).

On the other end of the scale Farrell et al are reporting low levels of pepsin, in 74 patients the median pepsin level was 0ng/ml with an inter-quartile range of 0-1.5ng/ml (Farrell et al. 2006). The group uses a sandwich ELISA with two porcine pepsin antibodies as the capture and detection and the group shows mean recovery from spiking experiments to be 88.2%, however, they do not mention if the BAL was spiked with human or porcine pepsin. From my investigations I have shown it is possible to obtain good recovery of porcine pepsin in spiking experiments with a sandwich ELISA; however this is not the case for human pepsin. It is possible that the group are underestimating the concentration of pepsin in their samples due to human pepsin only having one available epitope for the porcine pepsin antibody to bind to; therefore once it is captured it can not be detected effectively (figure 4.7).

The differences in methods used to detect pepsin and the variation in levels reported make it impossible to allow direct comparisons across centres. If the assay was standardised data could then be shared between centres. This is only practical if the assay is sensitive and reproducible, and through a sequence of investigations I have shown this to be the case for the plate ELISA outlined in this research.

Chapter 5

Longitudinal analysis of pepsin present in BAL from lung transplant recipients

5.1 Introduction

The biggest limiting factor to long term survival in lung transplantation is the onset of bronchiolitis obliterans syndrome (BOS), and despite advances in surgical techniques and sophisticated immunosupression there have been no significant improvements in long term outcomes. Traditional treatments have focussed on the alloimmune mechanisms, however, some non-alloimmune mechanisms have now also been implicated in this chronic disease process, including the aspiration of gastric contents into the lungs.

Previous work from our group has shown that pepsin, a marker of gastric aspiration, can be measured in the bronchoalveolar lavage (BAL) from transplant patients in a cross-sectional cohort (Stovold et al. 2007). This cross-sectional study did not show an association between high levels of pepsin and BOS, however, it did show an association between acute rejection and high levels of pepsin.

From the above study it was not possible to investigate how pepsin levels in BAL vary with time, for example, a patient may experience fewer episodes of gastric aspiration the further post transplant they are due to the body adjusting to the operation and medication that may cause gastro-oesophageal reflux and

subsequent aspiration. It was also not possible to investigate whether high levels of pepsin in the lavage can predict for BOS. In order to attempt to address these questions a cohort of patients were recruited and longitudinal BAL samples were taken at 1 week, 1, 3, 6 and 12 months post-transplant and were analysed for pepsin.

A longitudinal approach allows a broader range of analysis to be performed, including a sequential cross sectional analysis (i.e. analysing pepsin levels at 1 week, 1, 3, 6 and 12 months) to investigate if high levels of pepsin are associated with different grades of rejection at different time points. Additionally, survival analyses can be performed, including whether high levels of pepsin at an early time point can predict for BOS. As far as I am aware this is the first longitudinal analysis of BAL pepsin levels in lung transplant recipients.

5.2 Patient demographics

Patient demographics are summarised in table 5.1. Forty patients were recruited to the study (17 male, age range 19-61 years) and fourteen of those patients went on to develop BOS during the follow-up time (last follow up June 2006). Between three and eight lavage samples were taken from each patient and were analysed for pepsin (table 5.2). In addition, five to seven transbronchial biopsies (TBB) were taken at each bronchoscopy to assess acute vascular and airway inflammation according to standard criteria (Yousem et al. 1996) by a pathologist.

An *a priori* decision was made by a consulting clinician to include an additional seven samples taken from stable lung transplant patients as a control group. These patients were optimally stable and had no evidence of acute rejection or BOS or any other problems commonly experienced by lung transplant recipients (table 5.3).

Subject no.	Age	Sex	Diagnosis	Operation	BOS ever	Date of BOS
1	20	m	cf	BLT	n	
2	59	f	emp	SLT	n	
3	61	f	lam	SLT	n	
4	31	f	cf	BLT	n	
5	50	f	emp	SLT	n	
6	57	m	emp	SLT	n	
7	55	f	lpf	SLT	y	07.11.03
8	36	f	emp	SLT	n	
9	27	m	cf	BLT	n	
10	20	f	cf	BLT	y	26.06.02
11	26	f	ob	BLT	y	03.09.03
12	41	f	ipf	BLT	y	20.10.04
13	41	f	lam	SLT	n	
14	29	m	cf	BLT	y	03.02.03
15	19	m	cf	BLT	y	29.01.03
16	39	m	emp	BLT	n	
17	31	f	cf	BLT	n	
18	31	f	cf	BLT	n	
19	48	f	lam	SLT	n	
20	35	f	pph	HLT	n	
21	48	f	ob	BLT	y	01.10.04
22	57	f	emp	SLT	y	05.01.04
23	24	f	cf	BLT	n	
24	41	m	cf	BLT	n	
25	25	f	pph	HLT	n	
26	21	m	cf	BLT	y	28.08.02
27	36	f	pph	HLT	n	
28	45	f	emp	BLT	n	
29	47	f	emp	SLT	n	
30	16	f	cf	BLT	y	21.02.03
31	26	m	cf	BLT	n	
32	28	m	cf	BLT	y	13.08.04
33	40	m	ipf	SLT	y	07.07.04
34	46	m	ipf	BLT	n	
35	28	m	cf	BLT	y	19.02.03
36	52	m	cf	SLT	y	12.02.02
37	37	m	cf	BLT	n	
38	48	m	ipf	BLT	n	
39	20	f	cf	BLT	n	
40	35	m	sar	SLT	n	

Table 5.1. Patient demographics. ipf - idiopathic pulmonary fibrosis, lam - lymphangioleiomyomatosis, cf-cystic fibrosis, sar-sarcoidosis, emp-emphysema, pph- primary pulmonary hypertension, ob- obliterative bronchiolitis. slt-single lung transplant, blt- bilateral lung transplant, hlt-heart lung transplant.

Id	DOT	Date of Sample	Pepsin (ng/ml)	Biopsy	BOS Score
1	01.01.02	11.01.02	23.82	A1B1/2	0
1		28.01.02	130.35	A2B1/2	0
1		08.04.02	6.61	A1B0	0
1		08.07.02	34.17	A1B0	0
1		06.01.03	7.31	A0BX	0
2	23.10.02	22.11.02	17.37	A0B1	0
2		22.01.03	20.07	A1B1	0
2		30.04.03	19.1	A1B1	0
2		27.10.03	22.3	A0B0	0
3	19.05.01	25.05.01	4.34	A1BX	0
3		11.06.01	18.53	A0B1	0
3		22.08.01	11.11	A1B1	0
3		14.11.01	21.7	A0B0	0
3		27.05.02	16.61	A0BX	0
4	14.10.01	24.10.01	21.92	A0B0	0
4		19.11.01	8.89	A2B1	0
4		15.04.02	13.66	A0B1	0
4		14.10.02	17.46	A1B1	0
5	16.06.03	24.06.03	12.8	A2BX	0
5		16.07.03	7.4	A2B1	0
5		17.09.03	17.4	A0	0
5		05.01.04	4.3	A1B1	0
5		14.06.04	15	A1/2B1	0
5		08.11.04	18.1	A1B1	0
6	18.05.01	25.05.01	16.61	A1	0
6		20.06.01	13.8	A1B2	0
6		18.07.01	20.46		0
6		05.09.01	8.41	A1B0	0
6		19.11.01	11.98	A1B1	0
6		11.09.02	31.08	A1B0	0
7	23.06.03	04.07.03	23.33	A2B1	0
7		25.07.03	17.9	A0B1	0
7		08.10.03	17.51	A2B2	0p
7		07.11.03	21.95	A1BX	2
7		22.12.03	18.84	A2B3	2
8	27.06.02	05.07.02	26.45	A2	0
8		24.07.02	25.58	A1B1	0
8		21.08.02	14.16	A 1 2	0
8		30.09.02	2.41	A1BX	0
8		29.04.03	14.86	A1B2	0
8		23.06.03	13.13	A1BX	0

Table 5.2 Pepsin levels (ng/ml) recovered from sequential BAL samples from 40 patients. Table also details biopsy and BOS scores associated with each sample. AX and BX denotes un-gradable biopsy. DoT-date of transplant. BOS-bronchiolitis obliterans syndrome.

Id	DOT	Date of Sample	Pepsin ng/ml	Biopsy	BOS
9	27.12.01	15.01.02	5.59	AXBX	0
9		30.01.02	9.90	A1B2	0
9		27.03.02	6.16	A1	0
9		24.06.02	15.49	A1B0	0
9		16.12.02	36.76	A1B1	0
9		10.11.04	21.81		0
10	21.09.01	22.10.01	20.65	A2B0	0
10		19.12.01	12.39		0
10		25.03.02	4.13	A0B1	0
10		26.06.02	2.86		1
10		21.10.02	12.20	A0B1	3
11	14.05.02	20.05.02	19.75	A2	0
11		10.06.02	14.22	AX	0
11		20.06.02	332.01	AX	0
11		05.08.02	15.94	A1BX	0
11		18.11.02	14.79	AXB0/1	0
11		02.06.03	14.79	A1BX	2
12	05.01.01	12.02.01	49.90	A2/3BX	0
12		26.03.01	27.54	A1B1	0
12		09.07.01	28.12	A1B1	0
12		06.01.03	11.42	A0B1	0
12		20.10.04	23.43	A0B1	1
13	27.06.02	05.07.02	17.53	A2	0
13		26.07.02	0	A2	0
13		25.09.02	18.17	A1 A2	0
13		18.12.02	1.05	A0B1	0
13		30.06.03	0	A0B1	0
14	12.11.02	19.11.02	33.25	A2B2	
14		09.12.02	5.17	A2B1	0
14		03.02.03	22.53	AXBX	1
14		04.03.03	19.53	AXB1/2	2
14		22.08.03	10.29	A1B2	3
14		17.11.03	17.71	A0B1/2	2
14		05.05.04	1.00	AXBX	2
14		22.06.04	8.00	AXBX	3
15	16.05.02	05.08.02	24.00	A2B1	0
15		20.11.02	23.29	A0B0/1	0
15		29.01.03	15.30	A1B0	1
16	01.05.03	09.05.03	5.15	A0B1	0
16		23.07.03	20.78	A0B0	0
16		03.11.03	28.60	A0B0	0
16		10.05.04	0	A2B1	0
16		09.08.04	15.51		0

Table 5.2 (continued). Pepsin levels (ng/ml) recovered from sequential BAL samples from 40 patients. Table also details biopsy and BOS scores associated with each sample. AX and BX denotes un-gradable biopsy. DoT-date of transplant. BOS-bronchiolitis obliterans syndrome.

Id	DOT	Date of Sample	Pepsin ng/ml	Biopsy	BOS
17	17.08.02	23.08.02	3.70	A2B1	0
17		16.09.02	0	A2B1/2	0
17		11.11.02	5.17	AXB0	0
17		24.02.03	25.63	A1B1	0
17		18.08.03	0	AXB0	0
17		26.04.04	11.75	A0B0	0
18	06.08.02	14.08.02	14.59	A2B1	0
18		06.09.02	12.94	A0BX	0
18		10.02.03	17.65	A1B1	0
18		15.08.03	18.59	A1B1	0
19	10.09.02	07.10.02	12.44	A2b0	0
19		11.12.02	0		0
19		12.03.03	0	A2B0	0
19		15.09.03	0	A1B0	0
20	23.06.01	02.07.01	5.85	A1/2B1	0
20		11.07.01	5.54	AXB1	0
20		19.09.01	8.26	A1B1	0
20		17.12.01	13.34	A2B2	0
20		24.06.02	15.20	A1B1	0
21	24.08.03	26.11.03	7.03	A0B0	0
21		23.02.04	18.84	A0BX	0
21		11.08.04	10.71	AxB1	0
21		01.10.04	25.23	A1OB	2
22	21.03.03	25.04.03	19.41	A2B1	0
22		18.06.03	18.48	A1B0	0
22		03.09.03	19.04	A2Bx	0
22		05.01.04	22.00	A0B0	1
23	15.05.03	16.06.03	24.30	A2B2	0
23		15.08.03	31.45	A1B1	0
23		10.11.03	10.89	A1B1	0
23		17.05.04	68.78	A1BX	0
24	09.01.02	16.01.02	37.70	A2/3B2	0
24		11.02.02	30.03	A2B1	0
24		11.03.02	31.37	A2B1	0
24		15.04.02	14.86	AXB0	0
24		15.07.02	18.35	A2	0
24		08.01.03	30.80	A1B0	0
25	22.05.02	24.06.02	5.10	A3	0
25		05.08.02	11.63	A2/3B1	0
25		02.09.02	16.54	A1B0	0
25		25.11.02	38.50	A2B1	0
25		15.11.04	4.30		0

Table 5.2 (continued). Pepsin levels (ng/ml) recovered from sequential BAL samples from 40 patients. Table also details biopsy and BOS scores associated with each sample. AX and BX denotes un-gradable biopsy. DoT-date of transplant. BOS-bronchiolitis obliterans syndrome.

Id	DOT	Date of Sample	Pepsin ng/ml	Biopsy	BOS
26	29.06.01	06.07.01	32.40	A2B1	0
26		19.07.01	21.60	A2B1	0
26		26.09.01	24.14	A1B1	0
26		17.12.01	23.82	A0BX	0
26		28.08.02	37.70	A1BX	3
27	30.10.01	05.11.01	0	A2B1	0
27		28.11.01	10.61	A2B2	0
27		06.02.02	13.20	A2B0	0
27		28.10.02	10.90	A1B0	0
27		17.10.03	2.37	A0BX	0
28	22.01.02	01.02.02	29.86	A2B2	0
28		22.02.02	25.42	A2B2	0
28		10.04.02	29.86	A0B0	0
28		29.07.02	13.48	A0B1	0
28		19.11.02	0	A2B1	0
28		02.04.03	25.26	A1B0	0
29	26.06.01	04.07.01	0	A1B1	0
29		01.10.01	0	A1/2B1	0
29		19.12.01	31.70	A2B0	0
29		22.07.02	15.20	A1B1	0
30	12.04.01	09.05.01	18.82	A0B0	0
30		06.06.01	0	A0B0	0
30		11.07.01	9.55	A2B2	0
30		10.10.01	5.21	A1B1	0
30		21.02.03	8.800	A1BX	3
31	27.11.01	03.12.01	32.97	A1B1	0
31		21.12.01	0	A2BX	0
31		20.02.02	17.32	A1	0
31		05.06.02	7.26	A1B0	0
31		18.11.02	15.36	A1B0	0
32	14.08.02	16.09.02	27.83	A1B1	0
32		13.11.02	19.89	A0B1	0
32		14.02.03	22.65	A0B0	0
32		11.08.03	18.32	A0B0	0
32		14.11.03	21.25	A0	0
32		13.08.04	20.39	AxBx	2
33	10.10.02	16.10.02	17.23	A2B1	0
33		18.11.02	21.47	AxBx	0
33		03.03.03	15.29	A1B0	0
33		16.07.03	19.07	A0BX	0
33		15.10.03	33.30	A0Bx	0
33		07.07.04	5.17	A0B0	2

Table 5.2 (continued). Pepsin levels (ng/ml) recovered from sequential BAL samples from 40 patients. Table also details biopsy and BOS scores associated with each sample. AX and BX denotes un-gradable biopsy. DoT-date of transplant. BOS-bronchiolitis obliterans syndrome.

Id	DOT	Date of Sample	Pepsin ng/ml	Biopsy	BOS
34	17.02.03	04.03.03	7.81	AXB1/2	0
34		21.03.03	21.15	AXBX	0
34		12.05.03	14.58	A2B0	0
34		20.08.03	13.46	A0B1	0
34		18.02.04	12.03	A0B0	0
35	27.11.01	16.12.02	13.17	A1B1	0
35		19.02.03	16.70	A1BX	1
35		04.03.03	0	A1BX	2
35		30.07.03	5.17	A1BX	2
36	02.05.01	30.05.01	13.13	A2	0
36		23.07.01	12.90	A3	0
36		21.11.01	5.70	A2	0
36		20.02.02	5.12		0
36		01.05.02	0		1
37	05.11.01	13.11.01	0	A0B0	0
37		14.12.01	0	A1B0	0
37		04.02.02	0	A0B0	0
37		08.05.02	0	A0B0	0
37		04.11.02	12.01	AXB0	0
38	11.08.04	10.09.04	26.00	A1B1	0
38		10.11.04	20.80	A2B2	0
38		02.02.05	22.10	A1B1	0
39	15.01.02	21.01.02	9.40	A2B1/2	0
39		13.02.02	0	A2B1	0
39		01.03.02	5.01	A2BO	0
39		24.04.02	13.27	A2BX	0
39		28.08.02	18.22	AX	0
39		13.01.03	16.51	AXB0	0
39		07.02.05	22.90		0
40	19.07.02	26.07.02	26.35	A2BX	0
40		16.08.02	23.14	A2BX	0
40		24.02.03	37.39	A1B1	0
40		21.07.03	23.08	A2B1	0
40		03.03.04	6.05	A1B1	0

Table 5.2 (continued). Pepsin levels (ng/ml) recovered from sequential BAL samples from 40 patients. Table also details biopsy and BOS scores associated with each sample. AX and BX denotes un-gradable biopsy. DoT-date of transplant. BOS-bronchiolitis obliterans syndrome.

Stable Subject no.	Age	Sex	Diagnosis	Operation	Time post-Tx (months)	Biopsy	BOS Score	Pepsin (ng/ml)
1	37	m	copd	blt	6	A0B0	0	10.38
2	51	f	copd	blt	6	A0B0	0	0
3	49	m	copd	blt	3	A0B0	0	5.21
4	22	f	cf	blt	6	A0B0	0	7.40
5	34	f	cf	blt	3	A0B0	0	5.20
6	37	m	ob	slt	6	A0B0	0	5.50
7	28	f	cf	blt	3	A0B0	0	12.56

Table 5.3 Demographics and pepsin levels (ng/ml) recovered from lavage of stable controls. copd-chronic obstructive pulmonary disease, cf-cystic fibrosis, ob-obliterative bronchiolitis. blt-bilateral lung transplant, slt-single lung transplant. Tx-transplant. BOS-bronchiolitis obliterans syndrome.

5.3 Cross-sectional analysis of BAL pepsin

Levels of pepsin present in BAL ranged from 0 to 332ng/ml. BAL samples were grouped into samples taken at 1 week, 1 month, 3 months, 6 months and 12 months, and were divided into 5 groups; A0, (no acute rejection), A1 (minimal acute rejection), ≥A2 (mild-moderate acute rejection), BOS and all transplant patients. A control group containing 7 patients who were clinically stable was also included for comparisons. For statistical analysis non parametric methods were used throughout using GraphPad Prism software. Groups were compared using the Kruskal Wallis test (non parametric one-way analysis of variance) with a *post hoc* Mann Whitney test.

5.3.1. Analysis of BAL pepsin at 1 week

Pepsin levels from all transplant patients (median 16.9ng/ml, range 0-37.7ng/ml) were not significantly higher than the stable controls (median 5.50ng/ml, range 0-12.56ng/ml, P= 0.055). Pepsin levels from the ≥A2 acute rejection group were significantly higher than the control group (median 17.5ng/ml range 0-37.7ng/ml P= 0.03, figure 5.1). The pepsin levels from A0 and A1 groups were not significantly different than the controls (P>0.05).

5.3.2 Analysis of BAL pepsin at 1 month

Pepsin levels from all transplant patients (median 17.9ng/ml, range 0-332.0ng/ml) were significantly higher than the stable controls (median 5.5ng/ml, range 0-12.56ng/ml, P= 0.02). In addition, pepsin levels from the A0 and the A1 groups were significantly higher than the control group (median 17.9 and 13.8ng/ml, range 12.9-18.8 and 0-27.8ng/ml, P= 0.003 and P=0.04 respectively, figure 5.2). The pepsin levels from ≥A2 group were not significantly different than the controls (P>0.05).

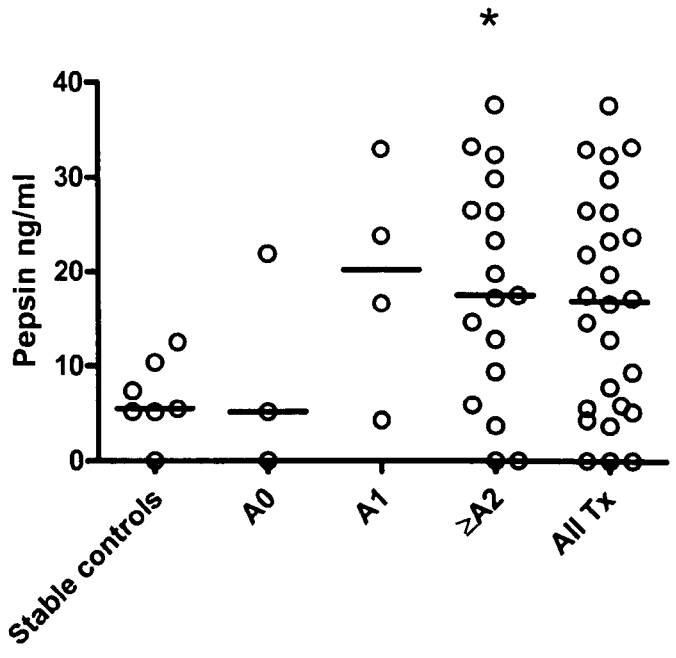


Figure 5.1 Values of pepsin (ng/ml) recovered from BAL at 1 week post transplant. Lines represent median values. Stable controls are patients with no evidence of rejection or infection, A0 are patients with no evidence of acute rejection, A1 are patients with minimal acute rejection and ≥A2 are patients with mild to moderate acute rejection. Tx-transplant. Stable controls n=7, A0 n=3, A1 n=4, ≥A2 n=17 and all Tx n=26

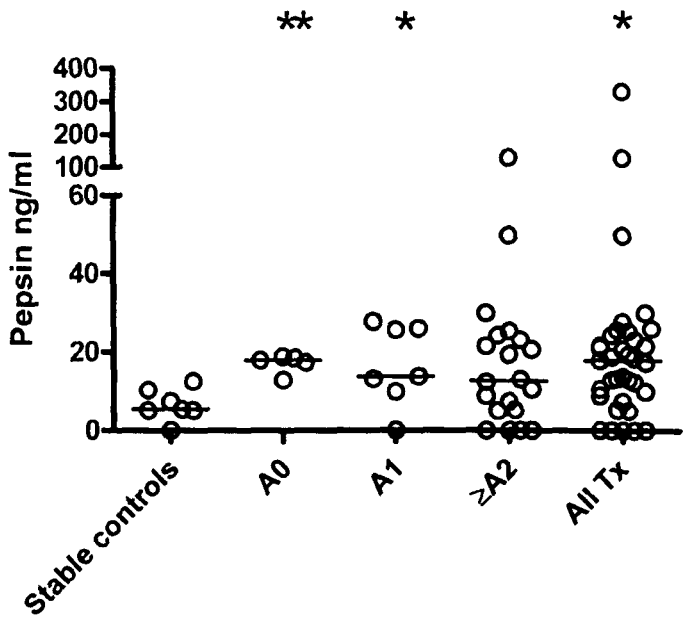


Figure 5.2 Values of pepsin (ng/ml) recovered from BAL at 1 month post transplant. Lines represent median values. Stable controls are patients with no evidence of rejection or infection, A0 are patients with no evidence of acute rejection, A1 are patients with minimal acute rejection and ≥A2 are patients with mild to moderate acute rejection. Tx-transplant. Stable controls n=7, A0 n=5, A1 n=7, ≥A2 n=20 and all Tx n=35

5.3.3 Analysis of BAL pepsin at 3 months

Pepsin levels from all transplant patients (median 15.4ng/ml, range 0-31.5ng/ml) were significantly higher than the stable controls (median 5.5ng/ml, range 0-0-12.6ng/ml, P= 0.01). In addition, pepsin levels from the A1 and ≥A2 groups were significantly higher than the control group (median 16.2 and 13.9ng/ml range 2.4-31.5 and 0-24.0ng/ml, P= 0.01 and 0.007 respectively, figure 5.3). Pepsin levels from the A0 group were not significantly different from the controls (P>0.05) and there were not enough patients in the BOS group to perform statistical analysis.

5.3.4 Analysis of BAL pepsin at 6 months

Pepsin levels from all transplant patients (median 18.2ng/ml, range 0-38.5ng/ml) were significantly higher than the stable controls (median 5.5ng/ml, range 0-0-12.6ng/ml, P= 0.009). In addition, pepsin levels from the A1 group were significantly higher than the control group (median 17.7ng/ml, range 4.3-37.4ng/ml, P= 0.02, figure 5.4). The pepsin levels from A0 and ≥A2 groups were not significantly higher than controls (P>0.05) and again, there were not enough patients in the BOS group to perform statistical analysis.

