Gastric aspiration, epithelial injury and chronic lung allograft rejection

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'For those who cannot be here,

for those who are here,

and those who are yet to come'

Dr Matthew D. Wilcox September 2010

Declaration

This thesis is based on research performed in the Institute for Cell and Molecular Biosciences, Newcastle University. I performed the laboratory work and analysis of the results. With the exception of the aspects of the work that are outlined below.

- Dr Malcolm Brodlie and Dr Chris Ward: Initial dissection and preparation of cells through to passage 1 into T25 flasks.
- Dr Andrew Robertson: Collection of gastric juice samples.
- Prof John Perry and Ms Audrey Nicholson: Provided pathogen results for gastric juice samples.
- Miss Shruti Parikh: Measuring bile levels and pH in gastric juice.
- Dr Bernard Verdon: Measuring of pepsin activity in gastric juice.

Abstract

Introduction

For patients with a variety of end stage lung diseases, lung transplantation has become an effective therapy. Chronic allograft rejection occurs in over 50% of patients 5 years post transplantation however. Although alloimmune-mediated injury directed against endothelial and epithelial structures were traditionally thought to be the major culprit, non-alloimmunologic inflammation after bile acid aspiration has been implicated in cystic fibrosis (CF) lung injury, after transplantation.

Hypothesis

Reflux with aspiration of bile acid is present in the lower airways of people with cystic fibrosis associated lung injury before and after transplantation. Bile acid challenge would cause cytoxicity and release of inflammatory mediators from patient derived primary epithelial cells (PBECs), before and after transplantation.

Methods

PBECs from lung transplantation patients, explanted CF patient cultures and a goblet cell line were used to perform proof of concept experiments. In these experiments the effect of individual primary and secondary bile acids, porcine pepsin, different patient derived gastric juices (whole or filtered and dialysed) samples and an artificial bile acid mixture were evaluated. Cell death, Interleukin 8 (IL-8), Interleukin 6 (IL-6) and Granulocyte Macrophage Colony Stumulating Factor (GMCSF) production were measured by Titer blue and multiplex ELISA.

Results

Epithelial cells can be cultured successfully from the bronchial brushings of lung transplant recipient, CF patient explanted lungs and a Goblet cell line.

In work connected with this study my research group has demonstrated that the lungs of people with advanced CF lung disease removed at the time of transplantation contained significant levels of bile acids higher than expected based on normal serum levels. I therefore tested the effects of bile acids on PBECs from lung transplant and CF patients. Challengesof ≥10μmol/l was associated with significant cell death. Potentially physiological challenges with 1, 5 and 10 μmol/l bile acids led to a significant release of pro-neutrophilic cytokines from lung transplant PBECs and CF PBECs .The goblet cell line HT-29 MTX was resistant to bile acids.

Conclusion

Aspiration of bile acids in CF lungs before and after transplantation may cause cell damage and inflammation. This injury may benefit from medical and surgical treatments for reflux, which may benefit the lung allograft generally.

Publications and reviewed abstracts

I.A. Brownlee, A. Aseeri, C. Ward, J.P. Pearson
From gastric aspiration to airway inflammation. Arch. Chest. Dis. (2010) 73-54-63 (Review)

A Aseeri*, M Brodlie*, J Pearson, J Lordan, J Dark, P Corris, C Ward**. *Equal contributions made
Bile acids are present in the lower airways of people with cystic fibrosis. American Journal of Respiratory and Critical Care Medicine. Provisionally accepted for publication Research letter. Provisional Acceptance Blue-201106-1067LE

A Aseeri*, M Brodlie*, J Pearson, J Lordan, J Dark, P Corris, C Ward**. *Equal contributions made
Aspiration of bile acids and inflammation in advanced cystic fibrosis lung disease.
In submission Thorax

AGN Robertson, C Ward, AJ Bredenoord, IA Brownlee, A Aseeri, M. Brodlie, JH Dark, PA Corris, SM Griffin, JP Pearson.
Aspiration secondary to gastro-oesophageal reflux but rarely duodenal reflux occurs in the immediate post-lung transplant period. In submission

A Aseeri*, M Brodlie*, J Pearson, J Lordan, J Dark, P Corris, C Ward**. *Equal contributions made
Aspiration of bile salts and inflammation in Cystic Fibrosis pre and post transplantation [Abstract] Presented at the European Respiratory Society 20. Oral presentation

A Aseeri, J Pearson and C Ward
Gastric aspiration, epithelial injury and chronic lung allograft rejection
[Abstract] Poster Presented at the North East Post Graduate Research Conference 2008

A Aseeri, M Brodlie, J Pearson and C Ward
Primary human bronchial epithelial cells from lung transplant recipients respond to a bile acid challenge by upregulating interleukin 8 (IL-8) productions.
[Abstract] Presented at the North East Post Graduate Research Conference 2009

A Aseeri, M Brodlie, J Pearson and C Ward
Primary human bronchial epithelial cells from lung transplant recipients respond to a bile acid challenge by upregulating interleukin 8 (IL-8) productions.
[Abstract] Presented at the Epithelial Biology Meeting Dundee 2010

Contents

List of figures

List of tables

List of Abbreviations

Abbreviation	Meaning
ABST	2,2-Azino-bis(3-ethylbenzothiazoline-6) sulfonic acid
AEC	Airway Epithelial Cell
APC	Antigen Presenting Cells
BALF	Bronchoalveolar lavage Fluid
BEBM	Bronchial Epithelial Basal Media
BEGM	Bronchial Epithelial Growth Media
BOS	Bronchiolitis Obliterans Syndrome
BSA	Bovine Serum Albumin
BM	Basement Membrae
CF	Cystic Fibrosis
CMV	Cyto-Megalo- Virus
COPD	Chronic Obstructive Pulmonary Disease
DCA	Deoxycholic acid
DEP	Diesel Exhaust Particle
DMEM	Dulbecco's Modified Eagles Medium
MHC	Major Histocompatibility Complex
ELISA	Enzyme Linked Immunosorbent Assay
EMTU	Epithelial Mesenchyme Trophic Unit
ERKs	Extracellular Signal Regulated Kinases
FCS	Foetal Calf Serum
FEV_1	Forced Expiratory Volume in 1 Second
GI	Gastrointestinal
GMCSF	Granulocyte Macrophage Colony Stimulating Factor
GOR	Gastro-oesophageal Reflux
GVHD	Graft Versus Host Diseases
HBEC	Human Bronchial Epithelial Cells
HLA	Human Leukocyte Antigens
HRP	Horseradish Peroxidase
JAK2	Janus Kinase 2
IL-6	Interleukin 6
IL-8	Interleukin 8
IPAH	Idiopathic Pulmonary Arterial Hypertension
IPF	Idiopathic Pulmonary Fibrosis
LCA	Lithochlic acid
LTx	Lung Transplantation
LPS	Lipopolysacharide
LOS	lower oesophageal sphincter
MMP	Matrix metalloproteinase
NF-κB	Nuclear Factor kappa B
NK	Natural Killer
PAMPs	Pathogen Associated Molecular Patterns
PBECs	Primary Bronchial Epithelial Cells

PBS	Phosphate Buffered Saline (Dulbecco's)
PBC	Primary Biliary Cirrhosis
PPIs	Proton Pump Inhibitors
RPMI	Roswell Park Memorial Institute Media
SDS	Sodium Dodecyl Sulphate
TBS	Tris Buffered Saline
TLN	Thoracic Lymph Nodes
TNF	Tumour Necrosis Factor

Chapter 1

Introduction

1. Introduction

Lung transplantation remains the only therapy that can restore selected patients with end stage lung disease towards better health (Meachery et al., 2008). In the last two decades lung transplantation has become a practical option for end stage lung diseae (Trulock et al., 2005). The survival of pulmonary patients at 5 years after lung transplantation is only 49% however, and is unsatisfactory (Trulock et al., 2005). Long-term patient survival of lung transplant recipients is limited by the development of bronchiolitis obliterans syndrome (BOS), a clinically measured decrease in allograft function. It is the major reason behind the death of adult lung transplant recipients more than one year post transplantation (Trulock et al., 2005). Therefore, there is an urgent need to understand the mechanisms by which BOS develops.

Peribronchiolar lymphocytic infiltrates, fibrous scarring and luminal narrowing of the small airways are the histological characteristics of the fibroproliferative progression of obliterative bronciolitis (OB), which is the histological cause of BOS as defined by Burke and colleagues (Burke et al., 1984). The development of OB and thus BOS is thought to be influenced by immune and non-immune mediated elements (Girgis et al., 1996, Fisher et al., 2002), with overall lung injury contributing to loss of function (Robertson et al., 2009). The potential link between chronic aspiration and OB after heart-lung transplantation was recognised in an early study (Reid et al., 1990). High rates of gastroesophageal reflux with resultant aspiration are found after pulmonary transplantation (Kirk et al., 1990, Rinaldi et al., 1995). It is thought that, the aspiration of gastric components might cause allograft injury and OB directly by cuasing airway injury, inflammation and scarring. Aspiration may also cause an increase in acute and chronic rejection by exacerbating the alloimmune response (Li et al., 2008).

1.1 Literature review

Between January 1995 and June 2009 the major indications leading to lung transplantation were, chronic obstructive pulmonary disease (COPD, 26%), idiopathic pulmonary fibrosis (IPF, 15%), cystic fibrosis (CF, 26%), α_1-anti-trypsin deficiency emphysema (7%) and re transplantation (2%) (Figure 1). In the last decade, all the above mentioned diseases (except for CF) were considered suitable for single lung transplantation, accounting for the increased number of these operations (Hertz et al., 2010).

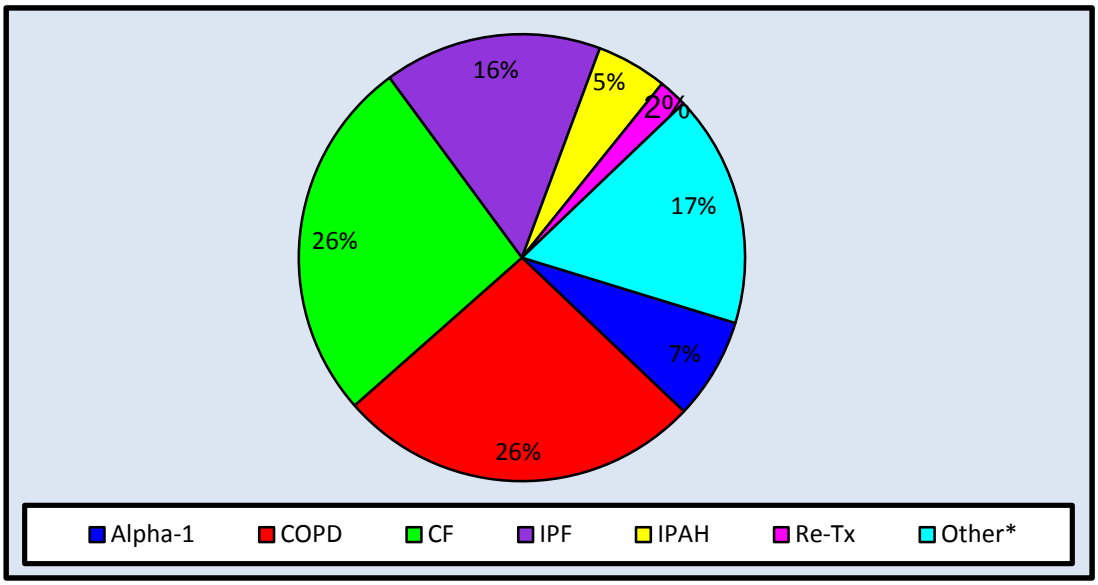

Figure 1: Indications for adult lung transplantations Alpha-1, α_1-anti-trypsin deficiency emphysema, (COPD) Chronic Obstructive Pulmonary Disease, (CF) Cystic fibrosis, (IPF) Idiopathic Pulmonary Fibrosis, (IPAH) Idiopathic Pulmonary Arterial Hypertension, (Re-Tx) Re-Transplantation, PPH, primary pulmonary hypertension, Other includes: Sarcoidosis 3.0%, Bronchiectasis 4.4%, Congenital Heart Disease1.3%, LAM 1.2% OB (non-ReTx) 1.2%, Miscellaneous 5.8% by year (Hertz et al., 2010).

Cystic fibrosis (CF) is a common reason for lung transplantation at our centre with 30% of the total lung transplantations performed in Newcastle being for CF (Meachery et al., 2008). Gastro-oesophageal reflux (GOR) is common in CF before and after transplantation and may be a particularly important contributor to chronic loss of lung function. In this population 31-81% of cystic fibrosis patients show increased acid GOR (Ledson et al., 1998, Brodzicki et al., 2002, Scott et al., 1985).

A reduced pressure of the lower oesophageal sphincter (LOS) (Ledson et al., 1998), increased number of transient LOS relaxations (Cucchiara et al., 1991), and postponed gastric emptying (Couturier et al., 2004), are some potential reasons for increased gastro-oesophageal reflux in CF. It has also been argued that increased abdominal-thoracic pressure gradients during cough and/or physiotherapy (Ledson et al., 1998) is a possible reason behind increased GOR in CF. Measurement of acid GOR has been achieved by 24h oesophageal pH monitoring in CF patients (Blondeau et al., 2008b). Acidity, volume and distribution of gastric contents are three factors which define the damaging potential of gastro-oesophageal reflux. A less acidic pH (between 7 and 4) in gastro-oesophageal reflux can also be found in the early postprandial period and during the night. The reflux make up of GOR could be changed because of the potential adjustability of the volume of gastric contents, acidity and concentration of bilio-pancreatic components in CF patients. The assessment of all kinds of gastro-oesophageal reflux incidents (acid and weakly acidic) (Sifrim et al., 2004) can only be made by the sensitive procedure of combined impedance-pH monitoring.

Heartburn, regurgitation and even oesophagitis are typical GOR findings that could be caused by increased GOR in CF patients. A study of CF patients with increased acid GOR showed that they have lower levels of lung function than patients without GOR (Navarro et al., 2001). This shows the possibility that reflux-induced lung aspiration of gastric components is associated with declining lung function. Few studies have shown the existence of gastric components in bronchial secretions of CF patients, and any correlation between GOR and poor lung function does not mean that there is a causal link and does not necessarily show aspiration has occurred. In order to detect CF patients with increased GOR and risk of lung aspiration, the detection of gastric indicators in sputum is suggested as a surrogate marker for the increased proximal extent of GOR (Potluri et al.,

2003). This may be a useful relatively non-invasive method e.g compared to bronchoscopic based BAL.

Indicators of gastric contents in bronchoalveolar lavage (BAL) have been shown in patients after lung transplant (LTx) including transplanted CF patients during bronchoscopy (Stovold et al., 2007). It is known that lung transplant patients transplanted because CF have higher levels of pepsin and bile acid in their BAL fluid than other lung transplant recipients (Blondeau et al., 2008b).

1.2 Pepsin as a marker of reflux and aspiration

Pepsins are acidic proteases with two aspartate residues at the active site (Szecsi, 1992). Pepsins have two fold symmetry of two almost identical lobes. In between the two lobes is a central cleft that contains the aspartate residues Asp32 and Asp215. The mechanism of peptide bond hydrolysis involves nucleophilic attack (Power, 1977).

Pepsin is secreted in the stomach as an inactive zymogen (pepsinogen) which is spontaneously activated to pepsin at pHs below 5 with the loss of an N-terminal peptide. This results in a metastable molecule (Dee et al., 2006). The loss of peptide on pHs activation and the instability of pepsin explain the irreversible inactivation of pepsin at PHs above 7. Pepsin becomes unfolded at pHs above 7 and although it will refold to some extent if the pH is returned to acidic it is misfolded and inactive. It fails to refold correctly due to the loss of absolute symmetry because the N terminal lobe has missing peptides compared to the C terminal lobe (Pearson, 2010).

As a gastric protease pepsin is an important biomarker of reflux and because it retains activity up to pH 6.0 an important damaging agent in the aerodigestive tract (Bulmer et al., 2010a).

In addition pepsin can be refluxed into the airways in an active form which will lose activity as the pH rises but will not be irreversibly inactivated as the pH will not rise much above pH 7. Therefore it will remain stable and will regain activity as the Ph falls with the net reflux/aspiration event.

Many reflux-related disorders indicate a major role for pepsin (Koufman, 1991). The majorty of reflux that leaves the oesophagus is likely to be adove pH4.0 and gastroesophageal refluxate, will always contain pepsin (Dunn, 2002, Johnston et al., 2003, Piper and Fenton, 1965) It is also noteworthy that compared to the esophageal epithelium, the laryngeal epithelium, and in particular the regions that contain respiratory

epithelium, are far more sensitive to damage by pepsin in the presence of acidic conditions, both strongly and weakly acidic (Johnston et al., 2003, Blondeau et al., 2010, Bulmer et al., 2010b).

One of the potential pathophysiologic mechanisms of reflux (GOR) related to respiratory symptoms is microaspiration of gastric contents (Kiljander et al., 2002, Farrell et al., 2006). Triggered after microaspiration, pulmonary clearance mechanisms and mucus bronchial secretion are more involved in the development of symptoms. Due to impaired clearance function, patients with cystic fibrosis and patients after lung transplantation (LTx) may have prolonged exposure of the bronchial epithelium to gastric contents (Veale et al., 1993). This exposure to gastric contents may cause bronchial epithelial cells to initiate an inflammatory reaction that further impairs lung function (Perng et al., 2007). Pepsin and bile acids luminal gastroduodenal components, have been detected in bronchial material of LTx recipients (D'Ovidio et al., 2005b, Ward et al., 2005). Damage to lung tissue could result due the presence of low pH along with pepsin and bile acids (Porembka et al., 1993, Knight et al., 2004)

Ward and colleagues have published on pepsin as a marker of aspiration. Pepsin is a proteolytic enzyme which is found in the stomach. Pepsin has been used as a biomarker of extra-oesophageal reflux in cystic fibrosis and bronchiectasis. Another use has been as a possible indicator for gastric aspiration in lung transplant patients, with the potential association with pulmonary damage and lung disease (Ward et al., 2005). Pepsin can also be used as an extra-oesophageal reflux biomarker in glue ear as well its ole in aspiration with pulmonary damage and lung disease (Tasker et al., 2002a, Tasker et al., 2002b).

Currently there is no consensus as to the best measurement technology for pepsin. Gastric juice contains 0.1-0.6 mg/ml of pepsin, so if it was aspirated neat then the alveolar fluid when sampled by broncho alveolar lavage might include about 1-6 µg/ml assuming (100

fold dilution from the wash out with (saline). Different values, from different studies on lung

pepsin have been published (Metheny et al., 2002) for example, recorded levels of upto 9.5µg/ml in suctioned tacheal secretions from patients recieving mechanical ventilation with no saline dilution. Implying that gastric juice has been diluted ≈ 60 times in the lungs, and the lungs do not contain neat gastric juice. Whereas (Blondeau et al., 2008a) recorded levels of 2µg/ml in BAL. This would fit with a 100x dilution of whole gastric juice, suggesting aspiration of whole gastric juice. The variability in the literature makes it difficult to compare results between groups (Haslam et al., 1999). In addition assay strategies vary and pepsin has been measured by both enzyme linked immunosorbent assay (ELISA) and by enzymatic activity (Badellino et al., 1996).

Work in our lab to assess pepsin levels in BAL samples, used ELISA, in these studies transplant patients were compared with control volunteers, median BAL pepsin levels were, 8.3 vs 1.1ng/ml respectively (P=0.02). This demonstrated that pepsin can be present in transplanted lungs before significant airflow limitation develops (Stovold et al., 2007) . The study included 36 patients who went through a lung transplant procedure and were divided into three groups: medically stable, patients with acute vascular rejection, and BOS patients.

Acute vascular rejection patients had the highest levels of pepsin as well as inflammation, suggesting a possible relationship relationship between non-alloimmunue injury (aspiration) and alloimmune elements of injury (acute rejection). Proton Pump Inhibitor (PPI) treatment did not affect pepsin levels. It is important to note that in this study the effect of time post transplant was not considered when measuring pepsin levels (Stovold et al., 2007). Consequently a longitudinal study is required.

Finding pepsin in BAL samples in the afore mentioned patients indicates the presence of micro-aspiration and this is also true in patients on proton pump inhibitor therapy. This

therapy does not preclude reflux but at least it should decrease the acidity (Wise and Murray, 2007). Consequently pepsin is a good candidate as a biomarker of reflux/aspiration if a sensitive and reproducible assay is widley available.

1.3 Bile acids as a marker of reflux and aspiration

The bile acids are modified steroids and therefore can be named as acidic sterols (Klyne, 1959). The principal bile acids synthesized by the human liver (Bjorkhem et al., 1983, Russell and Setchell, 1992) have hydroxyl groups substituted at the carbon positions C-3, C-7, and C-12. Relatively high proportions of bile acids hydroxylated at the C-1, C-2, C-4, and C-6 positions have indicated that alternative pathways for bile acid synthesis and metabolism become quantitatively important during early development (Lester et al., 1983, Setchell et al., 1988). The two bile acids referred to as 'primary' bile acids and synthesized by the liver are cholic acid (3a,7a,12-atrihydroxy- 5b-cholanoic acid) and chenodeoxycholic acid (3a,7a-dihydroxy-5b-cholanoic acid). In vivo these bile acids are greater than 90% conjugated to the amino acids glycine and taurine (Sjovall, 1959). The biosynthetic pathway for bile acids is depicted in (Figure 2).

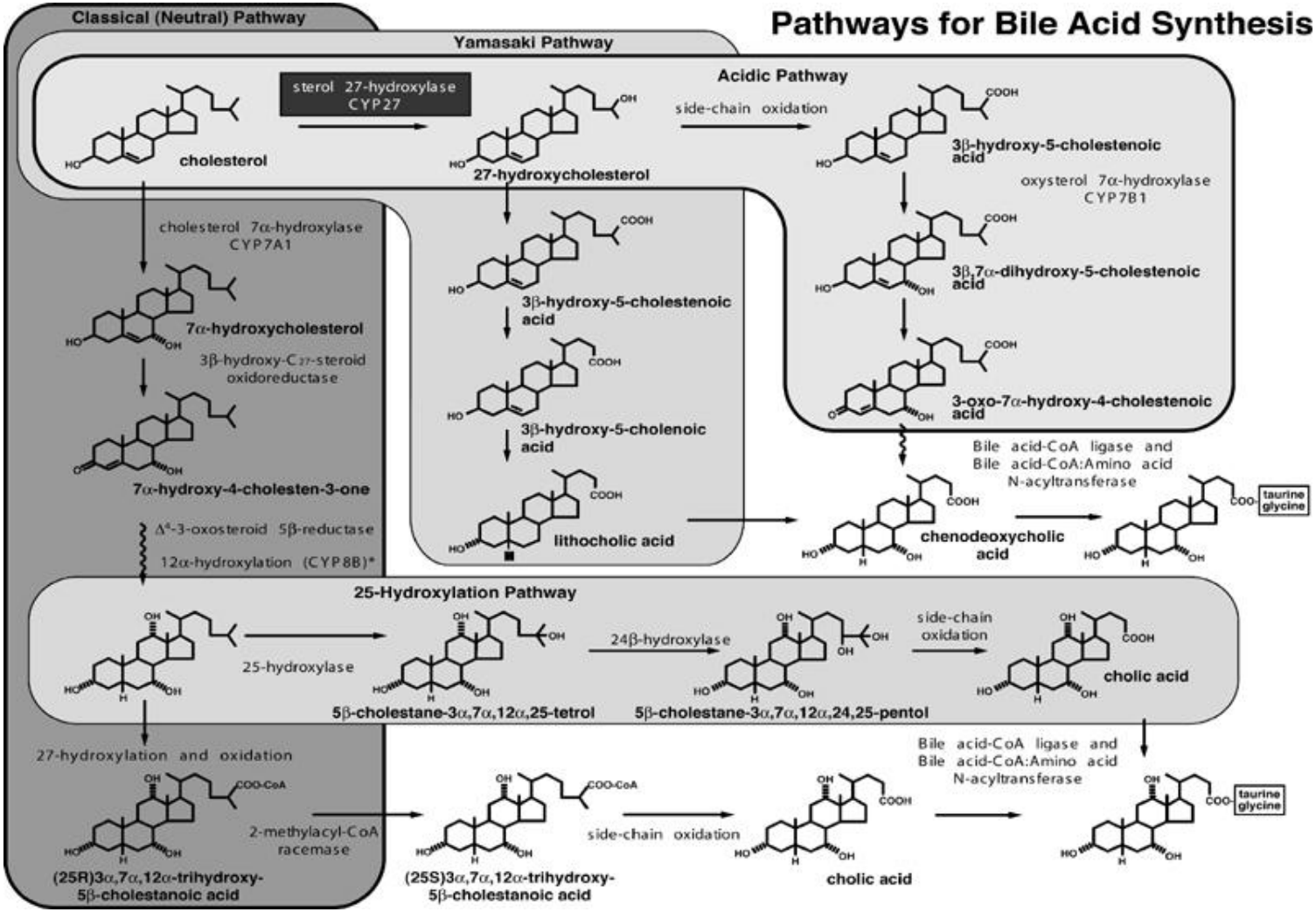

Figure 2: The biosynthetic pathway for the primary bile acids pathway and the alternative or acidic pathway taken from (Heubi et al., 2007).

In humans, the major solutes in bile consist of bile acids conjugated with glycine and taurine while unconjugated bile acids are almost non-detectable in normal bile (Bjorkhem et al., 1983). Conjugated bile acids have a less toxic character because they are less lipid soluble and remain more efficient promoters of intestinal absorption of dietary lipid when compared with unconjugated bile acids (Vessey et al., 1977). The relative amounts of taurine- and glycine-conjugated bile acids differs significantly among species (Johnson et al., 1991). Taurine conjugation dominates the newborn in humans; while in the adulthood, dependending on the dietary intake of amino acids, the ratio of glycine– to taurine–bile acid conjugates in bile is about 3.5:1 (Hardison and Grundy, 1983).