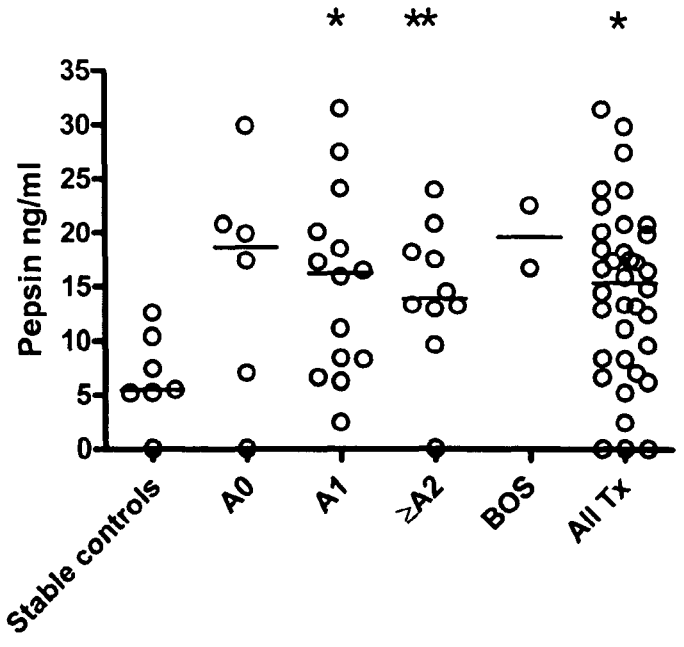


Figure 5.3 Values of pepsin (ng/ml) recovered from BAL at 3 months post transplant. Lines represent median values. Stable controls are patients with no evidence of rejection or infection, A0 are patients with no evidence of acute rejection, A1 are patients with minimal acute rejection, ≥A2 are patients with mild to moderate acute rejection and BOS are patients diagnosed with bronchiolitis obliterans syndrome at the time the sample was collected. Tx-transplant. Stable controls n=7, A0 n=6, A1 n=14, ≥A2 n=10 and all Tx n=36

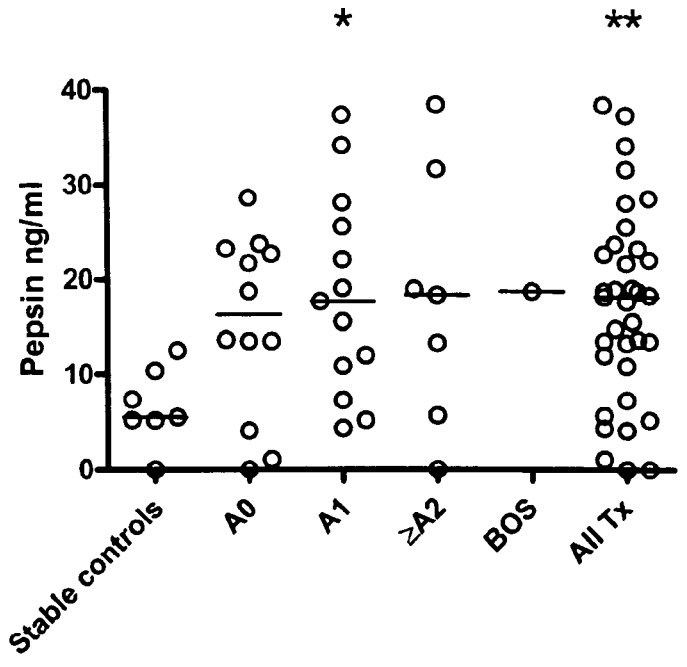


Figure 5.4 Values of pepsin (ng/ml) recovered from BAL at 6 months post transplant. Lines represent median values. Stable controls are patients with no evidence of rejection or infection, A0 are patients with no evidence of acute rejection, A1 are patients with minimal acute rejection, ≥A2 are patients with mild to moderate acute rejection and BOS are patients diagnosed with bronchiolitis obliterans syndrome at the time the sample was collected. Tx-transplant. Stable controls n=7, A0 n=12, A1 n=13, ≥A2 n=7, BOS n=1 and all Tx n=35

5.3.5 Analysis of BAL pepsin at 12 months

Pepsin levels from all transplant patients (median 15.0ng/ml, range 0-68.8ng/ml) were significantly higher than the stable controls (median 5.5ng/ml, range 0-0-12.6ng/ml, P= 0.02). In addition, pepsin levels from the A1 group were significantly higher than the control group (median 15.4ng/ml range 0-68.8ng/ml, P= 0.006, figure 5.5). Pepsin levels in the A0, ≥A2 and BOS groups were not significantly higher than the controls.

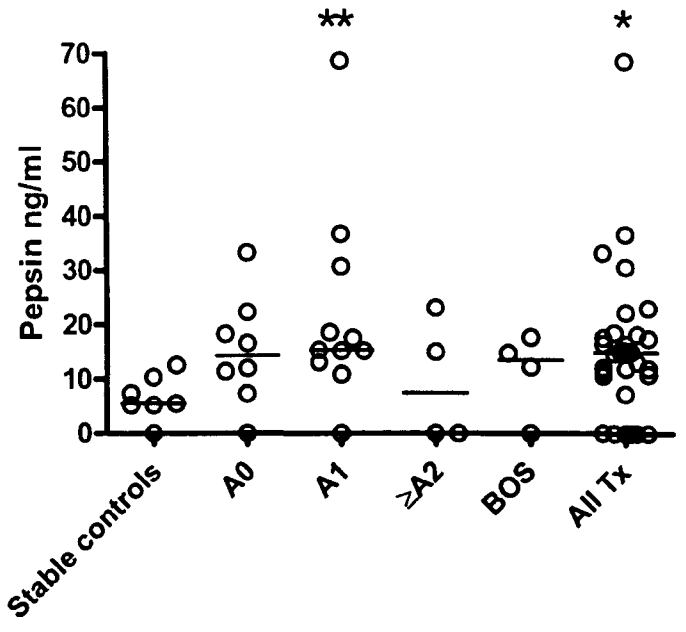


Figure 5.5 Values of pepsin (ng/ml) recovered from BAL at 12 months post transplant. Lines represent median values. Stable controls are patients with no evidence of rejection or infection, A0 are patients with no evidence of acute rejection, A1 are patients with minimal acute rejection, ≥A2 are patients with mild to moderate acute rejection and BOS are patients diagnosed with bronchiolitis obliterans syndrome at the time the sample was collected. Tx-transplant. Stable controls n=7, A0 n=8, A1 n=11, ≥A2 n=4, BOS n=4 and all Tx n=31.

5.4 Longitudinal analysis of BAL pepsin

The chosen format of analysis was to decide on a time point and investigate whether high levels of pepsin at this time point can predict for the development of BOS. As there is evidence of gastric aspiration being problematic early post-transplant (Cantu et al. 2004) three months was chosen. Three months is still relatively early post-transplant, however, patients will be more 'clinically stabilised' than they would be at one week or one month.

There were 36 patients with a BAL sample collected at 3 months (± 2 weeks) that were included in the analysis. Patients 4, 18, 33 and 40 were excluded from the analysis as they did not have a sample taken at 3 months. Patient 33 did develop BOS during the follow up time; therefore the number of patients included in the study that went on to develop BOS was 13.

The statistical analysis of data contained in this chapter was performed with the help of a statistician.

5.4.1 BOS vs. no BOS-pepsin levels at 3 months

The patients were split into two groups, patients who did not develop BOS in the follow up time and those who did develop BOS within the follow up time. The descriptive statistics for the two groups can be seen in table 5.4 and a dotplot of pepsin levels in figure 5.6. There was a trend for a higher BAL pepsin level in the group who developed BOS, however this was not statistically significant (using the Mann Whitney test, P=0.07).

	n	Mean	SEM	Minimum	Median	Maximum
No BOS development within follow up	23	12.9	1.8	0	13.3	31.5
BOS development within follow up	13	17.9	1.7	7.0	17.5	27.5

Table 5.4 The mean, standard error of the mean (SEM), median and range of pepsin levels (ng/ml) recovered from BAL taken at 3 months post-transplant from patients who did not develop BOS and patients who did develop BOS within the follow up time of the study.

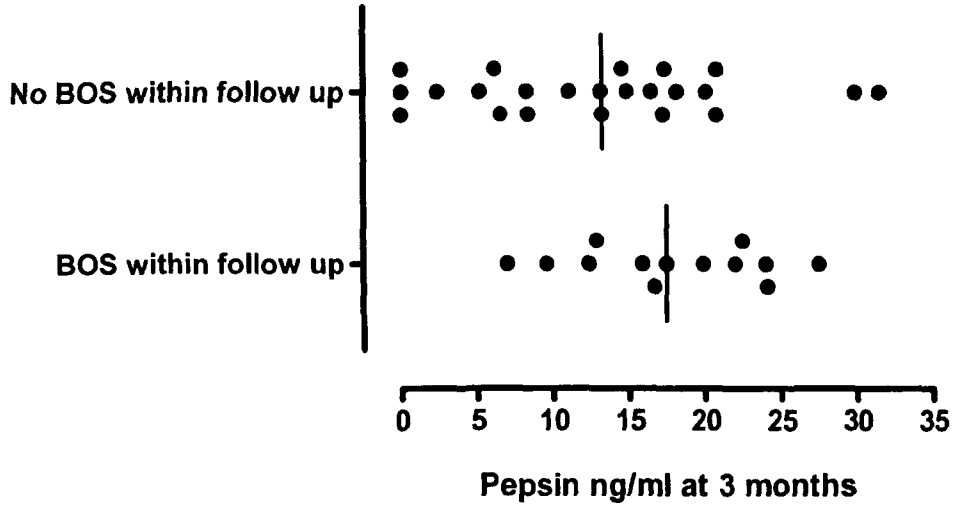


Figure 5.6 Dotplot of pepsin (ng/ml) recovered from BAL collected at 3 months post-transplant from patients at who did not develop BOS (n=23) within the follow up time of the study and those who did develop BOS (n=13). Lines represent median values.

5.4.2 Percentage BOS by pepsin level

The general hypothesis is that 'high' values of pepsin in the BAL at around 3 months post-transplant may be predictive for time to BOS. As there is no general consensus of what high levels of pepsin are in bronchoalveolar lavage a number of 'cut-offs' were used based on levels measured in the stable controls. These were the median, 5.5ng/ml, the maximum, 12.6ng/ml and the two values between the median and the maximum, 7.4 and 10.4ng/ml.

The percentage of patients with BOS by 'high' or 'low' BAL pepsin at 3 months was calculated and the percentage of patients with BOS was higher in patients with 'high' levels of pepsin for all the cut of levels (table 5.5).

In addition to the percentage of patients with BOS, the rate ratios were also calculated for the cut off values. The rate ratio estimates the rate the patient is likely to develop BOS and the confidence interval indicates the range of possible underlying true values for the rate ratio. For example patients with BAL pepsin levels above 7.4ng/ml at 3 months are estimated to develop BOS at 4.1 times the rate of patients with 7.4ng/ml or below, however, the width of the confidence interval suggests that it could be as high as 31.6 times, or conversely twice as slow (0.5 times). Patients with levels above 10.4ng/ml are estimated to develop BOS at 3.0 times the rate of those with 10.4ng/ml or below (CI 0.7-13.6) and patients with levels above 12.6ng/ml are estimated to develop BOS at 2.3 times the rate of patients with 12.6ng/ml or below (CI 0.6-8.3) (table 5.6).

A rate ratio of 1.0 indicates no difference between the groups and as all the confidence intervals include a rate ratio of 1.0, this demonstrates that there are no significant differences in the rate of BOS development between the two groups. This is also reflected by the P values.

Variable	Level	Number of patients (total=36)	Number and percentage with BOS by level	
Pepsin at 3 months	≤ 5.5 ng/ml	5	0	0.0%
	> 5.5 ng/ml	31	13	41.9%
Pepsin at 3 months	≤ 7.4 ng/ml	8	1	12.5%
	> 7.4 ng/ml	28	12	42.9%
Pepsin at 3 months	≤ 10.4 ng/ml	11	2	18.2%
	> 10.4 ng/ml	25	11	44.0%
Pepsin at 3 months	≤ 12.6 ng/ml	13	3	23.1%
	> 12.6 ng/ml	23	10	43.5%

Table 5.5 Percentage of patients with bronchiolitis obliterans syndrome (BOS) based on whether they have 'high' or 'low' levels of pepsin present in their bronchoalveolar lavage at 3 months.

Variable	Level	Rate ratio	95% CI	*P*-value (score test)
Pepsin at 3 months	≤ 5.5 ng/ml > 5.5 ng/ml			0.1
Pepsin at 3 months	≤ 7.4 ng/ml > 7.4 ng/ml	1.0 4.1	0.5 - 31.6	0.1
Pepsin at 3 months	≤ 10.4 ng/ml > 10.4 ng/ml	1.0 3.0	0.7 -13.6	0.1
Pepsin at 3 months	≤ 12.6 ng/ml > 12.6 ng/ml	1.0 2.3	0.6 -8.3	0.2

Table 5.6 Rate ratios and 95% confidence intervals for BOS development by 'high' or 'low' bronchoalveolar lavage pepsin at 3 months. There is no rate ratio for 5.5ng/ml as when using Cox proportional hazards, the rate ratio can not be calculated if there are 0 events in one of the groups (refer to table 5.5). CI-confidence interval.

5.4.3 Survival analysis of patients with 'high' and 'low' BAL pepsin at 3 months

Kaplan-Meier estimates of survivor functions (i.e. remaining BOS free) were plotted using a cut off of 10.4 and 12.6ng/ml. Using a cut off of 10.4ng/ml patients with BAL pepsin at 3 months of 10.4ng/ml or below are approximately 80% free from BOS at 3 years post-transplant compared to approximately 60% with levels above 10.4ng/ml. Using a cut off of 12.6ng/ml patients with BAL pepsin at 3 months of 12.6ng/ml or below are approximately 75% free from BOS at 3 years post-transplant compared to approximately 60% with levels above 12.6ng/ml (figures 5.7 and 5.8).

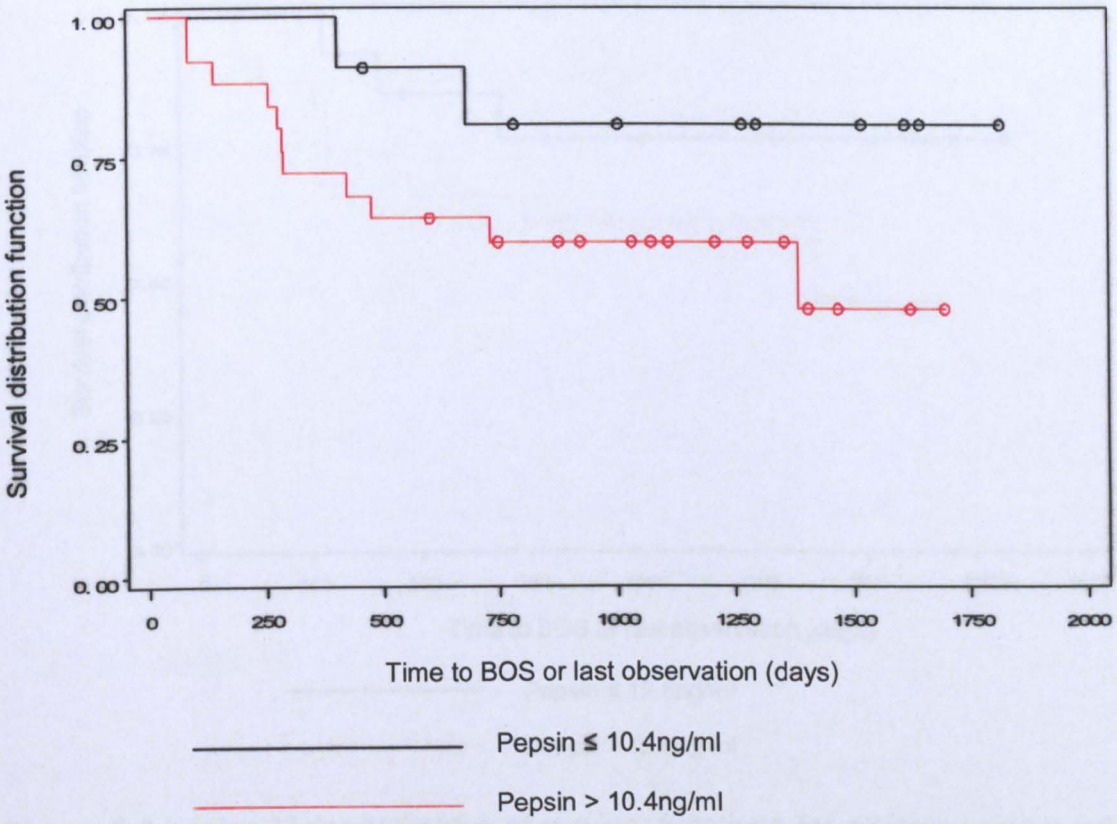


Figure 5.7 Kaplan-Meier estimates of survivor functions for patients using a cut off of 10.4ng/ml at 3 months post transplant to define 'high' pepsin levels. Survival function–remaining BOS free. BOS -bronchiolitis obliterans syndrome.

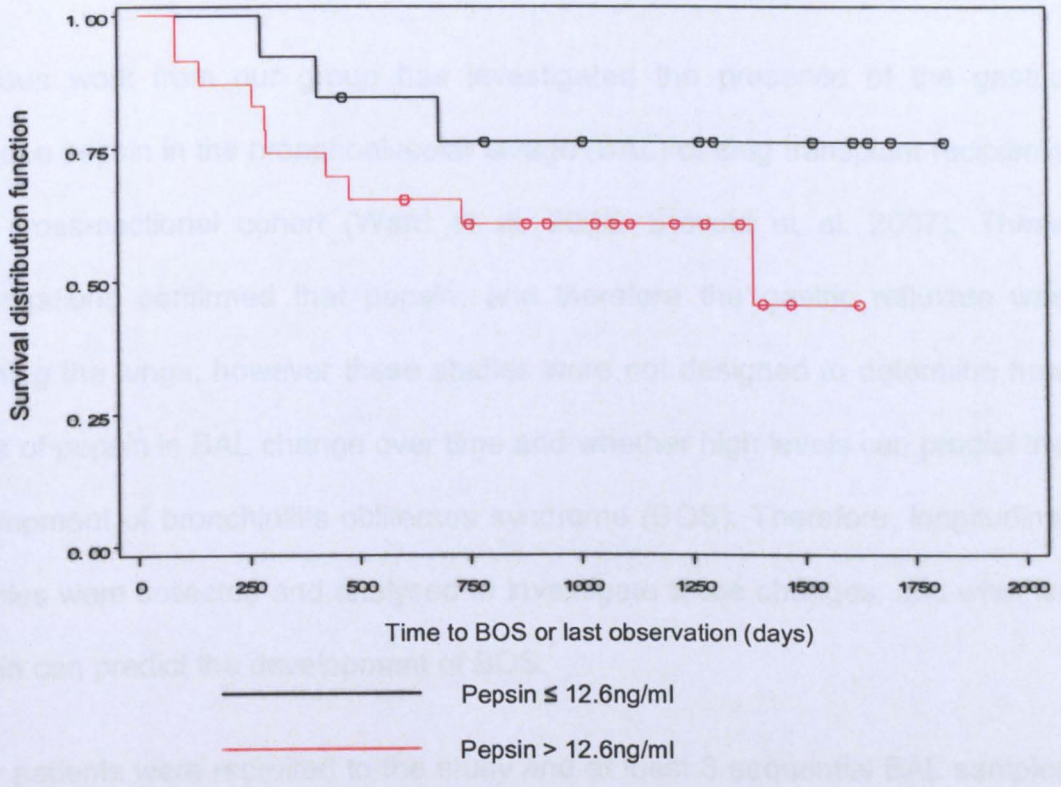


Figure 5.8 Kaplan-Meier estimates of survivor functions for patients using a cut off of 12.6ng/ml at 3 months post transplant to define 'high' pepsin levels. Survival function–remaining BOS free. BOS -bronchiolitis obliterans syndrome.

5.5 Discussion

Previous work from our group has investigated the presence of the gastric protease pepsin in the bronchoalveolar lavage (BAL) of lung transplant recipients in a cross-sectional cohort (Ward et al. 2005; Stovold et al. 2007). These investigations confirmed that pepsin, and therefore the gastric refluxate was reaching the lungs, however these studies were not designed to determine how levels of pepsin in BAL change over time and whether high levels can predict the development of bronchiolitis obliterans syndrome (BOS). Therefore, longitudinal samples were collected and analysed to investigate these changes, and whether pepsin can predict the development of BOS.

Forty patients were recruited to the study and at least 3 sequential BAL samples were taken and analysed for pepsin. Initially, the data was split into 5 cross sectional time points: 1 week, 1 month, 3 months, 6 months and 12 months post-transplant to investigate whether high levels of pepsin are associated with different grades of rejection at different time points. Samples were divided into 5 groups; A0, (no acute rejection), A1 (minimal acute rejection), ≥A2 (mild-moderate acute rejection), BOS and all transplant patients. In addition, a control group containing 7 patients who were clinically stable was also included for comparisons.

Pepsin levels from all transplant patients were significantly higher than the control group at 1 month, 3 months, 6 months and 12 months. The samples taken at 1 week did not contain significantly higher levels of pepsin than the controls,

however, this may be explained by the fact that there were a large number of patients (n=14) without a BAL sample taken at 1 week.

The pepsin levels in the A0 group were only significantly higher than the controls at 1 month post transplant. A difference between the controls and A0 group was not expected, as the two groups are similar by definition. Both groups have no evidence of acute rejection, however the A0 group may have evidence of infection. In addition, the difference at 1 month may be explained by the A0 patients not being clinically stabilised, the patients may still be adjusting to the transplant itself and also the medication they would be taking, factors which may promote gastro-oesophageal reflux and consequent aspiration. The patients in the stable control group were at least 3 months post-transplant, therefore will have had more time to adjust to these factors.

The A1 group showed significantly higher levels of pepsin than the controls at 1, 3, 6 and 12 months, suggesting a link between minimal acute rejection and gastric aspiration. This was not identified in the previous study, however, this could be due to the smaller numbers of patients with A1 rejection in the earlier work. In addition, comparisons were made against a normal volunteer control group, not stable transplant controls. The decision not to include a normal (non-transplant) control group in the current analysis was made on the basis that for survival analysis lung transplant patients are not comparable to normal healthy subjects, therefore deciding what a high level of BAL pepsin is based on normal subjects would not be appropriate. In addition, the normal control data has been included in two previous studies (Ward et al. 2005; Stovold et al. 2007) and

considering no new 'normal' samples were taken it would not be good practice to include them in a third study.

There is some evidence that minimal acute rejection is important in the development of obliterative bronchiolitis (OB). Studies have shown that multiple episodes of histologically graded A1 acute rejection can be a risk factor for OB/BOS (Hopkins et al. 2004; Khalifah et al. 2005), however, the evidence is not as strong as it is for ≥A2 rejection and should therefore be further investigated.

The A2 or greater acute rejection group was significantly different at 1 week and 3 months post-transplant, suggesting a link between ≥A2 rejection and gastric aspiration early post transplant. Acute rejection is consistently linked with the development of OB/BOS (Sharples et al. 2002; Scott et al. 2005). In addition, the Duke University group have published data suggesting that gastric aspiration is a problem early post-transplant and needs to be treated early to see any survival benefit (Cantu et al. 2004).

Finally, statistical analysis could only be performed on the BOS group at 12 months post-transplant, as there were too few numbers at the other time points. There was no significant difference between the stable controls and the BOS group, however further investigations are required to increase the numbers in this group to allow a more detailed analysis.

For the survival analysis it was decided to look at pepsin levels at 3 months post-transplant, as this is still an early time point, however patients will have clinically stabilised, i.e. adjusted to factors that may promote gastric aspiration for example

certain medications and the transplant surgery itself. The patients were divided into two groups, one including patients who developed BOS during the follow up time and the other group included patients who remained BOS free throughout the study.

For the survival analysis the hypothesis was that 'high' values of pepsin in the BAL at three months post-transplant may be predictive of time to BOS. As there is no general consensus of what a 'high' level of BAL pepsin is a number of possible 'cut-offs' were used based on values recovered from stable transplant controls. These were the median (5.5ng/ml), the maximum (12.6ng/ml) and the two values between the median and the maximum (7.4 and 10.4ng/ml).

For all cut-off values the percentage of patients with BOS was greater in the high BAL pepsin group, suggesting that high levels of pepsin in the BAL at 3 months may be associated with the development of BOS. The rate ratios were also calculated for the groups using 7.4, 10.4 and 12.6ng/ml as a cut of for high pepsin. Using all three values the estimated rate of developing BOS in the high pepsin group was at least twice that of the low pepsin group (4.1, 3.0 and 2.3 times respectively), again suggesting a link between high pepsin levels and the development of BOS. However, the confidence intervals (representing the range of possible values) are wide and include a value of 1.0, which indicates that there is not enough evidence from this data to conclude that there is a statistically significant difference in the rate of development of BOS between the two groups. However, to demonstrate no difference between the two groups the confidence interval should be 'tight' around 1.0, and as this is not the case it would also be

incorrect to assume no difference between the two groups. In this case it is possible that the width of the confidence intervals reflect the small number of patients and the small number of BOS events in the two groups.

Based on the Kaplan-Meier survival curves using a cut off of 10.4ng/ml the low pepsin group (≤10.4ng/ml) are approximately 80% BOS free at 3 years, compared to approximately 60% in the high pepsin group. This difference in survival is also demonstrated when using 12.6ng/ml as a cut off (75% BOS free in the low pepsin group and 60% BOS free in the high pepsin group).

Although all the data outlined in this chapter is suggesting a link between early high levels of pepsin in BAL and the development of BOS there is a lack of statistical significance, which could be explained by the small numbers of patients and the small number of BOS events in the groups. To detect a statistically significant difference of 20% in BOS free survival at 3 years post-transplant (i.e. 80% vs. 60% BOS free for a 10.4ng/ml cut off) using a logrank test would require 173 patients and 53 'events' (Freedman 1982). This number may be reduced, however, with longer follow-up and/or a difference in percentage of BOS free survival. These sample-size calculations should be considered in any future analysis.

Gastric aspiration is becoming increasingly recognised as a contributing factor for the development of BOS. A study by Li et al has shown that the chronic aspiration of gastric fluid can induce the development of OB in a rat model of transplantation. Allografts that were instilled with gastric juice once a week for at

least 2 months showed evidence of cellular fibro-proliferative tissue, significant reduction in the size of the bronchiolar lumens and mild degrees of peri-bronchiole inflammatory cell infiltrates, consistent with the development of OB. This was not seen in allografts and isografts that were not treated with gastric juice, showing that gastric aspiration can accelerate the development of OB/BOS (Li et al. 2008).

As previously discussed, Cantu et al have also shown that the treatment of gastro-oesophageal reflux can lead to a survival benefit in terms of BOS, however any intervention must be carried out early in the transplant process to see any improvement (Cantu et al. 2004). Using Kaplan-Meier survival analysis Cantu et al showed that patients who received an early fundoplication (up to 87 days post-transplant) were 100% free from BOS at 3 years post-transplant, compared to 60% in patients with a history of reflux and no fundoplication and 47% in those with a history of reflux and a late fundoplication (106 to 2999 days post transplant.

This is consistent with the work outlined in this chapter, as there is a trend for patients with 'high' BAL pepsin (>10.4ng/ml) at 3 months post-transplant to show a survival disadvantage compared to those with 'low' BAL pepsin (≤10.4ng/ml) at 3 months post-transplant (60% vs. 80% BOS free at 3 years post-transplant respectively). This suggests that treatment is required before this time point to reduce the development of BOS.

As gastric aspiration is becoming increasingly recognised as a contributing factor for the development of BOS measuring biomarkers is becoming ever more important in identifying which patients are most likely to benefit from anti-reflux treatment. There are two biomarkers currently being utilised by transplant groups world wide; pepsin and bile salts (D'Ovidio et al. 2005; Ward et al. 2005; Blondeau et al. 2008). However, over the past 3 years many groups have chosen to focus on bile salts rather than pepsin. A possible explanation for this is the commercial availability of kits designed to measure bile salts, combined with the difficulty in setting up an assay to accurately measure pepsin.

Our group chose to continue measuring pepsin as a biomarker, mainly due to the fact that the gastric refluxate will not always contain bile acids, as duodenal-gastro-oesophageal reflux may occur less frequently than gastro-oesophageal reflux, therefore, some aspiration episodes may be overlooked. Pepsin is more likely to be consistently present in the gastric refluxate and as a result less aspiration episodes would be missed.

More recently, our group has also investigated the presence of bile salts in the BAL of lung transplant patients (1 and 3 months post-transplant) using mass spectrometry, with a lower limit of detection of 0.1μmol/l and found no evidence of bile salts. This does not necessarily mean that bile salts are not reaching the lungs, it may be that once the saline used to perform the lavage is instilled the bile salts become too diluted to measure using these methods. Again, this implies that pepsin may be a more sensitive marker of aspiration.

Further investigations are required to understand the mechanisms behind this injury. Neutrophil levels have also been investigated in this patient cohort by another member of the group as they are consistently implicated in the pathogenesis of OB/BOS (DiGiovine et al. 1996; Elssner and Vogelmeier 2001). Neutrophils are part of the innate immune system and in infection are recruited to the lung and activated, mainly by macrophage-derived mediators, such as IL-8, TNF-α and GM-CSF. Neutrophils can cause damage to the tissue in their region of activity by the reactive oxygen species they produce, chiefly via the action of myeloperoxidase (MPO). It is possible that the lung epithelial cells are releasing neutrophilic chemokines such as TNF-α and GM-CSF in response to stress or damage caused by refluxed pepsin and/or acid. If this was the case there may be a correlation between neutrophil and pepsin levels in the BAL and as both have been measured in the same patients statistical analysis could be performed. This would help to identify if neutrophils are implicated and whether or not they should be further investigated as a potential mechanism of damage caused by gastric aspiration.

The investigations outlined in this chapter have confirmed previous work by demonstrating that pepsin is present in the BAL of lung transplant patients and that there is an association between high pepsin levels and acute rejection (minimal to moderate acute rejection). There is also a trend towards high BAL pepsin levels at 3 months and the development of BOS, however the difference is not statistically significant, which may be reflecting the small number of patients and also the small number of BOS 'events', therefore further

investigations involving a larger patient cohort are required to confirm these results. This is the first longitudinal study investigating BAL pepsin levels and it supports the hypothesis that gastric aspiration may be an important injury in lung transplantation and that pepsin is potentially a useful biomarker.

Chapter 6

The effect of pepsin on goblet and epithelial cells

6.1 Introduction

Extra-oesophageal reflux has been shown to have adverse effects on the upper respiratory tract, including the trachea, larynx and pharynx (Vaezi 2003; Farrokhi and Vaezi 2007). In addition aspiration of gastric contents into the lungs has been associated with the development of chronic rejection in lung transplantation (Blondeau et al. 2008; Li et al. 2008). Chronic lung rejection manifests itself histologically as obliterative bronchiolitis (OB) and clinically as bronchiolitis obliterans syndrome (BOS).

Early OB is characterised by an influx of inflammatory cells into the lung, including T lymphocytes, monocytes and neutrophils (Boehler et al. 1998). Chemokines such as interleukin 8 (IL-8) and interleukin 17 (IL-17) have been shown to be important in the recruitment of such inflammatory cells, especially neutrophils which are repeatedly elevated in the lung lavage of transplant patients with BOS. This suggests that IL-8 along with other such chemokines plays a crucial role in the pathogenesis of OB.

IL-8 is an important component of the innate immune system and can be produced by immune cells, such as macrophages as well as the epithelium itself in response to many pathogens or stimuli present in the lung. The potential for

pepsin, an important component of the gastric refluxate, to stimulate IL-8 production from epithelial cells was therefore investigated.

In addition, epithelial surfaces (including the respiratory, gastrointestinal and reproductive tracts) are coated with mucus, a mixture of water, ions, glycoproteins, proteins and lipids, which provides a protective barrier against pathogens and toxins. Mucin glycoproteins (mucins) are the major macromolecular constituent of mucus and the predominant mucins in the airway appear to be those expressed by the MUC5AC and MUC5B genes (Callaghan-Rose and Voynow 2006). MUC5AC is mainly produced by the goblet cells present in the epithelium and MUC5B by the secretory cells of the submucosal glands.

It is possible that airway goblet cells could secrete mucus in response to pepsin aspirated into the lung. This could be receptor mediated, as the presence of a receptor on laryngeal cells that has the ability to recognise pepsin has been suggested (Johnston et al. 2007). Hypersecretion of mucus has been reported in some airway diseases for example COPD, CF and asthma (diseases also linked with gastro-oesophageal reflux) and has been observed in lung transplant patients, along with poor airway clearance and altered cough (Veale et al. 1993). Therefore, in addition to analysing media collected from epithelial cells, media from goblet cells challenged with pepsin was also collected and assayed for mucin (MUC5AC).

Pro-inflammatory cytokines have also been shown to stimulate mucus production from goblet cells. Studies by Smirnova et al have shown that IL-8 and TNF-α both stimulate MUC5AC mucin production from these cells *in vitro* (Smirnova et al. 2000; Smirnova et al. 2002). Pro-inflammatory cytokines, especially IL-8, have been consistently implicated in the pathophysiology of BOS. The possible stimulation of epithelial cells by pepsin emphasises the potential for complex interactions between epithelial and goblet cells.

6.2 Stimulation of epithelial cells with porcine pepsin

Primary bronchial epithelial cells from transplant patients were grown in 24 well plates until confluent. Cells were seeded at approximately 50000 per well. The cells were then incubated with either 10ng/ml, 50ng/ml or 50µg/ml pepsin in basal media (serum-free, BEBM at pH 7.4, Lonza, Switzerland) or basal media adjusted to pH 7.0 with 1M HCl. Media was collected at 24, 48 and 72h and assayed for interleukin-8 (IL-8) with a commercially available kit (R&D systems, USA). Data was plotted for each pepsin concentration at either pH 7.4 or 7.0 at 24, 48 and 72h.

6.2.1 Cells challenged with 10ng/ml porcine pepsin

As the production of IL-8 from the primary cells varied between patients data was analysed with the Wilcoxon matched pairs test (non-parametric) using GraphPad prism software. IL-8 production was not significantly altered when challenged with 10ng/ml pepsin at either pH 7.4 or pH 7.0 over 24, 48 or 72h (figures 6.1.1, 6.1.2 and 6.1.3). Data was also grouped to allow comparisons between pH 7.4 and pH 7.0 (figure 6.1.4). IL-8 production increased over each 24h time period (i.e. from 24h to 48h and again from 48h to 72h) for each challenge condition, however the increase was not significant ($P>0.05$). There was also no significant difference in IL-8 production between the challenge conditions when analysed using a non-parametric one-way analysis of variance with a *post-hoc* Mann Whitney test (median values in pg/ml for 72h; pH 7.4 947, pH 7.4 & pepsin 1191, pH 7.0 822 and pH 7.0 & pepsin 1545).

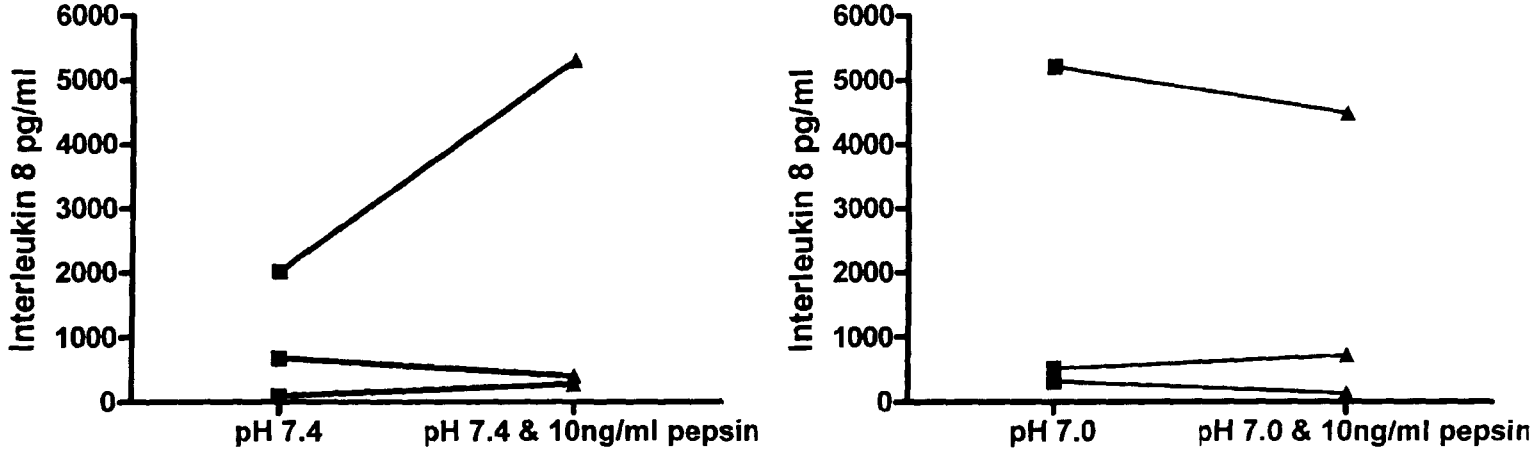


Figure 6.1.1 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 10ng/ml pepsin. Cells were seeded at approximately 50000 per well. The 3 lines represent 3 different patients and experiments were performed in duplicate. Media was collected at 24h.

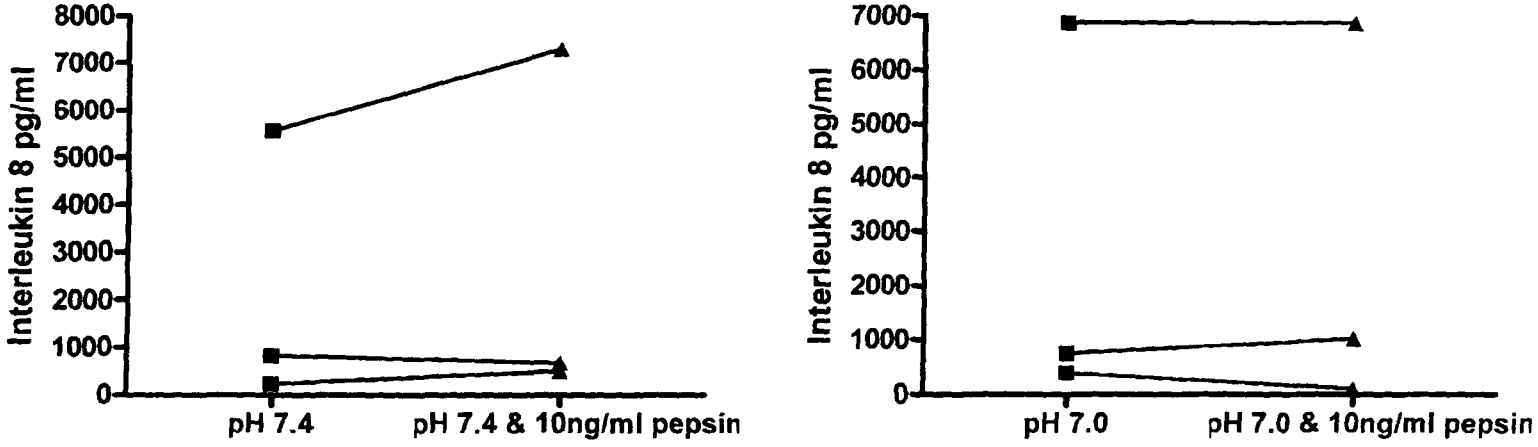


Figure 6.1.2 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 10ng/ml pepsin. Cells were seeded at approximately 50000 per well. The 3 lines represent 3 different patients and experiments were performed in duplicate. Media was collected at 48h.

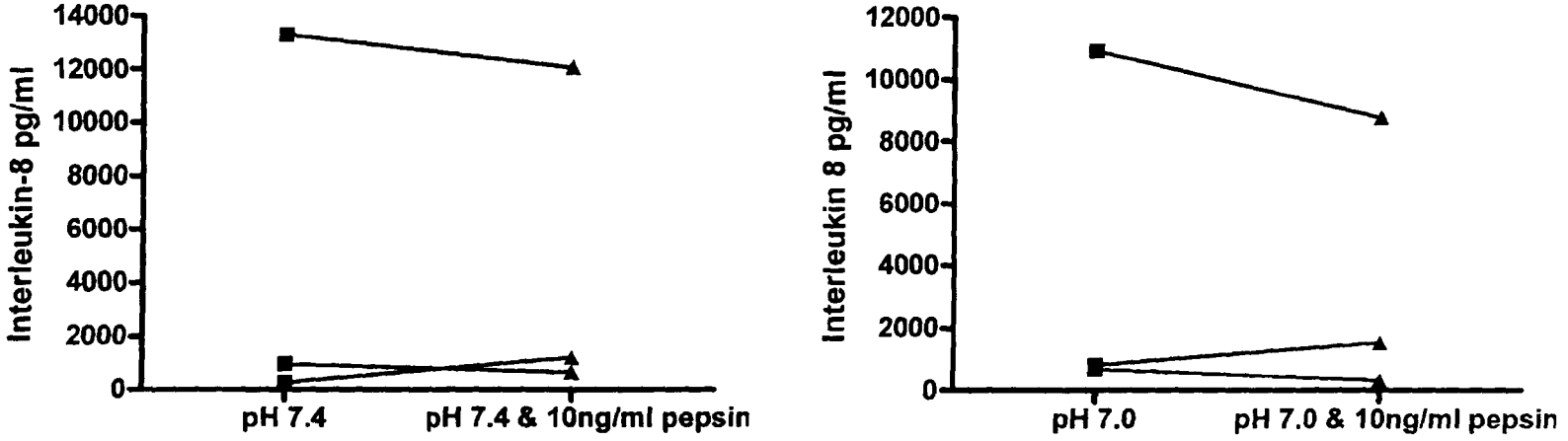


Figure 6.1.3 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 10ng/ml pepsin. Cells were seeded at approximately 50000 per well. The 3 lines represent 3 different patients and experiments were performed in duplicate. Media was collected at 72h.

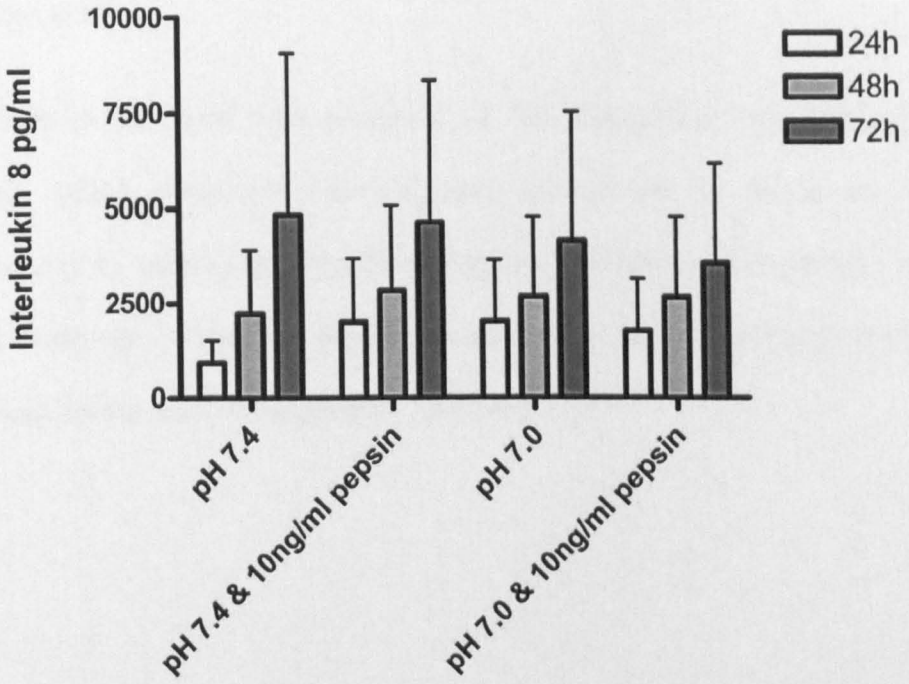


Figure 6.1.4 Interleukin 8 production from primary bronchial epithelial cells challenged with 10ng/ml pepsin at either pH 7.4 or pH 7.0. Bars represent mean with SEM and shows the average of 3 experiments.

6.2.1.1 Cell viability

The viability of the cells was analysed at 72h using the Cell TiterBlue assay (Promega, USA). Negative controls were performed by fixing the cells in methanol prior to adding the TiterBlue reagent. Viability remained fairly constant over the challenge conditions. Pepsin caused a decrease in viability at pH 7.0 to 72%, however this was not significant (figure 6.1.5).

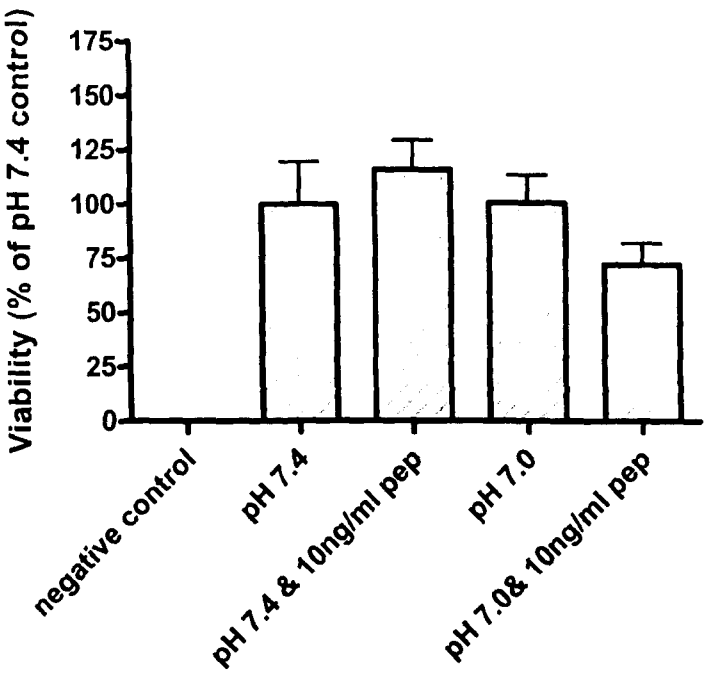


Figure 6.1.5 Primary bronchial epithelial cell viability after 72h exposure to 10ng/ml pepsin at either pH 7.4 or pH 7.0. Data is displayed as a percentage of the positive control (pH 7.4). Bars represent mean with SEM and shows the average of 3 experiments. Negative controls were performed by fixing cells in methanol prior to adding the TiterBlue solution.

6.2.2 Cells challenged with 50ng/ml porcine pepsin

Again, data was analysed with the Wilcoxon matched pairs test (non-parametric) using GraphPad prism software. IL-8 production was not significantly different when challenged with 50ng/ml pepsin at either pH 7.4 or pH 7.0 over 24, 48 or 72h (figures 6.2.1, 6.2.2 and 6.2.3). Data was also grouped to allow comparisons between pH 7.4 and pH 7.0 (figure 6.2.4). As with the 10ng/ml pepsin challenge IL-8 production increased over each 24h time period (i.e. from 24h to 48h and again from 48h to 72h) for each condition; however the increase was not significant ($P>0.05$). There was still no significant difference in IL-8 production when comparing the different challenge conditions when analysed using a non-parametric one-way analysis of variance with a *post-hoc* Mann Whitney test (median values in pg/ml for 72h; pH 7.4 1608, pH 7.4 & pepsin 2911, pH 7.0 2348 and pH 7.0 & pepsin 2411).

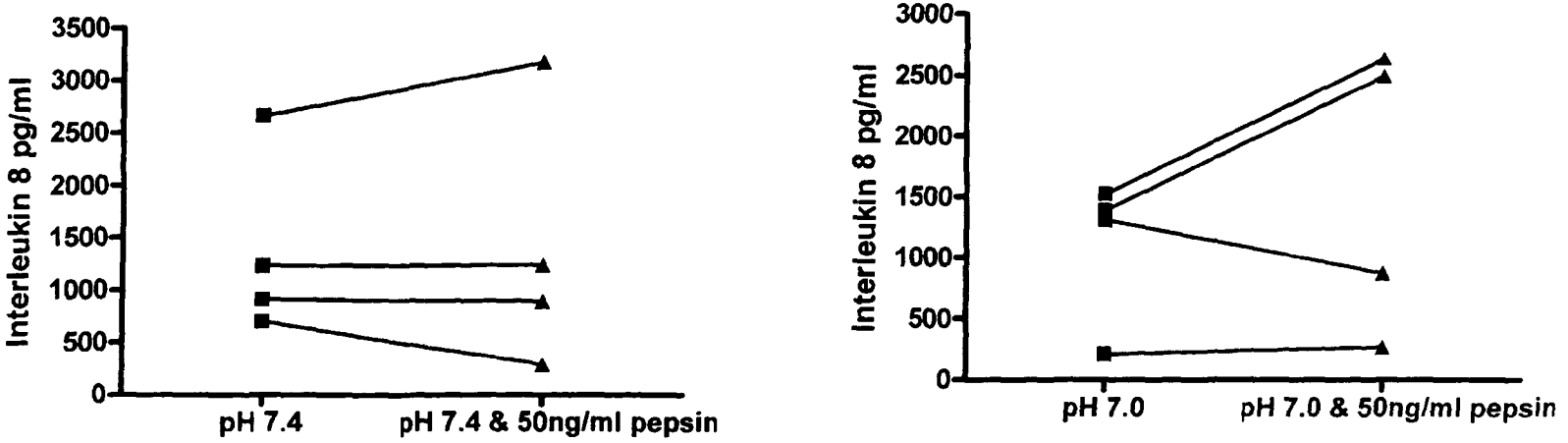


Figure 6.2.1 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 50ng/ml pepsin. Cells were seeded at approximately 50000 per well. The 4 lines represent 4 different patients and experiments were performed in duplicate. Media was collected at 24h.

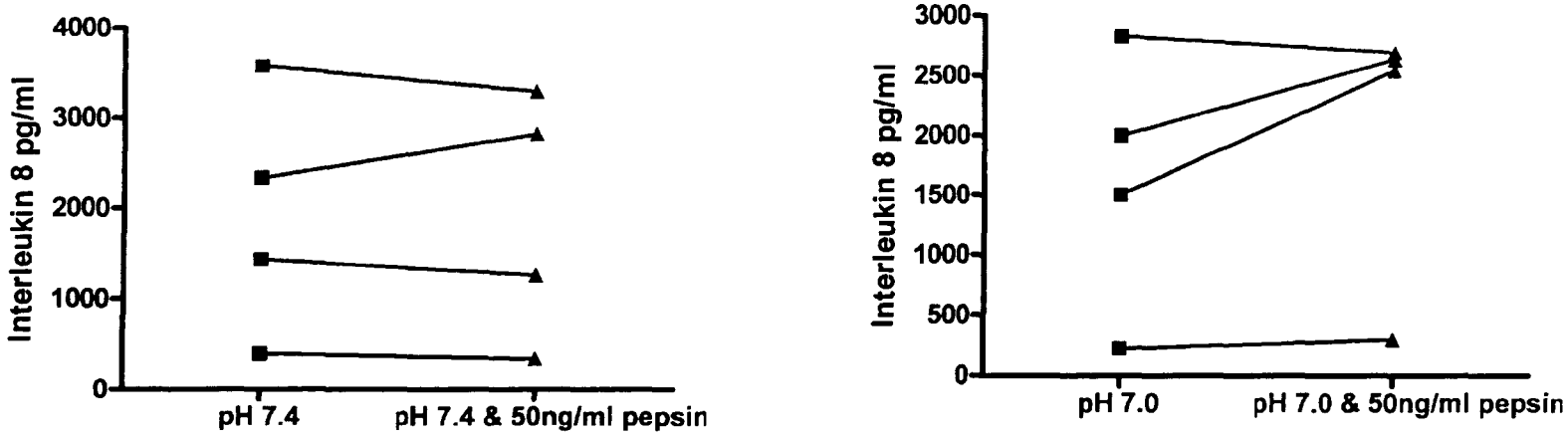


Figure 6.2.2 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 50ng/ml pepsin. Cells were seeded at approximately 50000 per well. The 4 lines represent 4 different patients and experiments were performed in duplicate. Media was collected at 48h.