It is now widely believed that refluxed bile acids play a significant role in the initiation and promotion of Barrett's oesophagus and cancer (Attwood et al., 1992, Stamp, 2002, Gillen et al., 1988). Barrett's metaplasia patients have higher levels of bile exposure and the proportions of secondary bile acids have been found at increased levels in their refluxate (Nehra et al., 1998). Furthermore, complicated cases of Barrett's patients with early adenocarcinoma have relatively higher exposure to bile acids compared to uncomplicated Barrett's patients (Stein et al., 1998). It has also been suggested that unconjugated bile acids play an important role in the progression of the neoplastic sequence in oesophageal cancers (Jankowski et al., 1993, Kauer et al., 1995). However, the relative roles that bile acids and stomach acid play are subject to further investigation and debate (Triadafilopoulos, 2001). Due to the fact that despite undergoing acid suppression therapy, certain patients continue their progress to adenocarcinoma, it has been suggested that factors other than acid could be equally important in Barrett's carcinogenesis.

Bile acids are known to be capable of causing multiple injuries such as inducing DNA damage (Venturi et al., 1997) chromosome aberrations (Jenkins et al., 2004), and causing gene expression abnormalities (Tselepis et al., 2003, Kaur et al., 2000). Hence bile acids

may be considered as potential causal factors for oesophageal carcinogens. Bile acid induced apoptosis (Lapre et al., 1992) coupled with the compensatory proliferation occuring in vivo has implicated bile as both an initiator and promoter of cancer development.

Deoxycholic acid (DCA) derived from the bacterial degradation of the primary bile acid cholic acid (CA) in the colon, has been proposed as a factor in the aetiology of breast cancer (Hill et al., 1971, Lewis and Heaton, 1999). DCA concentrations found in human breast cyst fluid are (17-160 /µmol/L) (Raju et al., 1990, Javitt et al., 1994), the mean concentration for DCA in plasma was (0.43 µmol/l) (Costarelli and Sanders, 2001).

Since the early 1960s, the effects of the monohydroxy bile acid lithocholic acid (LCA) has been studied as a hepatotoxic factor in the pathogenesis of cholestatic liver injury in rodents i.e., rat, hamster, and rabbit (Hofmann, 2004, Palmer and Ruban, 1966, Miyai et al., 1977, Hofmann, 1999b). This has led to increased understanding of the pathogenesis of LCA-induced cholestasis in rodents, the key concepts have included biochemical alterations of the bile canalicular membrane (Kakis and Yousef, 1978, Kakis et al., 1980) the development of crystalline plugs in bile canaliculi due to the poor solubility of LCA (Miyai et al., 1977, Bonvicini et al., 1978) and impaired trafficking or increased retrieval of canalicular export pumps to and from the canalicular membrane (Kubitz et al., 2004, Beuers et al., 2003). In addition, the role of hepatic metabolic phase I and II detoxifying enzymes and their regulatory nuclear receptors in the hepatic defense against toxic bile acids has been investigated extensively in gene knockout mice. (Staudinger et al., 2001, Xie et al., 2001, Sinal et al., 2000, Kitada et al., 2003, Schuetz et al., 2001, Saini et al., 2004). However, the cholestatic phenotype and the pathogenesis of hepatobiliary injury of LCA-treated mice are not well understood. Hepatocytes, as well as bile duct epithelial cells (cholangiocytes) are affected by toxic bile acids (Lazaridis et al., 2004). Under physiological conditions, transportation of bile acids and biliary phospholipids into bile

takes place via the bile salt export pump (Bsep/Abcb11) and the canalicular phospholipid flippase (multidrug resistance gene 2 [Mdr2]/Abcb4) respectively, and subsequently it forms mixed micelles which protects cholangiocytes from bile acid toxicity (Trauner and Boyer, 2003).

Several epithelial diseases such as bronchial asthma, atopic dermatitis, and inflammatory bowel diseases have in common eosinophils as important effectors cells (Kita H, 1998, Gleich et al., 1993, Gleich, 2000). Epithelial injury induced by activated eosinophils takes place through release of cytotoxic granule proteins, superoxide radicals, lipid mediators, and various cytokines (Gleich, 2000, Gleich et al., 1993, Kita H, 1998). Out of these, cytotoxicity of eosinophil granule proteins against various epithelial cells has been well established (Gleich et al., 1979, Frigas et al., 1991). However, there is increasing evidence for a role of eosinophils in the pathogenesis of immune-mediated diseases of bile duct epithelia (Neuberger, 1999, Yamazaki et al., 1996). Blood and tissue eosinophilia in the liver have been associated with primary biliary cirrhosis (PBC) (Yamazaki et al., 1996, Neuberger, 1999, Terasaki et al., 1993), primary sclerosing cholangitis (Neeman and Kadish, 1987, Noguchi et al., 1992), hepatic allograft rejection (de Groen et al., 1994), and chronic graft-versus host disease (Nonomura et al., 1996). Indeed, an observation regarding eosinophil degranulation in the vicinity of injured bile ducts in patients with PBC has been well-documented (Yamazaki et al., 2001, Terasaki et al., 1993). However, the mechanism(s) of eosinophil activation in these immune cholangiopathies, remains unknown. The most abundant biliary constituent, the bile acids are synthesized in the liver and secreted into the bile canaliculus, from where they are transported along the biliary and intestinal tracts (Nonomura et al., 1996). Sakisaka et al, have suggested that, luminal bile acids cross damaged epithelia and affect the functions of periductal immune cells after the structural integrity of biliary or intestinal epithelia has been impaired (Sakisaka et al., 1997). Interestingly, bile acids have heen shown to

possess diverse immunomodulatory actions toward lymphocytes (Yoshikawa et al., 1992), monocytes (Calmus et al., 1992), macrophages (Ljubuncic et al., 1996), NK cells (Nishigaki et al., 1996), neutrophils, (Beuers et al., 1990) and mast cells (Yamazaki et al., 2001). The relevance of this to the lung transplant situation is that many of these cell types are present in elevated levels (Gerhardt et al., 2003b). Bile salts have been shown to upregulate the tissue destructive potential of these cells e.g. Taurochenodeoxycholic acid stimulates Eosinophil degranulation, IL-8 and superoxide production (Yamazaki et al., 2001). Consequently the reflux and aspiration of bile salts will enhance the ability of the immune response to damage the transplanted lung.

1.4 Bile acids in cystic fibrosis patients.

The recent history of gastro-oesophageal reflux (GOR) in cystic fibrosis (CF) disease can be traced to 1975 when it was first reported in patients with CF. Today, its prevalence in CF is estimated to be between 35 and 81 %.(Brodzicki et al., 2002, Ledson et al., 1998, Scott et al., 1985, Vic et al., 1995). Increased GOR in CF could be a secondary phenomenon during cough and/or physiotherapy resulting from an increased abdominal–thoracic pressure gradient (Ledson et al., 1998). In CF patients, the characteristics of GOR might be affected by the modifications in the volume of gastric contents, acidity and concentration of bilio-pancreatic components. Combined impedance–pH monitoring is a technique that allows detection of all types of gastro-oesophageal reflux events i.e. both acid weakly acidic and alkaline (Sifrim et al., 2004). However, the incidence of weak acidic reflux in patients with CF is currently unknown (Blondeau et al., 2007, Sifrim et al., 2005, Tutuian et al., 2006). The most important concern about GOR in CF is the possible deterioration of lung function resulting from reflux induced lung aspiration of gastric contents. This assumption has been empirically supported by the observation that CF patients with increased acid GOR had poorer lung function than those without abnormal GOR (Navarro et al., 2001). However, prevalence of GOR and poor lung function together cannot be taken as the proof of causal relationship between them, some studies data have shown the abnormal presence of gastric contents in bronchial secretions of CF patients (Blondeau et al., 2008a). Some recent studies focussing on patients after lung transplant (LTx), including transplanted CF patients, have reported the presence of markers of gastric contents in bronchoalveolar lavage (BAL) obtained during bronchoscopy (Blondeau et al., 2008b). However, it remains to be determined whether or not CF patients had higher levels of lung aspiration than other LTx patients.

The most common genetic disorder of the Caucasian population is cystic fibrosis (CF) (Steinberg and Brown, 1960). The disease is characterized by the involvement of different exocrine glands (Park and Grand, 1981), making it an extremely heterogeneous disease. The prognosis of cystic fibrosis depends largely upon the extent of respiratory involvement and the presence of serious complications like biliary cirrhosis. Around thirty years ago there was a proliferation of research aimed at investigating bile acid metabolism in patients with cystic fibrosis (Goodchild et al., 1975, Roy et al., 1977, Watkins et al., 1977, Harries et al., 1979). However, a clear understanding of the mechanisms has been complicated by the fact that both intestinal absorption and hepatic extraction of bile acids are potentially impaired. CF patients with pancreatic insufficiency suffer from bile acid malabsorption (Weber et al., 1973, Weber et al., 1976, Smalley et al., 1978). This may suggest that overall there is a greater potential for reflux of bile acids in CF. The pathophysiological significance is uncertain and the consequences are rather vaguely understood however. Similarly, serum bile acid concentrations in patients with CF have been studied on a limited scale (Davidson et al., 1980, Colombo et al., 1983, Strandvik and Samuelson, 1985, Colombo et al., 1984, Setchell et al., 1985).

1.4.1 Bile acids in sputum

Patients with CF had higher level of bile acids in sputum, median 3.3µmol/l, (25^{th} to 75^{th} percentile 2.4-6.1) than chronic cough patients, median 0.72µmol/l (0.2 – 1.2) and more than patients with Gastro-oesophageal reflux disease median 1.23mmol/l, (1.2–2.3) ($p<0.05$). A study of 38 patients was performed and only 16 who had DF508 homozygote genotype proved to have bile acids. On the other hand, bile acids were found in sputum of 4/10 chronic unexplained cough patients. No bile acids were found in healthy controls (Blondeau et al., 2008b).

1.4.2 Bile acids in bronchoalveolar lavage

CF transplanted patients had higher concentrations of bile acids [median 0.65mmol/l 25th to 75th percentile (0.4–0.7)] compared with non-CF lung transplanted patients [median 0.50mmol/l 25th to 75th percentile (0.3–1.3)]. 60% of CF transplanted patients (6/10) and 10/24 of non CF-transplanted patients (41%) had bile acids, while non transplanted patients (43%) had bile acids. No typical GOR symptoms were found in (59%) CF patients who had bile acids in BAL or saliva (Blondeau et al., 2008b)

1.4.3 Gastro-oesophageal reflux, gastric aspiration and respiratory status

CF patients who do not have bile acids in sputum had higher levels of lung function 71% (61–79%) than CF patients who have bile acids in sputum 61% (38–82%) (FEV1% predicted). Transplanted CF patients, with bile acids had a trend for a lower lung function.

Bronchiolitis obliterans syndrome (BOS) developed in 1/3 patients who had bile acids in BALF. No BOS was developed in patients who did not have bile acids in the BALF (Blondeau et al., 2008b).

1.5 Bile acids in lung transplantation

Bile acid aspiration can cause serious pulmonary damage (Henderson et al., 1975, D'Ovidio et al., 2005a). As confirmed by in vitro studies on Type II pneumocytes, bile aspiration injures the cell membrane and modifies cationic penetrability (Oelberg et al., 1990, D'Ovidio et al., 2005a, D'Ovidio and Keshavjee, 2006). Bile acids have different effects depending on the site they are in. If they are in the stomach, the mucosa wall could be injured but it is reasonably well protected by mucus. In addition solubility becomes a factor at low stomach pH levels. In the lungs, the mucosal layer might be disturbed as a result of the physical effect they can have on lipids in surfactant. Type II pneumocytes, which are responsible for lung homeostasis, phospholipid production and surfactant proteins could be damaged by bile acids. Bile acids may also cause downregulation of innate immunity receptors on monocytes and macrophages (D'Ovidio et al., 2006b). By reducing phagocytosis and LPS mediated cytokine production, bile salts can lead to the reduction of macrophage function, as shown in rabbits. Bile acids can also downregulate interferon-mediated signal transducers (D'Ovidio et al., 2006b).

Regional innate immunity can be disturbed by bile aspiration, and this can consequently have an influence on the balance of innate and adaptive immunity and promote local infection, causing a more hostile adaptive immunity (D'Ovidio et al., 2006b). Activated macrophages and injured epithelial cells can accelerate allo-immunity. (Davis et al., 2003a). This shows potential cross-talk between adaptive and innate immunity (D'Ovidio et al., 2006b).

Raised neutrophils and IL-8 levels, the presence of bacteria, fungi, lower levels of pulmonary surfactant and higher inflammatory grades on transbronchial biopsy, have all been linked to the presence of bile acids (D'Ovidio et al., 2006b). Cytokine production is

controlled by opsonins and lower levels of surfactant surface proteins A and D are associated with bile salt aspiration. The cytotoxic influences of bile acids are supported by augmented lipid sphingomyelin, a phospholipid which is related to tissue damage. This causes a modification in the mucosal defences (D'Ovidio et al., 2006b). An important problem with a lot of the studies on lung damage and bile acids are that the effects *in vitro* using very high levels of bile acids are being extrapolated to the lung where much lower levels may well be present *in vivo.*

BOS grades, trans bronchial biopsies, microbiology examinations, the variance of cell counts, cytokine concentrations, and bronchoalveolar lavage bile acids were all estimated in a study of 120 patients who underwent lung transplant (D'Ovidio et al., 2005a).

It was found that the average amount of bile acids in BOS positive patients was higher than BOS negative patients. The level was 1.6µmol/L (median) in BOS positive patients compared to 0.3µmol/L (median) in negative patients (p=0.002). Those who had late BOS (after one year) had lower levels of bile acids than early BOS patients (within one year). The level was 0.8µmol/L (median) compared to 2.6µmol/L (median) respectively (p=0.02) (D'Ovidio et al., 2005a).

Blondeau *et al* supported the link between bile salts and BOS (Blondeau et al., 2008a). Patients with negative inflammation scores on biopsy had lower average level of bile salts than those with positive biopsy (median 0.2µmol/l and median 1.1µmol/l respectively). The median level of bile salts in patients with negative microbiology samples was lower than patients with positive microbiology samples, median 0.3µmol/l and median 0.7µmol/l respectively. This supports a link between the development of infection and higher amounts of bile acid (D'Ovidio et al., 2005a).

The levels of neutrophils appear to depend on the levels of bile acids present. Patients with low levels of bile acids have a level of 2-5% neutrophils (BALF differential cell count percentage), compared to patients with no detectable levels of bile acid who show

2% or lower levels of neutrophils (D'Ovidio et al., 2005a). This could be important as neutrophil levels are associated with acute rejection and BOS (Riise et al., 1998, Zheng et al., 2005). Since the bronchoalveolar pH favours activity of duodenal pancreatic agents, the duodenal reflux could be more damaging than chronic acid aspiration. BALF bile acids and abnormal pH in lung transplant patients are linked (D'Ovidio et al., 2005a). A limitation of most of the current literature regarding bile acid aspiration is that insensitive spectrophotometric techniques have been used to quantify levels. In our lab the limit of quantification of such methods is ≈ 5μmol/L. It is therefore difficult to interpret many studies, which claim to measure lower levels (Klokkenburg et al., 2009, Mud et al., 1982).

1.6 Neutrophilic inflammation and cytokines

Many inflammatory lung diseases involve neutrophil inflammation, (Goya et al., 2003) as well as the CXC chemokine, interleukin (IL-8). Asthma (Vu et al., 1998), adult respiratory distress syndrome (McQuibban et al., 2002), chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) as well as BOS are good examples of these diseases. Lymphocyte chemoattractant factor, granulocyte-macrophage colony-stimulating factor (GMCSF), and members of the chemokine superfamily constitute chemotactic cytokines. Primarily their function relates to the migration of inflammatory cells to sites of injury and disruption. Depending upon the presence or absence of an amino acid between the first two of four conserved cytokines, members of the chemokine superfamily are further divided into A and B subgroups. Several studies have demonstrated that one of the most potent chemoattractants and activators of neutrophils, on a molar basis, is the chemokine interleukin 8 (IL-8), IL-8 is synthesized and released in great quantities from airway epithelial cells (Khair et al., 1995, Devalia et al., 1993, Baggiolini et al., 1992). IL-8 has been of particular interest in studies investigating the pathogenesis of chronic bronchitis and COPD due to its important effects on neutrophil cell biology (Keatings et al., 1996). IL-8 has also been shown to be a chemoattractant for eosinophils and therefore may also play a role in the pathophysiology of allergic airway diseases (Wang et al., 1996, Sousa et al., 1994). In humans, IL-8/CXC ligand 8 (CXCL8), the prototypical neutrophil specific.

CXC chemokine is known as key mediator of acute inflammation (Baggiolini and Clark-Lewis, 1992). IL-8/CXCL8 concentrations are associated with inflammatory conditions such as necrotizing enterocolitis in the developing intestine, where they are elevated in serum and tissue (Edelson et al., 1999, Viscardi et al., 1997). Biologically significant IL-8/CXCL8 concentrations in swallowed amniotic fluid (Denison et al., 1998, Kemp et al.,

2002), and breast milk (Srivastava et al., 1996, Michie et al., 1998, Maheshwari et al., 2002, Maheshwari et al., 2003), prove that the human fetal /neonatal gastrointestinal lumens are normally exposed to these chemicals. Tested by enzyme immunoassay, an *in vitro* model of neonatal gastric and proximal intestinal conditions has shown that IL-8/CXCL8 remains undigested (Maheshwari et al., 2002). In intestinal epithelial cell (IEC) lines, recombinant human IL-8/CXCL8 promotes cellular migration, proliferation, and differentiation as well as protecting these cells from chemical injury (Maheshwari et al., 2002). The intact survival of IL-8/CXCL8 through gastric digestion needs confirmation in assays for structural and functional integrity and it would be desirable to assess the physiologic relevance of the above listed effects.

Interleukin 6 (IL-6) is another multifunctional cytokine synthesized and released by airway epithelial cells. This cytokine has pleotropic proinflammatory effects on a number of target cells. It is also involved in the activation of B lymphocytes and monocytes. It is known to induce acute-phase protein synthesis (Devalia et al., 1993, Abdelaziz et al., 1997, Borish and Rosenwasser, 1996, Levine, 1995).

IL-6 has been shown to influence the expression of cell adhesion molecules (Hutchins and Steel, 1994).

Key roles of IL-6, IL-8 and GMCSF in the regulation of airway inflammation and pathophysiology of COPD have been documented in many studies (Ruiz et al., 2002). In vitro studies have shown that these pro-inflammatory cytokines inhibit apoptosis of granulocytes (Lee et al., 1993, Leuenroth et al., 1998, Brach et al., 1992, Castro-Alcaraz et al., 2002). In addition IL-8 has been of particular interest in studies investigating the pathogenesis of chronic bronchitis and COPD, due to its important effects on neutrophil cell biology (Keatings et al., 1996).

1.7 Alloimmune and non alloimmune injury

There have been some improvements in 1-year survival rates post lung transplant, however, compared with transplantation of other solid organs, the long-term outcome after lung transplantation remains poor. At 5 years, the survival rates of lung transplant recipients are only 49% which further decline to 25% at 10 years (Trulock et al., 2006). The main cause for late death after lung transplantation is a manifestation of chronic allograft rejection called bronchiolitis obliterans syndrome (Estenne and Hertz, 2002, Okazaki et al., 2007). The pathologic changes of bronchiolitis obliterans syndrome are referred to as bronchiolitis obliterans.

The human airway epithelium plays a major role in responding to injury, infection, or toxins by producing various cytokines and mediators which modulate airway inflammation. Certain exogenous or endogenous stimuli, such as granulocyte-macrophage colony-stimulating factor (GMCSF) regulate and activate normal T-cell expression and secretion (Wu et al., 2009). Tumour necrosis factor-α ; prostaglandin E2; or transforming growth factor-β1 can directly drive epithelial cells to increase the generation of IL-8 (Khalifah et al., 2004, D'Ovidio et al., 2005b, Groetzner et al., 2006, Novick et al., 1998, Brugiere et al., 2003, Hertz et al., 1993). Resulting damage to the airway epithelium may induce a fibrotic response (Holgate, 2000). The loss of airway epithelial cells and partial or complete occlusion of the lumina of terminal and respiratory bronchioles by inflammatory and fibrous tissue are considered as the histologic hallmarks of the bronchiolitis obliterans syndrome. Airway epithelial cells are thought to represent an important cellular target during lung allograft rejection; although the precise mechanisms leading to the development of bronchiolitis obliterans are poorly understood.

It has been hypothesized that excessive fibroproliferation is caused by injury and apoptosis of airway epithelial cells with subsequent aberrant tissue repair (Gourishankar

and Halloran, 2002). Development of bronchiolitis obliterans after lung transplantation has been attributed to risk factors which have been subdivided into alloimmune-dependent and alloimmune-independent factors. It has been suggested that the frequency and severity of acute rejection episodes and the subsequent development of chronic rejection could also be inter-related (Hachem et al., 2005). Ischemia / reperfusion injury, bacterial and viral respiratory infections are potential injuries (Daud et al., 2007, Khalifah et al., 2004, D'Ovidio et al., 2005b). Food particles, bile acids, pepsin, trypsin, other enzymes, and bacteria or their products, are all part of the non-acidic component of gastric juice (Mertens et al., 2010). Gastric juice therefore constitutes a potential alloimmune-independent risk factor if aspiration occurs shown in Figure 3 (Daud et al., 2007, Khalifah et al., 2004, D'Ovidio et al., 2005b). All gastric juice factors are potential pro- inflammatory stimuli, which directly damage the airway epithelium or indirectly influence the alloimmune response by altering the milieu in the lung allograft to increase antigenicity. Bronchiolitis obliterans is generally not reversible, therefore, once established, therapeutic options remain limited. In the majority of these patients, the augmentation of immunosuppression has failed to prevent the progression of fibrotic airway obliteration leaving retransplantation as the only option (Groetzner et al., 2006, Novick et al., 1998, Brugiere et al., 2003). Indicating that alloimmune driven process are not the only factors in BOS development.

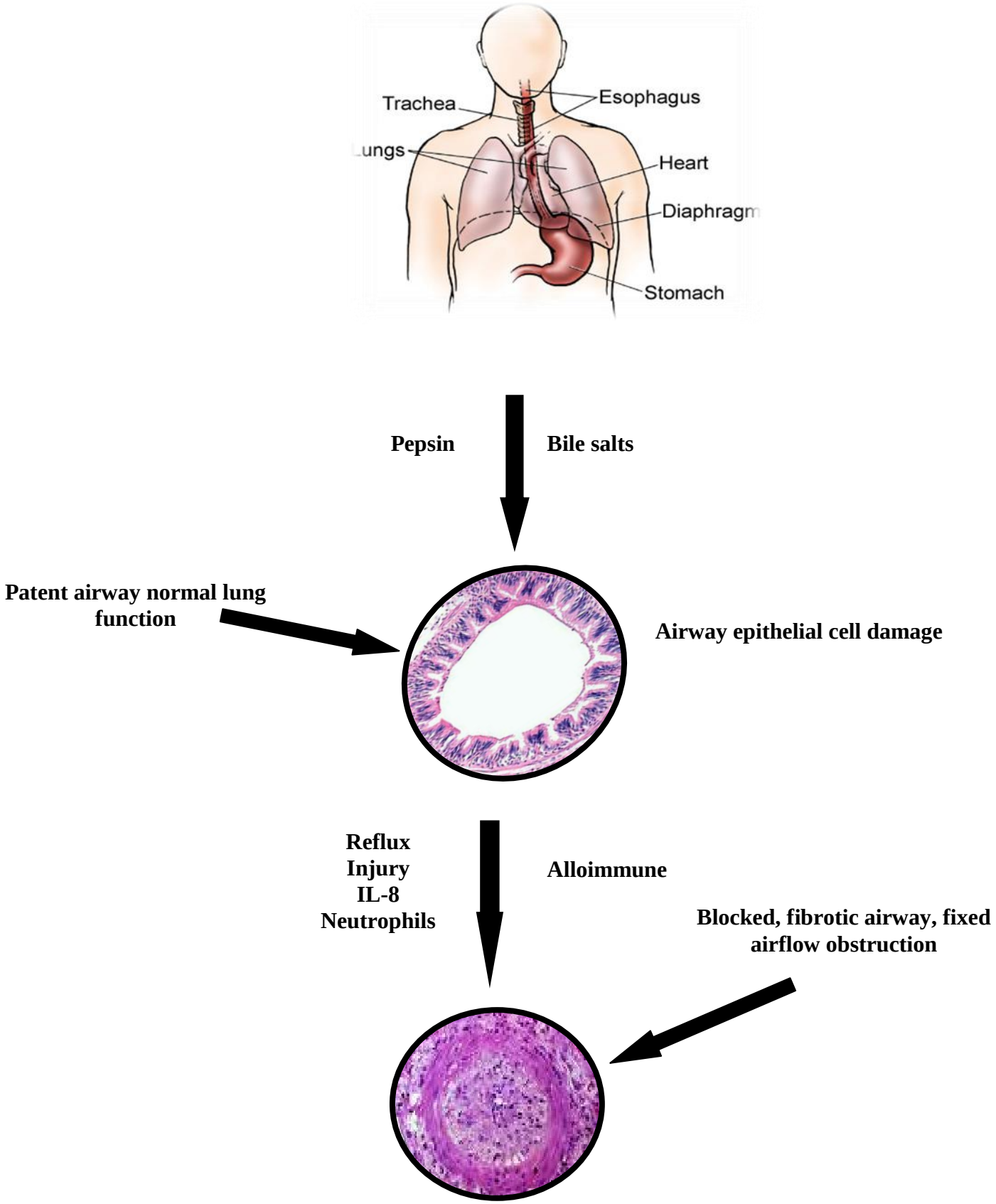

Figure 3: Potential model of epithelial cells and alloimmune non alloimmune injury infection Interactions leading to chronic rejection

The interface between the internal milieu and the external environment is established by the epithelium. This interface will form the first point of contact for inhaled substances such as respiratory viruses, airborne allergens and environmental or aspirated pollutants. It will also become a target of inhaled drugs (Gizycki et al., 1997, Folkerts and Nijkamp, 1998). The main role of the respiratory epithelium as previously recorded was of a physical barrier. However, the importance of the airway epithelial cells (AEC) in controlling various inflammatory responses observed in most respiratory diseases and especially in asthma is increasingly recognised. The complex nature of AEC interactions with a range of cells and processes can be well demonstrated in the airway response to injury (Busse et al., 1999). Additional functions of AEC has been identified because of the inflammatory cytokines and growth factors that AEC produce. These functions are:

1- A contribution to repair processes.
2- Remodelling, this can be marked in asthma and other diseases involving airway fibrosis.
3- Regulation of various immune responses (Holgate et al., 2000) .

Moreover, nitrogen dioxide (Vagaggini et al., 1996) and some particulate matters (Nordenhall et al., 2001) are environmental pollutants that are known to make asthma patients more sensitive to the proinflammatory effects. It was found that there is a difference between the cytokines released from AEC from asthma patients and healthy people. Cultured bronchial epithelial cells (HBEC) (Devalia et al., 1999) secrete various proinflammatory mediators which were examined by Devalia and co-workers both constitutively and following challenge with diesel exhaust particles (DEP). These studies showed that large quantities of interleukin (IL-8) (Lordan et al., 2002) are liberated from HBEC of asthma patients following DEP challenge. It is of interest that recent studies

have shown that there is an association between lung allograft failure, death and proximity to major road traffic (Nawrot et al., 2011).

1.8 Overall aims

Primary bronchial epithelial cells from explanted CF lungs, lung transplant patients' and a goblet cell line will be cultured to assess the effect of bile salt and gastric aspirate challenge. The effect of cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, taurodeoxycholic acid, taurocholic acid, porcine pepsin, gastric juice samples and a bile acid mixture will be measured by assessment of inflammatory cytokine production. Markers will include IL-8, IL-6 and GMCSF which are important in neutrophil inflammation, lung transplant rejection and epithelial homeostasis. Interleukin-8 in common with IL-6 is a significant driver of mucus hyper-secretion as well as neutrophilic inflammation. Cell viability will also be assayed.

1.8.1 Specific aims

- To quantify injury in cultured primary epithelia following incubation with gastric juice, the acid protease pepsin, and bile salts.
- To investigate the effect of incubation with gastric juice, the acid protease pepsin, and bile salts on a goblet cell line (HT29 MTX).

Chapter 2

Materials and Methods

2.1 Collection of samples

2.1.1 Collection of lung recipient samples

In order to facilitate the study of epithelial cell responses *in vivo* during the pathogenesis of BOS and end stage lung disease, the current study used methods for primary bronchial epithelial cell culture from lung allografts. Ethical approval for the study was granted from the Newcastle and North Tyneside 2 (reference number 2001/179), informed consent from individual patients was also obtained**.**

Patients

In brief the patients who were studied all underwent the following:

1- Lung function tests.

2- Standard assessment for BOS status (Estenne and Hertz, 2002).

3- Collection of bronchial brushings from lung transplant recipients during bronchoscopy (Forrest et al., 2005).

4- Bronchoalveolar lavage (BAL) of the right middle lobe or lingula, assessed routinely for microbiology (Yousem et al., 1996).

5- Transbronchial biopsies in order to diagnose acute vascular rejection according to international standards (Yousem et al., 1996).

	Gender	Date of birth	Reason for transplant	Date LTx	Age	One lung or two transplanted	Date BAL	BOS stage	Biopsy score	Microbiology
TW 1015	M	08.03.1957	IPF	05.04.2006	49	SL	03.10.2008	1	Ax B0	Negative
TW 1029	F	18.03.1961	LAM	06.11.2007	46	SSL	05.11.2008	NK	A0 B0 C0	Negative
TW 1080	F	06.06.1961	Emphysema	13.01.2008	47	SL	11.03.2009	1	A0 B1R(B2)	Negative
TW 1090	M	13.05.1948	Emphysema	02.09.1999	51	SL	01.05.2009	1	Ax BIR C2	Negative
									(Ax B1 Ca)	
TW 1131	M	12.05.1978	CF	08.05.2008	30	SSL	28.08.2009	1	A0 Bx Cx	Negative
TW 1140	M	27.04.1956	COPD	31.03.2009	53	SSL	28.09.2009	1	Ax Bx Cx	Negative
TW 1142	M	02.04.1961	COPD Bronch	12.07.2009	48	SSL	30.09.2009	0	Ax Bx C0	Pseudomonas aeruginosa
TW 1162	M	08.11.1962	Fib Lung Dis	02.06.2009	47	SL	02.12.2009	0	Ax Bx C0	Candida Albicans

Table 1: The clinical details of LTx patients

Key: TW numbers=individual patient number (anonymised), M=Male, F=Female, Reason for transplant indicate the lung disease that resulted in the need for lung transplamt. IPF=Idiopathic Pulmonary Fibrosis, LAM=Lymphangioleiomyomatosis, CF=Cystic Fibrosis, COPD=Chronic Obstructive Pulmonary Disease, Bronch=Bronchiectasis, Fib Lung Dis=Fibrotic lung disease. Biopsy score=Pathologist score for rejection derived from transbrobchial biosy scored according to ISHLT criteria (Hertz et al., 2010).

2.1.2 Collection of cystic fibrosis samples

Ethics and Consent

Approval was obtained for this study from the Newcastle and North Tyneside 2 Research Ethics Committee reference number 07/Q0906/47. The study was also approved by the Research and Development Department of the Newcastle upon Tyne Hospitals Foundation NHS Trust.

Dr Malcolm Brodlie obtained informed consent from all participants at the time ofacceptance on to the active lung transplantation list at the Freeman Hospital.

2.1.3 Collection of Gastric juice samples

Ethics and Consent

Ethical approval was obtained from the County Durham & Tyne Valley 2 Reserch Ethics Committe (reference number 07/H0908/70). Research & Development approval was granted by the Newcastle Upon Tyne Hospital Trust Research & Development Department.

After informed consent gastric juice was collected at routine endoscopy undertaken at a local gastroenterology clinic with Gastric juice obtained from both lung transplant recipients and non transplant related patients by Dr Andrew Robertson. The contents of these samples were then tested to determine total bile acids levels and pH by Miss Shruti Parikh. Pepsin activity was measured by Dr Bernard Verdon. The pathogen results were provided by the Freeman Road Hospital Microbiology lab (courtesy of Prof John Perry and Ms Audrey Nicholson).Data is presented in Table 9. Fractions of the samples were filtered to remove any remaining pathogens. This was done by pushing samples through 0.22μm sterile filters (Millipore) by sterile syringes (Millex). Small molecular weight molecules were removed by filtration and dialysis. To do this aliquouts of Gastric Juice were left overnight in deionised water, in 19mm dialysis membrane (Scientific

Laboratory Supplies ltd, UK), with this process removing molecules with a molecular weight below 14000 Dalton.

2.2 Cell culture methods

2.2.1 Bronchial epithelial cell isolation and culture from brushings

Bronchial epithelial cells were cultured from airway brushings taken from lung transplant patients at the time of clinically indicated bronchoscopies. This method was previously published by Forrest *et al.* and is outlined as follows (Forrest et al., 2005). Patients were pre-treated with intravenous midazolam and topical 4% lignocaine before undergoing bronchoscopy (Using an Olympus FB 45.5 bronchoscope; Olympus, Tokyo, Japan) (Mills et al., 1999). Lignocaine was applied to the vocal cords and tracheal lumen in 1ml aliquots to a maximum dose of 7mg per kg of body weight. Protected specimen single-sheathed nylon cytology brushes were used to collect bronchial brushings from subsegmental bronchi. These bushings were immediately placed into Roswell Park Memorial Institute Media (RPMI).

The suspended samples were centrifuged for seven minutes at 1100 rpm, and the cell pellet was than resuspended in 2ml of Clonetics bronchial epithelial cell basal medium (Clonetics BEBM, (Lonza), San Diego, CA, USA) together with bronchial epithelial cell growth medium (BEGM) single quots (Clonetics,) (Table 5), 50U/ml penicillin, 50mg/ml streptomycin (Sigma, UK), 50mg/ml gentamycin, and 50µg/ml amphotericin B (Lonza, USA). These were the final antimicrobial concentrations in the culture medium throughout the process.

Cell suspensions were put in to a T25cm flask pre-coated with collagen (Vitrogen 100, cohesion, Palo Alto, CA, USA) and placed in a CO_2 incubator (37C$^{\circ}$/5% CO_2). After the first 48 hours a further 3ml of supplemented medium was added with subsequent exchange (3 ml) every 48 hours, until primary bronchial epithelial cell cultures (PBECs)

reached 80%-95% confluence. Once confluent, PBECs were passaged using 3ml of trypsin, which was neutralised using an equal volume of RPMI supplemented with 10% of foetal calf serum (FCS). PBECs were then put in 10ml of culture medium and transfered to Vitrogen (Cohesion) coated T75cm^2 flasks, 24 well plates or to eight chamber slides, (Lab-Tek, Nunc, Naperville, IL, USA; Chamber-1). Which were cultured to 80-95% confluence.

2.2.2 Cystic fibrosis epithelial cell culture

Initial dissection and preparation of cells through to passage 1 into T25 flasks were carried out by Dr. M Brodlie and Dr. Chris Ward.

Cystic fibrosis cells were cultured from resected lungs, from patients with end stage disease, at the time of transplantation (Brodlie et al., 2010).

The lungs removed at the time of transplantation were examined by a consultant pathologist, Dr. Fiona Black, both macroscopically and histologically. This examination aimed at confirming the pre-identified disease and diagnosing other unidentified diseases, for example a malignancy before the transplantation.

After collecting clinical samples for microbiology and virology examinations, the explanted lungs were stored at 4^{o}C. The tissue was then processed, usually less than 60 minutes after the explantation. The later culturing work was done in a class II laminar flow hood using a meticulous aseptic technique. Around 0.5 cm to 2 cm pieces of the main bronchus were then resected from the connected lymphoid, alveolar and vascular tissues. Four pieces of bronchus were taken out of each lung, rinsed twice at 4^{o}C in 25 ml of sterile phosphate buffered saline (Sigma-Aldrich) (Figure 4 and Figure 4a). The rinsed tissue was then immersed in 20ml of washing solution A at 4^{o}C see (Table 2). The solution was agitated for 30 minutes, and then the pieces of bronchus were rinsed twice at 4^{o}C in 25ml of Dulbecco's Modified Eagle's Medium (DMEM) to be later immersed in

20 ml of the washing solution B at 4^{o}C (Table 3). The tissue was then agitated for 48 to 72 hours (4^{o}C) at 50 HZ on a rocker-shaker set.

Component	Concentration	Supplier
DMEM	Neat	Invitrogen
Dnase	10µg/ml	Sigma -Aldrich
Dithiothreitol	500µg/ml	Sigma- Aldrich
Designated Antimicrobials	See Table -3	

Table 4: Constituents of Washing Solution A

Expert advice from Professor John Perry, Clinical Scientist, Microbiology Department, of the Freeman Hospital helped in the choice of the antimicrobial combination used in Washing Solution B (Table 3). The organisms (and sensitivity results) recently taken from the sputum of a specific patient were taken into consideration where possible. All the used antimicrobics and their concentration are shown in (Table 4) . The data published by Randell *et. al* (2001) concerning the relative cytotoxicity of different agents in primary bronchial epithelial cell cultures and a discussion with Professor Perry determined the concentration of each antimicrobial used (Brodlie et al., 2010).

Component	Concentration	Supplier
DMEM	Neat	Invitrogen
Dnase	1µg/ml	Sigma -Aldrich
Protease	1%	Sigma- Aldrich
Designated Antimicrobials	See Table -4	

Table 5: Constituents of Washing Solution B based on (Fulcher et al., 2005)

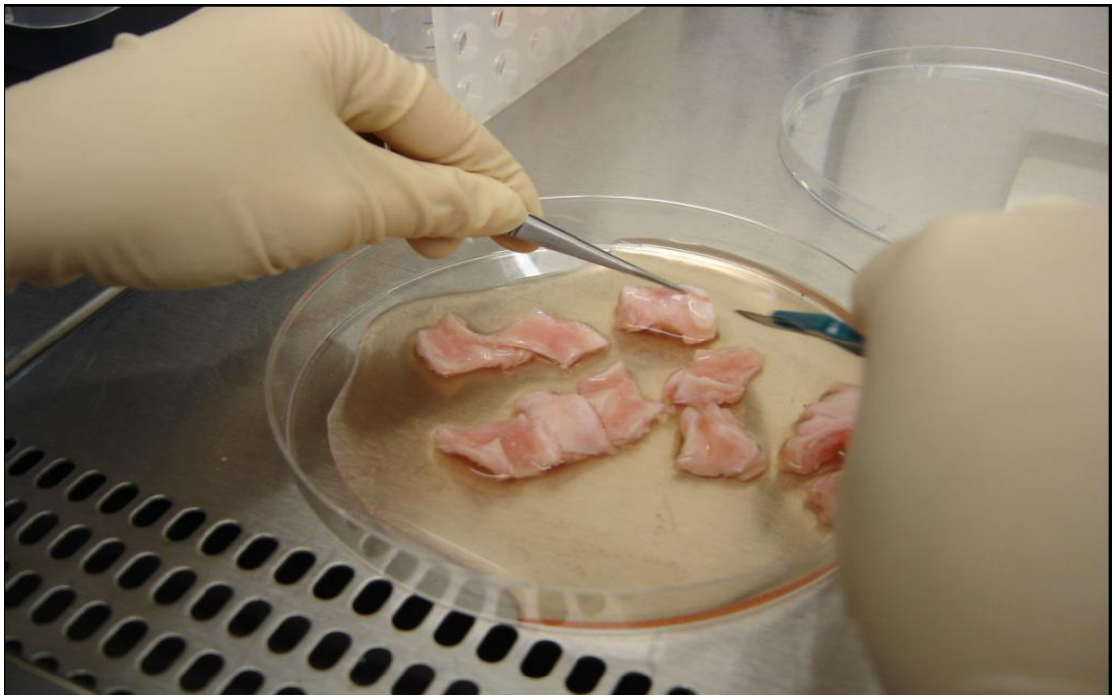

Figure 4a: Epithelial cell removal after protease dissociation. The concave, luminal airway surface is gently scraped with a convex scalpel blade to remove adherent cells (Fulcher et al., 2005).

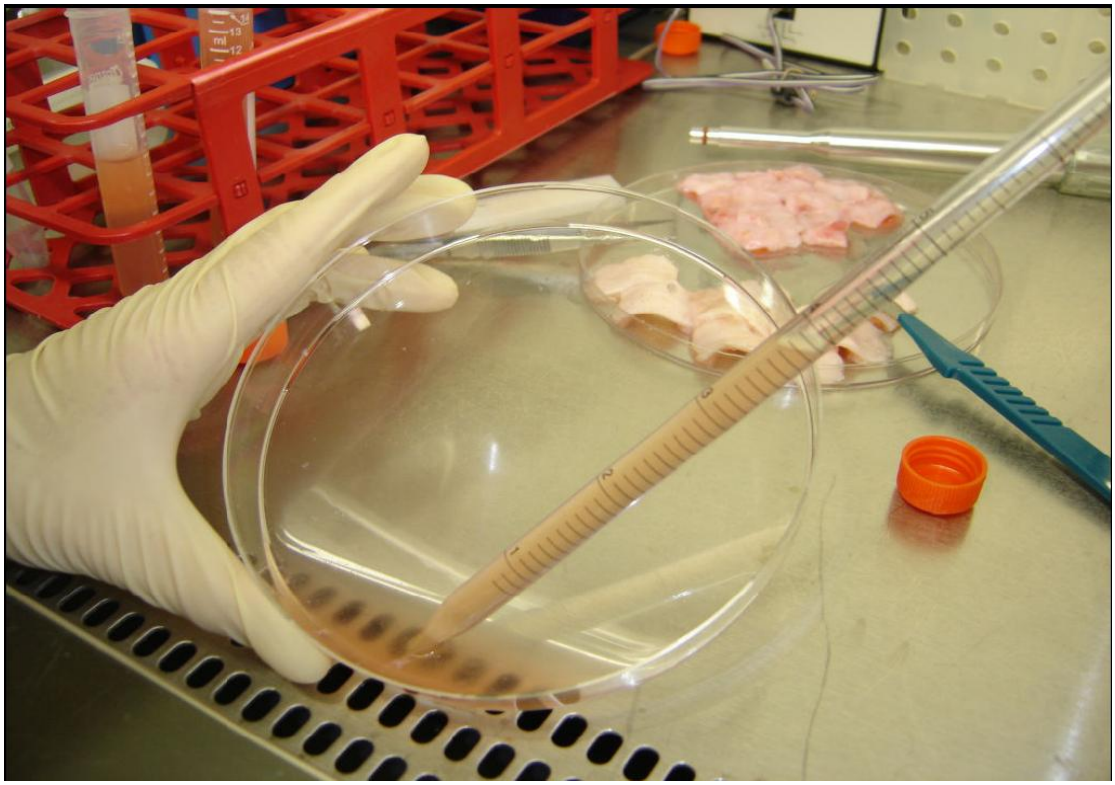

Figure 4b: Rinsing and collecting the scraped cells (Fulcher et al., 2005)

The bronchial cells were harvested after being put for 48 to 72 hours in washing solution B (Table 3). 5ml of Roswell Park Memorial Institute-1640 (RPMI) (Invitrogen) containing 10% Fetal Calf Serum (FCS) (Invitrogen) was added to the solution to neutralise any remaining protease. Subsequently, the pieces of bronchus were transferred to a sterile petri dish. The luminal side of the bronchi was lightly scraped using a scalpel blade to remove the epithelial cells (Figure 4a). The scraped cells were later immersed in 10ml of RPMI containing 10% FCS and centrifuged at 1000 rpm for 5 minutes. After discarding the supernatant, the cells were resuspended in 5ml of Bronchial Epithelial Basal Medium (BEBM) (Clonetics) warmed to 37° C and supplemented with the single quots described in (Table 5), 1% streptomycin , 1% penicillin and designated antibiotics as shown in(Table 4) (figure 4b). The resulting cell suspension was later seeded into 25cm^2 tissue culture flasks precoated with collagen (PureCol, Advanced BioMatrix, USA) and placed in a carbon dioxide (5%) enriched incubator at 37° C.

Antimicrobial	Working Concentration in BEGM
Ceftazidime	128 µg/ml
Tobramycin	16 µg/ml
Vancomycin	10 µg/ml
Colistin	5 µg/ml
Meropenem	100 µg/ml
Co-Trimoxazole	12 µg/ml
Ticarillin and Clavuantae	16 µg/ml
Amphotericin B	4 µg/ml
Voriconazole	10 µg/ml

Table 6: Antimicrobial Working Concentration in Media (Fulcher et al., 2005)

Component	Volume Added to 500 ml of BEBM
Bovine Pituitary Extract	2 ml
Insulin	500 µl
Hydrocortisone	500 µl
Retinoic Acid	500 µl
Transferrin	500 µl
Epinephrine	500 µl
Human Epidermal Growth Factor	500 µl
Tri- Iodothyronine	500 µl
Gentamicin/ Amphotericin	500 µl

Table 7: Bronchial epithelial cell growth medium (BEGM) Single Quots

A careful observation of the PBEC cultures was maintained to make sure that the growth of the cells was satisfactory and to note any signs of infection. If signs were detected the flask was instantly removed from the incubator and its contents sent for routine bacterial and fungal culture to determine the infecting organism and the relevant antimicrobial sensitivities. It was presupposed that early infection episodes were due to the presence of organisms, resistant to the antimicrobial cocktail, emanating from the patient rather than introduced during the culturing process. The BEBM was changed every 48 hours. The designated antibiotics were normally removed from the BEBM after approximately 96 hours of successful cell culturing free of overt infection.

After flasks were around 80% confluent, the PBECs were passaged using Trypsin/ethylene diamine tetraacetic acid (EDTA) [Sigma] by applying the following method. First, the culture medium was isolated from the flask and replaced with 2ml of

trypsin/EDTA. Second, the adherents PBECs were removed by a gentle shaking action. The level of trypsinisation was determined by light microscopy. After it was ascertained that a sufficient number of cells had been lifted from the flask, the trypsin was neutralised by the addition of an identical volume of RPMI containing 10% FCS. The resulting suspension was then removed and centrifuged at 1100 rpm for 7 minutes at 20°C. The cell pellet was either resuspended in BEGM and seeded into flasks (Corning) or 24-well plates (Corning) for further submerged culture, or cryopreserved. The supernatant was discarded.

2.2.3 Goblet cell line (HT 29MTX) culture

The HT29-MTX cell line although of colonic origin shows rheologic changes characteristic of respiratory goblet cells in that is secretes MUC5AC a mucin secreted by a respiratory goblet cells and not MUC2 the mucus secreted by colonic goblet cells. The human goblet cell line HT29-MTX, donated by Dr Thecla Lesuffler (INSERM U178, France), was grown at 37°C and 10% CO_2 in a T75 flask in 12ml of Dulbeco's modified eagles medium (Sigma, UK) supplemented with 10% FCS (Sigma, UK), 50U/ml penicillin, 50mg/ml streptomycin (Sigma, UK), 50mg/ml gentamycin, and 50µg/ml amphotericin B (both Lonza, USA). Media was changed every 48 hours until 75 to 85% confluence was reached. Cells were then passaged to a T75 flask, using the same method used for PBECs. Upon reaching confluence the cells were passaged to 24 well plates for experimental interventions. The wells contained 4-5x10^5 cells.

2.2.4 Epithelial Cell passage

2.2.4.1 Epithelial Cell passage (common to CF samples and lung Tx brushing)

When the cells grew to near confluence in the cell culture flask they required passage, for further growth or to allow experimentation. To do this the cell culture flask had 2.5 ml of trypsin EDTA added and was then incubated at 37 ºC for 3 to 5 minutes, 5ml of RPMI media was then used to resuspend the cells, and to stop the function of trypsin. The cells were pelleted by centrifugation for seven minutes at 1100 rpm and 20 ºC. Finally 12 ml of complete epithelial media was added and mixed gently. 24 well plates were then seeded with 0.5 ml of the cell suspension per well, the well contains between 3.5 to 4 $x10^5$ cells.

2.2.5. Primary bronchial epithelial Cell (PBECs) and goblet cell (HT29 MTX) stimulation.

When PBECs were 80-95% confluent 24 well plates were rested for 24 hours by the addition of serum free medium (BEBM, 50mg/ml gentamycin, and 50µg/ml amphotericin B (Lonza, USA), 50U/ml penicillin, 50mg/ml streptomycin (Sigma, UK)). Goblet cells were rested for 24 hours in serum free medium (DMEM, 50U/ml penicillin, 50mg/ml streptomycin (Sigma, UK), 50mg/ml gentamycin, and 50µg/ml amphotericin B (Lonza, USA)). Cells were stimulated with bile salts, pepsin or whole, filtrated and dialysed human gastric juice made up in resting medium. The initial solubilisation of bile salts cholic acid, chenodeoxycholic acid, deoxycholic acid, lithocholic acid, taurodeoxycholic acid, and taurocholic acid bile salts required methanol. All bile salts were solubilised in methanol then diluted with resting media to give required concentrations.

Different concentrations of the bile salts, (0.25, 0.50, 75, 1, 5, 10, 25, 50, 100 and 200µmol/L) as well as different incubation times 24, 48 hours were used. PBECs were

stimulated with porcine pepsin at concentrations of 25µg/ml, 50µg/ml and 100µg/ml for different incubation times 24, 48 hours.

Samples of medium were collected at 24 and 48 hour intervals; control samples were also collected at these times. Controls consisted of cells incubated with resting media and methanol. The data presented in the thesis are all 48 hour results because the stimulations at 48 hours were more effective compared to 24 hour results.

PBECs were stimulated for 24 hours, with 8 different gastric juice samples, gastric juice samples were diluted 1/1000, 1/2000 and 1/3000 in 500µl of the resting media. The eight human gastric juice samples were also filtered, dialysed as well as diluted by the same method. Gastric juice with pH between7-8 and diluted 1/1000 have previously been shown to stimulate large IL-8 levels from PBECs, (Mertens et al., 2010), I therefore standardised the pH of my gastric juice samples (pH 7.4), using 1/1000 dilution.

PBECs were also stimulated with a bile acid mixture; the composition of this was based on concentrations of bile salts present in human bile (50% taurocholic acid, 15% taurodeoxycholic acid, 30% chenodeoxycholic acid and 5% lithocholic acid) (Allan et al., 1976) (10µmol/l) for 48 hours. The conjugated bile acids were originally going to be used in the experiments, however the free acids were used as the conjugated forms were prohibitively expensive.

2.3 Cryopreservation of epithelial and goblet cells

2.5 ml or 5 ml of trypsin (depending on the size of the flask) were added to the cell culture flask in the hood. It was then incubated at 37 ºC for 2-4 minutes after which 5-10 ml of RPMI media was added to resuspend the cells and to stop the action of trypsin. The cells were then transferred to a 25 ml universal tube and centrifuged for 7 minutes at 1100 rpm, 20ºC. Then 0.5 ml of freezing media (80% BEBM 10% FCS and 10% dimethyl sulfoxide (DMSO)) was added to the cells which were left at -80ºC for 24 hours, in cryotubes in a cell freezer filled with isopropanol (Nunc). Finally they were stored in liquid nitrogen at -180 ºC

2.4 Epithelial cell culture after cryopreservation

The sample was removed from liquid nitrogen and incubated at room temperature for 10 minutes. The sample was then put in 2-3 ml of complete BEGM and mixed gently then added to a cell culture flask coated by 0.5% of Vitrogen (PureCol, Advanced BioMatrix, USA). The cells were then incubated at 37 ºC for 24 hours in a 5% CO_2 atmosphere and the media was changed at 24 hours. After 48 hours the samples were checked under the microscope and the media changed.