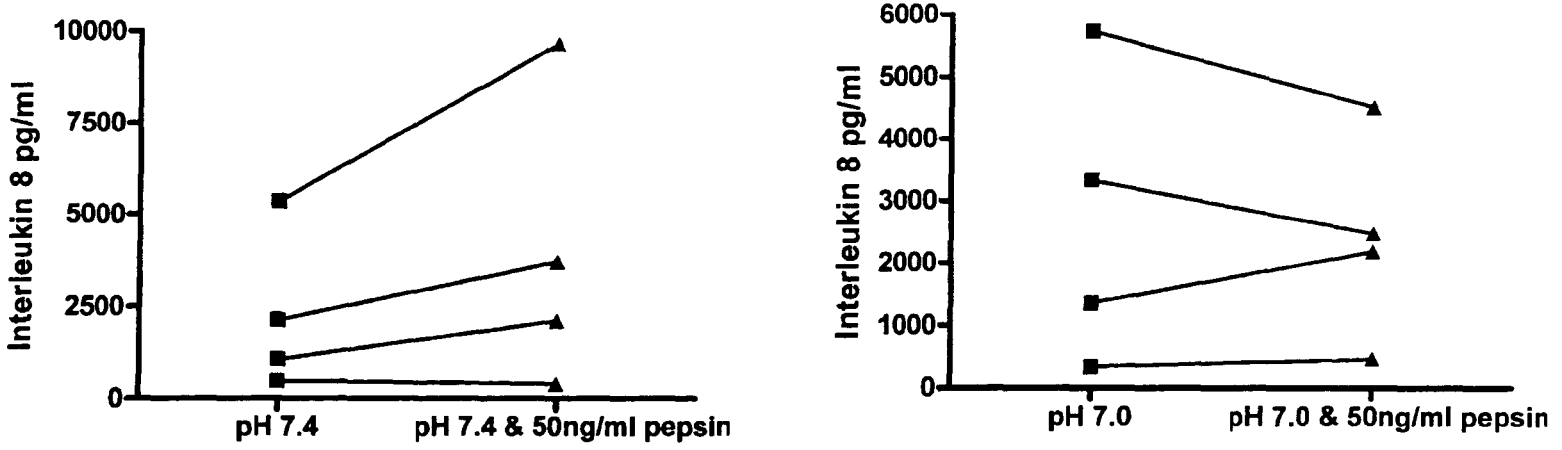


Figure 6.2.3 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 50ng/ml pepsin. Cells were seeded at approximately 50000 per well. The 4 lines represent 4 different patients and experiments were performed in duplicate. Media was collected at 72h.

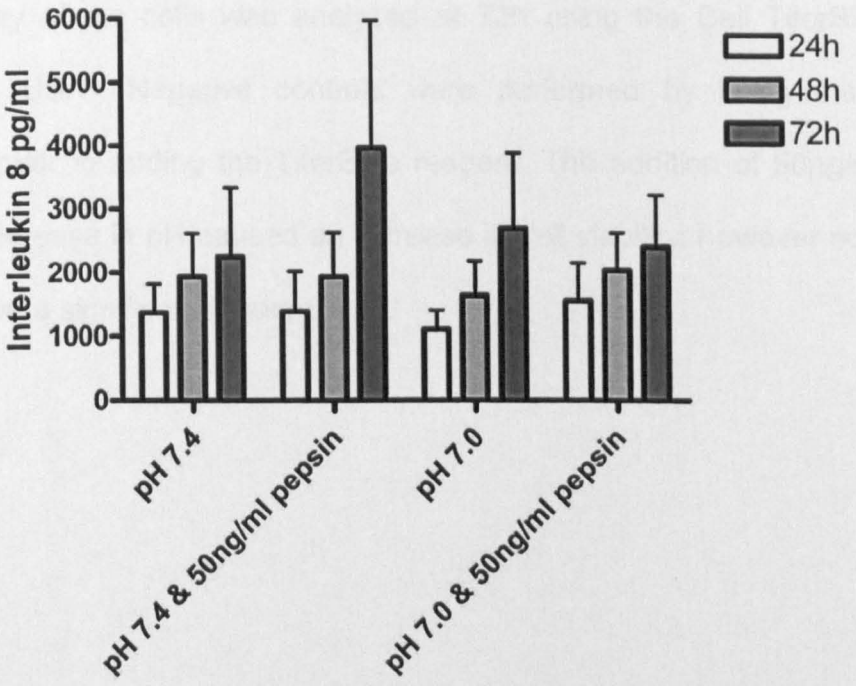


Figure 6.2.4 Interleukin 8 production from primary bronchial epithelial cells challenged with 50ng/ml pepsin at either pH 7.4 or pH 7.0. Bars represent mean with SEM and show the average of 4 experiments.

6.2.2.1 Cell viability

The viability of the cells was analysed at 72h using the Cell TiterBlue assay (Promega, USA). Negative controls were performed by fixing the cells in methanol prior to adding the TiterBlue reagent. The addition of 50ng/ml pepsin and the decrease in pH caused an increase in cell viability; however none of the changes were significant (figure 6.2.5).

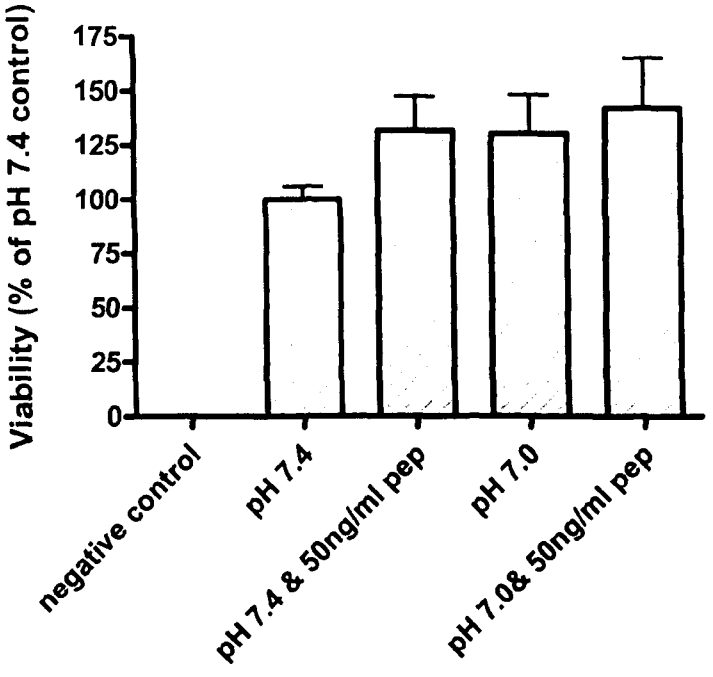


Figure 6.2.5 Primary bronchial epithelial cell viability after 72h exposure to 50ng/ml pepsin at either pH 7.4 or pH 7.0. Data is displayed as a percentage of the positive control (pH 7.4). Bars represent mean with SEM and show the average of 4 experiments. Negative controls were performed by fixing cells in methanol prior to adding the TiterBlue solution.

6.2.3 Cells challenged with 50µg/ml porcine pepsin

Again data was analysed with the Wilcoxon matched pairs test using GraphPad prism software. IL-8 production was still not significantly different when the pepsin concentration was increased 1000 fold to 50µg/ml pepsin at either pH 7.4 or pH 7.0 over 24, 48 or 72h (figures 6.3.1, 6.3.2 and 6.3.3). Data was also grouped to allow comparisons between pH 7.4 and pH 7.0 (figure 6.3.4) and at pH 7.4, pH 7.4 & pepsin and pH 7.0 & pepsin there was an increase in IL-8 production over time, which was not significant ($P>0.05$). At pH 7.0 there was an increase from 24 to 48h, however from 48 to 72h IL-8 production remains fairly constant. Again, there was no significant difference in IL-8 production between the challenge conditions when analysed using a non-parametric one-way analysis of variance with a *post-hoc* Mann Whitney test (median values in pg/ml for 72h; pH 7.4 1350, pH 7.4 & pepsin 1153, pH 7.0 1174 and pH 7.0 & pepsin 1575).

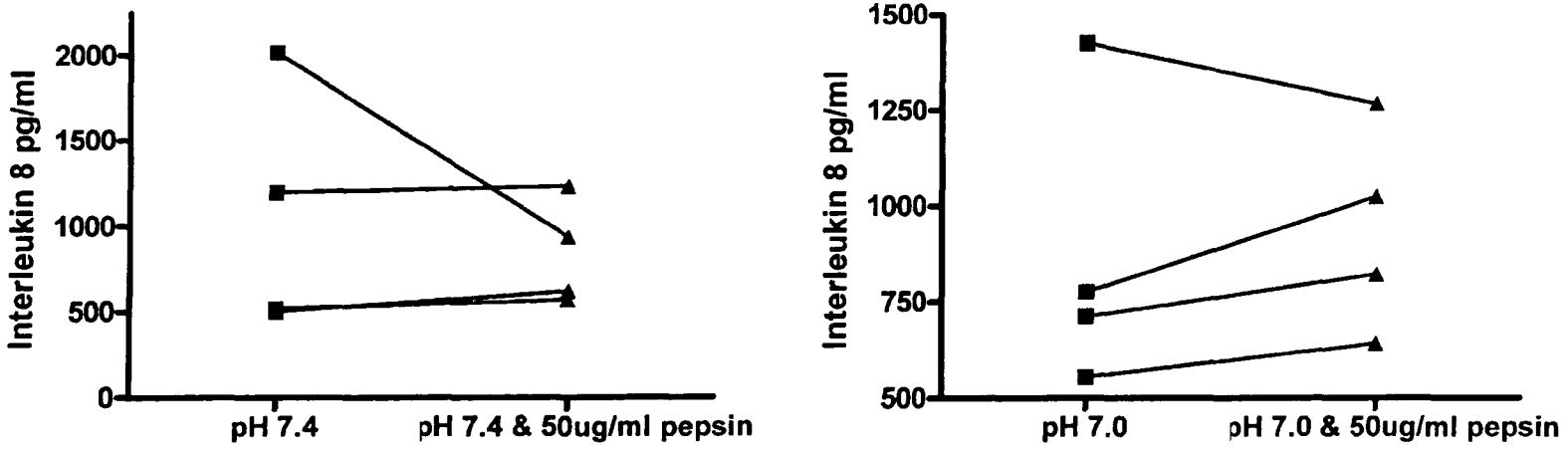


Figure 6.3.1 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 50µg/ml pepsin. Cells were seeded at approximately 50000 per well. The 4 lines represent 4 different patients and experiments were performed in duplicate. Media was collected at 24h.

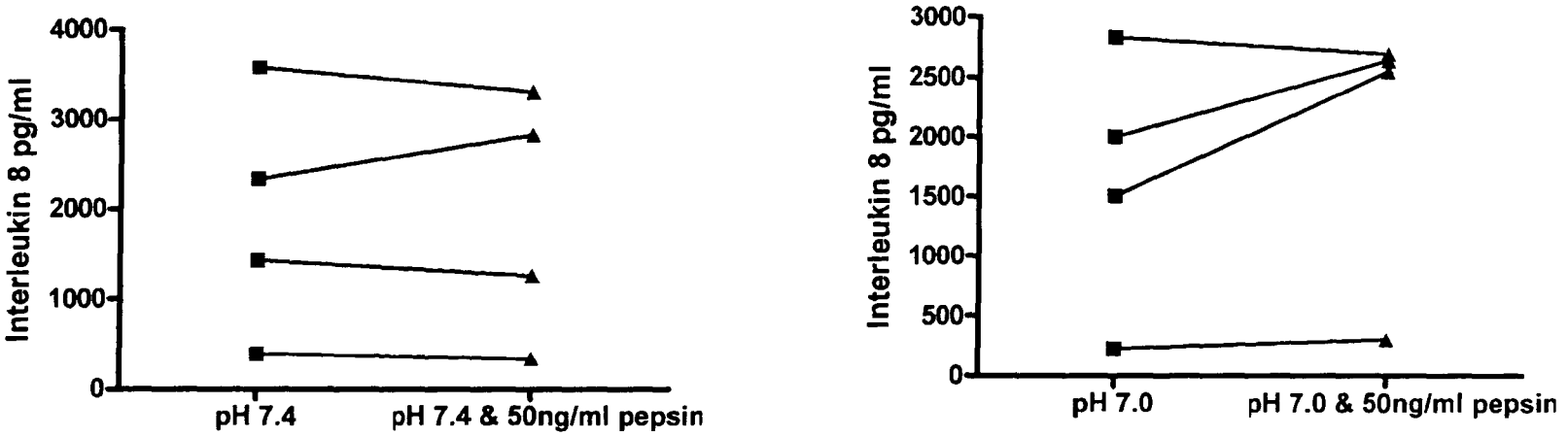


Figure 6.3.2 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 50µg/ml pepsin. Cells were seeded at approximately 50000 per well. The 4 lines represent 4 different patients and experiments were performed in duplicate. Media was collected at 48h.

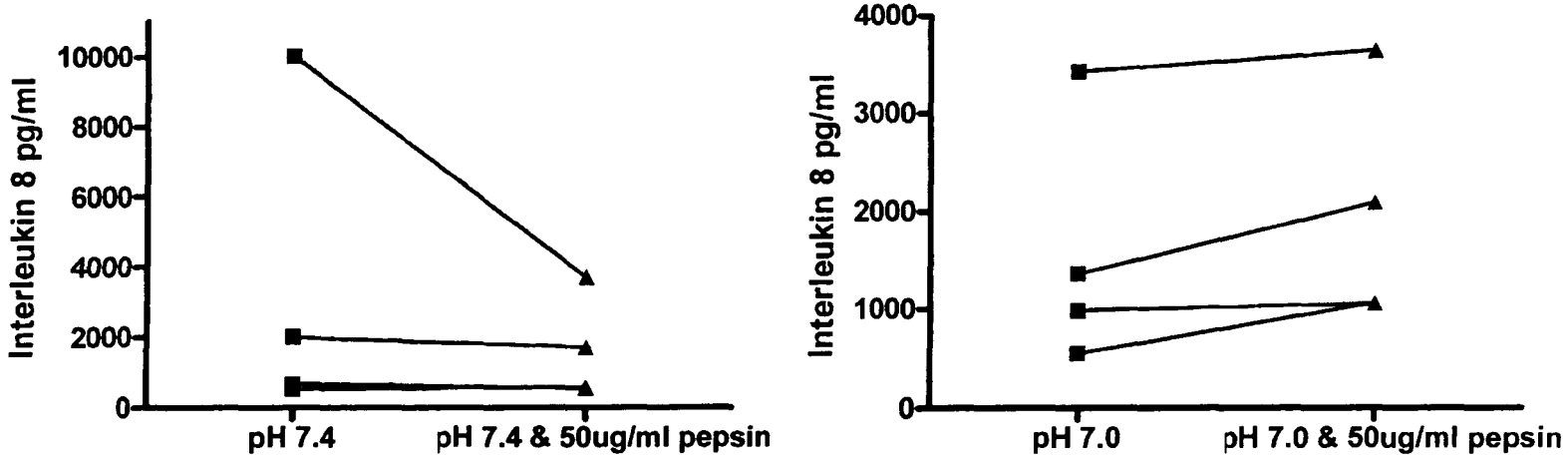


Figure 6.3.3 Mean interleukin 8 production from primary human bronchial epithelial cells challenged with 50µg/ml pepsin. Cells were seeded at approximately 50000 per well. The 4 lines represent 4 different patients and experiments were performed in duplicate. Media was collected at 72h.

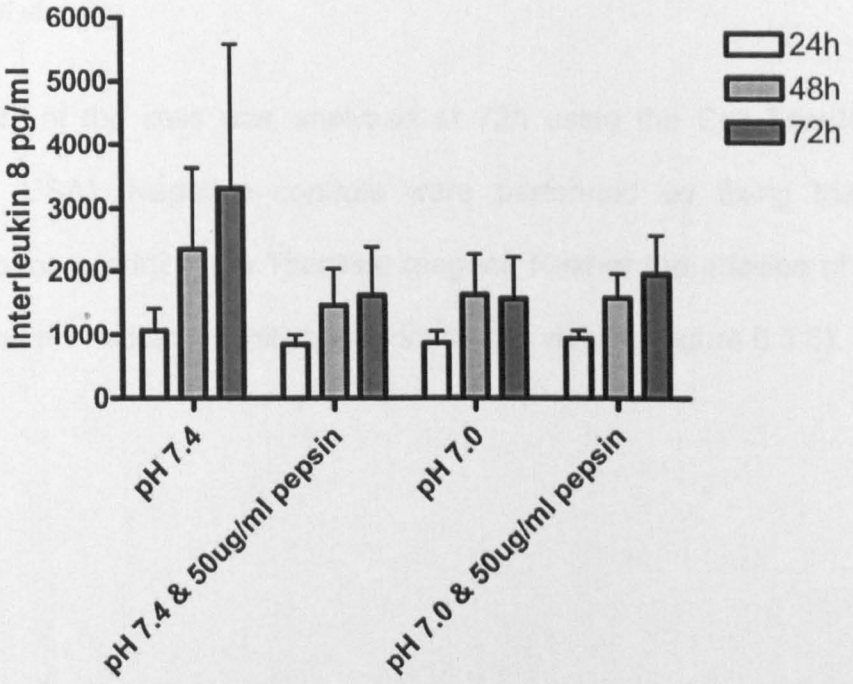


Figure 6.3.4 Interleukin 8 production from primary bronchial epithelial cells challenged with 50µg/ml pepsin at either pH 7.4 or pH 7.0. Bars represent mean with SEM and show the average of 4 experiments.

6.2.3.1 Cell viability

The viability of the cells was analysed at 72h using the Cell TiterBlue assay (Promega, USA). Negative controls were performed by fixing the cells in methanol prior to adding the TiterBlue reagent. Neither the addition of pepsin or reducing the pH had any significant effect on cell viability (figure 6.3.5).

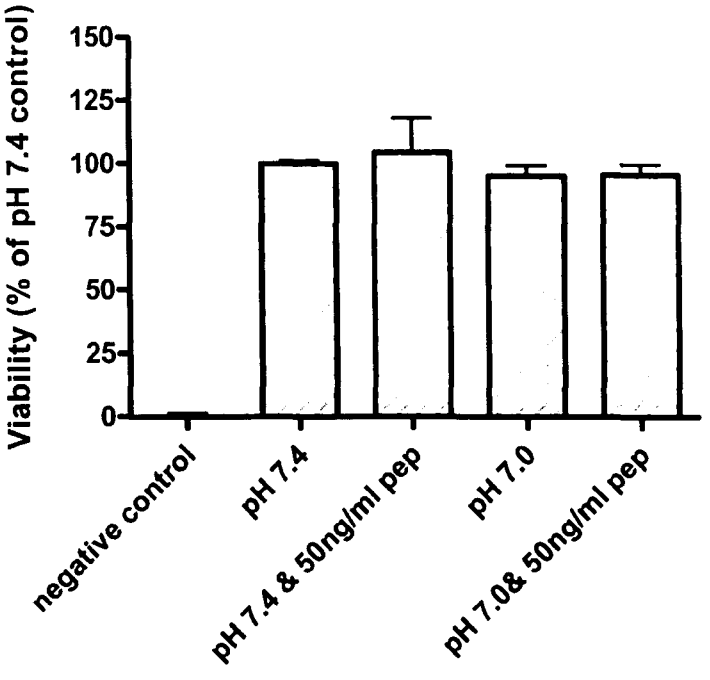


Figure 6.3.5 Primary bronchial epithelial cell viability after 72h exposure to 50µg/ml pepsin at either pH 7.4 or pH 7.0. Data is displayed as a percentage of the positive control (pH 7.4). Bars represent mean with SEM and show the average of 4 experiments. Negative controls were performed by fixing cells in methanol prior to adding the TiterBlue solution.

6.3 Viability of epithelial cells challenged with gastric juice

In a preliminary experiment the epithelial cells were also challenged with basal human gastric juice collected from patients undergoing endoscopy. The patients were taking proton pump inhibitors and as a result the pH of the gastric juice was abnormally high (pH 5).

The epithelial cells were challenged with neat or diluted gastric juice (1/4, 1/20, 1/100, 1/1000, 1/5000 or 1/10000 diluted in serum free basal epithelial media, Lonza, Switzerland). The viability was calculated as a percentage of the control (cells grown in serum free basal media) after 24h and was significantly reduced in all test conditions when analysed using a one-way analysis of variance with a *post-hoc* Mann Whitney test ($P< 0.001$ for all dilutions, figure 6.4).

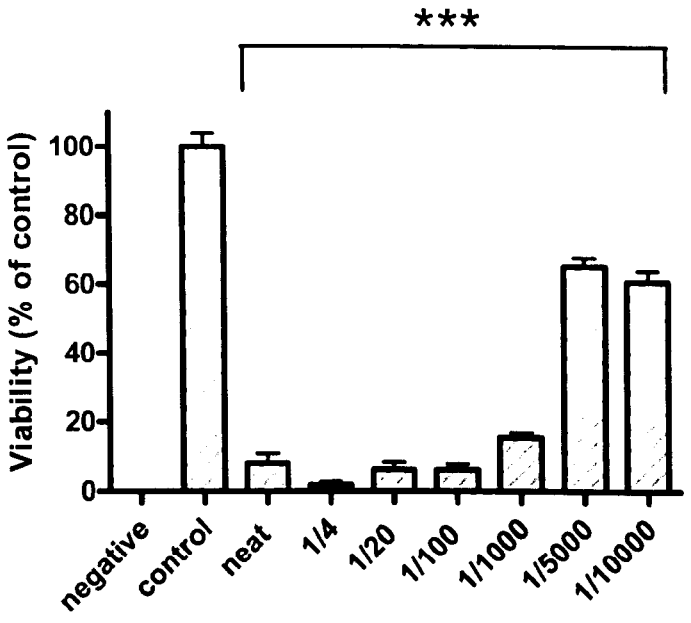


Figure 6.4 Viability of epithelial cells challenged with a range of dilutions of human gastric juice (diluted in serum free basal epithelial media, Lonza, Switzerland). Viability is displayed as a percentage of the normal control and was measured after 24h. Bars represent mean & SEM. Negative controls were performed by fixing the cells in methanol prior to adding the TiterBlue reagent. Viability is significantly reduced with all dilutions of gastric juice ($P < 0.001$ for all conditions). This data represents a single experiment.

6.4 Stimulation of goblet cells with porcine pepsin

Mucus secreting goblet cells (cell line HT29-MTX) were grown until confluent in 24 well plates. The cells were then incubated with 10ng/ml, 50ng/ml or 50µg/ml pepsin in serum free Dulbecco's modified eagles medium (DMEM) or serum free DMEM adjusted to pH 7.0 with 1M HCl. Media was collected at 24, 48 and 72h and assayed for MUC5AC mucin with a slot/blot ELISA.

6.4.1 Cells challenged with 10ng/ml pepsin

Data was analysed using a one-way analysis of variance with a *post-hoc* Mann Whitney test. Mucin production increased over each 12h period and was significantly increased from 24 to 72h within each condition ($P<0.05$). At pH 7.4 there was a significant increase in mucin production between 48 and 72h ($P= 0.026$). In addition at pH 7.0 and 7.0 & pepsin mucus production was also increased over 24h to 48h ($P= 0.004$ and 0.009 respectively). Mucus production was not significantly altered between the challenge conditions (figure 6.5). Viability was also measured using the TiterBlue assay (Promega, USA) and was not significantly altered by lowering the pH or with the addition of 10ng/ml pepsin (figure 6.6).

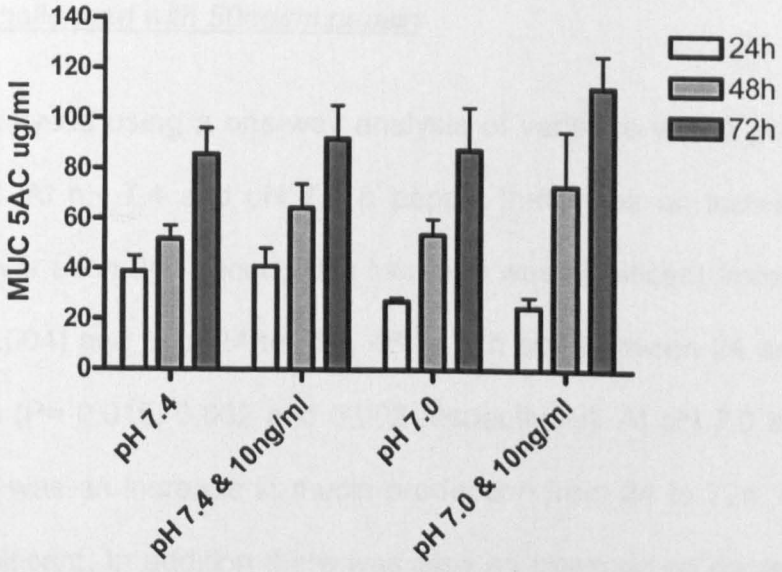


Figure 6.5 MUC5AC mucin production from goblet cell line HT29-MTX challenged with 10ng/ml pepsin. Bars represent mean with SEM and show the average of 2 experiments.

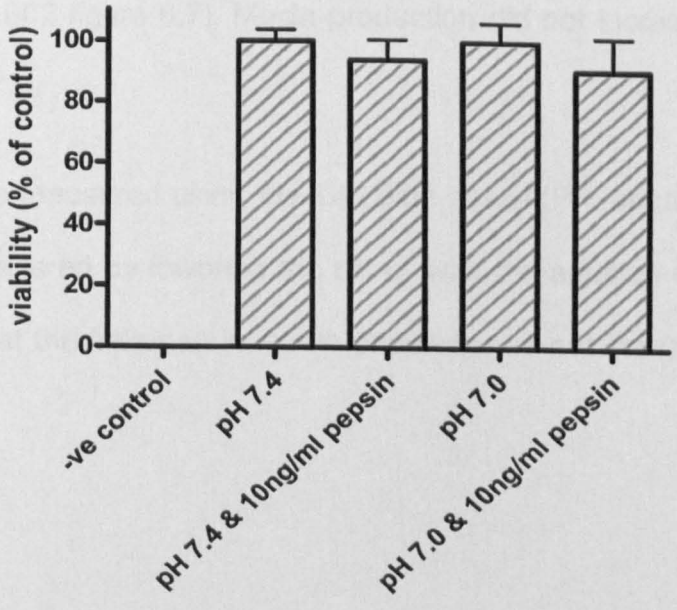


Figure 6.6 Goblet cell viability after 72h challenge with 10ng/ml pepsin at pH 7.4 and pH 7.0. Data is displayed as a percentage of the pH 7.4 control. Bars represent mean with SEM and show the average of 2 experiments..