2.6 Cell viability assay

Cell viability was assessed by the Titre-blue Assay (Promega, USA) which assessed metabolically active cells after 48 hour incubations under experimental conditions. The percentages of viable cells were determined from a standard curve which was generated from mixtures of live and dead cells (dead cells were produced by ice cold methanol treatment for 5 minutes). 5 points were used to create the standard curve, 100% live cells resting media, 25% dead cells with 75% live cells, 50% dead cells with 50% live cells, 75% dead cells with 25% live cells and 100% dead cells (positive control).

2.7 Enzyme-Linked Immunosorbent Assay (ELISA) MSD

To measure cell culture supernatant IL-8, IL-6 and GMCSF, a multiplex enzyme-linked immunosorbent assay (ELISA) was performed using manufacturer's instructions (Meso Scale Discovery, Maryland, USA). Cytokines were measured by MSD cytokine assays. One to four cytokines can be measured in a MSD 96-well MULTI-SPOT plate, competitive immunoassay (sandwich), single spot, or patterned array with capture antibodies (Figure 5).

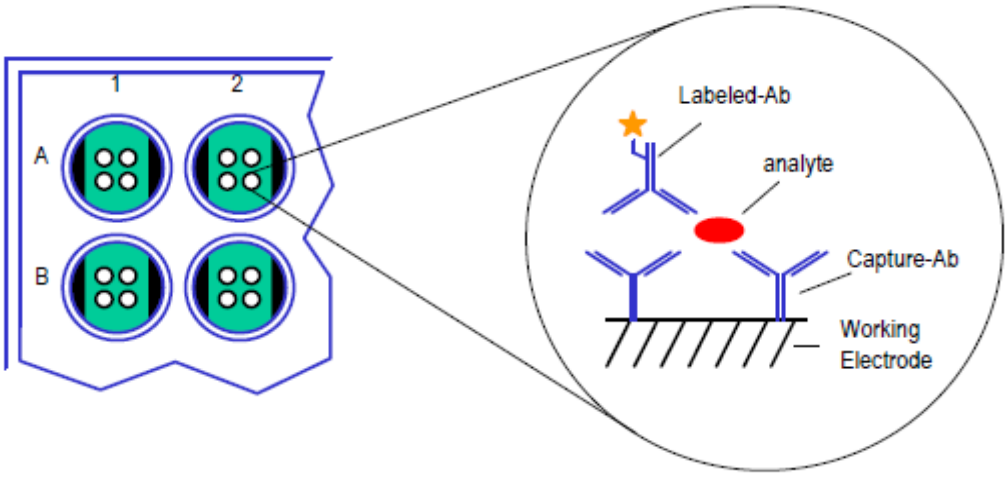

Figure 5: M-CSF Capture Antibody is pre-coated on a Single Spot MULTI-ARRAY plate Or on specific spots of a 4-Spot MSD MULTI-SPOT plate. Calibrator solutions and Samples are incubated in the plate, and M-CSF binds to corresponding Capture antibody spot. M-CSF is detected using an M-CSF-specific Detection Antibody labelled with MSD SULFO-TAGTM reagent. This figure has been modified from MSD

All reagents were used at room temperature. 25 µL of diluents 2 (protein and animal derived material medium) was added into plates well, covered with adhesive strip and incubated with vigorous shaking for 30 minutes at room temperature. 25 µL of standards and samples were added; with vigorous shaking and incubated for 2 hours at room temperature. Plates were washed three times with PBS + 0.05% Tween-20 at pH 7. Detection antibody solution (25 µL) was added (mix 2.94 mL of diluents 3 with 60 µL

aliquot of the stock detection antibody) and the plates were then incubated for 2 hours at room temperature with vigorous shaking. A wash with wash buffer was repeated three times. 150 µL of read buffer was then added, and the plate was then read by the MSD plate reader. The lowest levels for cytokines quantitation by the MSD plate reader was 0.6pg/mL.

2.8 Statistical analysis

GraphPad Prism version 4 for Windows was used to carry out analyses; Non-parametric statistical tests were used throughout on the basis that "Non-parametric" implies that there was no assumption of a specific distribution for the population data gathered in the thesis. The 1-Sample Wilcoxon was used for paired data comparisons. This is the nonparametric alternative to 1-sample (paired) t-tests, used when data is known to be normally distributed. P values of 0.05 or less were considered to be significant as is convention.

Chapter 3

Effect of refluxate components on primary cells cultured from lung transplant recipients

3.1 Introduction

Lung transplantation (LTx) has attained the status of a viable therapeutic option for increasing survival as a treatment option for end-stage, pulmonary disorders, improving the quality of life (Trulock et al., 2006). The development of chronic allograft rejection is believed to be a significant reason for the reduced long-term survival of lung transplant recipients (Trulock et al., 2006). The clinical manifestation of chronic rejection is termed bronchiolitis obliterans syndrome (BOS) which is defined as a persistent drop after transplantation in the forced expiratory volume in one second (FEV_1). Obliteration of the terminal bronchioles is the pathological cause for the development of this syndrome (Verleden et al., 2005). The pathophysiology of BOS is not well-understood; however, it is increasingly recognised that both immunological and non-immunological mechanisms influence it (Boehler et al., 1998, Blondeau et al., 2008b, Verleden and Dupont, 2006). A potential non-alloimmune cause of BOS has been identified as gastro-oesophageal reflux (GOR) and oesophageal pH-metry has shown a greater oesophageal acid exposure in 70% of LTx patients (Benden et al., 2005, Hadjiliadis et al., 2003). Pepsin and bile acids have been identified as important luminal gastric components present, in bronchial material of LTx recipients (D'Ovidio et al., 2005b, Ward et al., 2005, D'Ovidio et al., 2006a). D'Ovidio et al have demonstrated that increased bile acids levels in (BALF) in 22% of LTx patients was associated with reduced freedom from BOS (D'Ovidio et al., 2006a). All LTx recipients had increased levels of pepsin in BALF, suggesting that aspiration after LTx might be widespread even in the patients with normal GOR indices (Ward et al., 2005, O'Halloran et al., 2004). Bile acids have also been detected in BALF in 49% of LTx patients but not in the non-transplant samples (D'Ovidio et al., 2005b, D'Ovidio et al., 2006a). The absolute levels of BALF pepsin was thought to be a general marker of aspiration of gastric contents, while bile acids were found to be a more specific marker of

gastric aspiration, possibly related specifically to the pathophysiology of aspiration induced BOS. Increased oesophageal acid exposure does not seem to be discriminating, as similar levels have been reported in patients both with and without BOS as described by Davis et al and Hadjilidis et al (Davis et al., 2003a, Hadjiliadis et al., 2003)

The potential pathophysiological, mechanistic relationships between the primary epithelial cells from lung recipients and the aspiration markers that have been associated with the development chronic rejection have been studied within this thesis. Specifically, the responses of primary epithelial cells have been examined when stimulated with different bile acids, porcine pepsin and different human gastric juice samples. The response of the epithelial cells has been assessed by cytokine output, concentrating on cytokines that are known to be involved in neutrophilia; such as interleukins 6, 8 and GMCSF as BOS is known to have a neutrophilic pathophysiology (Devouassoux et al., 2002, D'Ovidio et al., 2005b).

3.2 Methods and statistical analysis

When PBECs were 80-95% confluent in 24 well plates they were rested for 24 hours as (2.2.5). After that, individual primary and secondary bile acids, porcine pepsin, and different patient derived gastric juice (whole, filtered and dialysed) samples were used to stimulate the cells. All concentrations are reported in section (2.2.5).

Cell viability was assessed by the Titre-blue Assay (2.6). An MSD 96-well multiplex ELISA was used to measure IL-8, IL-6 and GMCSF (2.7). GraphPad Prism version 4 was used for data analysis as outlined in (2.8).

3.3 Results and Discussion

3.3.1 Epithelial cell culture:

One aim of my project was to process and culture primary epithelial cells from lung transplant recipients. Although the culture of these cells is very difficult in this research field, approximately 70% success rate was achieved. This experience was favourable compared to other studies, e.g other study success rate of 39% (Forrest et al., 2005)

The cells were cultured in different culture conditions, Figure 6(A) shows 24 well cell cultures of primary epithelial cells from patients in complete media (BEBM) at 37°C. In addition, it was possible to culture cells in a 24 well cell culture to a defined time point (6 days), stimulate with different bile acids, porcine pepsin and different human gastric juice samples (Figure 6(B)). After cryopreservation it was possible to re-establish these cells in culture as shown in Figure 6(C) after four days. Epithelial cells cultured in complete media (BEBM) in 24 well cell cultures and stimulated with 200 µmol/l lithocholic acid are shown in Figure 6(D) after 48 hours. Epithelial cells cultured in complete media (BEBM) in 24 well cell cultures stimulated with lithocholic acid 50 µmol/ l are shown in Figure 6(E). Epithelial cells cultured in complete media (BEBM) in 24 well cell cultures and stimulated with lithocholic acid 25 µmol/ l are shown in Figure 6(F). Epithelial cells cultured in complete media (BEBM) in 24 well cell cultures and stimulated with lithocholic acid 1µmol/ l are shown in Figure 6(G).

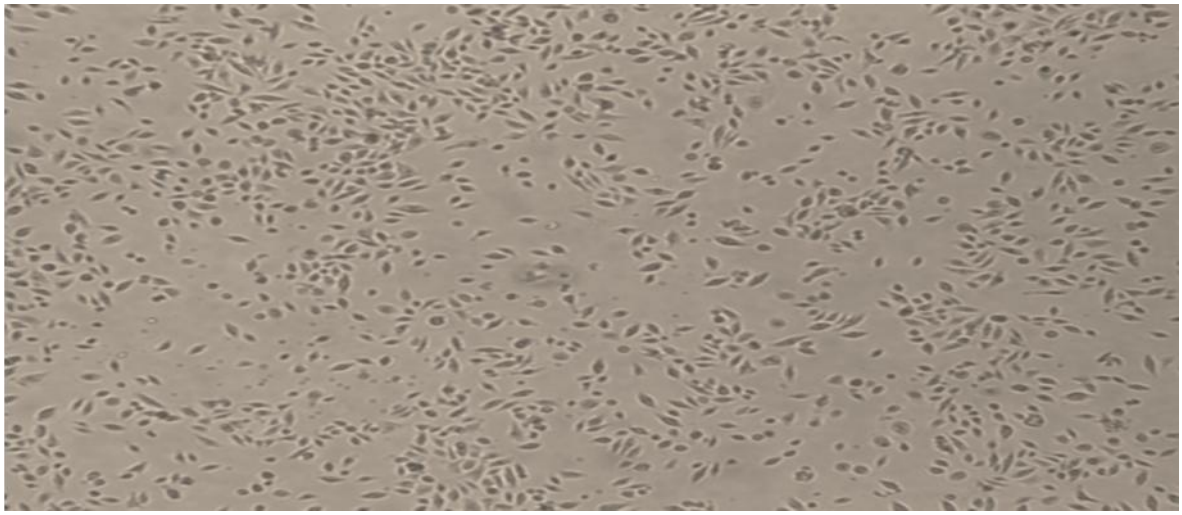

Figure 6A: Epithelial cells cultured in complete media (BEBM) in 24 well cell cultures, at 37°C after three days from passage (X100 magnification). These cells have the typical appearance of healthy, primary epithelial cells in submerged culture. This is shown by the patches of 'cobblestone' morphology. The cells are clearly adherent, with minimal numbers of floating or fibroblast like cells.

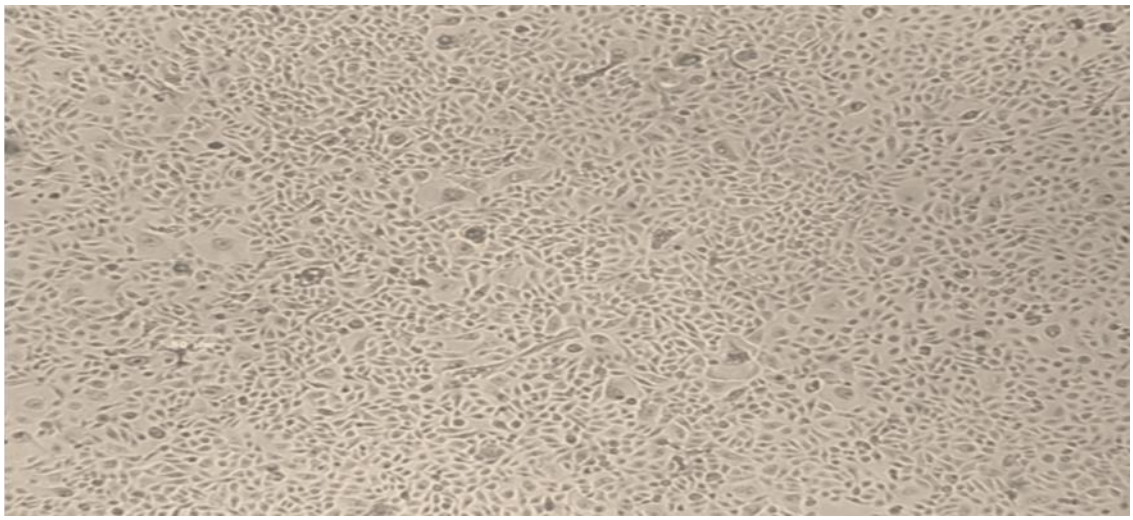

Figure 6B: Epithelial cells cultured in complete media (BEBM) of 24 well cell cultures after six days from passage, ready for stimulation by bile acid or vehicle alone (X100 magnification). As in 6 (A) these cells have the typical appearance of healthy, primary epithelial cells in submerged culture. This is shown by the 'cobblestone' morphology. The cells are clearly adherent, with minimal numbers of floating or fibroblast like cells. The difference between Fig 6(A) and 6 (B) is that there are more cells and no obvious gaps. The cells are therefore confluent and ready for stimulation experiments

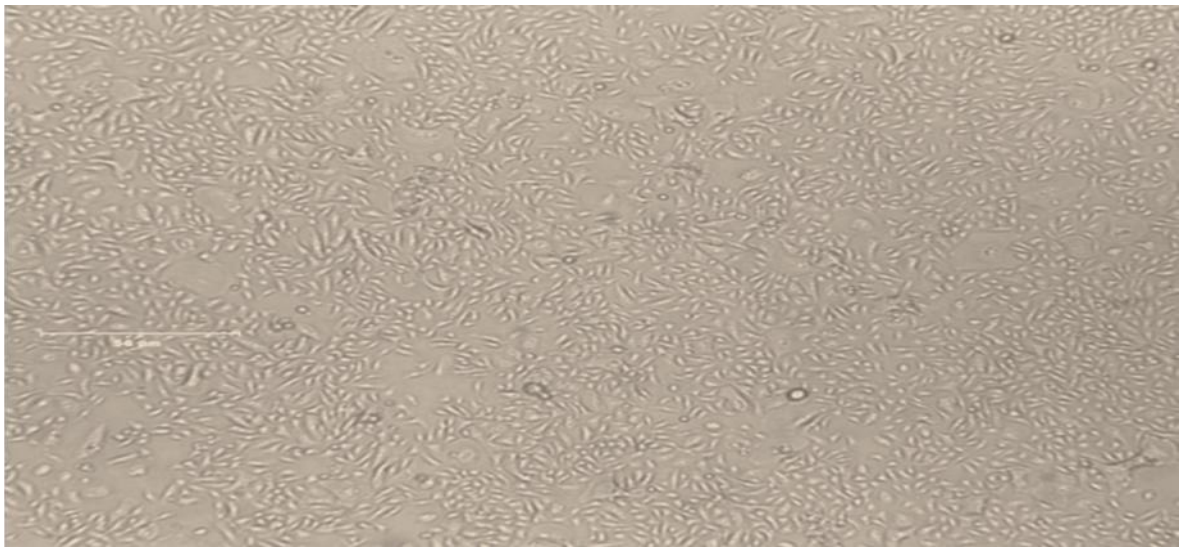

Figure 6(C): Epithelial cells cultured in complete media (BEBM) after storage in liquid nitrogen,cultured at 37°C, 24 well cell culture, for (6 days) (X100 magnification). As in figure 6(A) and figure 6 (B) these cells have the typical appearance of healthy, primary epithelial cells in submerged culture. This is shown by the 'cobblestone' morphology. The cells are clearly adherent, with minimal numbers of floating or fibroblast like cells. The cells are therefore confluent and ready for stimulation experiments. This shows that cells stored in liquid nitrogen are similar in appearance to freshly cultured cells.

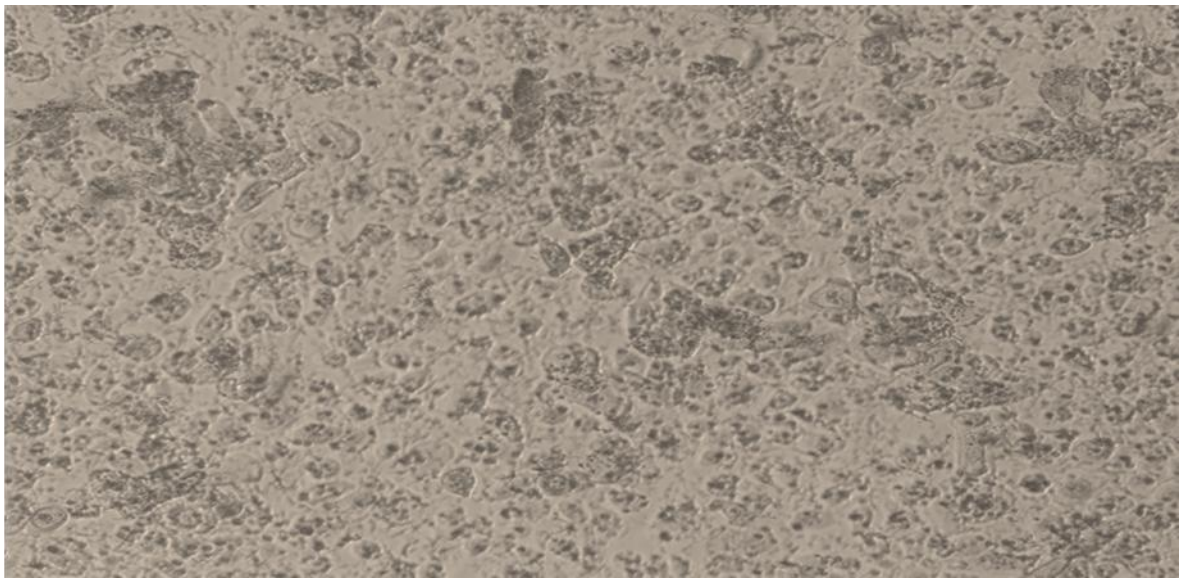

Figure 6 (D): Epithelial cells cultured in complete media (BEBM) of 24 well cell cultures after two days of stimulation with 200 µmol/l lithocholic acid (X100 magnification). In contrast to Figures 6 (A) - 6 (C), these cells have lost the normal cobblestone appearance of normal healthy cells. There is evidence of cell death as the cells appear more rounded.

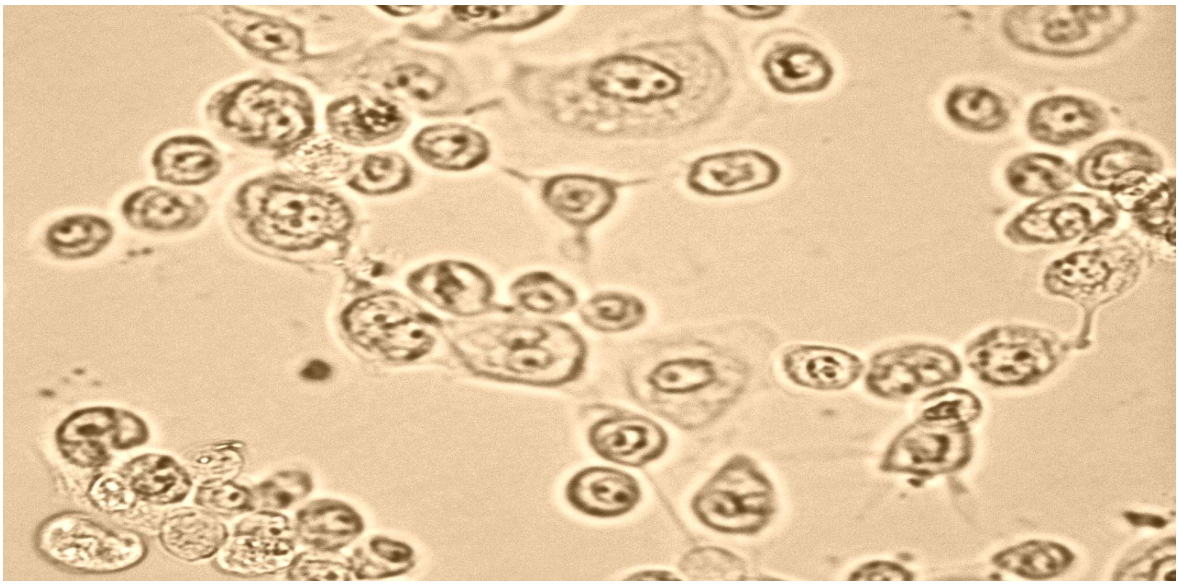

Figure 6(E): Epithelial cells cultured in complete media (BEBM) of 24 well cell cultures after stimulation with lithocholic acid 50 µmol/l for two days (X300 magnification). This shows obvious cell damage. In contrast to Figures 6(A) - 6(C) these cells have lost the normal cobblestone appearance of cells not incubated with lithocholic acid. Following lithocholic acid 50 µmol/l for two days, the cells are rounded, with gaps in between cells. Cell detachment has occurred leading to floating, non adherent cells.

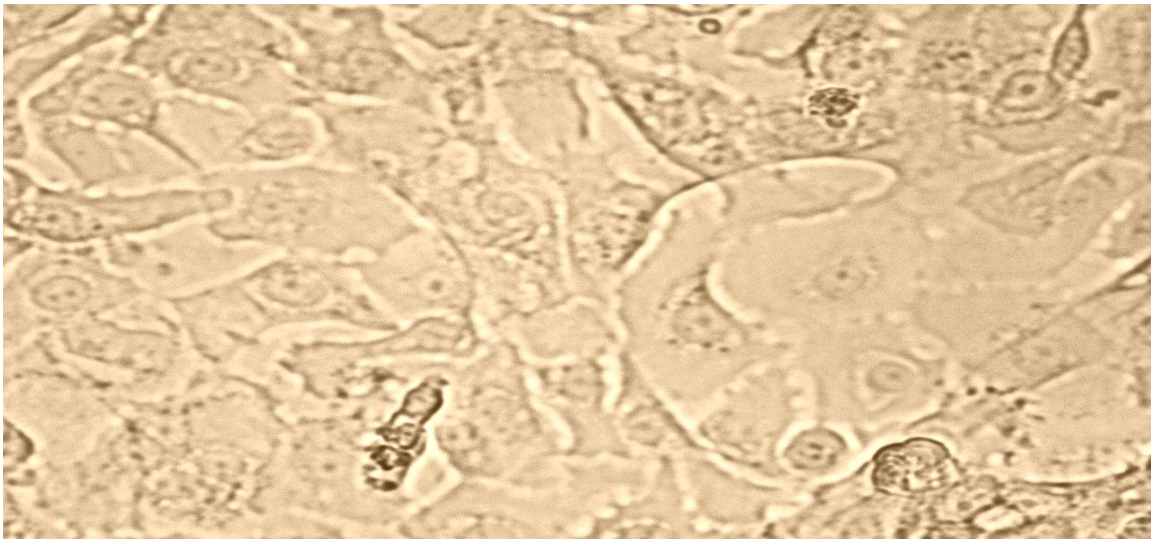

Figure 6(F): Epithelial cells cultured in complete media (BEBM) of 24 well cell cultures after stimulation with lithocholic acid (25µmol/l) for two days (X300 magnification). The cells do not appear normal (i.e. some cobblestone appearance but the cells are more rounded) as in Figures 6(A) - 6(C), but there is less clear damage than in figure 6(E)

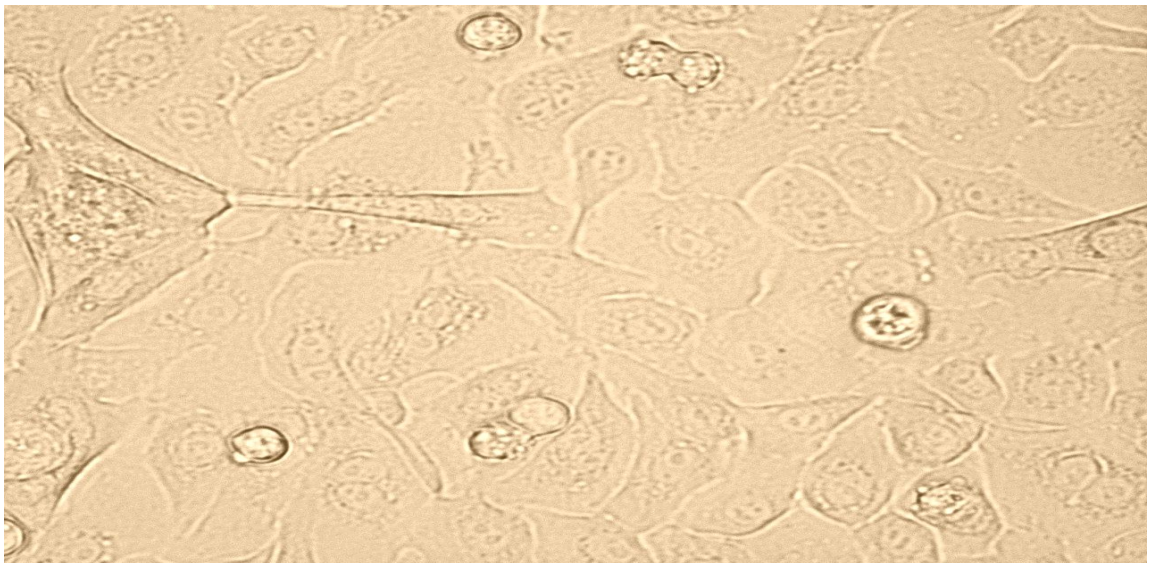

Figure 6(G): Epithelial cells cultured in complete media (BEBM) of 24 well cell cultures after stimulation with lithocholic acid (1µmol/l) for two days (X300 magnification). The cells appear normal with an obvious cobblestone appearance and the cells are in close association with each other. Similar in appearance to figures 6A - 6C

A further aim of the project was to determine whether primary bronchial epithelial cells from lung recipients were able to produce significant increases in IL-8, IL-6 and GMCSF after challenge by bile acids, porcine pepsin, and different human gastric juice (whole, filtered and dialysed) samples as outlined in (2.2.5).