6.4.2 Cells challenged with 50ng/ml pepsin

Data was analysed using a one-way analysis of variance with a *post-hoc* Mann Whitney test. At pH 7.4 and pH 7.4 & pepsin there was an increase in mucin production over each 24h period. This increase was significant from 24 to 72h at pH 7.4 (P=0.004) and from 24 to 48h, 48 to 72h and between 24 and 72h at pH 7.4 & pepsin (P= 0.015, 0.002 and 0.002 respectively). At pH 7.0 and pH 7.0 & pepsin there was an increase in mucin production from 24 to 72h, however this was not significant. In addition there was also an unexpected decrease from 24 to 48h for both pH 7.0 and 7.0 & pepsin (P=0.009 for both conditions).

Mucin production was also significantly increased at 72h when challenged with 50ng/ml pepsin at pH 7.4 (medians: pH 7.4 61.4µg/ml and pH 7.4 & pepsin 92.2µg/ml, P= 0.002 figure 6.7). Mucin production did not increase on exposure to pepsin at pH 7.0.

Viability was also measured using the TiterBlue assay (Promega, USA) and was not significantly altered by lowering the pH or with the addition of pepsin (figure 6.8), showing that the increase in mucin production is not due to increased cell numbers.

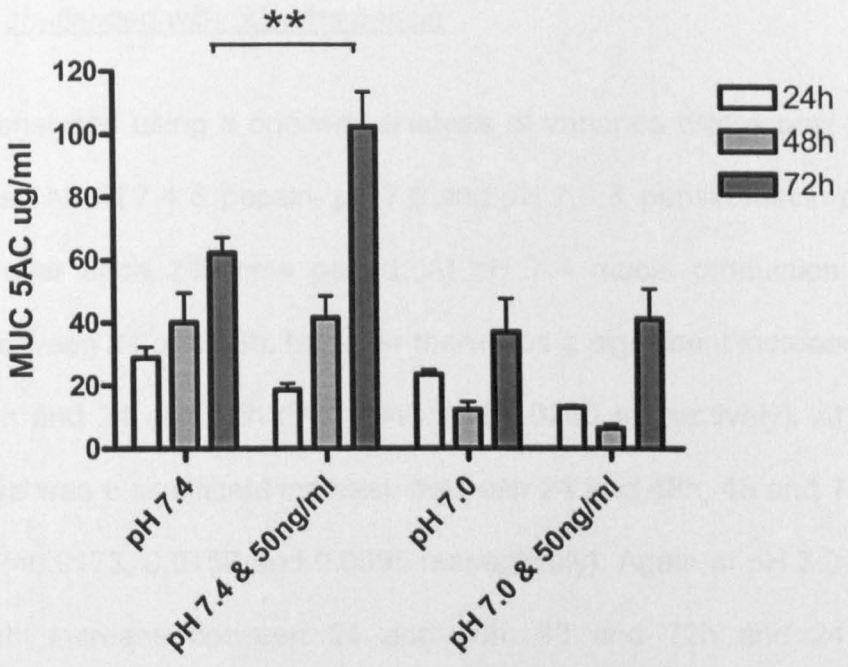


Figure 6.7 MUC5AC mucin production from goblet cell line HT29-MTX challenged with 50ng/ml pepsin. Bars represent mean with SEM and show the average of 2 experiments.

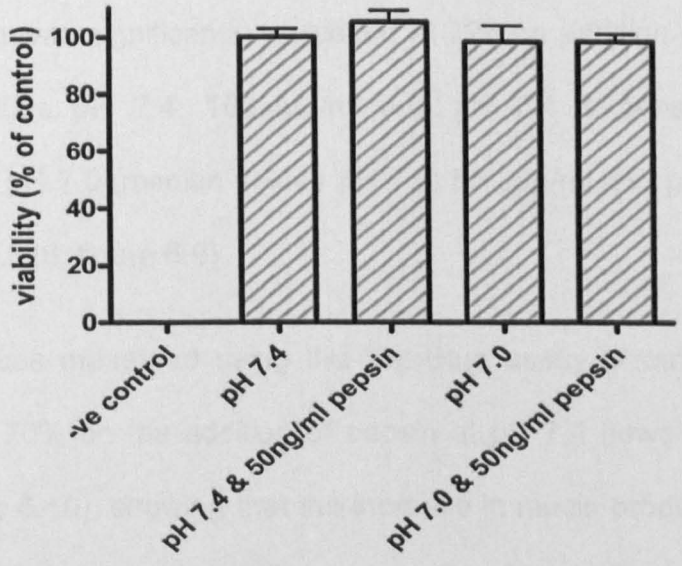


Figure 6.8 Goblet cell viability after 72h challenge with 50ng/ml pepsin at pH 7.4 and pH 7.0. Data is displayed as a percentage of the pH 7.4 control. Bars represent mean with SEM and show the average of 2 experiments.

6.4.3 Cells challenged with 50µg/ml pepsin

Data was analysed using a one-way analysis of variance with a *post-hoc* Mann Whitney test. At pH 7.4 & pepsin, pH 7.0 and pH 7.0 & pepsin mucin production increased over each 24h time period. At pH 7.4 mucin production remained constant between 24 and 48h, however there was a significant increase between 48 and 72h and 24 and 72h (P=0.0043 and 0.0260 respectively). At pH 7.4 & pepsin there was a significant increase between 24 and 48h, 48 and 72h and 24 and 72h (P=0.0173, 0.0159 and 0.0095 respectively). Again at pH 7.0 there was a significant increase between 24 and 48h, 48 and 72h and 24 and 72h (P=0.0043 for all time points). Finally, at pH 7.0 & pepsin there was a significant increase in mucin production from 48 to 72h and from 24 to 72h (P=0.0159 and 0.0043 respectively) (figure 6.9).

Mucin production was significantly increased at 72h on addition of pepsin at pH 7.4 (median values pH 7.4; 163.4µg/ml and pH 7.4 & pepsin; 448.9µg/ml, P=0.038) and at pH 7.0 (median values pH 7.0; 55.3µg/ml and pH 7.0 & pepsin; 327.2µg/ml, P=0.016, figure 6.9).

Again, viability was measured using the TiterBlue assay (Promega, USA) and was reduced to 70% on the addition of pepsin at pH 7.4 however this was not significant (figure 6.10), showing that the increase in mucin production is not due to increased cell numbers.

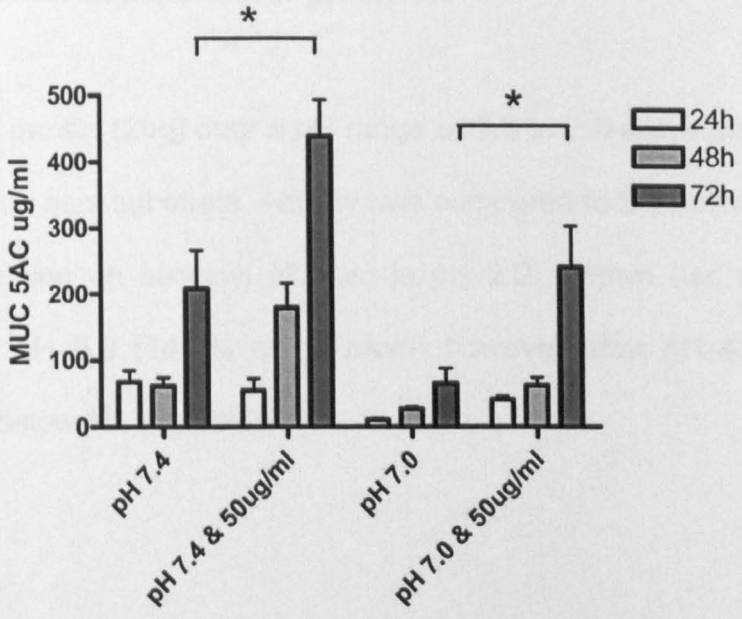


Figure 6.9 MUC5AC mucin production from goblet cell line HT29-MTX challenged with 50µg/ml pepsin. Bars represent mean with SEM and show the average of 2 experiments.

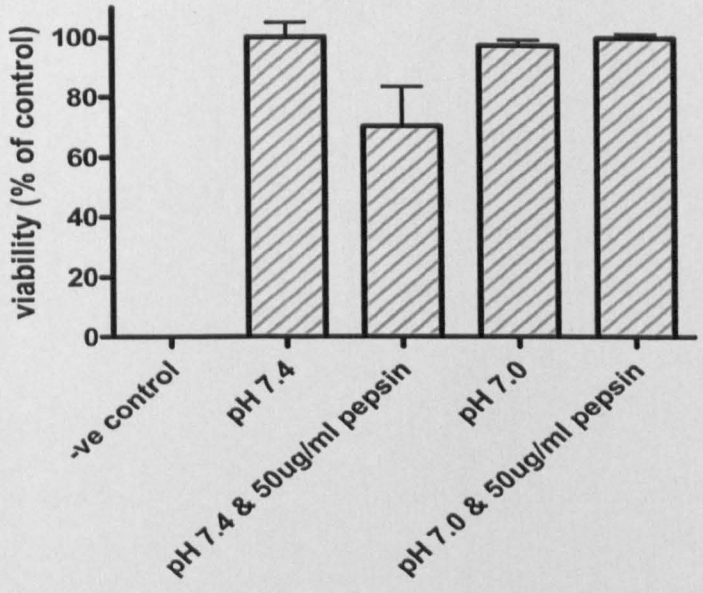


Figure 6.10 Goblet cell viability after 72h challenge with 50µg/ml pepsin at pH 7.4 and pH 7.0. Data is displayed as a percentage of the pH 7.4 control. Bars represent mean with SEM and show the average of 2 experiments.

6.5 Activity profile of pepsin over pH 5.0-7.0

The activity of pepsin (2ug) over a pH range of 5.5 to 7.0 was investigated using succinyl albumin as a substrate. Activity was compared to the optimum pH, which for pepsin working on succinyl albumin is pH 2.2. Pepsin had some residual activity up to pH 6.0 (14.5% of optimum) however after pH 6.5 the activity decreases to below 1% (figure 6.11).

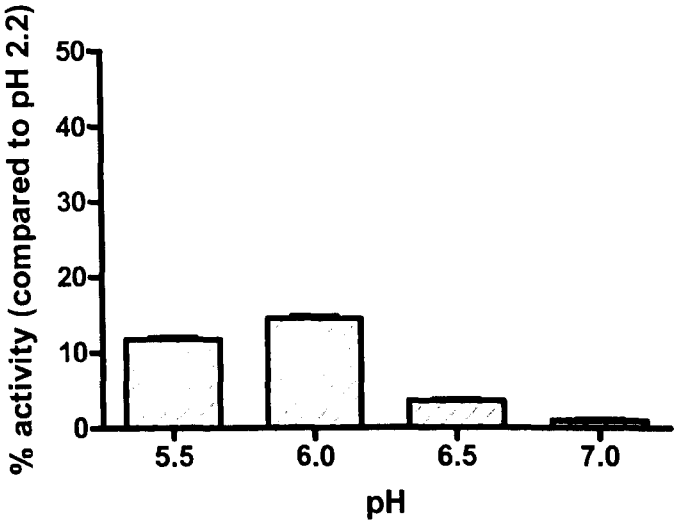


Figure 6.11 Activity profile for pepsin (2µg) over a pH range of 5.5 to 7.0. Values are displayed as a percentage of optimum (pH 2.2) and bars represent mean with SEM and are representative of 3 experiments.

6.6 Discussion

Mucus is an important part of the innate immune system and helps to protect the lungs against pathogens and/or toxins. However, mucus hypersecretion can be a problem in certain lung diseases, particularly asthma, COPD and CF (Voynow 2002; Callaghan-Rose and Voynow 2006). Aspiration of gastric contents into the lungs has also been associated with these pathologies; therefore the current study aimed to investigate the effect of pepsin, an important component of the gastric refluxate, on the production of mucus from goblet cells. In addition, interleukin 8 (IL-8) has been implicated in the chronic rejection of lung transplants and has also been shown to stimulate mucus production from goblet cells, therefore the potential for pepsin to stimulate IL-8 release from primary human lung epithelial cells has also been investigated.

The addition of pepsin or the reduction in pH did not significantly reduce the viability of the goblet cells over 72h; however, mucus production was significantly increased when the cells were exposed to 50ng/ml and 50µg/ml pepsin at pH 7.4 and with 50µg/ml pepsin at pH 7.0. Previous work has shown that pepsin has little or no activity above pH 6.5 (Piper and Fenton 1965). This has been confirmed by the current investigation (figure 6.11) and suggests that any pepsin remaining adhered to the airway tissue could stimulate mucus hypersecretion even after it has been neutralised.

There was also an unexpected decrease in mucin production at 48h when the cells were exposed to 50ng/ml pepsin at pH 7.0 and with pH 7.0 alone (figure

6.7). This may be explained by low mucin production in one of the experiments, reducing the average value. This suggests that the result is an anomalous one and that the experiments should be repeated.

The regulation of MUC genes has been previously investigated *in vitro* and a number of cytokines have been shown to up-regulate MUC5AC expression, for example, TNF-α, IL-6 and IL-17 (Chen et al. 2003; Callaghan-Rose and Voynow 2006). Smirnova et al have also shown that IL-8 can stimulate MUC5AC secretion from goblet cells (Smirnova et al. 2002). In addition, the group has shown that bacterial LPS up-regulates both IL-8 and MUC5AC mRNA expression and secretion from goblet cells, suggesting that the up-regulative effect of LPS is partially mediated through an IL-8 dependant mechanism (Smirnova et al. 2003).

Other factors known to stimulate MUC5AC expression *in vitro* include prostaglandins, matrix matallo-proteinases (MMPs), neutrophil elastase, reactive oxygen species and also exogenous toxins, such as tobacco smoke and environmental pollutants (Voynow et al. 2006).

Such factors are thought to stimulate the up-regulation of mucins by binding to specific surface cell receptors, for example, P2Y2 and toll like receptors (Voynow et al. 2006). Additionally, the epidermal growth factor (EGF) receptor has been implicated in mucin gene regulation through a variety of stimuli (Takeyama et al. 1999). Binding of these receptors is then thought to activate mitogen-activated protein kinase (MAPK) pathways, which in turn activate the transcription factor, nuclear factor kappa B (NFκB), which will regulate mucin gene expression

(Callaghan-Rose and Voynow 2006). Other transcription factors can also mediate mucin gene expression, for example SP1 and AP-1 (Perrais et al. 2002; Gensch et al. 2004).

To further understand the link between the presence of pepsin and the increase in mucus production the effect of pepsin on interleukin 8 release from epithelial cells was also investigated. IL-8 has been shown to play a key role in the recruitment of inflammatory cells in obliterative bronchiolitis (OB), the histological manifestation of chronic rejection in lung transplantation. IL-8 is a neutrophilic chemokine, and neutrophils are repeatedly elevated in transplant patients with BOS. Neutrophils may not only damage/kill invading micro-organisms, but also the tissue in their region of activity due to the reactive oxygen species they produce. IL-8 can be produced by the epithelium itself in response to any pathogens or toxins present in the lung, therefore the potential for any aspirated pepsin to stimulate IL-8 production by the epithelial cells was investigated.

Pepsin did not significantly alter IL-8 production at either pH 7.4 or pH 7.0. Levels of IL-8 measured in this study compare to levels previously measured from other groups, including primary bronchial epithelial cell cultures (Weihler et al measured levels of approximately 500pg/ml over 24h under control conditions) and also an epithelial cell line (BEAS-2B, Gulliot et al also measured levels of approximately 500pg/ml) (Wiehler and Proud 2007; Guillot et al. 2008). Additionally the viability of the cells was not altered with the addition of pepsin or when the pH was lowered.

Studies by Smirnova et al have shown that LPS can stimulate IL-8 and mucus production from goblet cells *in vitro*, and potentially that the mucus production is mediated through an IL-8 dependant mechanism (Smirnova et al. 2003). The potential for pepsin to stimulate IL-8 secretion from the goblet cells themselves was also investigated, however IL-8 production was negligible (data not shown). Whilst the epithelial or goblet cells did not produce IL-8 in response to pepsin it is possible that *in vivo* other sources could, for example macrophages.

It is also possible that other cytokines could contribute to the increase in mucin production. In a study by Chen et al differentiated human primary tracheobronchial epithelial cells in an air liquid interface were treated with a variety of cytokines (interleukins-1α&β, 2-13, 15-18 and TNF-α). The group showed that a significant increase in MUC5AC and 5B expression was seen with the addition of IL-6 and IL-17. As IL-17 is known to stimulate IL-6 production from bronchial epithelial cells the group also investigated whether the stimulatory effect of IL-17 on mucin expression was mediated through an IL-6 paracrine/autocrine loop. Using a neutralising IL-6 antibody they showed that IL-17 mediated MUC5B expression was significantly decreased (Chen et al. 2003). IL-6 can also be produced by airway epithelial cells so it is possible that an increase in IL-6 production from these epithelial cells (in response to pepsin or IL-17 from T cells) could cause an additional increase in mucus production from goblet cells causing further airflow obstruction in already injured airways.

The results from the current investigation provide evidence that the increase in mucin production from goblet cells in response to pepsin is not mediated entirely

through IL-8. One other potential explanation is the presence of a pepsin receptor on the goblet cells. A recent study by Johnston et al has indicated that pepsin can be taken up by laryngeal cells through receptor mediated endocytosis (Johnston et al. 2007). This could also be the case in goblet cells, and once pepsin has bound to a receptor it could stimulate mucus production via a MAPK pathway as previously described, or it could be taken up by the cell and stimulate mucus production through an alternative pathway.

It is of interest that mucus production is increased only with 50µg/ml pepsin at pH 7.0 when at pH 7.4 both 50µgml and 50ng/ml pepsin can stimulate mucus production. This could be explained by the reduction in pH causing certain cell processes to slow down. This might also explain why the result is only seen after 72h of simulation. The decrease in pH may be causing an increase in time taken for the cells to respond to pepsin i.e. introducing a 'time lag'.

Another previous study by Johnston et al has shown that although pepsin has little or no activity above pH 6.5, it remains stable for at least 24h at pH 7.0 and 37°C and can retain approximately 79% of its original activity upon reactivation (Johnston et al. 2007). This is clinically important as any pepsin remaining in the goblet cells after it has been taken-up could be reactivated by a decrease in pH that would occur after a subsequent reflux event. This could also be true of the lung epithelial cells, meaning that any refluxed pepsin could lay dormant until reactivation or it may also be possible that once taken up by the cells inactive pepsin could be transported to an intracellular compartment of lower pH and once activated could cause further damage.

Whether or not this is the case can not be determined from this study, therefore further investigation into the mechanisms behind this increase in mucus production on exposure to pepsin is required.

Viability was not significantly reduced with the addition of pepsin at either pH 7.4 or pH 7.0; however it was significantly reduced with the addition of whole gastric juice. This suggests that there are other components of the refluxate with the potential to cause epithelial damage, possibly bile salts as duodenal-gastro-oesophageal reflux has also been implicated in chronic lung rejection. It is likely that the gastric juice used to stimulate the cells in this study did contain bile, as the pH was abnormally high (approximately pH 5) and appeared green in colour.

There are certain limitations to this study. The cells were grown in separate cultures, meaning that any observations were only associative, for example, if pepsin had stimulated the epithelial cells to produce IL-8 it could only be assumed that this would then stimulate the goblet cells to produce mucin. IL-8 was not increased on exposure to pepsin, however other cytokines may have been, for example IL-6, and therefore future experiments could include incubating the goblet cells with media taken from epithelial cells challenged with pepsin. Another approach would be to set up an air-liquid interface of the primary epithelial cells. This would allow the cells to differentiate into ciliated epithelial cells and goblet cells within the same culture, therefore mucins and cytokines could be measured in the same experiment. This technique was not available in our laboratory when this study began; however now it is available the experiments could be repeated to strengthen the results.

To summarise, mucus production was significantly increased when the cells were exposed to 50ng/ml and 50µg/ml pepsin at pH 7.4 and with 50µg/ml pepsin at pH 7.0. This increase is unlikely to be IL-8 dependant, as pepsin did not alter IL-8 production from the goblet or epithelial cells. The addition of pepsin at either pH 7.4 or pH 7.0 did not reduce the viability of the epithelial cells, however the addition of whole gastric juice, whether neat or diluted did significantly reduce the viability of epithelial cells *in vitro*. This suggests there are other components present in the gastric juice with the potential to cause further damage to the lung epithelium.

Chapter 7

General Discussion

Lung transplantation has become a viable therapeutic option for patients with end-stage lung disease, however, despite improvements in surgical techniques and post-operative management, long term survival is poor when compared to those of other solid organ transplants. The long term success is limited by the onset of obliterative bronchiolitis (OB) and its clinical correlate bronchiolitis obliterans syndrome (BOS). Obliterative bronchiolitis is thought to occur as a response to both immunological and non-immunological mechanisms, and there is increasing evidence to suggest that gastro-oesophageal reflux with subsequent aspiration is a contributing factor (D'Ovidio and Keshavjee 2006; Li et al. 2008).

There were three overall aims of this study. The first was to investigate whether aspiration was occurring in lung transplant recipients by measuring levels of pepsin, as a biomarker of gastric aspiration, in the bronchoalveolar lavage (BAL) of transplant patients. The second was to measure BAL pepsin in a longitudinal cohort to investigate the variation in levels over time and also if high levels of pepsin in the BAL at a particular, early time point can predict for or predispose a patient to chronic rejection (OB/BOS). The final aim was to investigate the possible links between any pepsin present in the lung and damage caused to the epithelium using goblet cells and primary bronchial epithelial cell cultures taken from lung transplant patients.

When this project began gastro-oesophageal reflux (GOR) had been implicated in the development of BOS (Palmer et al. 2000; Davis et al. 2003; Cantu et al. 2004), however, there were few, if any studies investigating the underlying mechanisms. In 2005 Ward et al showed for the first time that pepsin can be found in the BAL of lung transplant patients, at levels higher than could be accounted for by serum pepsinogen, providing evidence that gastric contents could in fact reach the lungs. This has now been confirmed in a larger group of patients with more control data. In the cross-sectional study outlined in this thesis pepsin levels were higher in all lung transplant patients compared to normal and disease controls suggesting that gastric aspiration is an ongoing source of injury in this patient population. In addition, pepsin levels were highest in recipients with histologically verified A2 or greater acute rejection. Furthermore, these patients had the highest grades of airway inflammation, suggesting a possible link between aspiration, acute rejection and inflammation (Stovold et al. 2007).

Another point of interest is that this study has shown that patients who are being treated with maintenance proton pump inhibitors (PPI) still show evidence of the aspiration of gastric contents into the lung. On initial consideration this may seem unexpected, however, approximately 12-20% of gastro-oesophageal reflux disease (GORD) patients are resistant to acid suppression therapy (Ahlawat et al. 2005). In addition, PPIs act to reduce acidic reflux, but will not prevent mildly acidic, neutral or alkaline reflux, which may still contain pepsin. Gastric aspiration is not the target of PPI therapy, therefore patients may continue to aspirate whilst taking this medication.

The second aim of the study was to further investigate the role of gastric aspiration in lung transplant rejection using longitudinal BAL samples collected at 1 week, 1, 3, 6 and 12 months post-transplant. Initially, the data was split into cross-sectional time points to investigate whether high pepsin levels are associated with different grades of rejection at different times. Again, there was some evidence that transplant patients with histologically verified A2 or greater acute rejection had higher levels of pepsin compared to stable controls, confirming earlier work. In this study there were also significantly higher levels of BAL pepsin in patients with minimal acute rejection (graded A1), which was not the case in the previous cross-sectional study. This may be due to the smaller numbers in the A1 group used in the earlier investigation where there were only 6 patients with A1 acute rejection.

For the longitudinal assessment of BAL pepsin the chosen format of analysis was to decide on a time point and investigate whether high levels of pepsin at this time point can predict for the development of BOS. There is evidence of gastric aspiration being problematic early post-transplant (Cantu et al. 2004) and so three months was prospectively chosen. Three months is still relatively early post-transplant, however, patients will be more clinically stabilised than they would be at one week or one month.

As there is no general consensus of what are high levels of pepsin in bronchoalveolar lavage a number of 'cut-offs' were used based on levels measured in stable transplant controls. Concentrating on the 75th percentile (10.4ng/ml) as a cut off, patients with high levels of BAL pepsin at 3 months (i.e.

>10.4ng/ml) are estimated to develop BOS at 3 times the rate of those with low BAL pepsin (≤10.4ng/ml). In addition, using Kaplan-Meier estimates of survival patients with BAL pepsin above 10.4ng/ml at 3 months are 50% free from BOS at 4 years post-transplant compared to 80% in patients with BAL pepsin ≤10.4ng/ml.

Alloimmune mechanisms have been the traditional focus of research and therapeutic intervention for lung transplant rejection, however the investigations outlined here add to the growing evidence that supports the role of non-alloimmune mechanisms as risk factors for the development of chronic lung rejection.

Throughout these investigations there is suggestion of a link between acute rejection and high levels of BAL pepsin. This has also been shown in a rat model of lung transplantation from the Duke group. Allografts challenged with aspiration demonstrated severe grade 4 acute rejection with significant monocyte infiltration, fibrosis, and lung destruction (Hartwig et al. 2006). Aspiration was also associated with increases in CD8+ T cells and this study indicates that gastric aspiration may lead to pathological changes previously attributed to T cell, alloimmune based mechanisms (Takehisa et al. 2002; Boehler and Estenne 2003). Acute rejection is consistently linked with the development of BOS (Sharples et al. 2002; Scott et al. 2005) so it is possible that gastric aspiration is promoting injury (acute rejection) which in turn is accelerating the development of BOS.

In these investigations there was a trend towards patients with early elevated levels of BAL pepsin developing BOS at a greater rate than those with lower levels, however this was not significant at 95% confidence levels. Further investigations with an increased sample size are required to achieve conventional levels of adequate power.

As a result of this research and other studies, lung transplant patients at the Freeman hospital (Newcastle, UK) now have the option to be monitored for reflux post-operatively, and if appropriate fundoplicative surgery will be considered.