The primary bile acid chenodeoxycholic acid and secondary bile acids deoxycholic acid and lithocholic acid were each used to stimulate cultured PBECs at different concentrations from 0.25μmol/l to 10μmol/l for 48 hours and compared to basal incubations. Figures 7 to 9 shows the levels of IL-8, IL-6 and GMCSF released from PBECs from lung transplant recipients after stimulation by chenodeoxycholic acid for 48 hours. Table 6 shows that chenodeoxycholic acid caused a significant increase in the release of IL-8 at 1, 5 and 10μmol/l. However the IL-6 levels produced by the cells were only increase significantly by 5μmol/l chenodeoxycholic acid. GMCSF levels increased significantly after exposure to 0.75, 5 and 10μmol/l of chenodeoxycholic acid.

Figures 10 to 12 show the different levels for IL-8, IL-6 and GMCSF produced after the challenge of PBECs with deoxycholic acid. Table 7 show that IL-8 levels at 1 and 10 μmol/l deoxycholic acid were significantly stimulated. IL-6 levels were not elevated by any of the concentrations of this bile acid. Interestingly GMCSF levels were significantly elevated by all the concentrations of deoxycholic acid except 0.75μmol/l. Figures 13 to 15 show the different levels for IL-8, IL-6 and GMCSF produced by PBECs after they were stimulated with lithocholic acid. Table 8 shows that IL-8 levels were significantly higher than basal at concentrations of 1 μmol/l and above of lithocholic acid. IL-6 levels were only elevated when stimulated by 5μmol/l lithocholic acid, GMCSF levels were significantly elevated by concentrations of lithocolic acid of 1μmol/l and above.

In general these bile acid stimulation studies demonstrate that IL-6 production is the least effected by the bile acids with levels of 5μmol/l or above needed to achieve significant elevation in levels.

In figures 16 to 18 we can see the concentration of chenodoxycholic acid above 10µmol/l cause substation cell death, with 25µmol/l causing 50% cell death. 1µmol/l or above of deoxycholic acid and lithocholic acid also effect the cells viability, suggesting that secondary bile acids are more cytotoxic than primary bile acids.

Figures 19 to 21 show the different levels of IL-8, IL-6 and GMCSF produced after challenge with different concentrations of porcine pepsin at pH 7.4. Those figures show no significant stimulation of cytokine production from PBECS from lung recipients after testing across a range of porcine pepsin concentrations. The pepsin used in this study would be inactive and probably irreversibly denatured at pH 7.4. Therefore any effect would be attributed to the protein and not the active enzyme. Figures 22 to 24 show the different levels for IL-8, IL-6 and GMCSF after challenge of PBECs with 8 different human gastric juices derived from patients, diluted 1:1000. The production of cytokines by the cells depends on which gastric juices are used (tables 8 and 9), indicating marked variability between different gastric juices. As shown by CR52 which produces significant down- regulation of IL-8 as compared to CR45 which produces significant up-regulation of IL-8. This variability might be because of the variations in human gastric juice components. For example the gastric juice sample CR45 which contained no bile acids, and very low levels of pepsin (57µg/ml). However this particular gastric juice caused a significant increase in IL-8 production at 1:1000 dilution. This is potentially due to its content of pathogens, with α haemolytic streptococcus demonstrated on culture, though other organisms may also be present in the gastric juice. Interestingly this juice also caused a significant increase in IL-6 levels (the only juice to do so), but there was no significant GMCSF stimulation. The pepsin level in CR52 was much higher (1319 µg/ml) with no pathogens and it caused a significant down regulation of IL-8 levels. CR38 contained 250µmol/L of total bile acids, Candida sp, *A. lwoffii* and α haemolytic streptococcus and also led to significant IL-8 stimulation, but not IL-6 or GMCSF. In

addition CR34 (which contained high levels of pepsin and 240µmol/l bile acids but no pathogens) caused a significant reduction in GMCSF levels (Table 10). Figures 25 to 27 detail the finding that after stimulation with filtered and dialysed gastric juice samples PBECs produced no significant increase in levels of IL-8, IL6 and GMCSF. This may indicate that pathogens as well as bile acids are causing significant increases in IL-8, IL-6 production. The whole pathogens will be removed by filtration and some bacterial products such as LPS (depending on size) will be removed by dialysis. Bile acids are mainly removed by dialysis as show in Table 9. It is interesting to note that 4 gastric juice samples that had no stimulating affect on IL-6, after dialysis and filtration down regulated IL-6 levels. Figures 28 to 30 show that the PBECs from lung recipients were not significantly stimulated to produce IL-8, IL-6 or GMCSF after gastric juices were modified by filtration and dialysis of the samples. The comparison with the same samples stimulated by whole gastric juice samples indicates that the PBEC inflammatory/damaging components *in vitro* are molecules that can be removed by dialysis and filtration.

3.4.1 IL-8, IL6 and GMSF levels, after cell stimulation with the individual primary bile acid (chenodoxeycholic acid) at 37 ºC for 48hours in 5% CO2.

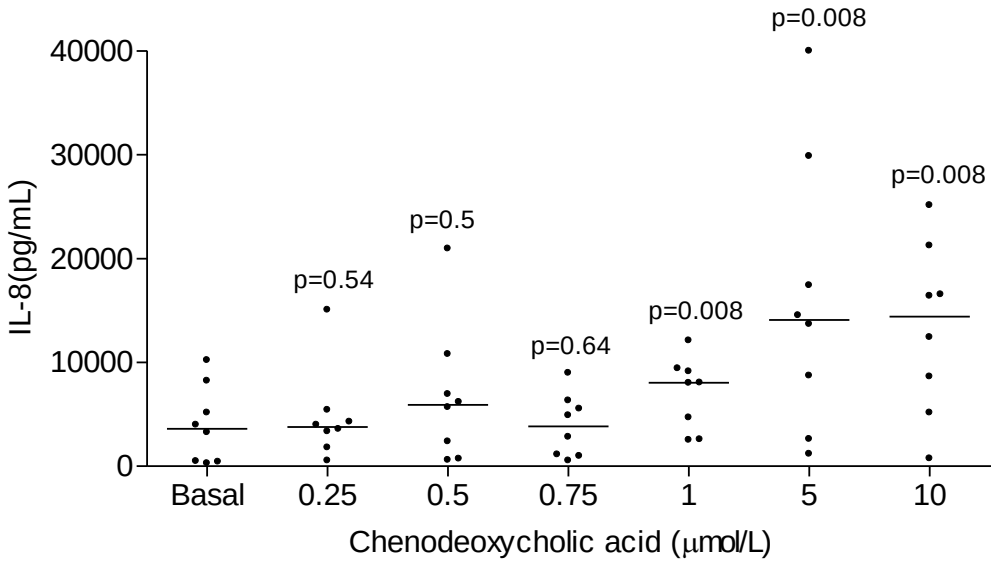

Figure 7: The effect of stimulating PBECs from lung transplantation recipients with chenodeoxycholic acid and its effect on IL-8 release, following stimulation for 48 hours. Basal represents the background production of cytokine without chenodeoxycholic acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

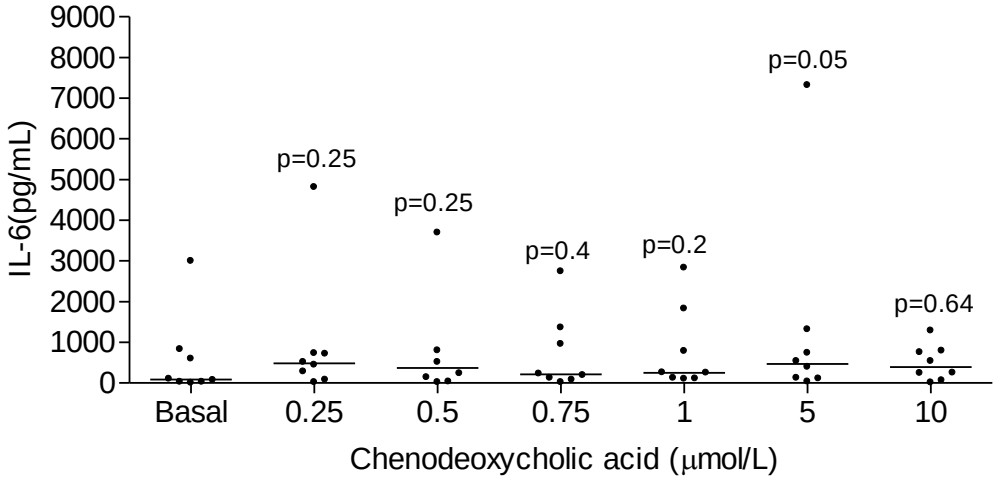

Figure 8: The effect of stimulating PBECs from lung transplantation recipients with chenodeoxycholic acid and its effect on IL-6 release, following stimulation for 48 hours. Basal represents the background production of cytokine without chenodeoxycholic acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

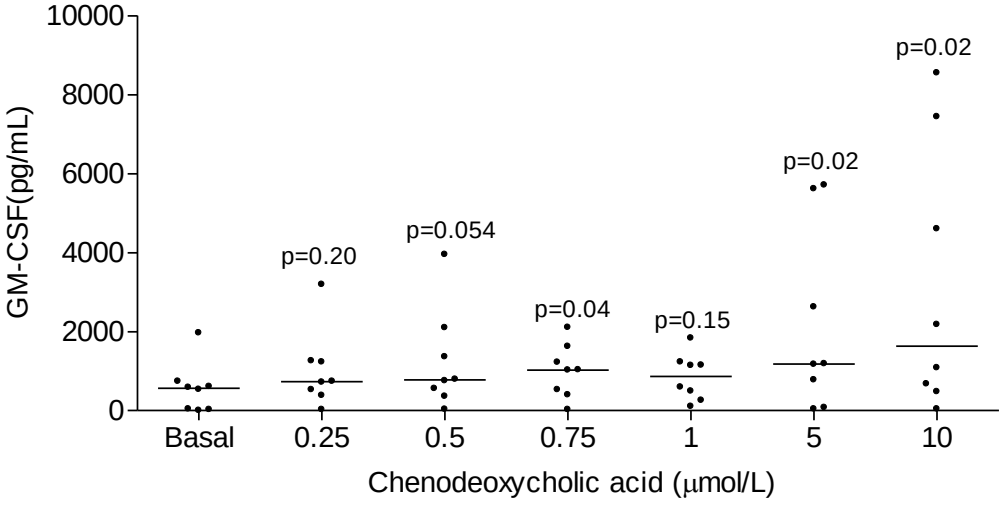

Figure 9: The effect of stimulating PBECs from lung transplantation recipients with chenodeoxycholic acid, and its effect on GMCSF release, following stimulation for 48 hours. Basal represents the background production of cytokine without chenodeoxycholic acid stimulation Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

Table 6: Levels of chenodeoxycholic acid that demonstrated significant stimulation of cytokine release from lung transplant derived PBECS.

	Chenodeoxycholic acid µmol/l					
	0.25	0.5	0.75	1	5	10
IL-8	_____	_____	_____	↑*	↑*	↑*
IL-6	_____	_____	_____	_____	↑*	_____
GMCSF	_____	_____	↑*	_____	↑*	↑*

Table 8: The levels of chenodeoxycholic acid challenge for PBECs from lung transplant recipient, which demonstrated significant stimulation of cytokine release.

↑ Increase

↓ Decrease

3.4.2 IL-8, IL-6 and GM-CSF levels, after cell stimulation with the individual secondary bile acid (Doxeycholic acid) at 37 ºC for 48hours in 5% CO2.

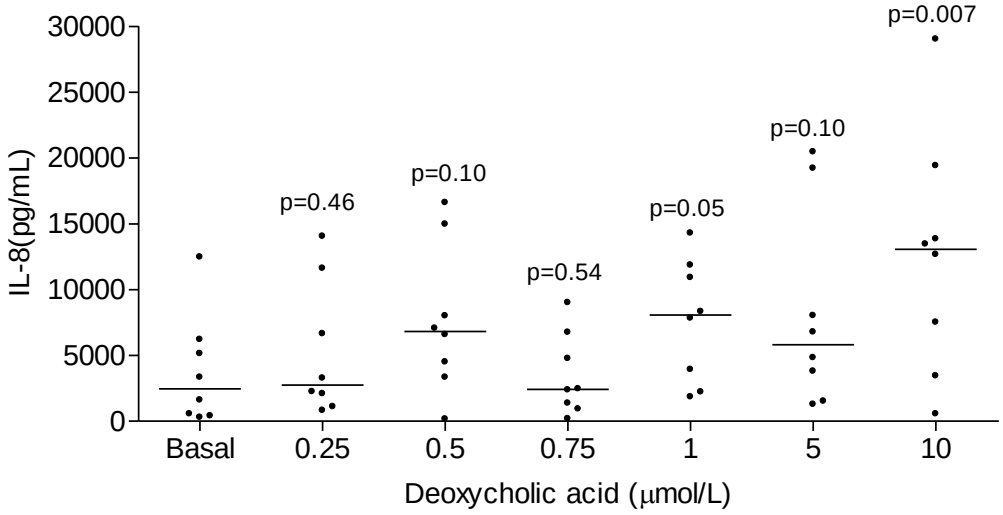

Figure 10: The effect of stimulating PBECs from lung transplantation recipients with deoxycholic acid and its effect on IL-8 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

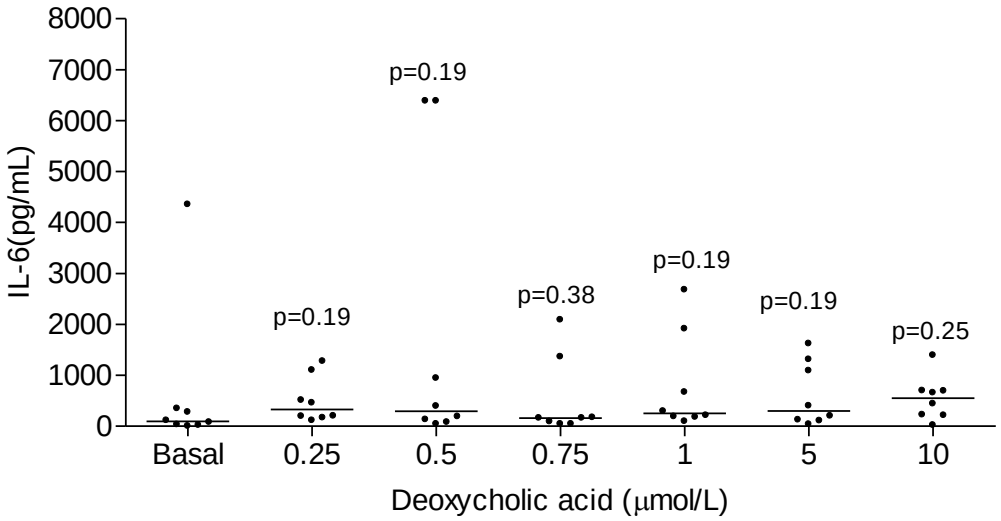

Figure 11: The effect of stimulating PBECs from lung transplantation recipients with deoxycholic acid and its effect on IL-6 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

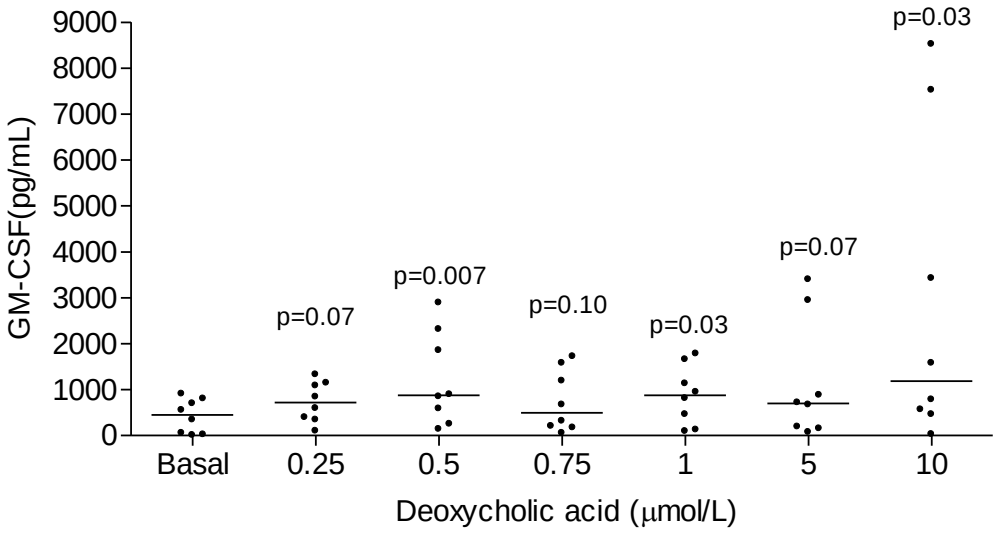

Figure 12: The effect of stimulating PBECs from lung transplantation recipients with deoxycholic acid and its effect on GMCSF release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

Table 7: Levels of deoxycholic acid that demonstrated significant stimulation of cytokine release from lung transplant derived PBECS.

	deoxycholic acid µmol/l					
	0.25	0.5	0.75	1	5	10
IL-8	____	____	____	↑*	____	↑*
IL-6	____	____	____	____	____	____
GMCSF	↑*	↑*	____	↑*	↑*	↑*

Table 9: The levels of deoxycholic acid challenge for PBECs from lung transplant recipient, which demonstrated significant stimulation of cytokine release.

↑Increase

↓Decrease

3.4.2 IL-8, IL-6 and GM-CSF levels, after cell stimulation with the individual secondary bile acid (Lithocholic acid) at 37 ºC for 48hours in 5% CO_2.

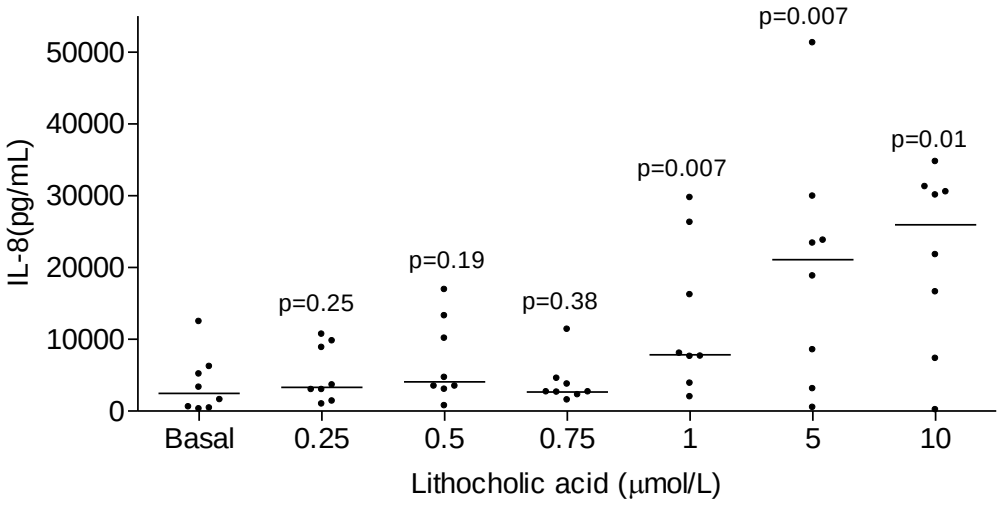

Figure 13: The effect of stimulating PBECs from lung transplantation recipients with lithocholic acid and its effect on IL-8 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

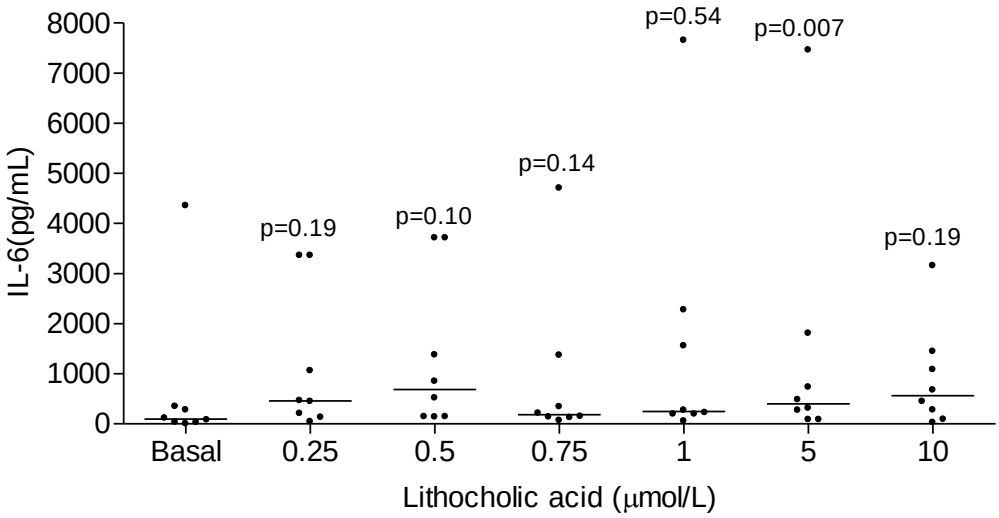

Figure 14: The effect of stimulating PBECs from lung transplantation recipients with lithocholic acid and its effect on IL-6 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

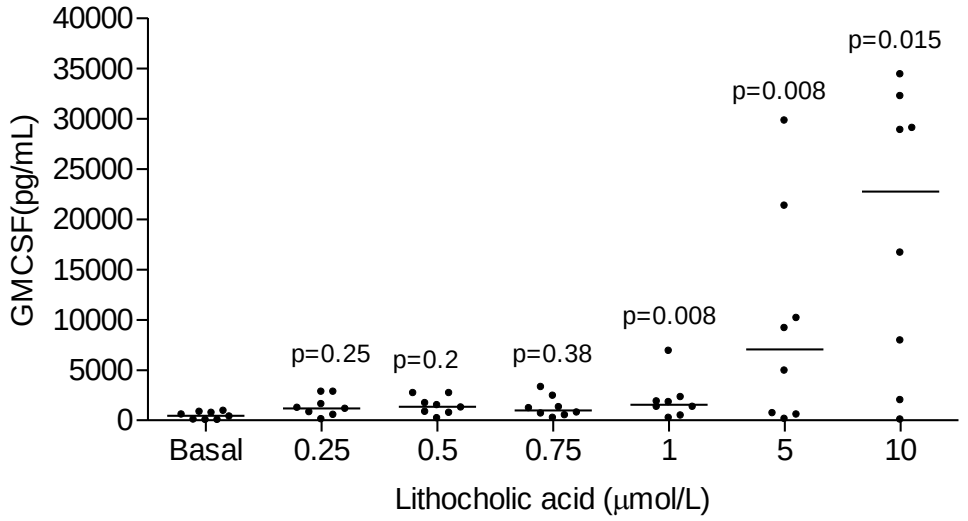

Figure 15: The effect of stimulating PBECs from lung transplantation recipients with lithocholic acid and its effect on GMCSF release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

Table 8: Levels of lithocholic acid that demonstrated significant stimulation of cytokine release from lung transplant derived PBECS.

	lithocholic acid µmol/l					
	0.25	0.5	0.75	1	5	10
IL-8	_____	_____	_____	↑*	↑*	↑*
IL-6	_____	_____	_____	_____	↑*	_____
GMCSF	_____	_____	_____	↑*	↑*	↑*

Table 10: The levels of lithocholic acid challenge for PBECs from lung transplant recipient, which demonstrated significant stimulation of cytokine release.

↑ Increase

↓ Decrease

3.4.3 Cell viability for primary bronchial epithelial cells from explanted CF patients after stimulation by individual bile acid

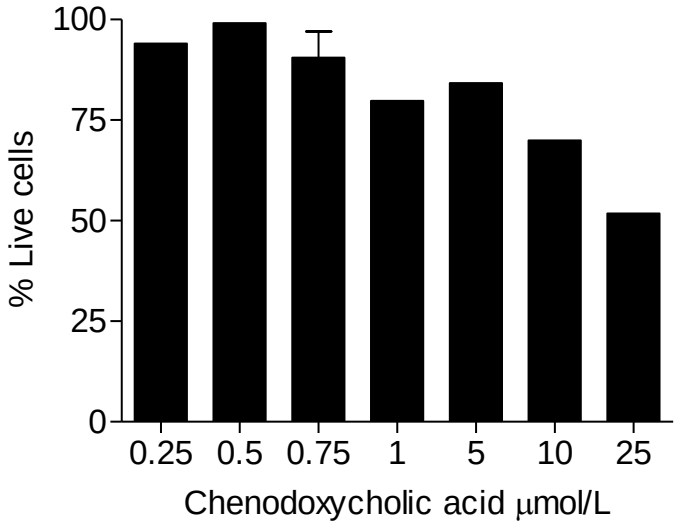

Figure 16: The viability of primary bronchial epithelial cells from lung recipients after challenge with different concentrations of chenodoxycholic acid for 48 hours. Cell viability was assessed by CellTiter- Blue cell viability assay (Promega. Madison), n=5.