During the project it was necessary to develop an ELISA for the measurement of pepsin. Although the development process took a significant amount of time the assay has now been shown to be sensitive and reliable, which is important as since this work began a number of other centres have started using biomarker approaches to monitor aspiration. Many of these centres are using bile salts as a marker rather than pepsin, and this may be due to the commercial availability of bile acid kits and the time required to develop an assay to measure pepsin. Some preliminary work from our group has shown that bile may not be detectable in BAL using mass spectrometry, suggesting the kits are not sensitive enough to accurately measure levels of bile you may expect to find in BAL. This implies that pepsin may be a more suitable biomarker of gastric aspiration and as a result of this research our group is one of the few that can accurately measure pepsin in BAL from lung transplant patients.

The final aim of the project was to investigate the possible links between any pepsin present in the lung and damage caused to the epithelium which may lead to the development of OB. Early OB is characterised by an influx of inflammatory cells into the lung (Boehler et al. 1998) and chemokines such as interleukin 8 (IL-8) have been shown to be important in the recruitment of such inflammatory cells, especially neutrophils which are repeatedly elevated in the lavage of transplant patients with BOS (DiGiovine et al. 1996; Elssner and Vogelmeier 2001). This suggests that IL-8 along with other such chemokines play a crucial role in the pathogenesis of OB.

IL-8 is an important part of the innate immune system and can be produced by the epithelium itself in response to many pathogens or stimuli potentially present in the lung. The potential for pepsin to stimulate IL-8 production from epithelial cells was therefore investigated.

Mucus production is also an important part of the innate immune response against any pathogens present in the respiratory tract. The cells responsible for mucus production in the airways are goblet cells and the secretory cells of the submucosal glands and one of their main products is the mucin expressed from the MUC5AC gene.

It is also possible that airway goblet cells could secrete mucus in response to pepsin aspirated into the lung, therefore, in addition to looking at the effects of pepsin on IL-8 production from epithelial cells, mucus production from goblet cells was also investigated.

Mucus production was significantly increased when goblet cells were exposed to 50ng/ml and 50µg/ml pepsin at pH 7.4 and with 50µg/ml pepsin at pH 7.0. This increase is unlikely to be IL-8 dependant, as pepsin did not alter IL-8 production from the goblet or epithelial cells. The effect of incubating the epithelial cells with whole gastric juice was also investigated and was found to significantly reduce cell viability, even when diluted 1/10000, an effect not seen with pepsin alone up to a concentration of 50µg/ml. This suggests there are other components present in the gastric juice with the potential to cause further damage to the lung epithelium, for example bile salts or some chemical constituents of food.

This work has shown that pepsin, a biomarker of gastric aspiration is detectable in BAL from lung transplant recipients, and that it is associated with injury (acute rejection, a known risk factor for BOS). In addition, it has shown a trend for early elevated BAL pepsin to be associated with an increased risk of developing BOS. Therefore, this thesis supports the hypothesis that gastric aspiration may be an important injury in lung transplantation and that pepsin is a potentially useful biomarker that may be associated with chronic allograft damage. Further investigations involving a larger patient cohort are required to confirm these findings.

Future Areas for Research

Future directions of the project could include setting up an air-liquid interface of the primary bronchial epithelial cells, as this would allow the cells to differentiate into ciliated epithelial cells and goblet cells within the same culture. The pepsin and gastric juice challenges could then be repeated and a number of cytokines and mucins could be measured within the same experiment. This could involve measuring MUC5B, as well as MUC5AC, seeing as there is some evidence to show that goblet cells can produce MUC5B in disease states (Chen et al. 2001). It could also involve measuring cytokines, including IL-6 and IL-17 (known to stimulate mucin production) as well as TNF-α and GM-CSF (known neutrophilic chemokines).

There is some evidence that pepsin is taken up by laryngeal epithelial cells through receptor-mediated endocytosis (Johnston et al. 2007) and this may also be occurring in the lung. It may therefore be of interest to stain biopsies and epithelial cells taken from lung transplant recipients for pepsin, in an attempt to further understand what happens to the pepsin once it has been aspirated into the lungs.

Additionally, neutrophil levels have already been investigated in this patient cohort by another member of the group as they are consistently implicated in the pathogenesis of OB/BOS (DiGiovine et al. 1996; Elssner and Vogelmeier 2001).

It is possible that the lung epithelial cells are releasing neutrophilic chemokines in response to stress or damage caused by refluxed pepsin and/or acid. If this was the case there may be a correlation between neutrophil and pepsin levels in the BAL and as both have been measured in the same patients statistical analysis could be performed. This would help to identify if neutrophils are implicated and whether or not they should be further investigated as a potential mechanism of damage caused by the aspiration of gastric contents into the lungs.

Bibliography

Adhami, T., Goldblum, J. R., Richter, J. E. and Vaezi, M. F. (2004). "The Role of Gastric and Duodenal Agents in Laryngeal Injury: An Experimental Canine Model." American Journal of Gastroenterology **99**(11): 2098-2106.

Ahlawat, S., Mohi-Ud-Din, R., Williams, D., Maher, K. and Benjamin, S. (2005). "A Prospective Study of Gastric Acid Analysis and Esophageal Acid Exposure in Patients with Gastroesophageal Reflux Refractory to Medical Therapy." Digestive Diseases & Sciences **50**(11): 2019-2024.

Aikawa, T., Shimura, S., Sasaki, H., Ebina, M. and Takishima, T. (1992). "Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack." Chest **10**(6): 916-921.

Andreeva, N. S., Gustchina, A. E., Fedorov, A. A., Shutzkever, N. E. and Volnova, T. V. (1977). "X-ray crystallographic studies of pepsin." Advances in Experimental Medicine & Biology. **95**: 23-31.

Arts, J., Caenepeel, P., Verbeke, K. and Tack, J. (2005). "Influence of erythromycin on gastric emptying and meal related symptoms in functional dyspepsia with delayed gastric emptying." Gut **54**(4): 455-460.

Arts, J., Lerut, T., Rutgeerts, P., Sifrim, D., Janssens, J. and Tack, J. (2005). "A One-Year Follow-up Study of Endoluminal Gastroplication (Endocinch) in GERD Patients Refractory to Proton Pump Inhibitor Therapy." Digestive Diseases & Sciences **50**(2): 351-356.

Badreddine, R. J. and Wang, K. K. (2008). "Barrett's Esophagus: Pathogenesis, Treatment, and Prevention." Gastrointestinal Endoscopy Clinics of North America **18**(3): 495-512.

Bammer, T., Hinder, R. A., Klaus, A. and Klingler, P. J. (2001). "Five- to eight-year outcome of the first laparoscopic nissen fundoplications." Journal of Gastrointestinal Surgery **5**(1): 42-48.

Becker, D. J., Sinclair, J., Castell, D. O. and Wu, W. C. (1989). "A Comparison of High and Low Fat Meals on Postprandial Esophageal Acid Exposure." American Journal of Gastroenterology **84**(7): 782-786.

Belperio, J. A., Keane, M. P., Burdick, M. D., Gomperts, B., Xue, Y. Y., Hong, K., Mestas, J., Ardehali, A., Mehrad, B., Saggar, R., Lynch, J. P., Ross, D. J. and Strieter, R. M. (2005). "Role of CXCR2/CXCR2 ligands in vascular remodeling during bronchiolitis obliterans syndrome." Journal of Clinical Investigation **115**(5): 1150-1162.

Benden, C., Aurora, P., Curry, J., Whitmore, P., Priestley, L. and Elliott, M. J. (2005). "High prevalence of gastroesophageal reflux in children after lung transplantation." Pediatric Pulmonology **40**(1): 68-71.

Besancon, M., Simon, A., Sachs, G. and Shin, J. M. (1997). "Sites of Reaction of the Gastric H,K-ATPase with Extracytoplasmic Thiol Reagents." Journal of Biological Chemistry **272**(36): 22438-22446.

Blondeau, K., Mertens, V., Vanaudenaerde, B. A., Verleden, G. M., Van Raemdonck, D. E., Sifrim, D. and Dupont, L. J. (2008). "Gastro-oesophageal reflux and gastric aspiration in lung transplant patients with or without chronic rejection." European Respiratory Journal **31**(4): 707-713.

Boehler, A. and Estenne, M. (2003). "Post-transplant bronchiolitis obliterans." European Respiratory Journal **22**(6): 1007-1018.

Boehler, A., Kesten, S., Weder, W. and Speich, R. (1998). "Bronchiolitis obliterans after lung transplantation: a review." Chest **114**(5): 1411-1426.

Borel, J. F., Feurer, C., Gubler, H. U. and Stähelin, H. (1976). "Biological effects of cyclosporin A: a new antilymphocytic agent." Agents Actions **6**(4): 468-475.

Borro, J. M., Sole, A., Torre, M. D. l., Pastor, A. and Tarazona, V. (2005). "Steroid Withdrawal in Lung Transplant Recipients." Transplantation Proceedings **37**(9): 3991-3993.

Bowdish, M. E., Arcasoy, S. M., Wilt, J. S., Conte, J. V., Davis, R. D., Garrity, E. R., Hertz, M. L., Orens, J. B., Rosengard, B. R. and Barr, M. L. (2004). "Surrogate Markers and Risk Factors for Chronic Lung Allograft Dysfunction." American Journal of Transplantation **4**(7): 1171-1178.

Brodzicki, J., Trawinska-Bartnicka, M. and Korzon, M. (2002). "Frequency, consequences and pharmacological treatment of gastroesophageal reflux in children with cystic fibrosis." Medical Science Monitor. **8**(7): 529-537.

BTS Bronchoscopy Guidelines Committee (2001). "British Thoracic Society guidelines on diagnostic flexible bronchoscopy." Thorax **56**(Suppl 1): 1-21.

Callaghan-Rose, M. and Voynow, J. A. (2006). "Respiratory Tract Mucin Genes and Mucin Glycoproteins in Health and Disease." Physiological Reviews **86**(1): 245-278.

Cantu, E., Appel, J. Z., Hartwig, M. G., Woreta, H., Green, C., Messier, R., Palmer, S. M. and Davis, R. D. (2004). "Early Fundoplication Prevents Chronic Allograft Dysfunction in Patients with Gastroesophageal Reflux Disease." The Annals of Thoracic Surgery **78**(4): 1142-1151.

Chan-Tompkins, N. H. and Babinchak, T. J. (1996). "Cardiac arrhythmias associated with coadministration of azole compounds and cisapride." Clinical Infectious Diseases. **23**(6): 305-313.

Chen, Y., Thai, P., Zhao, Y.-H., Ho, Y.-S., DeSouza, M. M. and Wu, R. (2003). "Stimulation of Airway Mucin Gene Expression by Interleukin (IL)-17 through IL-6 Paracrine/Autocrine Loop." Journal of Biological Chemistry **278**(19): 17036-17043.

Chen, Y., Zhao, Y. H., Di, Y.-P. and Wu, R. (2001). "Characterization of Human Mucin 5B Gene Expression in Airway Epithelium and the Genomic Clone of the Amino-Terminal and 5'-Flanking Region." American Journal of Respiratory Cell & Molecular Biology **25**(5): 542-553.

Cooper, J. D., Billingham, M., Egan, T., Hertz, M. I., Higenbottam, T., Lynch, J., Mauer, J., Paradis, I., Patterson, G. A., Smith, C. and et al. (1993). "A working formulation for the standardization of nomenclature and for clinical staging of chronic dysfunction in lung allografts. International Society for Heart and Lung Transplantation." The Journal of Heart & Lung Transplantation **12**(5): 713-6.

Corley, D. A., Katz, P., Wo, J. M., Stefan, A., Patti, M., Rothstein, R., Edmundowicz, S., Kline, M., Mason, R. and Wolfe, M. M. (2003). "Improvement of gastroesophageal reflux symptoms after radiofrequency energy: a randomized, sham-controlled trial." Gastroenterology **125**(3): 668-676.

D'Ovidio, F. and Keshavjee, S. (2006). "Gastroesophageal reflux and lung transplantation." Diseases of the Esophagus **19**(5): 315-320.

D'Ovidio, F., Mura, M., Ridsdale, R., Takahashi, H., Waddell, T. K., Hutcheon, M., Hadjiliadis, D., Singer, L. G., Pierre, A., Chaparro, C., Gutierrez, C., Miller, L., Darling, G., Liu, M., Post, M. and Keshavjee, S. (2006). "The Effect of Reflux and Bile Acid Aspiration on the Lung Allograft and Its Surfactant and Innate Immunity Molecules SP-A and SP-D." American Journal of Transplantation **6**(8): 1930-1938.

D'Ovidio, F., Mura, M., Tsang, M., Waddell, T. K., Hutcheon, M. A., Singer, L. G., Hadjiliadis, D., Chaparro, C., Gutierrez, C., Pierre, A., Darling, G., Liu, M. and Keshavjee, S. (2005). "Bile acid aspiration and the development of bronchiolitis obliterans after lung transplantation." Journal of Thoracic & Cardiovascular Surgery **129**(5): 1144-52.

Dallemagne, B., Weerts, J. M., Jehaes, C., Markiewicz, S. and Lombard, R. (1991). "Laparoscopic Nissen fundoplication: preliminary report." Surgical Laparoscopy & Endoscopy **1**(3): 138-143.

Davis, R. D., Lau, C. L., Eubanks, S., Messier, R. H., Hadjiliadis, D., Steele, M. P. and Palmer, S. M. (2003). "Improved lung allograft function after fundoplication in patients with gastroesophageal reflux disease undergoing lung transplantation." Journal of Thoracic & Cardiovascular Surgery **125**(3): 533-42.

DeVault, K. R. and Castell, D. O. (1995). "Guidelines for the diagnosis and treatment of gastroesophageal reflux disease. Practice Parameters Committee of the American College of Gastroenterology." Archives of Internal Medicine **155**(20): 2165-2173.

DeVault, K. R. and Castell, D. O. (2005). "Updated Guidelines for the Diagnosis and Treatment of Gastroesophageal Reflux Disease." American Journal of Gastroenterology **100**(1): 190-200.

Devière, J., Costamagna, G., Neuhaus, H., Voderholzer, W., Louis, H., Tringali, A., Marchese, M., Fiedler, T., Darb-Esfahani, P. and Schumacher, B. (2005). "Nonresorbable copolymer implantation for gastroesophageal reflux disease: A randomized sham-controlled multicenter trial." Gastroenterology **128**(3): 532-540.

DiGiovine, B., Lynch, J. P., 3rd, Martinez, F. J., Flint, A., Whyte, R. I., Iannettoni, M. D., Arenberg, D. A., Burdick, M. D., Glass, M. C., Wilke, C. A., Morris, S. B., Kunkel, S. L. and Strieter, R. M. (1996). "Bronchoalveolar lavage neutrophilia is associated with obliterative bronchiolitis after lung transplantation: role of IL-8." Journal of Immunology **157**(9): 4194-4202.

Dobhan, R. and Castell, D. O. (1992). "Prolonged intraesophageal pH monitoring with 16-Hr overnight recording." Digestive Diseases & Sciences **37**(6): 857-864.

Dykes, C. W. and Kay, J. (1976). "Conversion of pepsinogen into pepsin is not a one-step process." Biochemical Journal **153**(1): 141-144.

Elssner, A. and Vogelmeier, C. (2001). "The role of neutrophils in the pathogenesis of obliterative bronchiolitis after lung transplantation." Transplant Infectious Disease **3**(3): 168-176.

Equi, A., Balfour-Lynn, I. M., Bush, A. and Rosenthal, M. (2002). "Long term azithromycin in children with cystic fibrosis: a randomised, placebo-controlled crossover trial." The Lancet **360**(9338): 978-984.

Estenne, M., Maurer, J. R., Boehler, A., Egan, J. J., Frost, A., Hertz, M., Mallory, G. B., Snell, G. I. and Yousem, S. (2002). "Bronchiolitis obliterans syndrome 2001: an update of the diagnostic criteria." The Journal of Heart & Lung Transplantation **21**(3): 297-310.

Etherington, D. J. and Taylor, W. H. (1969). "The Pepsins of Normal Human Gastric Juice." Biochemical Journal **113**(4): 663-668.

Eubanks, T. R., Omelanczuk, P., Richards, C., Pohl, D. and Pellegrini, C. A. (2000). "Outcomes of laparoscopic antireflux procedures." The American Journal of Surgery **179**(5): 391-395.

Farhath, S., He, Z., Nakhla, T., Saslow, J., Soundar, S., Camacho, J., Stahl, G., Shaffer, S., Mehta, D. I. and Aghai, Z. H. (2008). "Pepsin, a Marker of Gastric Contents, Is Increased in Tracheal Aspirates From Preterm Infants Who Develop Bronchopulmonary Dysplasia." Paediatrics **121**(2): e253-259.

Farrell, S., McMaster, C., Gibson, D., Shields, M. D. and McCallion, W. A. (2006). "Pepsin in bronchoalveolar lavage fluid: a specific and sensitive method of diagnosing gastro-oesophageal reflux-related pulmonary aspiration." Journal of Pediatric Surgery **41**(2): 289-293.

Farrokhi, F. and Vaezi, M. F. (2007). "Extra-esophageal manifestations of gastroesophageal reflux." Oral Diseases **13**(4): 349-359.

Fass, R., Naliboff, B., Higa, L., Johnson, C., Kodner, A., Munakata, J., Ngo, J. and Mayer, E. A. (1998). "Differential effect of long-term esophageal acid exposure on mechanosensitivity and chemosensitivity in humans." Gastroenterology **115**(6): 1363-1373.

Feigelson, J., Girault, F. and Pecau, Y. (1987). "Gastro-oesophageal reflux and esophagitis in cystic fibrosis." Acta Paediatrica Scandinavica. **76**: 989-990.

Fernando, H. C., Schauer, P. R., Rosenblatt, M., Wald, A., Buenaventura, P., Ikramuddin, S. and Luketich, J. D. (2002). "Quality of life after antireflux surgery compared with nonoperative management for severe gastroesophageal reflux disease." Journal of the American College of Surgeons **194**(1): 23-27.

Finlayson, S. R. G., Laycock, W. S. and Birkmeyer, J. D. (2003). "National trends in utilization and outcomes of antireflux surgery." Surgical Endoscopy **17**(6): 864-867.

Fisher, A. J., Rutherford, R. M., Bozzino, J., Parry, G., Dark, J. H. and Corris, P. A. (2005). "The Safety and Efficacy of Total Lymphoid Irradiation in Progressive Bronchiolitis Obliterans Syndrome After Lung Transplantation." American Journal of Transplantation **5**(3): 537-543.

Foltman, B. (1981). "Gastric proteinases--structure, function, evolution and mechanism of action." Essays in Biochemistry **17**: 52-84.

Forrest, I. A., Murphy, D. M., Ward, C., Jones, D., Johnson, G. E., Archer, L., Gould, F. K., Cawston, T. E., Lordan, J. L. and Corris, P. A. (2005). "Primary airway epithelial cell culture from lung transplant recipients." European Respiratory Journal **26**(6): 1080-1085.

Freedman, L. S. (1982). "Tables of the number of patients required in clinical trials using the logrank test." Statistics in Medicine **1**(2): 121-129.

Galmiche, J. P., Zerbib, F. and Bruley Des Varannes, S. (2008). "Review article: respiratory manifestations of gastro-oesophageal reflux disease." Alimentary Pharmacology & Therapeutics **27**(6): 449-464.

Gamble, E., Grootendorst, D. C., Brightling, C. E., Troy, S., Qiu, Y., Zhu, J., Parker, D., Matin, D., Majumdar, S., Vignola, A. M., Kroegel, C., Morell, F., Hansel, T. T., Rennard, S. I., Compton, C., Amit, O., Tat, T., Edelson, J., Pavord, I. D., Rabe, K. F., Barnes, N. C. and Jeffery, P. K. (2003). "Antiinflammatory Effects of the Phosphodiesterase-4 Inhibitor Cilomilast (Ariflo) in Chronic Obstructive Pulmonary Disease." American Journal of Respiratory & Critical Care Medicine **168**(8): 976-982.

Ganzini, L., Casey, D. E., Hoffman, W. F. and McCall, A. L. (1993). "The prevalence of metoclopramide-induced tardive dyskinesia and acute extrapyramidal movement disorders." Archives of Internal Medicine **153**(12): 1469-1475.

Gensch, E., Gallup, M., Sucher, A., Li, D., Gebremichael, A., Lemjabbar, H., Mengistab, A., Dasari, V., Hotchkiss, J., Harkema, J. and Basbaum, C. (2004). "Tobacco Smoke Control of Mucin Production in Lung Cells Requires Oxygen Radicals AP-1 and JNK." Journal of Biological Chemistry **279**(37): 39085-39093.

Gerhardt, S. G., McDyer, J. F., Girgis, R. E., Conte, J. V., Yang, S. C. and Orens, J. B. (2003). "Maintenance Azithromycin Therapy for Bronchiolitis Obliterans Syndrome: Results of a Pilot Study." American Journal of Respiratory & Critical Care Medicine **168**(1): 121-125.

Gotfried, M. H. (2004). "Macrolides for the Treatment of Chronic Sinusitis, Asthma, and COPD." Chest **125**(Suppliment 2): 52S-61.

Gottlieb, J., Szangolies, J., Koehnlein, T., Golpon, H., Simon, A. and Welte, T. (2008). "Long-Term Azithromycin for Bronchiolitis Obliterans Syndrome After Lung Transplantation. ." Transplantation **85**(1): 36-41.

Grey, J. P. (2007). Nissen Fundoplication. Wikipedia.

Guillot, L., Carroll, S., Badawy, M. and Qureshi, S. (2008). "Cryptococcus neoformans induces IL-8 secretion and CXCL1 expression by human bronchial epithelial cells." Respiratory Research. **9**(1): 9.

Hadjiliadis, D., Davis, R. D. and Palmer, S. M. (2002). "Is Transplant Operation Important in Determining Posttransplant Risk of Bronchiolitis Obliterans Syndrome in Lung Transplant Recipients?" Chest **122**(4): 1168-1175.

Hamilton, J. A. and Anderson, G. P. (2004). "Mini Review: GM-CSF Biology." Growth Factors **22**(4): 225 - 231.

Hartwig, M. G., Appel, J. Z., Li, B., Hsieh, C.-C., Yoon, Y. H., Lin, S. S., Irish, W., Parker, W. and Davis, R. D. (2006). "Chronic aspiration of gastric fluid accelerates pulmonary allograft dysfunction in a rat model of lung transplantation." The Journal of Thoracic & Cardiovascular Surgery **131**(1): 209-217.

Hatlebakk, J. G. and Berstad, A. (1996). "Gastro-oesophageal reflux during 3 months of therapy with ranitidine in reflux oesophagitis." Scandinavian Journal of Gastroenterology **31**(10): 954-958.

Havemann, B. D., Henderson, C. A. and El-Serag, H. B. (2007). "The association between gastro-oesophageal reflux disease and asthma: a systematic review." Gut **56**(12): 1654-1664.

Higenbottam, T., Jackson, M., Woolman, P., Lowry, R. and Wallwork, J. (1989). "The cough response to ultrasonically nebulized distilled water in heart-lung transplantation patients." American Review of Respiratory Disease **140**(1): 58-61.

Hohfield, J., Neidermeyer, J. and Hamm, H. (1996). "Seasonal Onset of Bronchiolitis Obliterans Syndrome in Lung Transplant Recipients." Journal of Heart & Lung Transplantation **15**: 888-894.

Hopkins, P. M., Aboyoun, C. L., Chhajed, P. N., Malouf, M. A., Plit, M. L., Rainer, S. P. and Glanville, A. R. (2004). "Association of Minimal Rejection in Lung Transplant Recipients with Obliterative Bronchiolitis." American Journal of Respiratory & Critical Care Medicine. **170**(9): 1022-1026.

Hunter, J. G. M. D., Trus, T. L. M. D., Branum, G. D. M. D., Waring, J. P. M. D. and Wood, W. C. M. D. (1996). "A Physiologic Approach to Laparoscopic Fundoplication for Gastroesophageal Reflux Disease." Annals of Surgery **223**(6): 673-687.

Husain, A. N., Siddiqui, M. T., Holmes, E. W., Chandrasekhar, A. J., McCabe, M., Radvany, R. and ER., G. (1999). "Analysis of Risk Factors for the Development of Bronchiolitis Obliterans Syndrome." American Journal of Respiratory & Critical Care Medicine **159**(3): 829-833.

Hutton, D. A., Allen, A., Pearson, J. P., Ward, R. and Venables, C. W. (1986). "Separation of Pepsins in Human Gastric Juice: Analysis of Proteolytic and Mucolytic Activity." Biochemical Society Transactions **14**: 735-736.

Ing, A. J., Ngu, M. C. and Breslin, A. B. (1994). "Pathogenesis of chronic persistent cough associated with gastroesophageal reflux." American Journal of Respiratory & Critical Care Medicine **149**(1): 160-167.

Irwin, R. S., Ownbey, R., Cagle, P. T., Baker, S. and Fraire, A. E. (2006). "Interpreting the Histopathology of Chronic Cough: A Prospective, Controlled, Comparative Study." Chest **130**(2): 362-370.

Jackson, A. D. (2001). "Airway goblet-cell mucus secretion." Trends in Pharmacological Sciences **22**(1): 39-45.

James, M. N. and Sielecki, A. R. (1986). "Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 A resolution." Nature **319**(6048): 33-38.

Jeffery, P. K. and Li, D. (1997). "Airway mucosa: secretory cells, mucus and mucin genes." European Respiratory Journal **10**(7): 1655-1662.

Johnston, N., Dettmar, P. W., Bishwokarma, B., Lively, M. O. and Koufman, J. A. (2007). "Activity/stability of human pepsin: implications for reflux attributed laryngeal disease." Laryngoscope. **117**(6): 1036-1039.

Johnston, N., Knight, J., Dettmar, P. W., Lively, M. and Koufman, J. (2004). "Pepsin and carbonic anhydrase isoenzyme III as diagnostic markers for laryngopharyngeal reflux disease." Laryngoscope. **114**(12): 2129-2134.

Johnston, N., Wells, C. W., Blumin, J. H., Toohill, R. J. and Merati, A. L. (2007). "Receptor-mediated uptake of pepsin by laryngeal epithelial cells." Annals of Otology, Rhinology & Laryngology **116**(12).

Kalluri, R. and Neilson, E. G. (2003). "Epithelial-mesenchymal transition and its implications for fibrosis." Journal of Clinical Investigation **112**(12): 1776-1784.

Katz, P. O. and Tutuian, R. (2001). "Histamine receptor antagonists, proton pump inhibitors and their combination in the treatment of gastro-oesophageal reflux disease." Best Practice & Research Clinical Gastroenterology **15**(3).

Kauer, W. K. H., Peters, J. H., DeMeester, T. R., Feussner, H., Ireland, A. P., Stein, H. J. and Siewert, R. J. (1997). "Composition and concentration of bile acid reflux into the esophagus of patients with gastroesophageal reflux disease." Surgery **122**(5): 874-881.

Kempainen, R., Savik, K., Whelan, T., Dunitz, J., Herrington, C. and Billings, J. (2007). "High prevalence of proximal and distal gastroesophageal reflux disease in advanced COPD." Chest **131**(6): 1666-71.

Khajanchee, Y. S., O'Rourke, R. W., Lockhart, B., Patterson, E. J., Hansen, P. D. and Swanstrom, L. L. (2002). "Postoperative Symptoms and Failure After Antireflux Surgery." Archives of Surgery **137**(9): 1008-1014.

Khalifah, A. P., Hachem, R. R., Chakinala, M. M., Schechtman, K. B., Patterson, G. A., Schuster, D. P., Mohanakumar, T., Trulock, E. P. and Walter, M. J. (2004). "Respiratory Viral Infections Are a Distinct Risk for Bronchiolitis Obliterans Syndrome and Death." American Journal of Respiratory & Critical Care Medicine **170**(2): 181-187.

Khalifah, A. P., Hachem, R. R., Chakinala, M. M., Yusen, R. D., Aloush, A., Patterson, G. A., Mohanakumar, T., Trulock, E. P. and Walter, M. J. (2005). "Minimal acute rejection after lung transplantation: a risk for bronchiolitis obliterans syndrome." American Journal of Transplantation **5**(8): 2022-30.

Knoop, C., Haverich, A. and Fischer, S. (2004). "Immunosuppressive therapy after human lung transplantation." European Respiratory Journal **23**(1): 159-171.

Kricka, L. J. (1999). "Human Anti-Animal Antibody Interferences in Immunological Assays." Clinical Chemistry **45**(7): 942-956.

Kroshus, T. J., Kshettry, V. R., Savik, K., John, R., Hertz, M. I. and Bolman, R. M., 3rd (1997). "Risk factors for the development of bronchiolitis obliterans syndrome after lung transplantation." Journal of Thoracic & Cardiovascular Surgery **114**(2): 195-202.

Kudoh, S., Azuma, A., Yamamoto, M., Izumi, T. and Ando, M. (1998). "Improvement of Survival in Patients with Diffuse Panbronchiolitis Treated with Low-dose Erythromycin." American Journal of Respiratory & Critical Care Medicine **157**(6): 1829-1832.

Lafullarde, T., Watson, D. I., Jamieson, G. G., Myers, J. C., Game, P. A. and Devitt, P. G. (2001). "Laparoscopic Nissen Fundoplication: Five-Year Results and Beyond." Archives of Surgery **136**(2): 180-184.

Laube, B. L., Karmazyn, Y. J., Orens, J. B. and Mogayzel, P. J. (2007). "Albuterol Improves Impaired Mucociliary Clearance After Lung Transplantation." The Journal of Heart & Lung Transplantation **26**(2): 138-144.

Li, B., Hartwig, M. G., Appel, J. Z., Bush, E. L., Balsara, K. R., Holzknecht, Z. E., Collins, B. H., Howell, D. N., Parker, W., Lin, S. S. and Davis, R. D. (2008). "Chronic Aspiration of Gastric Fluid Induces the Development of Obliterative Bronchiolitis in Rat Lung Transplants." American Journal of Transplantation **8**(8): 1614-1621.

Lin, Y., Means, G. E. and Feeney, R. E. (1969). "The Action of Proteolytic Enzymes on N,N-Dimethyl Proteins. Basis for a microassay for proteolytic enzymes." Journal of Biological Chemistry **244**(4): 789-793.

Lind, T., Rydberg, L., Kylebäck, A., Jonsson, A., Andersson, T., Hasselgren, G., Holmberg, J. and Röhss, K. (2000). "Esomeprazole provides improved acid control vs. omeprazole in patients with symptoms of gastro-oesophageal reflux disease." Alimentary Pharmacology & Therapeutics **14**(7): 861-867.

Locke, G. R., Talleya, N. J., Fetta, S. L., Zinsmeistera, A. R. and Melton, L. J. (1997). "Prevalence and clinical spectrum of gastroesophageal reflux: A population-based study in Olmsted County, Minnesota." Gastroenterology **112**(5): 1448-1456.

Mantle, M. and Allen, A. (1978). "A colorimetric assay for glycoproteins based on the periodic acid/Schiff stain." Biochemical Society Transactions **6**(3): 607-609.

Metheny, N. A., Clouse, R. E., Chang, Y.-H., Stewart, B. J., Oliver, D. A. and Kollef, M. H. (2006). "Tracheobronchial aspiration of gastric contents in critically ill tube-fed patients: Frequency, outcomes, and risk factors." Critical Care Medicine **34**(4): 1007-1015.

Meyers, B. F. M. D., Lynch, J. M. D., Trulock, E. P. M. D., Guthrie, T. J. B. S. N., Cooper, J. D. M. D. and Patterson, G. A. M. D. (1999). "Lung Transplantation: A Decade of Experience." Annals of Surgery **230**(3): 362.

Moffatt, S. D., Demers, P., Robbins, R. C., Doyle, R., Wienacker, A., Henig, N., Theodore, J., Reitz, B. A. and Whyte, R. I. (2005). "Lung transplantation: A decade of experience." The Journal of Heart & Lung Transplantation **24**(2): 145-151.

Morice, A. H. and Kastelik, J. A. (2003). "Cough * 1: Chronic cough in adults." Thorax. **58**(10): 901-907.

Murphy, D. M., Ward, C., Forrest, I. A., Pritchard, G., Jones, D., Stovold, R., Fisher, A. J., Cawston, T. E., Lordan, J. L. and Corris, P. A. (2006). "The Phosphodiesterase Type IV Inhibitor Cilomilast Decreases Pro-inflammatory Cytokine Production From Primary Bronchial Epithelial Cells in Lung Transplantation Patients." The Journal of Heart & Lung Transplantation **25**(12): 1436-1440.

Niimi, A. and Chung, K. F. (2004). "Airway inflammation and remodelling changes in patients with chronic cough: do they tell us about the cause of cough? ." Pulmonary Pharmacology & Therapeutics **17**(6): 441-446.

Norgaard, M. A., Andersen, C. B. and Pettersson, G. (1998). "Does bronchial artery revascularization influence results concerning bronchiolitis obliterans syndrome and/or obliterative bronchiolitis after lung transplantation?" European Journal of Cardiothoracic Surgery **14**(3): 311-318.

Palmer, S. M. (2006). "Reflux of Bile Acids: An Innate Problem of Lung Transplantation." American Journal of Transplantation **6**(8): 1757-1758.

Palmer, S. M., Miralles, A. P., Howell, D. N., Brazer, S. R., Tapson, V. F. and Davis, R. D. (2000). "Gastroesophageal Reflux as a Reversible Cause of Allograft Dysfunction After Lung Transplantation." Chest **118**(4): 1214-1217.

Pearson, J. P. and Brownlee, I. A. Structure & function of the stomach. In:The Encyclopedia of Human Nutrition 2; 2005 Academic Press. Editors.M. J. Sadler, J. J. Strain and B. Caballero. p. 180-190.

Pearson, J. P., Ward, R., Allen, A., Roberts, N. B. and Taylor, W. H. (1986). "Mucus degradation by pepsin: comparison of mucolytic activity of human pepsin 1 and pepsin 3: implications in peptic ulceration." Gut **27**(3): 243-248.

Perlmann, G. E. (1963). "The optical rotatory properties of pepsinogen." Journal of Molecular Biology **6**: 452-464.

Perrais, M., Pigny, P., Copin, M.-C., Aubert, J.-P. and Van Seuningen, I. (2002). "Induction of MUC2 and MUC5AC Mucins by Factors of the Epidermal Growth Factor (EGF) Family Is Mediated by EGF Receptor/Ras/Raf/Extracellular Signal-regulated Kinase Cascade and Sp1*." Journal of Biological Chemistry **277**(35): 32258-32267.

Pessaux, P., Arnaud, J. P., Ghavami, B., Flament, J. B., Trebuchet, G., Meyer, C., Huten, N., Tuech, J. J., Champault, G. and Laparoscopique., S. F. d. C. (2002). "Morbidity of laparoscopic fundoplication for gastroesophageal reflux: a retrospective study about 1470 patients." Hepatogastroenterology. **49**(44): 447-450.

Piper, D. W. and Fenton, B. H. (1965). "pH stability and activity curves of pepsin with special reference to their clinical importance." Gut **6**(5): 506-508.

Powers, J. C., A.D., H. and Myers, D. V. (1977). "Subsite Specificity of Porcine Pepsin." Advances in Experimental Medicine & Biology. **95**: 141-157.

Prescott, E., Lange, P. and Vestbo, J. (1995). "Chronic mucus hypersecretion in COPD and death from pulmonary infection." European Respiratory Journal **8**(8): 1333-1338.

Quantz, M. A., Bennett, L. E., Meyer, D. M. and Novick, R. J. (2000). "Does human leukocyte antigen matching influence the outcome of lung transplantation? an analysis of 3,549 lung transplantations." The Journal of Heart & Lung Transplantation **19**(5): 473-479.

Raghu, G. (2003). "The role of gastroesophageal reflux in idiopathic pulmonary fibrosis." American Journal of Medicine. **115 Suppl 1**: 60-64.

Riise, G., Andersson, B., Kjellstrom, C., Martensson, G., Nilsson, F., Ryd, W. and Schersten, H. (1999). "Persistent high BAL fluid granulocyte activation marker levels as early indicators of bronchiolitis obliterans after lung transplant." European Respiratory Journal **14**(5): 1123-1130.

Rogan, M., Geraghty, P., Greene, C., O'Neill, S., Taggart, C. and McElvaney, N. (2006). "Antimicrobial proteins and polypeptides in pulmonary innate defence." Respiratory Research. **7**(1): 29.

Samloff, I. M. (1971). "Pepsinogens, pepsins, and pepsin inhibitors." Gastroenterology **60**(4): 586-604.

Sandler, R., Everhart, J., Donowitz, M., Adams, E., Cronin, K., Goodman, C., Gemmen, E., Shah, S., Avdic, A. and Rubin, R. (2002). "The burden of selected digestive diseases in the United States." Gastroenterology **122**(5): 1500-11.

Schreiber, S. L., Liu, J., Albers, M. W., Karmacharya, R., Koh, E., Martin, P. K., Rosen, M. K., Standaert, R. F. and Wandless, T. J. (1991). "Immunophilin-ligand complexes as probes of intracellular signaling pathways." Transplantation Proceedings **23**(6): 2839-2844.

Schwann, T. (1836). "Ueber das Wesen des Verdauungsprocessess." Mullers Archive Journal of Anatomy & Physiology: 90-138.

Schwartz, M. P. and Smout, A. J. P. M. (2007). "Review article: the endoscopic treatment of gastro-oesophageal reflux disease." Alimentary Pharmacology & Therapeutics **26**(s2): 1-6.

Schwartz, M. P., Wellink, H., Gooszen, H. G., Conchillo, J. M., Samsom, M. and Smout, A. J. P. M. (2007). "Endoscopic gastroplication for the treatment of gastro-oesophageal reflux disease: a randomised, sham-controlled trial." Gut **56**(1): 20-28.

Scott, A. I. R., Sharples, L. D. and Stewart, S. (2005). "Bronchiolitis Obliterans Syndrome: Risk Factors and Therapeutic Strategies." Drugs **65**(6): 761-771.

Serrick, C., Giaid, A., Reis, A. and Shennib, H. (1997). "Prolonged Ischemia Is Associated With More Pronounced Rejection in the Lung Allograft." The Annals of Thoracic Surgery **63**(1): 202-208.

Sharples, L. D., McNeil, K., Stewart, S. and Wallwork, J. (2002). "Risk factors for bronchiolitis obliterans: a systematic review of recent publications." The Journal of Heart & Lung Transplantation **21**(2): 271-281.

Sharples, L. D., Tamm, M., MacNeal, K., Higenbottam, T. W., Stewart, S. and Wallwork, J. (1996). "Development of Bronchiolitis Obliterans Syndrome in Recipients of Heart-lung Transplantation-Early Risk Factors." Transplantation **61**(4): 560-566.

Sifrim, D., Matsuo, H., Janssens, J. and Vantrappen, G. (1994). "Comparison of the effects of midecamycin acetate and azithromycin on gastrointestinal motility in man." Drugs Under Experimental & Clinical Research **20**(3): 121-6.

Smirnova, M. G., Birchall, J. P. and Pearson, J. P. (2000). "TNF-alpha in the regulation of MUC5AC secretion: some aspects of cytokine-induced mucin hypersecretion on the in vitro model." Cytokine **12**(11): 1732-1736.

Smirnova, M. G., Birchall, J. P. and Pearson, J. P. (2002). "In Vitro Study of IL-8 and Goblet Cells: Possible Role of IL-8 in the Aetiology of Otitis Media with Effusion." Acta Oto-Laryngologica **122**(2): 146 - 152.

Smirnova, M. G., Guo, L., Birchall, J. P. and Pearson, J. P. (2003). "LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells." Cellular Immunology **221**(1): 42-49.

Snell, G. I. and Westall, G. P. (2007). "Immunosuppression for lung transplantation: evidence to date." Drugs **67**(11): 1531-9.

Society of American Gastrointestinal Endoscopic, S. (1998). "Guidelines for surgical treatment of gastroesophageal reflux disease (GERD)." Surgical Endoscopy **12**(2): 186-188.

Speich, R., Boehler, A., Russi, E. W. and Weder, W. (1997). "A case report of a double-blind, randomized trial of inhaled steroids in a patient with lung transplant bronchiolitis obliterans." Respiration **64**(5): 375-380.

Stadaas, J. O. (1975). "Intragastric pressure/volume relationship before and after proximal gastric vagotomy." Scandinavian Journal of Gastroenterology **10**(2): 129-34.

Stein, H. J., Feussner, H. and Siewert, J. R. (1996). "Failure of antireflux surgery: causes and management strategies." The American Journal of Surgery **171**(1): 36-40.

Stewart, A. G. (2004). "Emigration and immigration of mesenchymal cells: a multicultural airway wall." European Respiratory Journal **24**(4): 515-517.

Stewart, S. Lung Transplant Pathology. In:Solid Organ Transplantion Pathology; 1994 W. B. Saunders Editors.E. Hammond, editor, 1st ed. p. 133-158.

Stovold, R., Forrest, I. A., Corris, P. A., Murphy, D. M., Smith, J. A., Decalmer, S., Johnson, G. E., Dark, J. H., Pearson, J. P. and Ward, C. (2007). "Pepsin, a Biomarker of Gastric Aspiration in Lung Allografts: A Putative Association with Rejection." American Journal of Respiratory & Critical Care Medicine **175**(12): 1298-1303.

Sundaresan, S., Mohanakumar, T., Smith, M. A., Trulock, E. P., Lynch, J., Phelan, D., Cooper, J. D. and Patterson, G. A. (1998). "HLA-A locus mismatches and development of antibodies to HLA after lung transplantation correlate with the development of bronchiolitis obliterans syndrome." Transplantation **65**(5): 648-53.

Swanson, S. J., Mentzer, S. J., Reilly, J. J., Bueno, R., Lukanich, J. M., Jaklitsch, M. T., Kobzik, L., Ingenito, E. P., Fuhlbrigge, A. and Donovan, C. (2000). "Surveillance transbronchial lung biopsies: Implication for survival after lung transplantation, ," The Journal of Thoracic & Cardiovascular Surgery **119**(1): 27-38.

Takao, M., Higenbottam, T. W., Audley, T., Otulana, B. A. and Wallwork, J. (1995). "Effects of inhaled nebulized steroids (budesonide) on acute and chronic lung function in heart-lung transplant patients." Transplantation Proceedings **27**(1): 1284-1285.

Takehisa, Y., Sakiyama, S., Uyama, T., Sumitomo, M., Tamaki, M., Hino, H., Takehisa, M., Liu, M., Kondo, K. and Monden, Y. (2002). "Progressive increase of CD4+/CD45RC- lymphocytes after allograft rat lung transplantation: A marker of acute rejection." Journal of Thoracic & Cardiovascular Surgery **124**(4): 675-683.

Takeyama, K., Dabbagh, K., Lee, H.-M., Agusti, C., Lausier, J. A., Ueki, I. F., Grattan, K. M. and Nadel, J. A. (1999). "Epidermal growth factor system regulates mucin production in airways." Proceedings of the National Academy of Sciences **96**(6): 3081-3086.

Tasker, A. (2003). Otitis media with effusion: Key factors, Newcastle University.

Tobin, R. W., Pope, C. E., Pellegrini, C. A., Emond, M. J., Sillery, J. and Raghu, G. (1998). "Increased prevalence of gastroesophageal reflux in patients with idiopathic pulmonary fibrosis." American Journal of Respiratory & Critical Care Medicine. **158**: 1804-1808.

Tochner, Z. and Slavina, S. (1988). "Immune modulation by ionized irradiation." Current Opinion in Immunology **1**(2): 261-268.

Tonini, M. (1996). "Recent Advances in the Pharmacology of Gastrointestinal Prokinetics." Pharmacological Research **33**(4-5): 217-226.

Toronto Lung Transplant Group (1986). "Unilateral lung transplantation for pulmonary fibrosis. Toronto Lung Transplant Group." New England Journal of Medicine **314**(18): 1140-1145.

Trulock, E. P., Edwards, L. B., Boucek, M. M., Taylor, D. O., Aurora, P., Christie, J., Dobbels, F., Rahmel, A. O., Keck, B. M. and Hertz, M. I. (2007). "Registry of the International Society for Heart and Lung Transplantation: Twenty-fourth Official Adult Heart Transplant Report-2007." The Journal of Heart & Lung Transplantation **26**(8): 769-781.

Trulock, E. P., Edwards, L. B., Taylor, D. O., Boucek, M. M., Keck, B. M. and Hertz, M. I. (2005). "Registry of the International Society for Heart and Lung Transplantation: Twenty-second Official Adult Lung and Heart-Lung Transplant Report--2005." The Journal of Heart & Lung Transplantation **24**(8): 956-967.

Tutuian, R. and Castell, D. O. (2006). "Review article: complete gastro-oesophageal reflux monitoring - combined pH and impedance." Alimentary Pharmacology & Therapeutics **24**(s2): 27-37.

Vaezi, M. F. (2003). "Extraesophageal manifestations of gastroesophageal reflux disease." Clinical Cornerstone **5**(4): 32-38.

Veale, D., Glasper, P. N., Gascoigne, A., Dark, J. H., Gibson, G. J. and Corris, P. A. (1993). "Ciliary beat frequency in transplanted lungs." Thorax **48**(6): 629-31.

Vestbo, J., Prescott, E. and Lange, P. (1996). "Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group." American Journal of Respiratory & Critical Care Medicine **153**(5): 1530-1535.

Victor, B. E. and Miller, T. A. Neural Factors and Gastric Mucosal Defence. In:Endogenous Mediators of Gatsrointestinal Disease; 1989 CRC Press Editors.J. L. Wallace, editor. p. 71-89.

Voynow, J. A. (2002). "What does mucin have to do with lung disease?" Paediatric Respiratory Reviews **3**(2): 98-103.

Voynow, J. A., Gendler, S. J. and Rose, M. C. (2006). "Regulation of Mucin Genes in Chronic Inflammatory Airway Diseases." American Journal of Respiratory Cell & Molecular Biology **34**(6): 661-665.

Ward, C., Forrest, I. A., Brownlee, I. A., Johnson, G. E., Murphy, D. M., Pearson, J. P., Dark, J. H. and Corris, P. A. (2005). "Pepsin like activity in bronchoalveolar lavage fluid is suggestive of gastric aspiration in lung allografts." Thorax **60**(10): 872-874.

Ward, C., Forrest, I. A., Murphy, D. M., Johnson, G. E., Robertson, H., Cawston, T. E., Fisher, A. J., Dark, J. H., Lordan, J. L., Kirby, J. A. and Corris, P. A. (2005). "Phenotype of airway epithelial cells suggests epithelial to mesenchymal cell transition in clinically stable lung transplant recipients." Thorax **60**(10): 865-871.

Waring, J. P., Eastwood, T. F., Austin, J. M. and Sanowski, R. A. (1989). "The immediate effects of cessation of cigarette smoking on gastroesophageal reflux." American Journal of Gastroenterology **84**(9): 1076-1078.

Whitford, H., Walters, E. H., Levvey, B., Kotsimbos, T., Orsida, B., Ward, C., Pais, M., Reid, S., Williams, T. and Snell, G. (2002). "Addition of inhaled corticosteroids to systemic immunosuppression after lung transplantation: a double-blind, placebo-controlled trial." Transplantation **73**(11): 1793-1799.

Wiehler, S. and Proud, D. (2007). "Interleukin-17A modulates human airway epithelial responses to human rhinovirus infection." American Journal of Physiology- Lung Cellular & Molecular Physiology **293**(2): L505-515.

Wong, W.-M. and Fass, R. (2004). "Extraesophageal and atypical manifestations of GERD." Journal of Gastroenterology & Hepatology **19**(s3): S33-S43.

Yates, B., Murphy, D. M., Forrest, I. A., Ward, C., Rutherford, R. M., Fisher, A. J., Lordan, J. L., Dark, J. H. and Corris, P. A. (2005). "Azithromycin Reverses Airflow Obstruction in Established Bronchiolitis Obliterans Syndrome." American Journal of Respiratory & Critical Care Medicine **172**(6): 772-775.

Young, L. R., Hadjiliadis, D., Davis, R. D. and Palmer, S. M. (2003). "Lung Transplantation Exacerbates Gastroesophageal Reflux Disease." Chest **124**(5): 1689-1693.

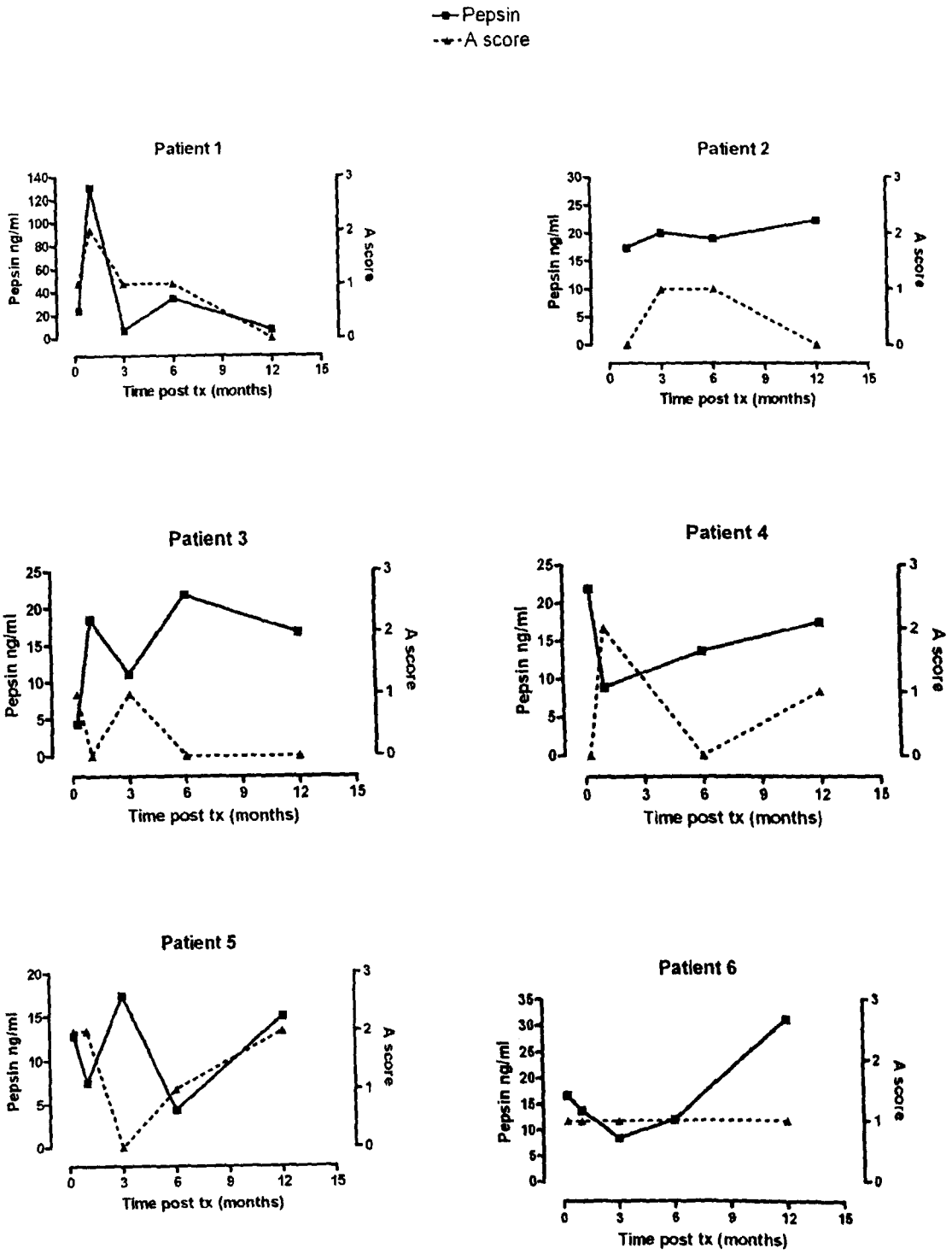
Yousem, S. A., Berry, G., Brunt, E., Chamberlain, D., Hruban, R., Sibley, R., Stewart, S. and Tazelaar, H. (1990). "A working Formulation for the Standardisation of Nomenclature in the Diagnosis of Heart and Lung Rejection." The Journal of Heart & Lung Transplantation **9**: 593-601.