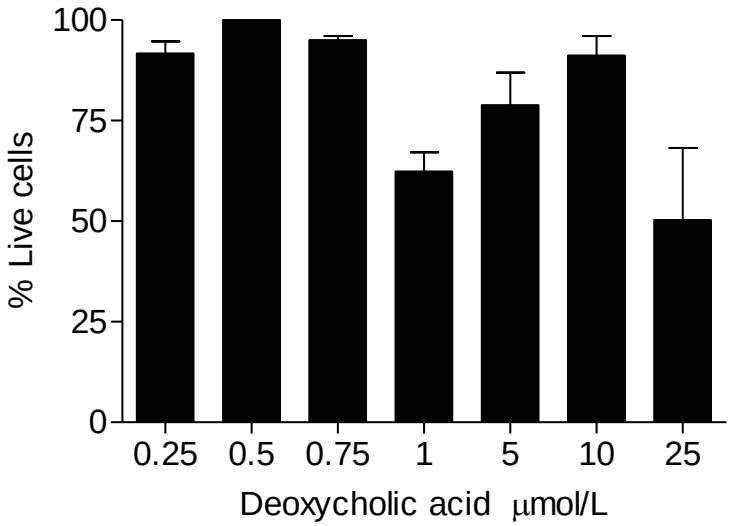

Figure 17: The viability of primary bronchial epithelial cells from lung recipients after challenge with different concentrations of deoxycholic acid for 48 hours. Cell viability was assessed by CellTiter- Blue cell viability assay (Promega. Madison), n=5.

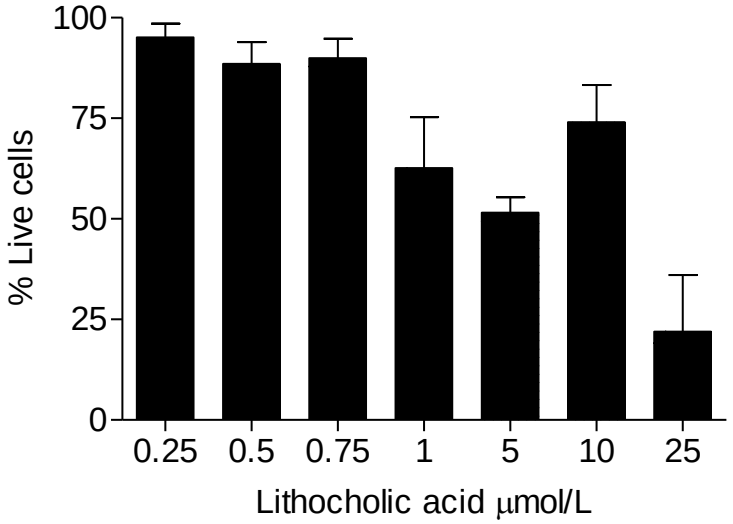

Figure 18: The viability of primary bronchial epithelial cells from lung recipients after challenge with different concentrations of lithocholic acid for 48 hours. Cell viability was assessed by CellTiter- Blue cell viability assay (Promega. Madison), n=5.

3.4.4 IL-8, IL-6 and GM-CSF levels, after cell stimulation with Pepsin at 37 ºC pH 7.4 for 48 hours in 5% CO_2.

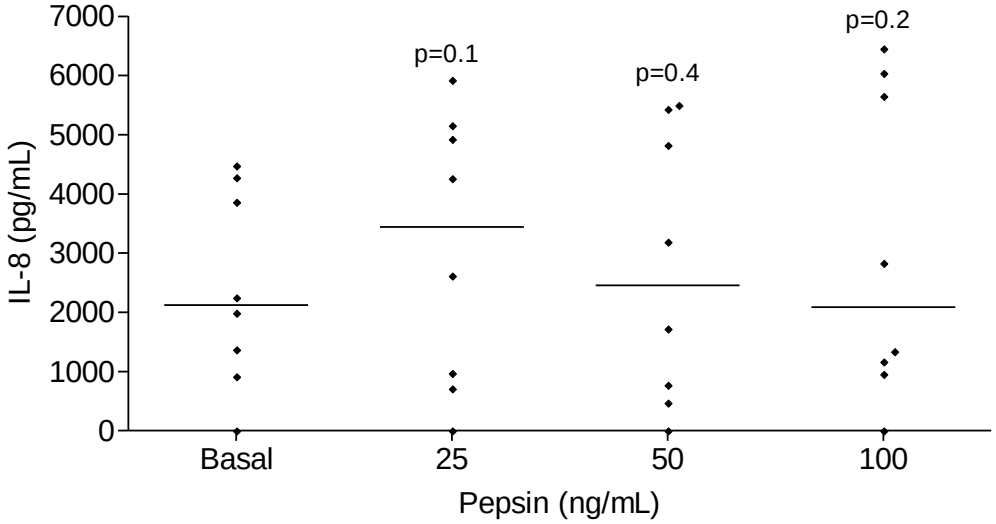

Figure 19: The effect of stimulating PBECs from lung transplantation recipients with porcine pepsin and its effect on IL-8 release, following stimulation for 48 hours. Basal represents the background production of cytokine without pepsin stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

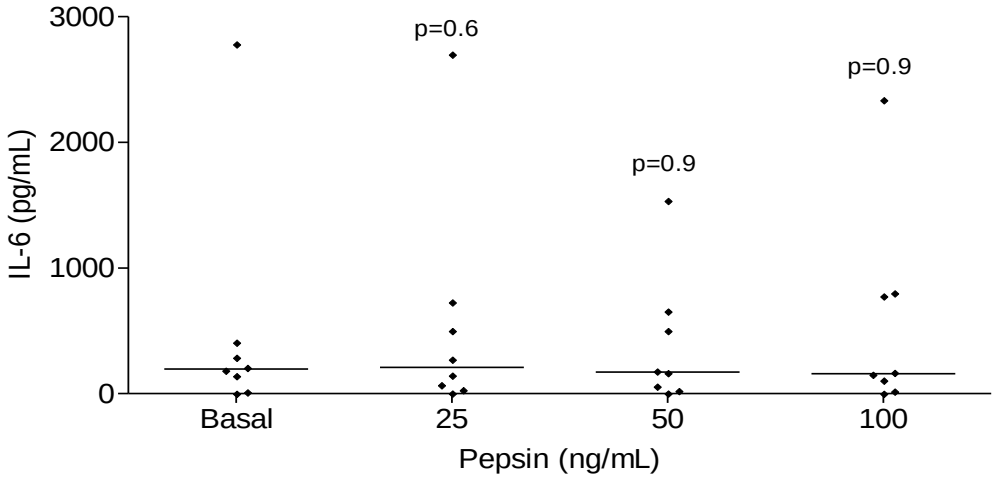

Figure 20: The effect of stimulating PBECs from lung transplantation recipients with porcine pepsin and its effect on IL-6 release, following stimulation for 48 hours. Basal levels (no pepsin stimulation). Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

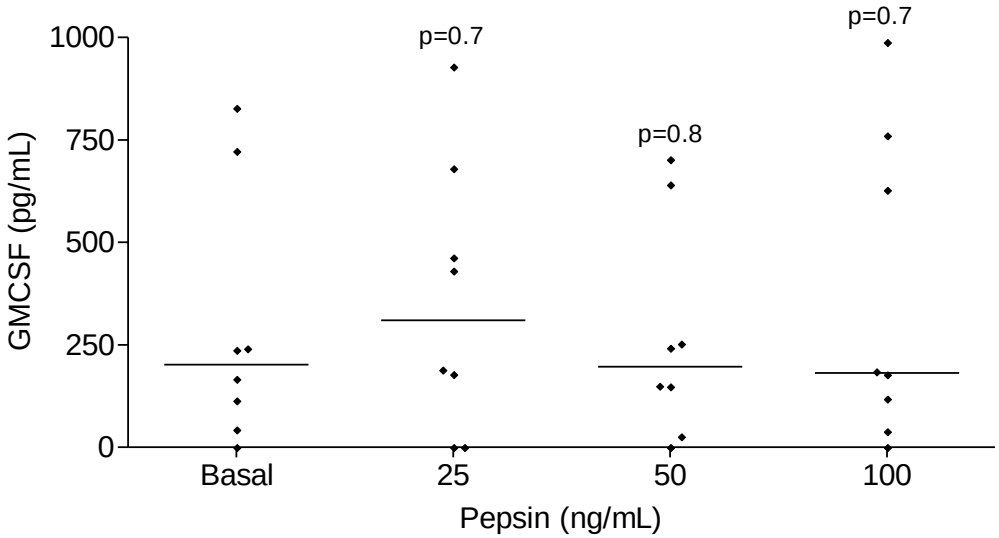

Figure 21: The effect of stimulating PBECs from lung transplantation recipients with porcine pepsin and its effect on GMCSF release, following stimulation for 48 hours. Basal levels (no pepsin stimulation). Cytokine secretions in cell supernatants were measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=8.

Gastric Juice	pH	Pepsin activity (µg/ml)	Total bile (µmol/L)	Total bile after filtered and dialysed(µmol/L)	Pathogens
LTx8	1.2	1346	320	60	Pseudomonas aeruginosa, Candida parapsilosis strains
CR34	1.6	1247	240	75	None
CR52	1.7	1319	20	0	None
CR47	5.1	460	0	0	Proteus Mirabilis, Klebsiella. ozaenae
CR50	5.2	3772	3060	290	Coagulase –ve Staphylococcus, Diptheroids
CR42	5.5	3153	480	0	Acinetobacter junii, Candida parapsilosis strains.
CR38	6.6	0	250	40	Candida parapsilosis strains., A.lwoffii,α haemolytic streptococcus
CR45	8.4	57	0	0	α haemolytic streptococcus

Table 11: The concentration of pepsin and bile acids of each gastric juice, and the pH of the gastric juice sample. LTx denotes lung transplant patient and CR samples are routine gastroenterology patients.

3.4.5 IL-8, IL-6 and GM-CSF levels, after cell stimulation with whole gastric juice at 37 ºC for 24 hours in 5% CO_2.

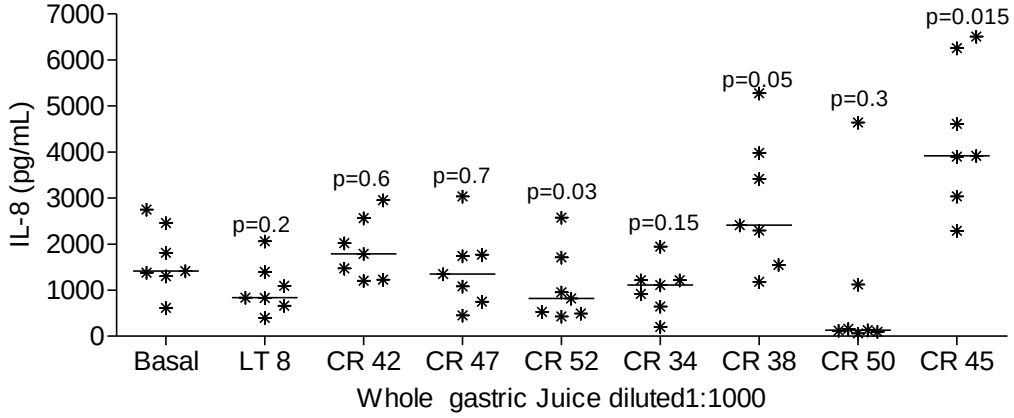

Figure 22: The effect of stimulating seven different sets of PBECs from lung transplantation recipients with whole gastric juice diluted 1:1000 and its effect on IL-8 release, following stimulation for 24 hours. Basal plotted for each gastric juice. Cytokine secretions in cell supernatants were measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=7.

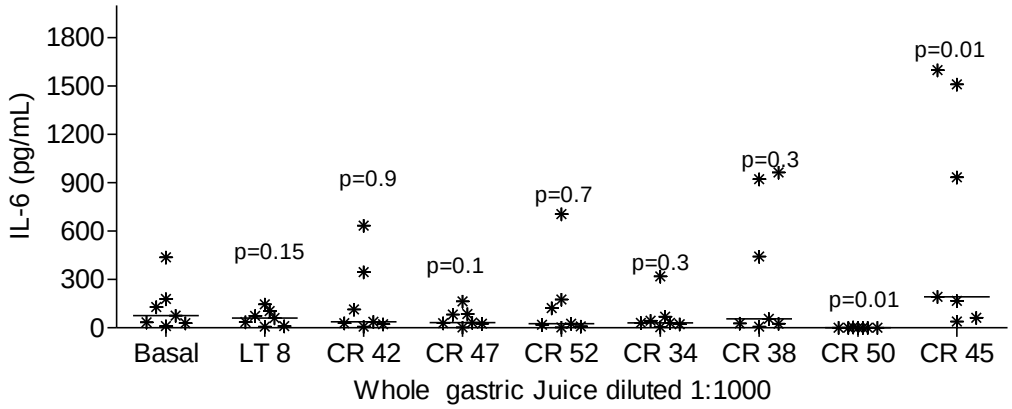

Figure 23: The effect of stimulating seven different sets of PBECs from lung transplantation recipients with whole gastric juice diluted 1:1000 and its effect on IL-6 release, following stimulation for 24 hours. Basal plotted for each gastric juice. Cytokine secretions in cell supernatants were measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=7.

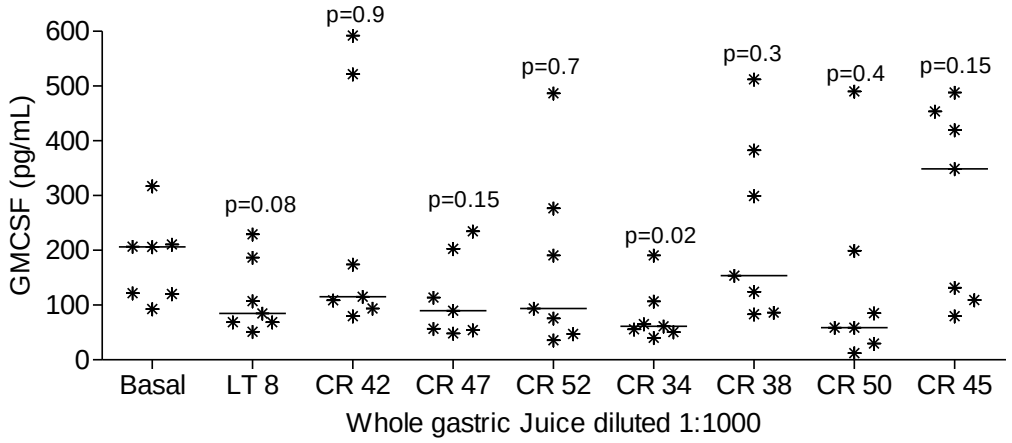

Figure 24: The effect of stimulating seven different sets of PBECs from lung transplantation recipients with whole gastric juice diluted 1:1000 and its effect on GMCSF release, following stimulation for 24 hours. Basal plotted for each gastric juice. Cytokine secretions in cell supernatants were measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=7.

Table 10: The whole diluted human gastric juice that demonstrates a significant effect of cytokine release.

	whole gastric juice patients 1:1000							
	LTx8	CR42	CR47	CR52	CR34	CR38	CR50	CR45
IL-8	____	____	____	↓*		↑*		↑*
IL-6	____	____	____	____	____	____	____	↑*
GMCSF	____	____	____	____	↓*	____	____	____

Table 12: Whole gastric juice (1:1000) challenge for PBECs from lung transplant recipient, which demonstrated significant changes to cytokine release.

↑ Increase

↓ Decrease

3.4.6 IL-8, IL-6 and GM-CSF levels, after cell stimulation with filtered and dialysed gastric juice at 37 ºC for 24 hours in 5% CO_2.

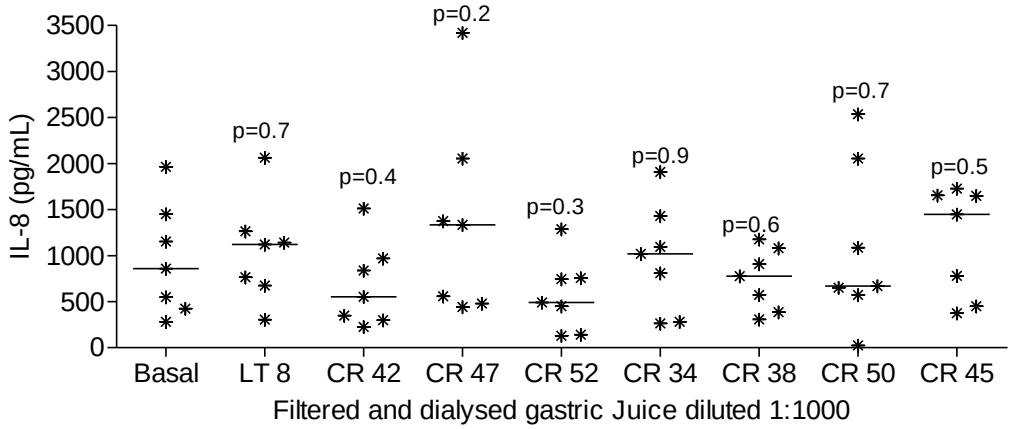

Figure 25: The effect of stimulating PBECs from lung transplantation recipients with filtrated and dialysed gastric juice at the same 1:1000 dilution and its effect on IL-8 release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=7.

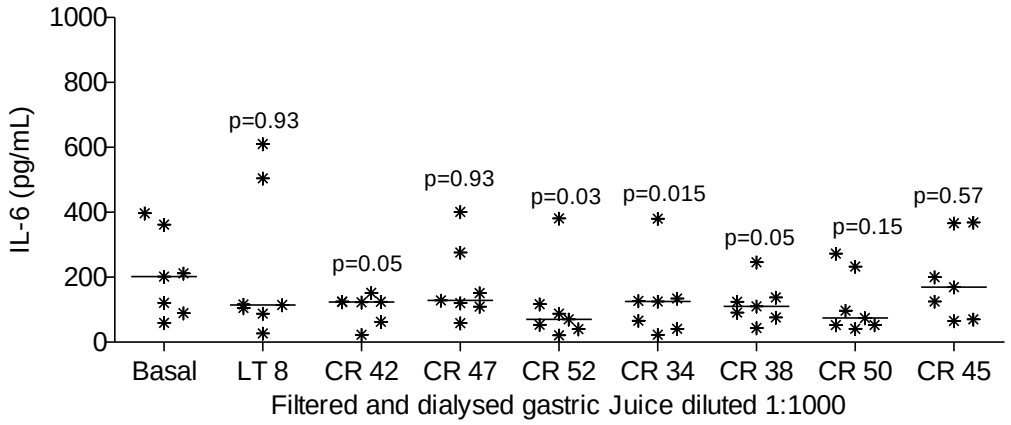

Figure 26: The effect of stimulating PBECs from lung transplantation recipients with filtrated and dialysed gastric juice at the same 1:1000 dilution and its effect on IL-6 release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=7.

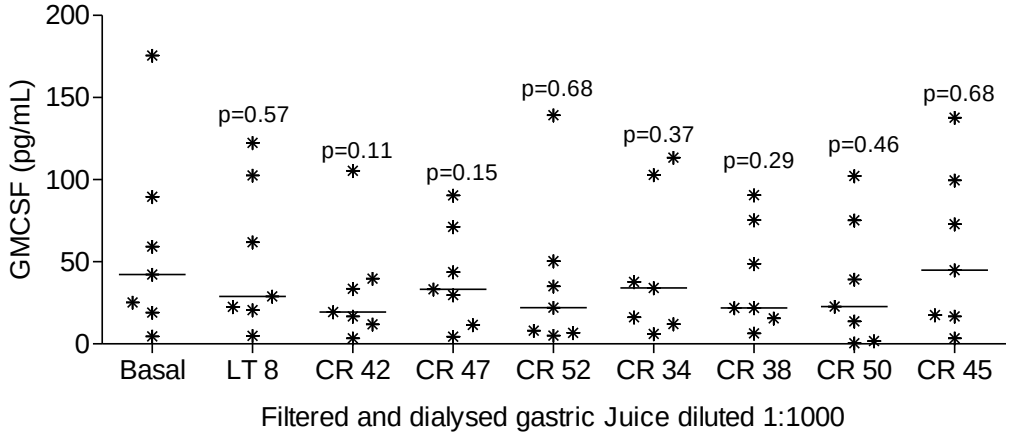

Figure 27: The effect of stimulating PBECs from lung transplantation recipients with filtrated and dialysed gastric juice at the same 1:1000 dilutions and its effect on GMCSF release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=7.

Table 11: The filtered and dialysed patient gastric juice that demonstrated a significant effect of cytokine release.

	whole gastric juice patients 1:1000							
	LTx8	CR42	CR47	CR52	CR34	CR38	CR50	CR45
IL-8	____	____	____	____	____	____	____	____
IL-6	____	↓*	____	↓*	↓*	↓*	____	____
GMCSF	____	____	____	____	____	____	____	____

Table 13: Whole gastric juice (1:1000) challenge for PBECs from lung transplant recipient, which demonstrated significant changes to cytokine release.

↑ Increase

↓ Decrease

3.4.7 Comparison IL-8, IL-6 and GM-CSF levels, after cell stimulation with whole gastric juice and dialyses gastric juice at 37 ºC for 24 hours in 5% CO_2.

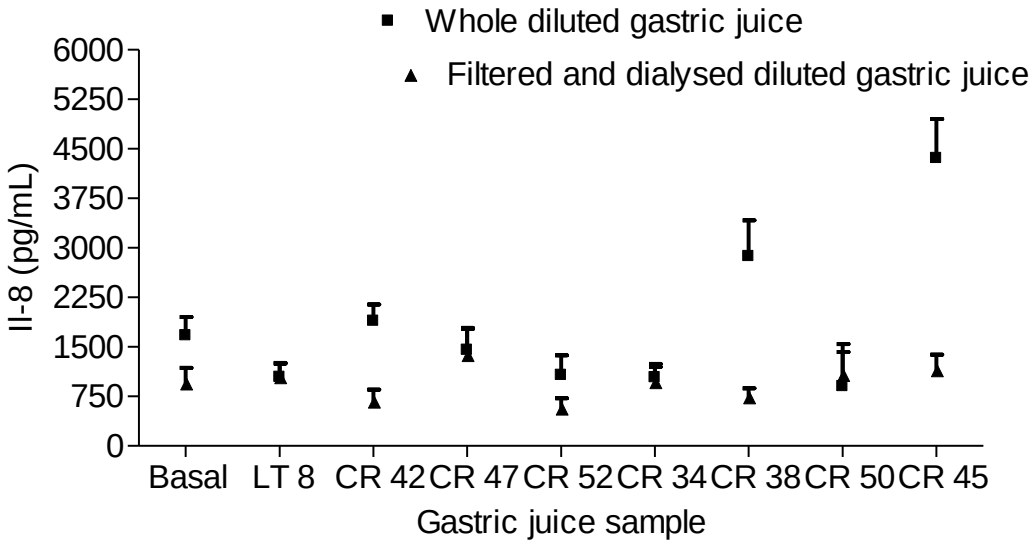

Figure 28: The effect of stimulating PBECs from lung transplantation recipients with whole gastric juice at the same 1:1000 dilution, filtered and dialysed gastric juice, its effect on IL-8 release, following stimulation for 24 hours. Cytokine secretions in cell supernatants were measured by MSD ELISA. The resulte shown are mean & SEM from experiments, n=7, for whole diluted and 7 for filtered gastric juice.

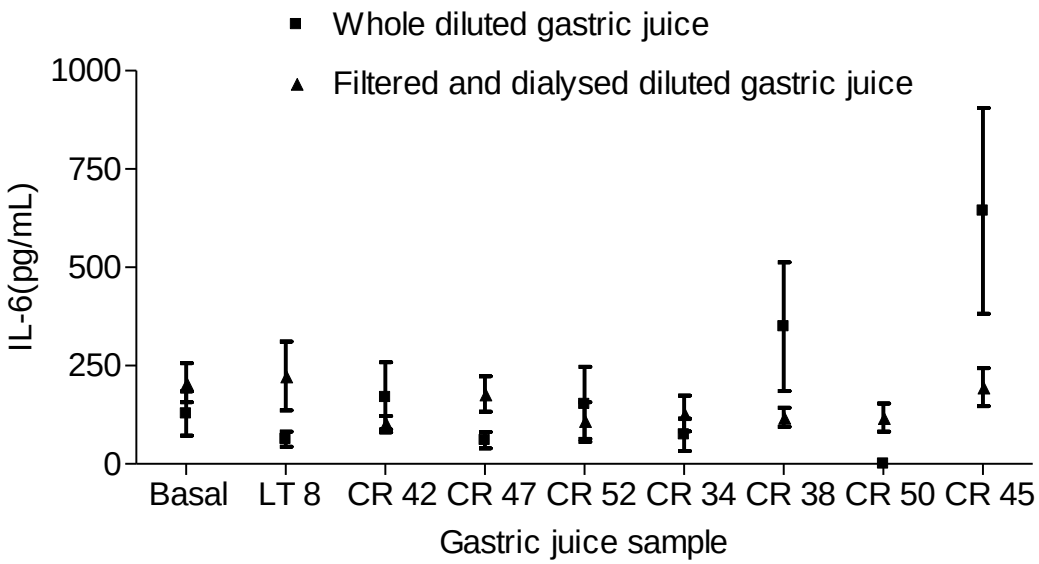

Figure 29: The effect of stimulating PBECs from lung transplantation recipients with whole gastric juice at the same 1:1000 dilution, filtered and dialysed gastric juice, its effect on IL-6 release, following stimulation for 24 hours. Cytokine secretions in cell supernatants were measured by MSD ELISA. The results shown are mean & SEM from experiments, n=7, for whole diluted and n=7 for filtered gastric juice.

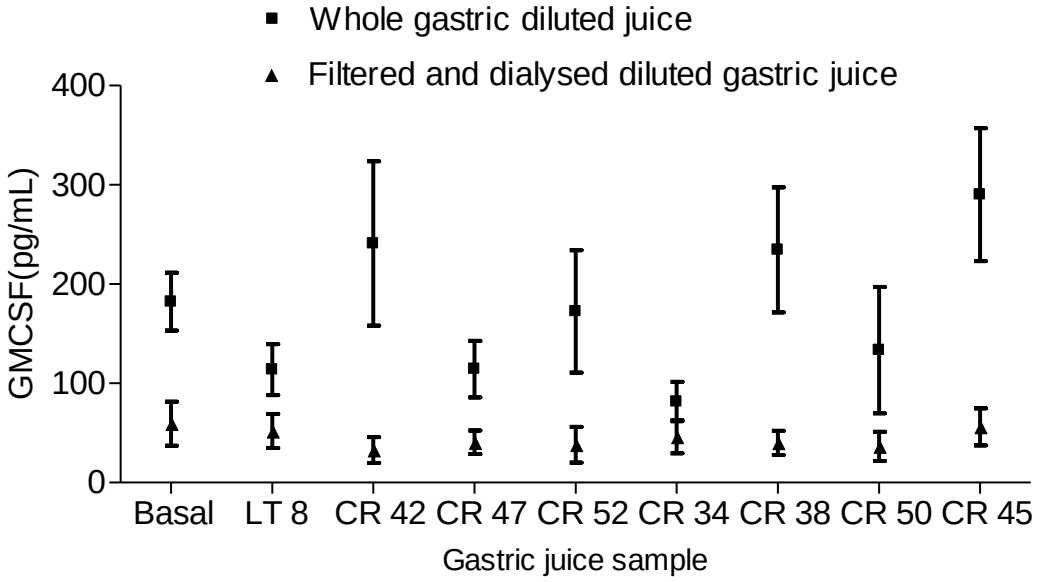

Figure 30: The effect of stimulating PBECs from lung transplantation recipients with whole gastric juice at the same 1:1000 dilution, filtered and dialysed gastric juice, its effect on GMCSF release, following stimulation for 24 hours. Cytokine secretions in cell supernatants were measured by MSD ELISA. The results shown are mean & SEM from experiments, n=7, for whole diluted and n=7 for filtered gastric juice.

Primary epithelial cells from lung transplant recipients were cultured, passaged and stored in liquid nitrogen, this method helped with the research because it allowed me to repeat experiements, and find the same sample to culture again after storage. PBECs stimulated with bile acids indicated the potential for a number of bile acids to cause inflammatory cytokine production with the hydrophobic lithocholic acid being particularly potent on a molar basis as can be seen with the cell viability data.

However, there was no significant cytokine release from PBECs, after stimulation with various concentrations of porcine pepsin (25-100 ng/ml).

Human gastric juices can stimulate inflammatory cytokine production from PBECs at potentially physiologically achievable dilutions (1:1000), i.e levels of gastric juice that could potentially be found in the lung. Filtration and dialysis of gastric juices indicate that a significant portion of the gastric juices' stimulatory potential is of small molecular weight origin such as bile acids.

The potential for microbiological content of gastric juice to be a stimulating factor was implied by the fact that gastric juice samples grew a range of bacteria and that the stimulatory potential of gastric juice was modifiable by filtration.

There are some study that suggests a relationship between bile acid structure and effect on PBECs *in vitro* (Resarcher, 2008). It may be that hydroxyl groups play a role in stimulation, as chenodeoxycholic acid and deoxycholic acid have two hydroxyl groups and were shown to be less toxic and also did not make PBECs produce significant levels of cytokines. Whereas lithocholic acid has one OH group and is more toxic to PBECs. Lithocholic acid caused significant stimulation of IL-8 and GMCSF with concentration of 1µmol/l and above.

Pepsin at pH 7.4 was found to have no effect on PBECs *in vitro* and did not cause significant stimulation of IL-8 IL-6 and GMCSF. At pH 7.4, pepsin is not active and cannot cause damage to the extracellular matrix of the lung tissues *in vitro*. Mertens et al,

suggest no relationship between concentration of pepsin and IL-8 released from PBECs *in vitro* (Mertens et al., 2010)

A hypothesis for the down regulation of inflammatory response would be the presence of *Helicobacter pylori* in gastric juice which has been shown to increase the production of IL-10 which can dampen the immune response (Bodger and Crabtree, 1998). Although the bacteria would be removed by filtration its endotoxins, vacuolating cytotoxin A (VacA), would not be. VacA is secreted and processed as a 33k and a 55k protein. These can induce apoptosis and down regulation of cellular activity (Isomoto et al., 2010). There was no effect on cell viability after stimulation with porcine pepsin, whole diluted human gastric juice, or filtered and dialysed gastric juice samples.

Chapter 4

Effect of refluxate components on primary cells cultured from the cystic fibrosis lung

4.1 Introduction

Defects in the cystic fibrosis transmembrane conductance regulator (CFTR) gene have been identified as the causal mechanism for cystic fibrosis (CF). Chronic CF leads to life-long morbidity and premature mortality (Ratjen, 2009). Lung disease accounts for more than 95% of morbidity and mortality in CF patients (Doring et al., 2007, Ratjen, 2009). During recent decades, promising improvements in the clinical care practices have led to an improved rate of survival for people suffering from CF, however knowledge about the exact pathogenesis of CF lung disease continues to be limited (Dodge et al., 2007).

Intense neutrophilic inflammation has been found to be a key histological characteristic of the CF airway, it is also well known that interleukin (IL-8) is a dominant cytokine in CF lung disease (Downey et al., 2009). Children and adults with CF have been reported to suffer from gastro-oesophageal reflux, however, the exact contribution of reflux to lung injury has not been evaluated extensively. The prevalence of reflux has been estimated between 55 to 90% (Blondeau et al., 2008a, Brodzicki et al., 2002, Button et al., 2005, Gustafsson et al., 1991, Heine et al., 1998, Ledson et al., 1998, Malfroot and Dab, 1991, Scott et al., 1985, Vic et al., 1995). It has been emphasized that certain primary and secondary mechanisms make CF patients prone to reflux. These mechanisms include reduced lower oesophageal sphincter pressure, increased transient lower oesophageal sphincter relaxations, delayed gastric emptying and altered abdominal-thoracic pressure dynamics during coughing and chest physiotherapy (Ledson et al., 1998, Cucchiara et al., 1991, Blondeau et al., 2008a). Reduced lung function in people with CF has been associated with the presence of reflux-induced cough (Blondeau et al., 2008a) and gastro-oesophageal reflux (Navarro et al., 2001, Blondeau et al., 2008a).

Another common finding in people with CF is abnormal gastrointestinal motility leading to reflux of duodenal contents back into the stomach (Hallberg et al., 2004). In line with

this observation, sputum of both adults and children with CF has been analyzed and found to contain high concentrations of bile acids (Blondeau et al., 2008a, Blondeau et al., 2010). Lung transplant recipients transplanted for CF have also been found to have higher concentration of bile acids in bronchoalveolar lavage (BAL) fluid, compared to non-CF lung transplant recipients and non-transplant controls (Blondeau et al., 2008a). It must be noted however, that non- or weakly-acidic refluxate, is more likely to be clinically silent, and therefore, under recognised. In order to detect it, oesophageal impedance is required in addition to pH monitoring alone (Sweet et al., 2009, Blondeau et al., 2008a). The present state of understanding about non-acidic reflux and aspiration in lung injury is limited and appropriate studies are warranted. The underlying hypothesis was that people with CF would have bile acids present in the lower airways and that bile acid challenge would cause release of inflammatory mediators and damage to primary bronchial epithelial cells (PBECs) cultured from CF lungs.

4.1.1 Method and Statistical analysis

CF lung derived PBEC cultures were used to perform proof of concept experiments. In these experiments the affect of individual primary and secondary bile acids, porcine pepsin, different patient derived gastric juice (whole or filtered and dialysed) samples and an artificial bile acid mixture (similar to that found in human bile) were evaluated. All concentrations are shown in section (2.2.5).

Cell viability was assessed by the Titre-blue Assay (2.6). An MSD 96-well multiplex cytokine kit was used to measure IL-8, IL-6 and GMCSF (2.7). GraphPad Prism version 4 was used for analysis of data as outlined in (2.8).

4.2 Results and Discussion

One of the project aims was to process and culture primary epithelial cells PBECs from the explanted lung of CF patients. Initial cultures were produced from CF lungs by Dr Malcolm Brodlie. Subsequently, an approximately 70% success rate was achieved for ongoing culture due to the methods developed (Brodlie et al., 2010). Another aim of the project was to establish whether primary epithelial cells PBECs from CF patients (CF PBECs) are able to produce significant increases in IL-8, IL-6 and GMCSF after stimulation with bile acids, porcine pepsin different human gastric juice (whole, filtered and dialysed) samples (Table 9) dilutions 1:1000 and a bile acid mixture (2.2.5)

The levels for inflammatory cytokine production IL-8, IL-6 and GMCSF from primary epithelial cells from explanted CF patients after stimulated by chenodeoxycholic acid for 48 hours are shown in Figures 31 to 33. Table 12 shows that chenodeoxycholic acid caused a significant increase in the release of IL-8 at 0.25, 0.5, 0.75, 1, 5 and 10µmol/l compared to basal levels. The IL-6 response threshold was higher than the IL-8 response becoming significant at 1µmol/l and 5µmol/l but not at 10µmol/l. The threshold for the GMCSF response was similar to IL-6 where significantly higher levels did not occur until the cells were exposed to concentration of 1µmol/l and above of chenodoxycholic acid. Figures 34 to 36 show the different levels for IL-8, IL-6 and GMCSF release from CF PBECs after being challenged with deoxycholic acid for 48 hours. Table 13 shows a significant increase in IL-8 levels at 0.25, 0.5, 0.75, 1, 5 and 10µmol/l deoxycholic acid. Concentration 1, 5 and 10µmol/l made CF PBECs release significantly higher levels of IL-6. Table 13 and figure 36 show that GMCSF release was significantly up regulated by levels of 1µmol/l and above of deoxycholic acid. Figures 37 to 39 show the levels for IL-8, IL-6 and GMCSF released from CF PBECs after stimulation with lithocholic acid for

48 hours. Table 14 shows that IL-8 levels at 0.25, 0.5, 0.75, 1, 5 and 10µmol/l are significantly different from basal. IL-6 and GMCSF levels are significantly elevated with lithocholic acid at concentrations of 1, 5 and 10µmol/l. These results demonstrate that the IL-8 cytokine response is invoked at much lower bile acid concentration than IL-6 and GMCSF. Cell viability for CF PBECs after stimulation with individual bile acids are shown in figures 40 to 42. Concentrations above 25µmol/l of chenodeoxycholic acid and deoxycholic acid cause 17 and 20 % cell death respectively, but with lithocholic acid the effects on cell viability at concentrations above 25µmol/l increased cell death to 55 %.

Figures 43 to 45 show the levels for IL-8, IL-6 and GMCSF after the cells were stimulated with different concentrations of porcine pepsin at pH 7.4. There was non-significant increase in IL-8 cytokine production from CF PBECs with no effect on IL-6 or GMCSF at concentrations of 25, 50 and 100 ng/ml of porcine pepsin. Figures 46 to 48 have shown the different levels of IL-8, IL-6 and GMCSF production after challenges with 8 different whole diluted human gastric juice samples. The cytokine production by the cells depends on which gastric juices used (Table 9), indicating marked variability between different gastric juices, and the variations in the whole human gastric juice components. IL-8 levels were significantly increased on exposure to four 1:1000 diluted gastric juices CR42, CR47, CR34 and CR38. Three out of four of these gastric juices also increased the levels of IL-6 but none of the gastric juices significantly altered GMCSF levels as seen in table 15. For example, the gastric juice sample CR47 which contained moderate levels of pepsin (460µg/ml) but no bile acids caused a significant increase in IL-8 production at 1:1000 dilution. This is potentially due to its content of pathogens, with *P. Mirabilis*, *K. ozaenae* demonstrated on culture (Table 9), though other organisms may be present in the gastric juice. The other three juices (CR 34, 38 and 42) that significantly increase IL-8 levels all contain bile acids ranging from 240 - 480µmol/l. CR34 and CR42 also contain pepsin activity, 1247µg/ml and 3153µg/ml respectively.

Pathogens were identified in CR38 and CR42 but not in CR34. This suggests that one of the above factors or a combination of them stimulates IL-8 release / production. Interestingly CR34 does not significantly stimulate IL-6 release and it does not contain any pathogens. This suggests that the IL-6, unlike the IL-8 response requires pathogens to be present in the gastric juice.

CF PBECs produced no significant increase of IL-8, IL-6 and GMCSF after stimulation with filtered and dialysed diluted human gastric juice samples.

There appears to be significant changes in IL-6 levels with the dialysed filtered gastric juices, with several showing a reduction in IL-6 levels. These were not considered further because the differences were close to the limit of quantisation of the ELISA for IL-6 i.e. 0.6pg/ml. A comparison of whole, filtered and dialysed gastric juices are provided in figure 52, 53 and 54 for IL-8, IL6 and GMCSF changes. Dialysis and filtration has removed the factors responsible for the induced changes to cytokine levels.

Figures 55, 56 and 57 show there is no significant changes in the levels of IL-8, IL-6 and GMCSF from CF PBECs after stimulation with an artificial bile acid mixture. IL-8 shows an upward trend and IL-6 and GMCSF a downward trend. It is surprising considering that the bile acid mixture had no effect on cytokine production considering that individual bile acids produce increases in cytokine output at levels below 10µmol/l (the concentration of the bile acid mixture). Porcine pepsin, different whole / filtered and dialysed diluted human gastric juice samples and bile acid mixture showed no significant effect on cell viability (data not shown).

4.2.1 IL-8, IL-6 and GM-CSF levels, after cell stimulation with the individual primary bile acid (chenodoxeycholic acid) at 37 ºC for 48hours in 5% CO2.

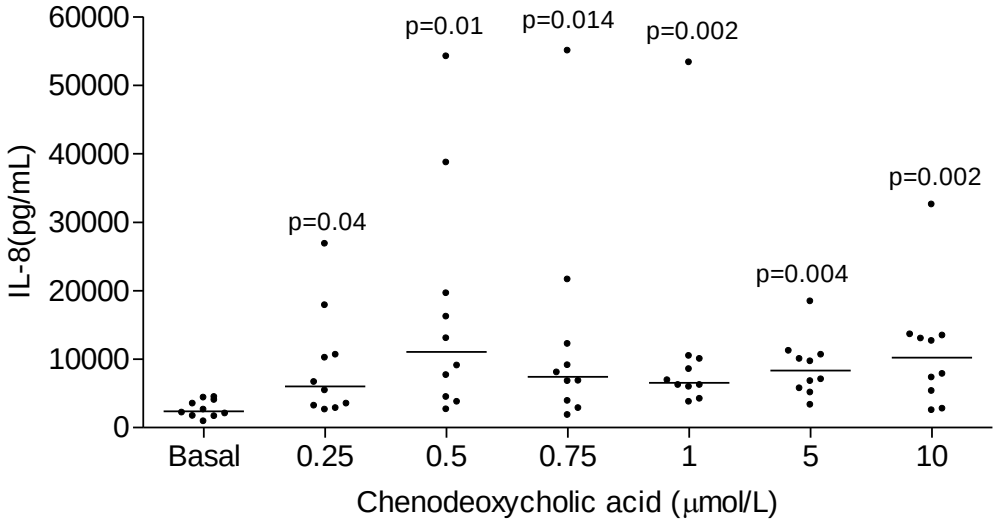

Figure 31: The effect of stimulating PBECs from explanted CF patients with chenodeoxycholic acid and its effect on IL-8 release, following stimulation for 48 hours. Basal represents the background production of cytokine without chenodeoxycholic acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=10.

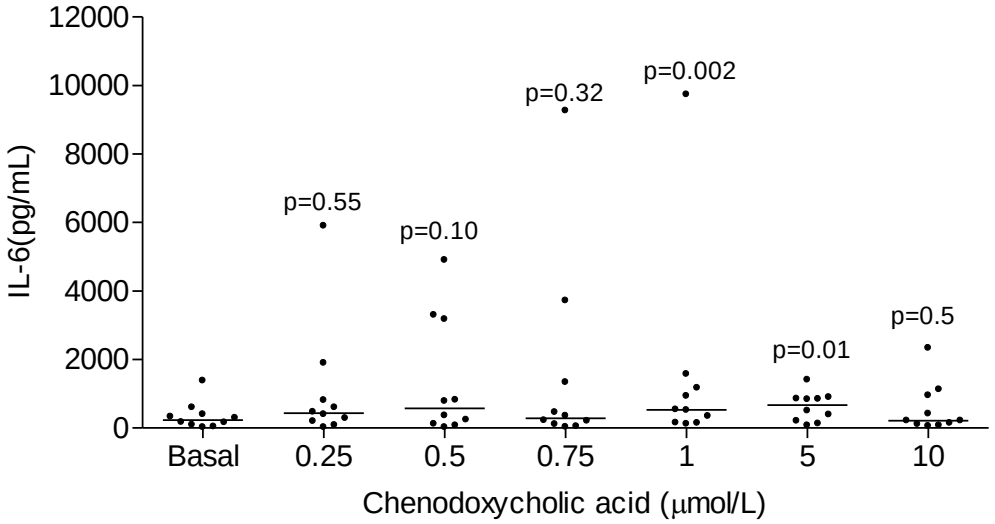

Figure 32: The effect of stimulating PBECs from explanted CF patients with chenodeoxycholic acid and its effect on IL-6 release, following stimulation for 48 hours. Basal represents the background production of cytokine without chenodeoxycholic acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=10.

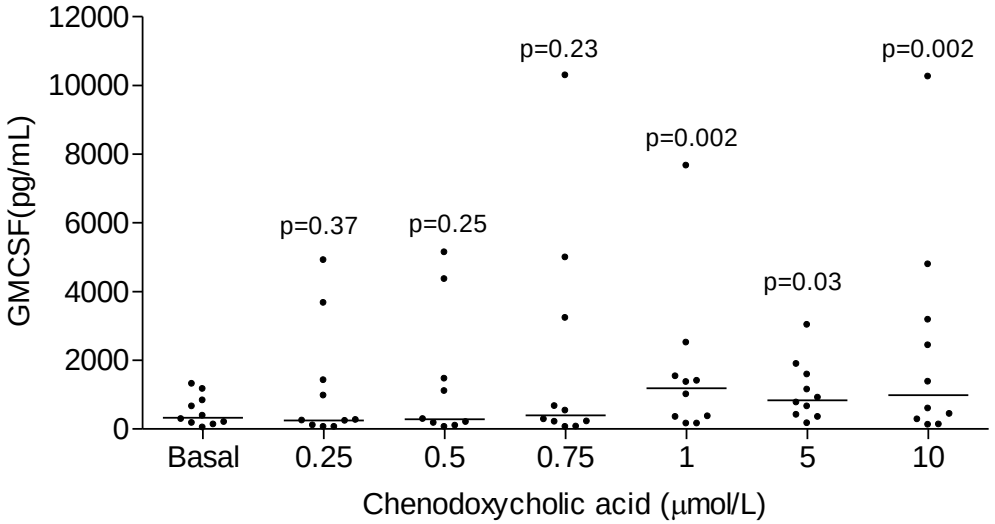

Figure 33: The effect of stimulating PBECs from explanted CF patients with chenodeoxycholic acid, and its effect on GMCSF release, following stimulation for 48 hours. Basal represents the background production of cytokine without chenodeoxycholic acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=10

Table 12: Levels of chenodeoxycholic acid that demonstrated significant stimulation of cytokine release.

	Chenodeoxycholic acid µmol/l					
	0.25	0.5	0.75	1	5	10
IL-8	↑*	↑*	↑*	↑*	↑*	↑*
IL-6	____	____	____	↑*	↑*	____
GMCSF	____	____	____	↑*	↑*	↑*

Table 14: The levels of chenodeoxycholic acid challenge for PBECs from explanted CF patients for 48 hours, which demonstrated significant stimulation of cytokine release.

↑ Increase

↓ Decrease

4.2.2 IL-8, IL-6 and GM-CSF levels, after cell stimulation with the individual secondary bile acid (doxeycholic acid) at 37 ºC for 48hours in 5% CO2.

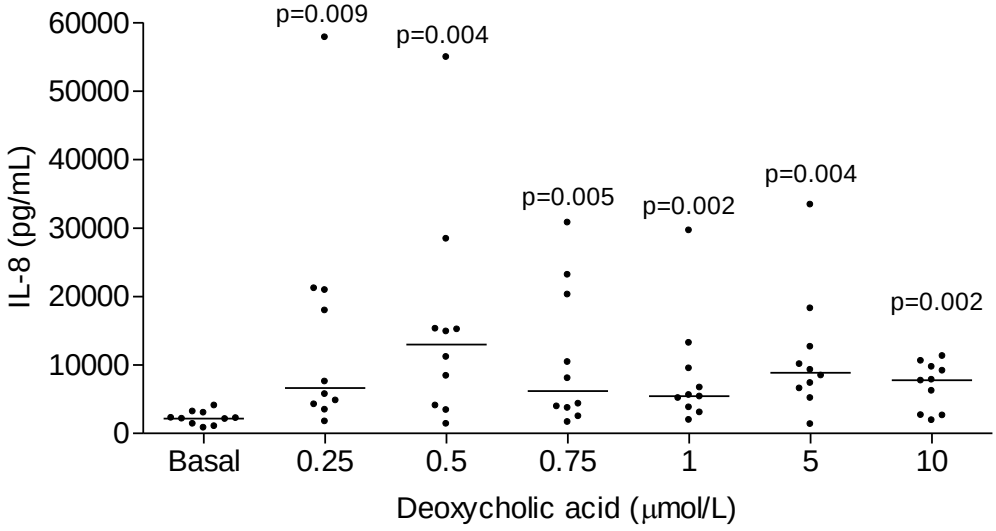

Figure 34: The effect of stimulating PBECs from explanted CF patients with deoxycholic acid and its effect on IL-8 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=10.

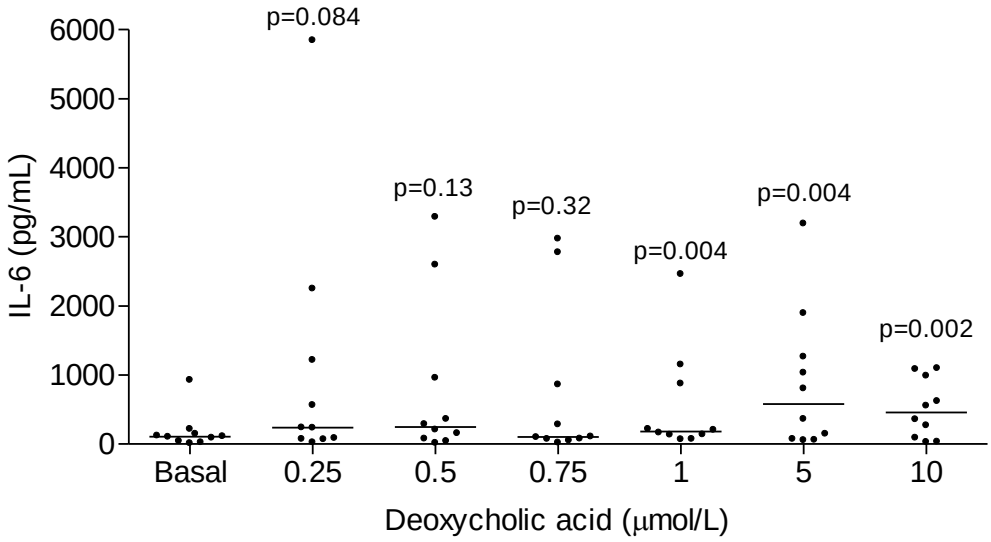

Figure 35: The effect of stimulating PBECs from explanted CF patients with deoxycholic acid and its effect on IL-6 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=10

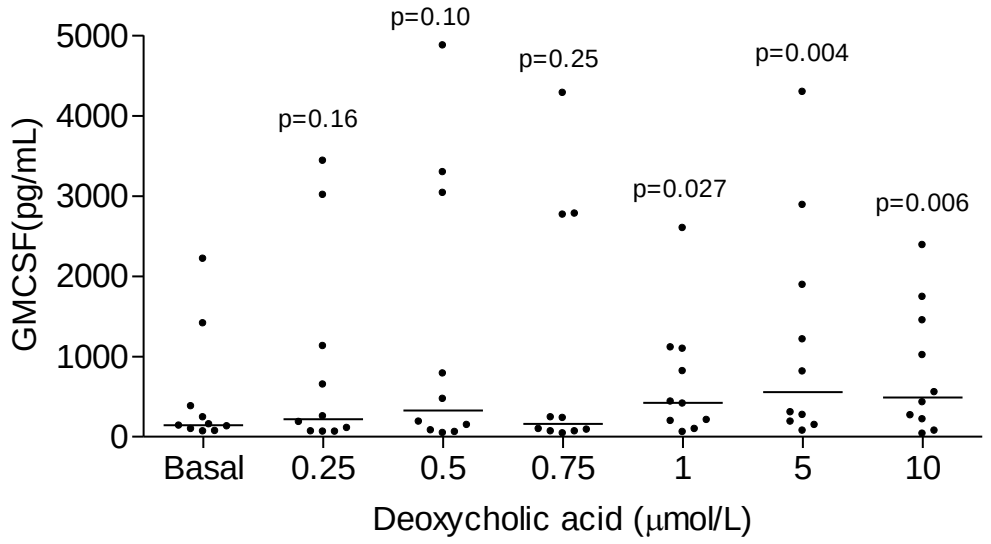

Figure 36: The effect of stimulating PBECs from explanted CF patients with deoxycholic acid and its effect on GMCSF release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=10.

Table 13: Levels of deoxycholic acid that demonstrated significant stimulation of cytokine release.

	deoxycholic acid µmol/l					
	0.25	0.5	0.75	1	5	10
IL-8	↑*	↑*	↑*	↑*	↑*	↑*
IL-6	____	____	____	↑*	↑*	↑*
GMCSF	____	____	____	↑*	↑*	↑*

Table 15: The levels of deoxycholic acid challenge for PBECs from explanted CF patients for 48 hour, which demonstrated significant stimulation of cytokine release.

↑ Increase
↓ Decrease

4.2.3 IL-8, IL-6 and GM-CSF levels, after cell stimulation with the individual secondary bile acid (Lithocholic acid) at 37 ºC for 48hours in 5% CO2.