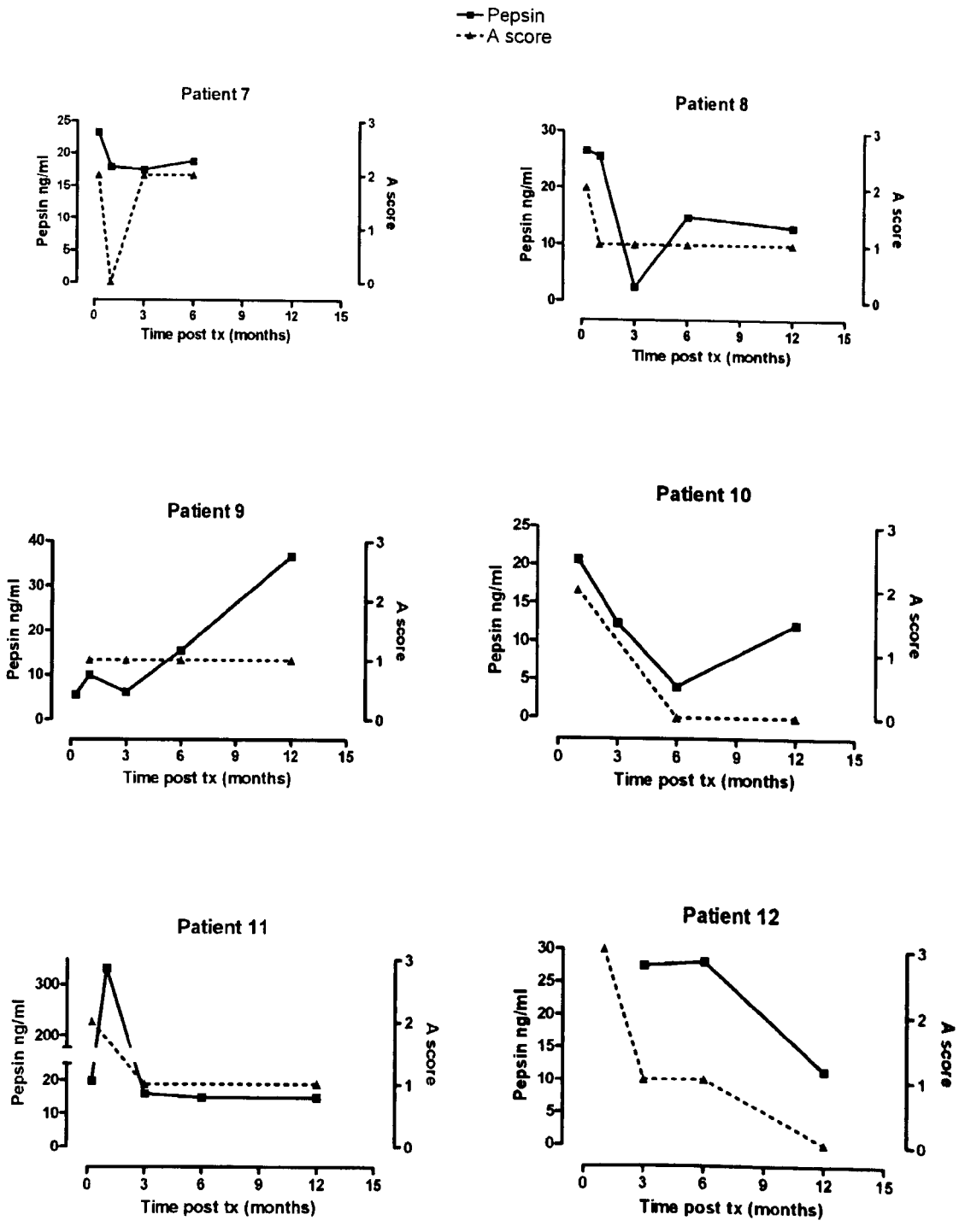
Yousem, S. A., Berry, G. J., Cagle, P. T., Chamberlain, D., Husain, A. N., Hruban, R., Marchevsky, A., Ohori, P., Ritter, J., Stewart, S. and Tazelaar, H. (1996). "Revision of the 1990 Working Formulation for the Classification of Pulmonary Allograft Rejection: Lung rejection Study Group." The Journal of Heart & Lung Transplantation **15**(number 1 part 1): 1-15.

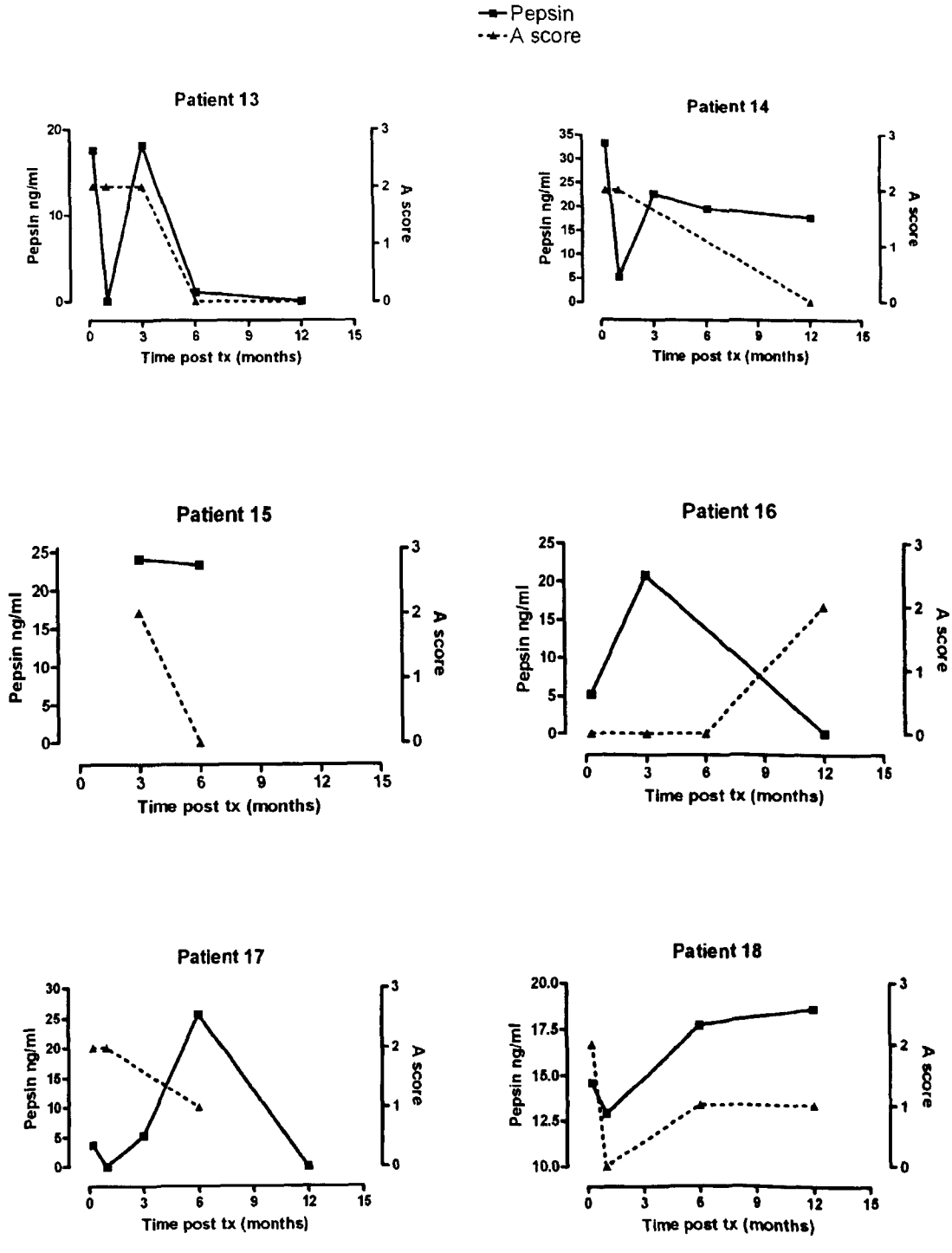
Zheng, L., Whitford, H. M., Orsida, B., Levvey, B. J., Bailey, M., Walters, E. H., Williams, T. J., Kotsimbos, T. and Snell, G. I. (2006). "The Dynamics and Associations of Airway Neutrophilia Post Lung Transplantation." American Journal of Transplantation **6**(3): 599-608.

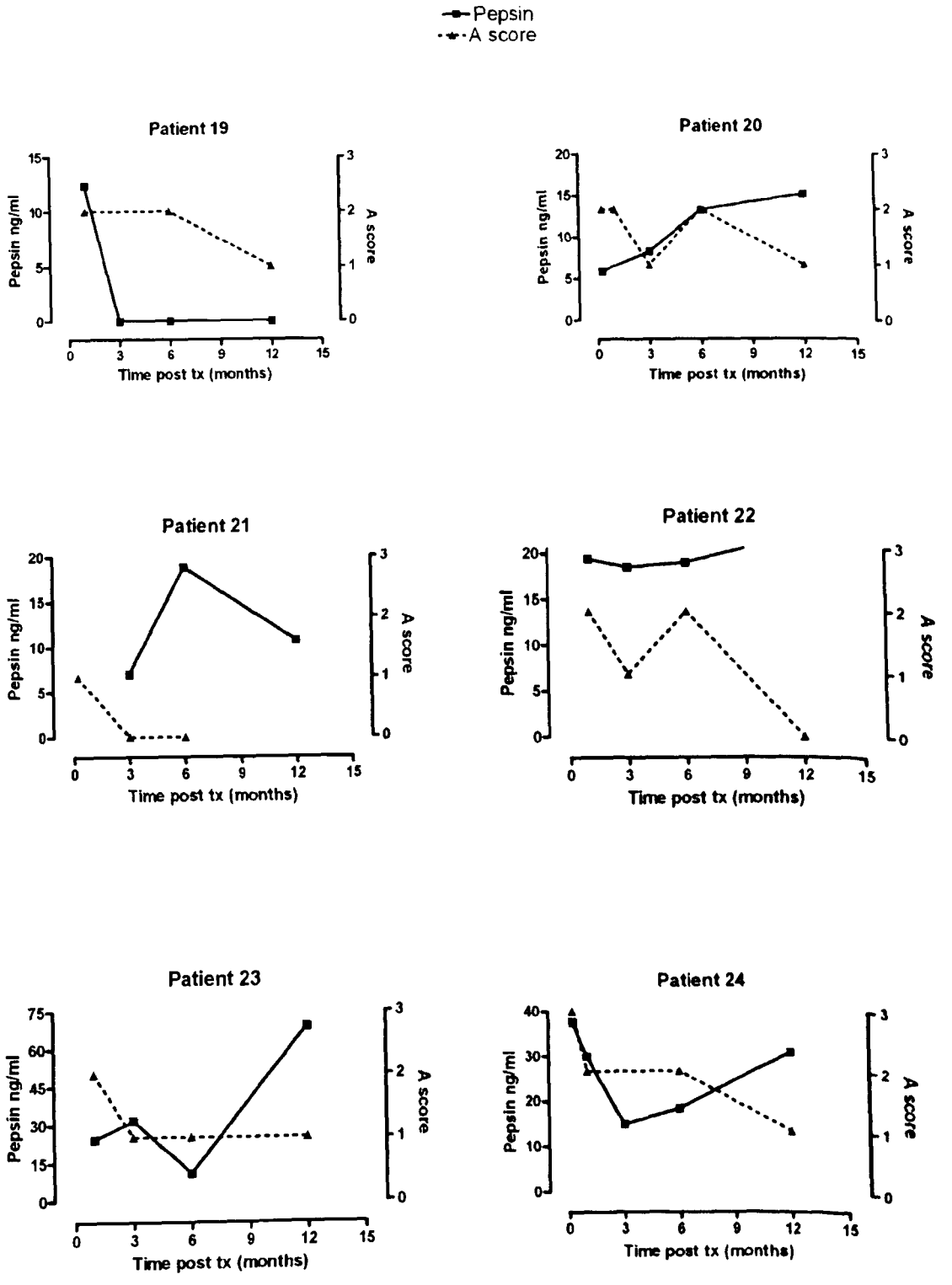
Appendix

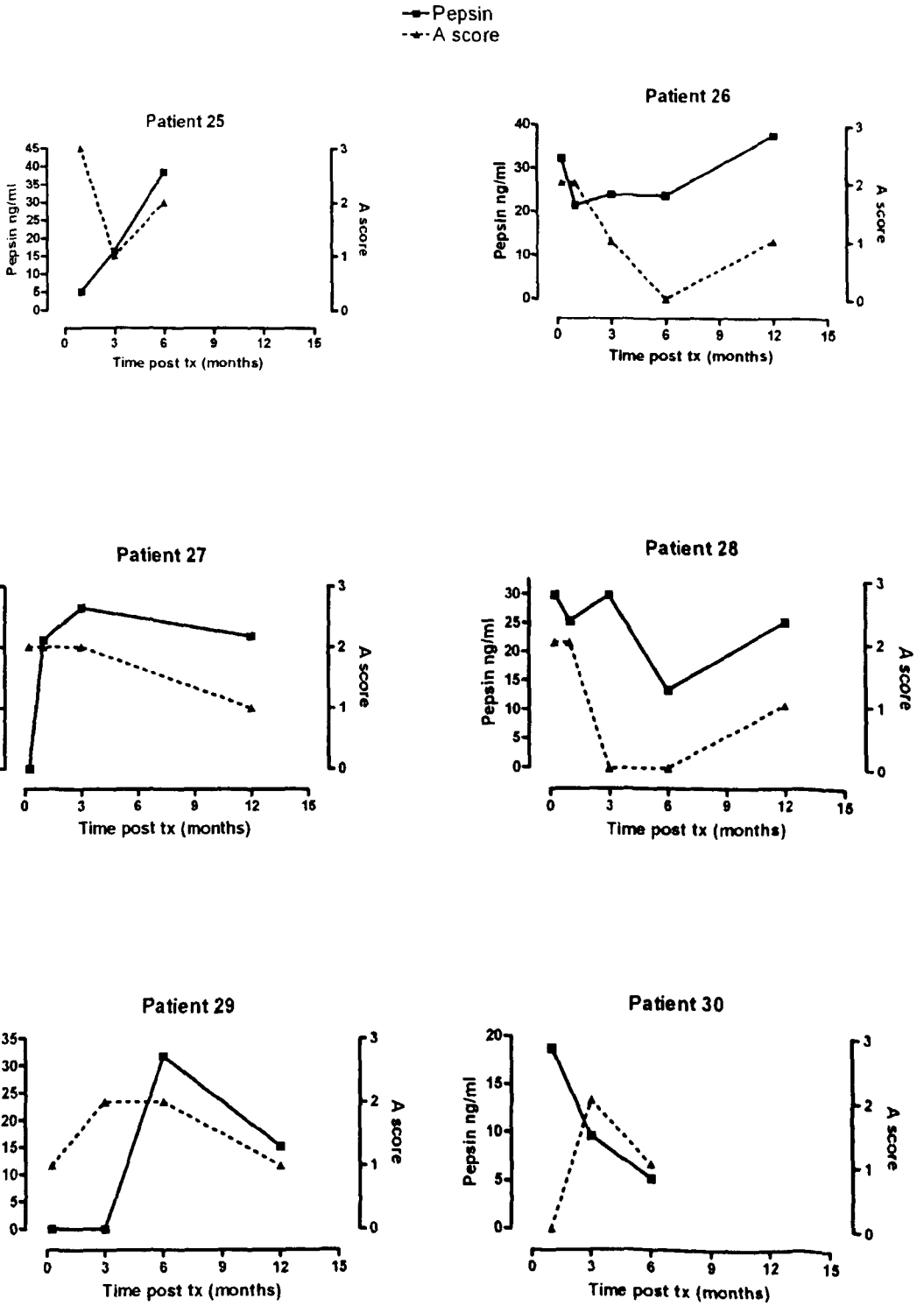
The following appendix contains graphs detailing individual patient's pepsin and acute rejection scores from chapter 5.

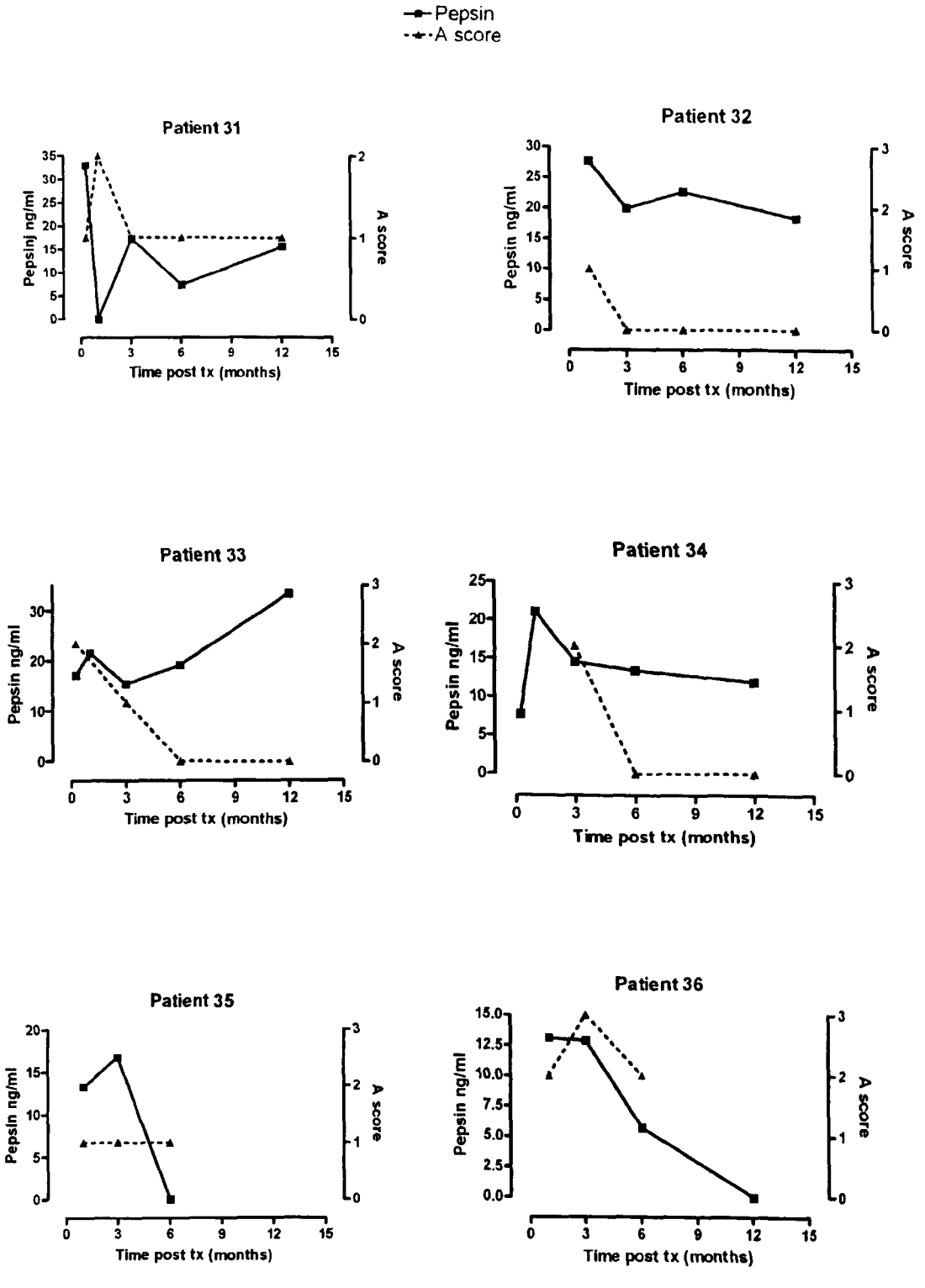

Pepsin
A score
Patient 1
Pepsin ng/ml
A score
Time post tx (months)
Patient 2
Pepsin ng/ml
A score
Time post tx (months)
Patient 3
Pepsin ng/ml
A score
Time post tx (months)
Patient 4
Pepsin ng/ml
A score
Time post tx (months)
Patient 5
Pepsin ng/ml
A score
Time post tx (months)
Patient 6
Pepsin ng/ml
A score
Time post tx (months)

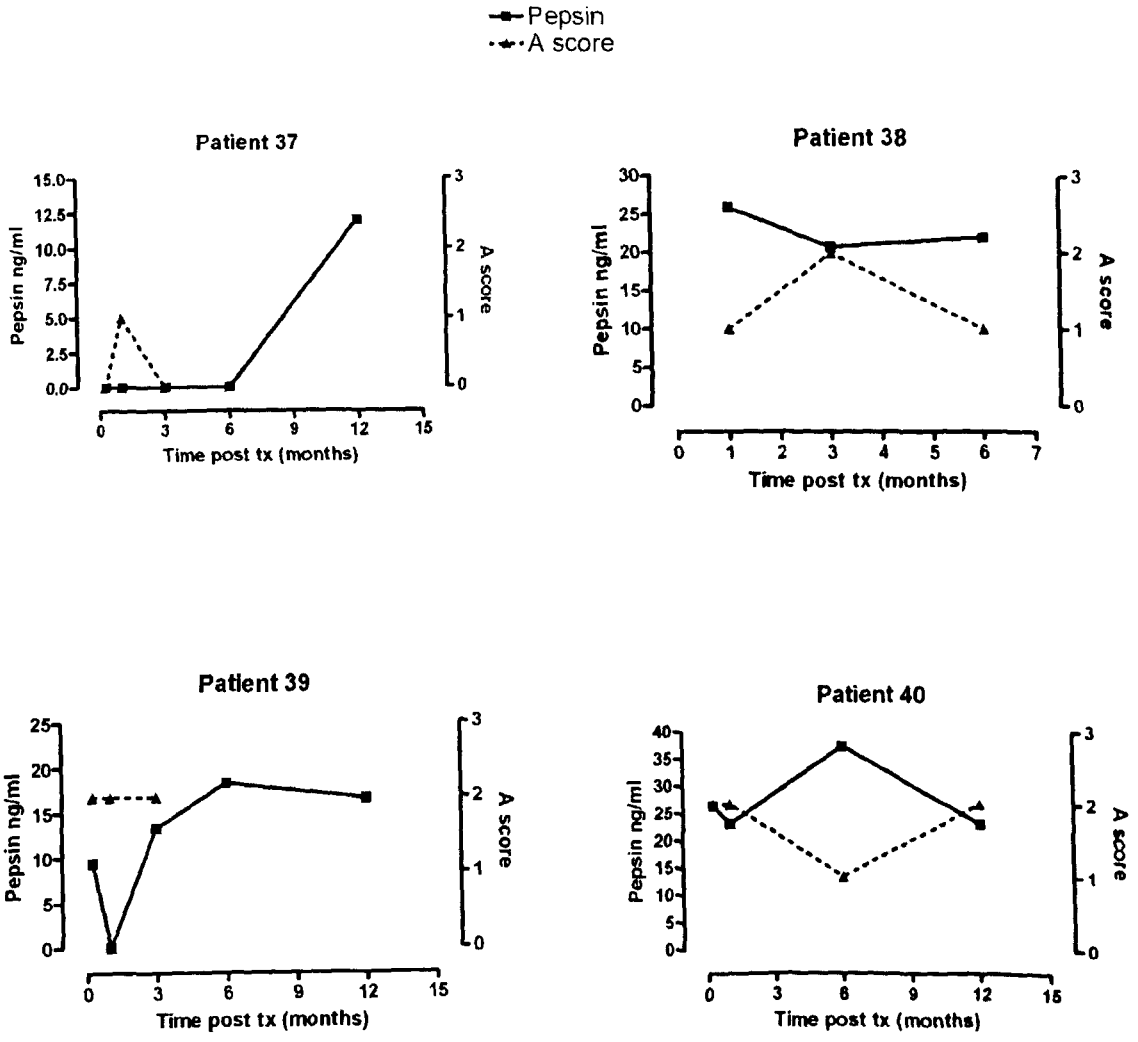

Pepsin
A score
Patient 7
Pepsin ng/ml
A score
Time post tx (months)
Patient 8
Pepsin ng/ml
A score
Time post tx (months)
Patient 9
Pepsin ng/ml
A score
Time post tx (months)
Patient 10
Pepsin ng/ml
A score
Time post tx (months)
Patient 11
Pepsin ng/ml
A score
Time post tx (months)
Patient 12
Pepsin ng/ml
A score
Time post tx (months)


Pepsin
A score
Patient 13
Pepsin ng/ml
A score
Time post tx (months)
Patient 14
Pepsin ng/ml
A score
Time post tx (months)
Patient 15
Pepsin ng/ml
A score
Time post tx (months)
Patient 16
Pepsin ng/ml
A score
Time post tx (months)
Patient 17
Pepsin ng/ml
A score
Time post tx (months)
Patient 18
Pepsin ng/ml
A score
Time post tx (months)


Pepsin
A score
Patient 19
Patient 20
Patient 21
Patient 22
Patient 23
Patient 24
Pepsin ng/ml
A score
Time post tx (months)


Pepsin
A score
Patient 25
Patient 26
Patient 27
Patient 28
Patient 29
Patient 30
Pepsin ng/ml
A score
Time post tx (months)


Pepsin
A score
Patient 31
Pepsinj ng/ml
A score
Time post tx (months)
Patient 32
Pepsin ng/ml
A score
Time post tx (months)
Patient 33
Pepsin ng/ml
A score
Time post tx (months)
Patient 34
Pepsin ng/ml
A score
Time post tx (months)
Patient 35
Pepsin ng/ml
A score
Time post tx (months)
Patient 36
Pepsin ng/ml
A score
Time post tx (months)


Pepsin
A score
Patient 37
Pepsin ng/ml
A score
Time post tx (months)
Patient 38
Pepsin ng/ml
A score
Time post tx (months)
Patient 39
Pepsin ng/ml
A score
Time post tx (months)
Patient 40
Pepsin ng/ml
A score
Time post tx (months)