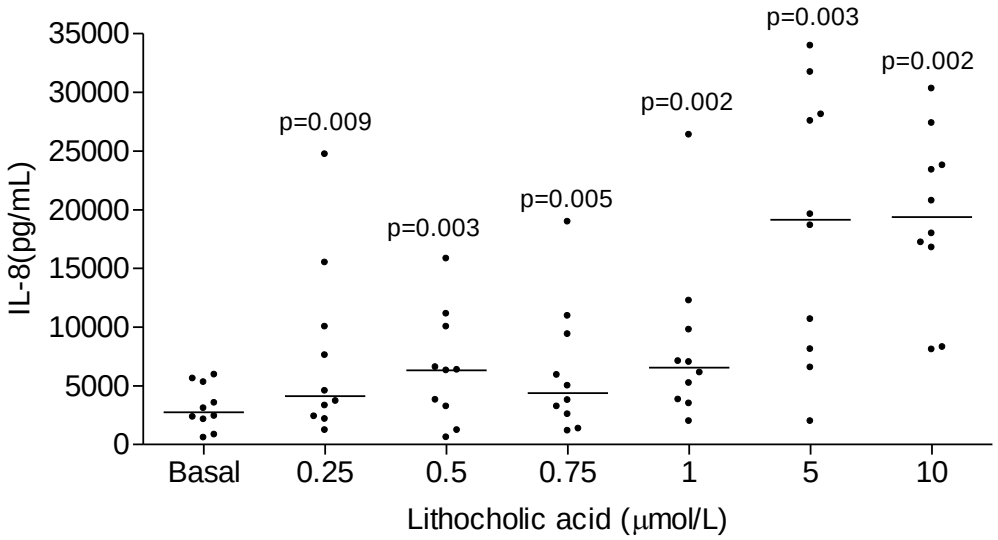

Figure 37: The effect of stimulating PBECs from explanted CF patients with lithocholic acid and its effect on IL-8 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=10

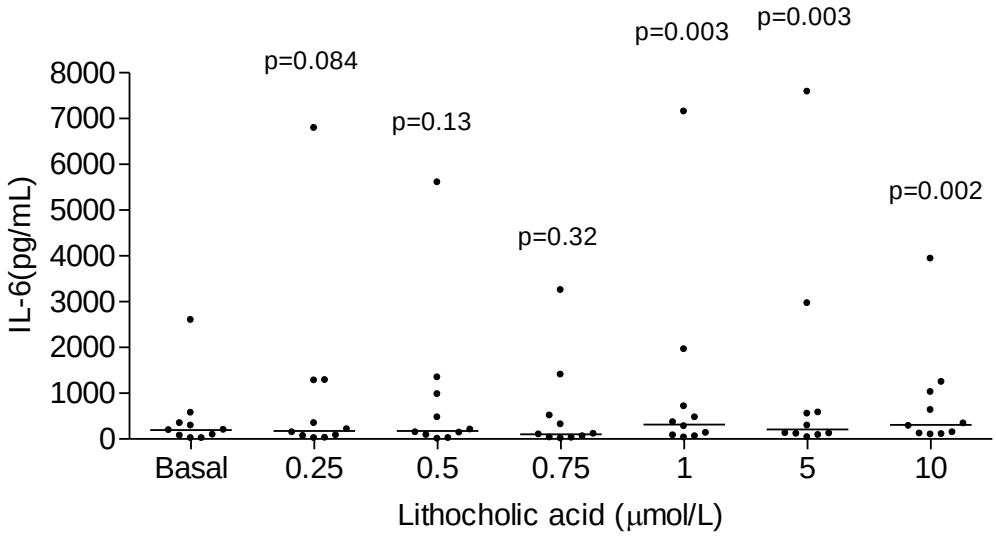

Figure 38: The effect of stimulating PBECs from explanted CF patients with lithocholic acid and its effect on IL-6 release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=10.

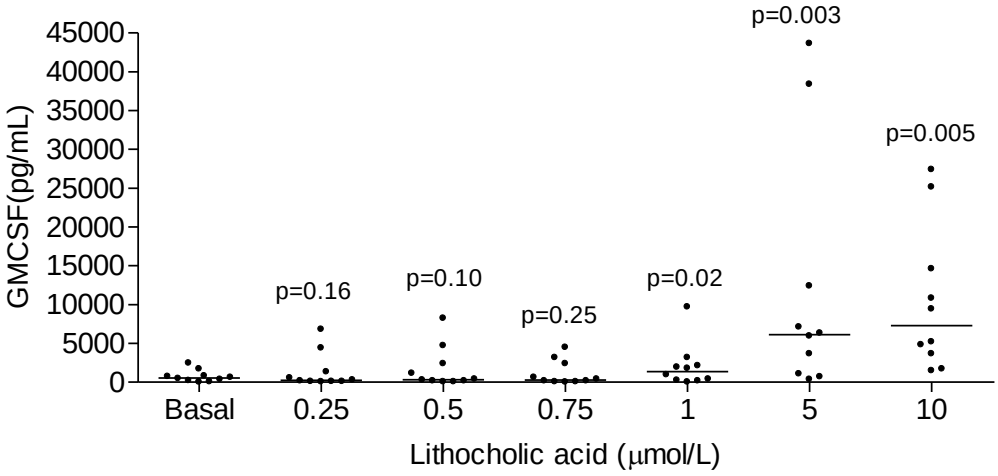

Figure 39: The effect of stimulating PBECs from explanted CF patients with lithocholic acid and its effect on GMCSF release, following stimulation for 48 hours. Basal represents the background production of cytokine without bile acid stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The horizontal bars represent the median values from experiments, n=10.

Table 14: Levels of lithocholic acid that demonstrated significant stimulation of cytokine release.

	lithocholic acid µmol/l					
	0.25	0.5	0.75	1	5	10
IL-8	↑*	↑*	↑*	↑*	↑*	↑*
IL-6	____	____	____	↑*	↑*	↑*
GMCSF	____	____	____	↑*	↑*	↑*

Table 16: The levels of lithocholic acid challenge for PBECs from explanted CF patients 48 hours, which demonstrated significant stimulation of cytokine release.

↑ Increase

↓ Decrease

4.2.4 Cell viability for primary bronchial epithelial cells from explanted CF patients after stimulation by individual bile acid.

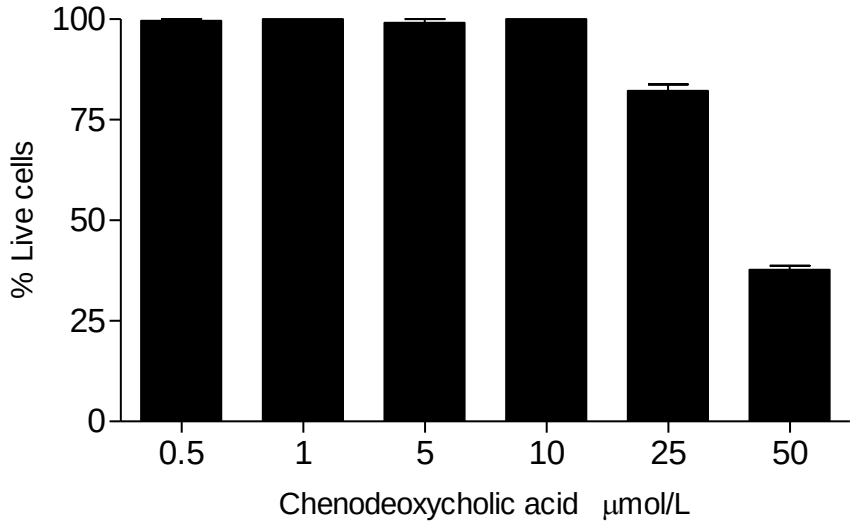

Figure 40: The viability of primary bronchial epithelial cells from explanted CF patients after challenge with different concentrations of chenodoxycholic acid for 48 hours. Cell viability was assessed by CellTiter- Blue cell viability assay (Promega. Madison), n=5.

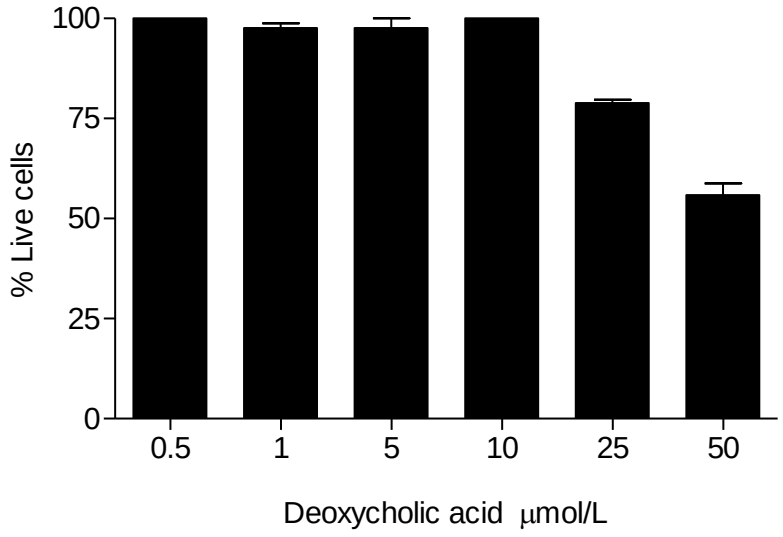

Figure 41: The viability of primary bronchial epithelial cells from explanted CF patients after challenge with different concentrations of deoxycholic acid for 48 hours. Cell viability was assessed by CellTiter- Blue cell viability assay (Promega. Madison), n=5.

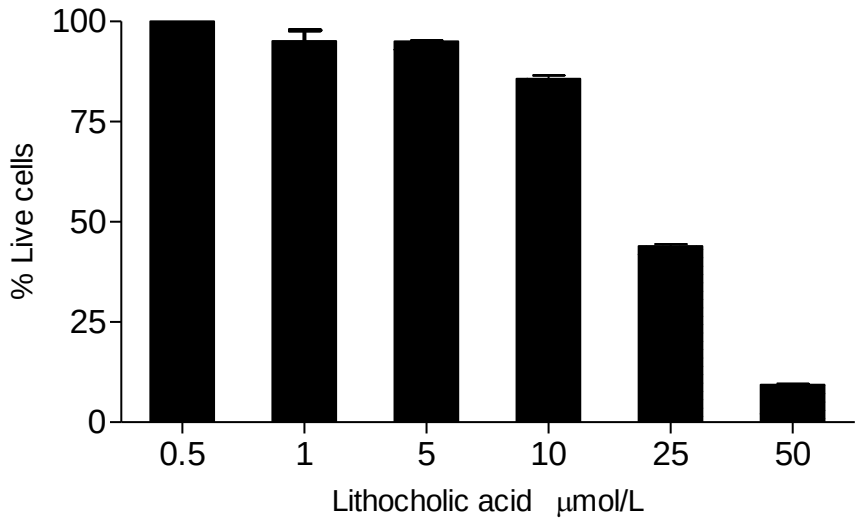

Figure 42: The viability of primary bronchial epithelial cells from explanted CF patients after challenge with different concentrations of lithocholic acid for 48 hours. Cell viability was assessed by CellTiter- Blue cell viability assay (Promega. Madison), n=5.

4.2.5 IL-8, IL-6 and GM-CSF levels, after cell stimulation with pepsin at 37 ºC (pH 7.4) for 48hours in 5% CO2.

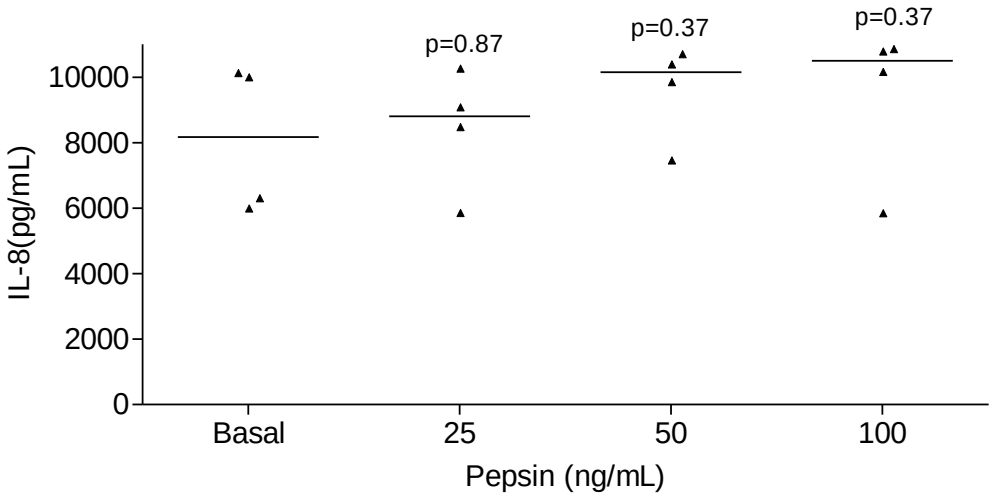

Figure 43: The effect of stimulating PBECs from explanted CF patients with porcine pepsin and its effect on IL-8 release as a log plot (log 10), following stimulation for 48 hours. Basal represents the background production of cytokine without pepsin stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=4.

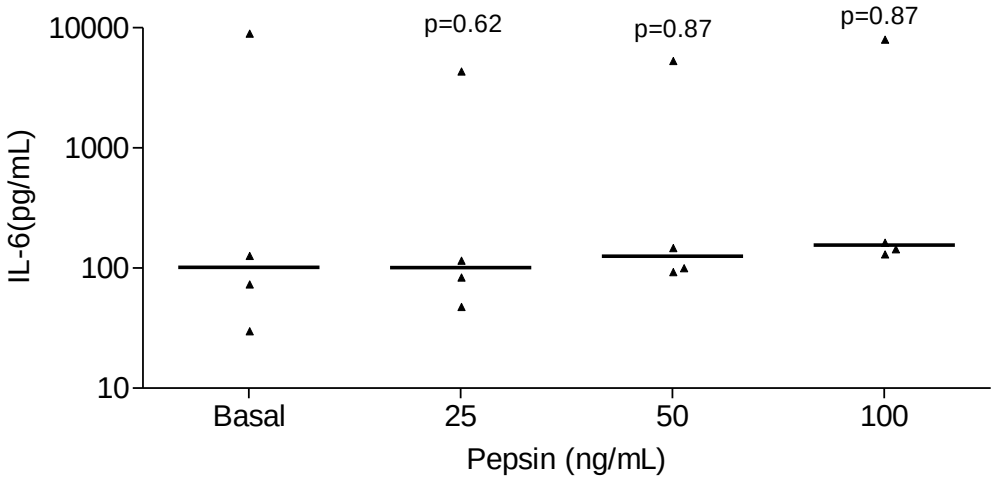

Figure 44: The effect of stimulating PBECs from explanted CF patients with porcine pepsin and its effect on IL-6 release as a log plot (log 10), following stimulation for 48 hours. Basal represents the background production of cytokine without pepsin stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=4.

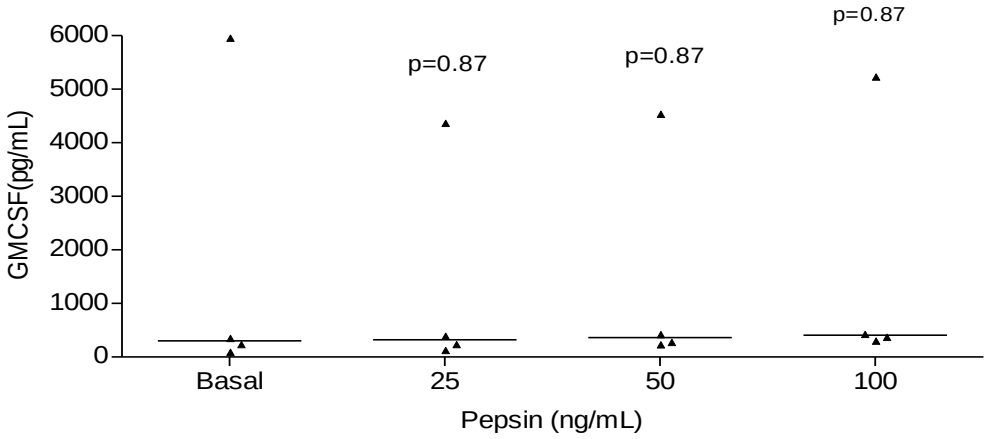

Figure 45: The effect of stimulating PBECs from explanted CF patients with porcine pepsin and its effect on GMCSF release, following stimulation for 48 hours. Basal represents the background production of cytokine without pepsin stimulation. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The resulte shown are median values from experiments, n=10.

4.2.6 IL-8, IL6 and GMSF levels, after cell stimulation with whole gastric juice at 37 ºC for 24 hours in 5% CO2.

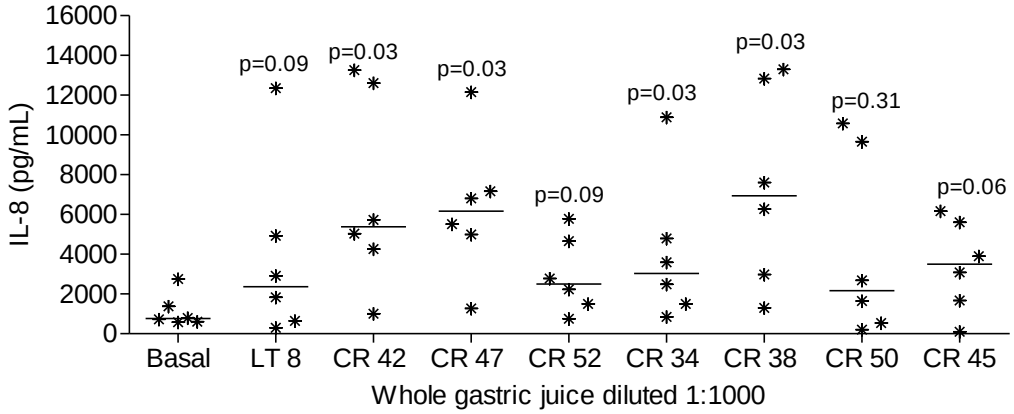

Figure 46: The effect of stimulating seven different sets of PBECs from explanted CF patients with whole gastric juice diluted 1:1000 and its effect on IL-8 release, following stimulation for 24 hours. Basal plotted for each gastric juice. Cytokine secretions in cell supernatants were measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=6.

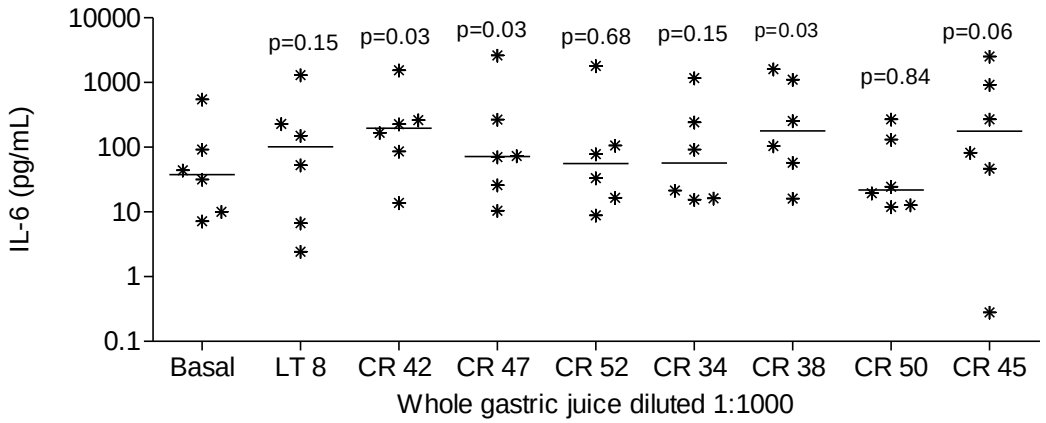

Figure 47: The effect of stimulating seven different sets of PBECs from explanted CF patients with whole gastric juice diluted 1:1000 and its effect on IL-6 release as a log plot (log 10), following stimulation for 24 hours. Basal plotted for each gastric juice. Cytokine secretions in cell supernatants were measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=6.

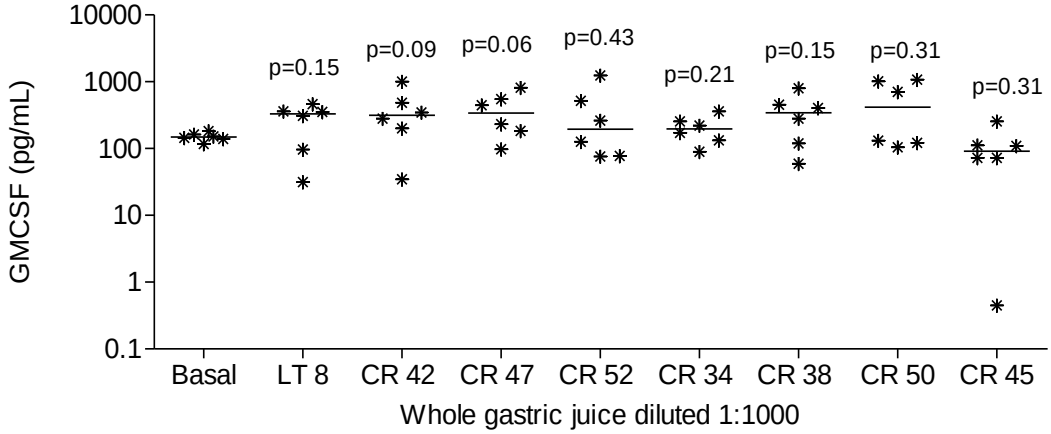

Figure 48: The effect of stimulating seven different sets of PBECs from explanted CF patients with whole gastric juice diluted 1:1000 and its effect on GMCSF release as a log plot (log 10), following stimulation for 24 hours. Basal plotted for each gastric juice. Cytokine secretions in cell supernatants were measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=6.

Table 15: Whole diluted gastric juice that demonstrated significant stimulation of cytokine release.

	whole gastric juice patients diluted 1:1000							
	LTx8	CR42	CR47	CR52	CR34	CR38	CR50	CR45
IL-8	____	↑*	↑*	____	↑*	↑*	____	____
IL-6	____	↑*	↑*	____	____	↑*	____	____
GMCSF	____	____	____	____	____	____	____	____

Table 17: The patients diluted whole gastric juice challenge for PBECs from explanted CF patients 24 hours, which demonstrated significant stimulation of cytokine release.
↑ Increase
↓ Decrease

4.2.7 IL-8, IL-6 and GM-CSF levels, after cell stimulation with filtered and dialysed human gastric juice at 37 ºC for 24 hours in 5% CO2

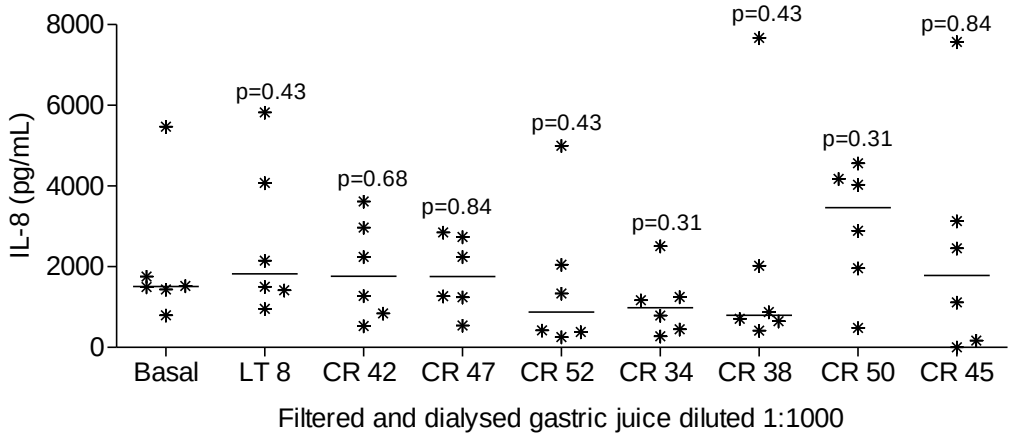

Figure 49 : The effect of stimulating PBECs from explanted CF patients with filtered and dialysed human gastric juice at the same 1:1000 dilution and its effect on IL-8 release as a log plot (log 10), following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=6.

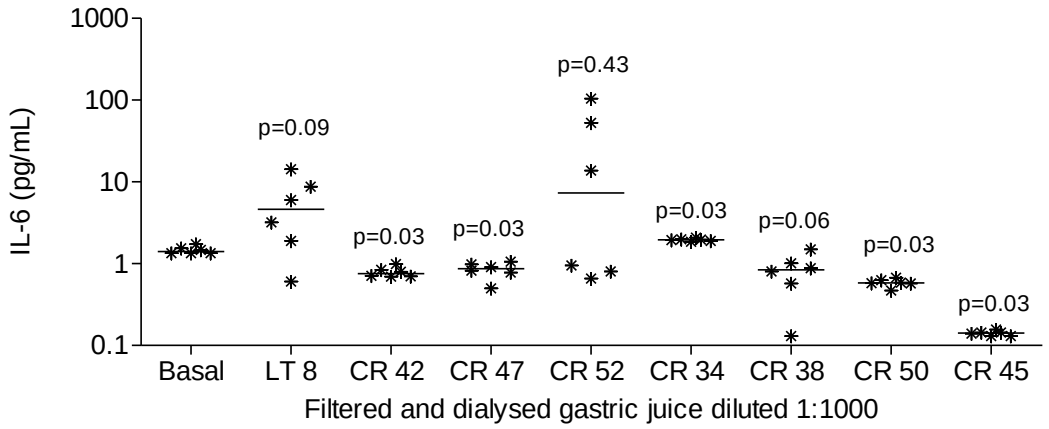

Figure 50: The effect of stimulating PBECs from explanted CF patients with filtered and dialysed human gastric juice at the same 1:1000 dilution and its effect on IL-6 release as a log plot (log 10), following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=6.

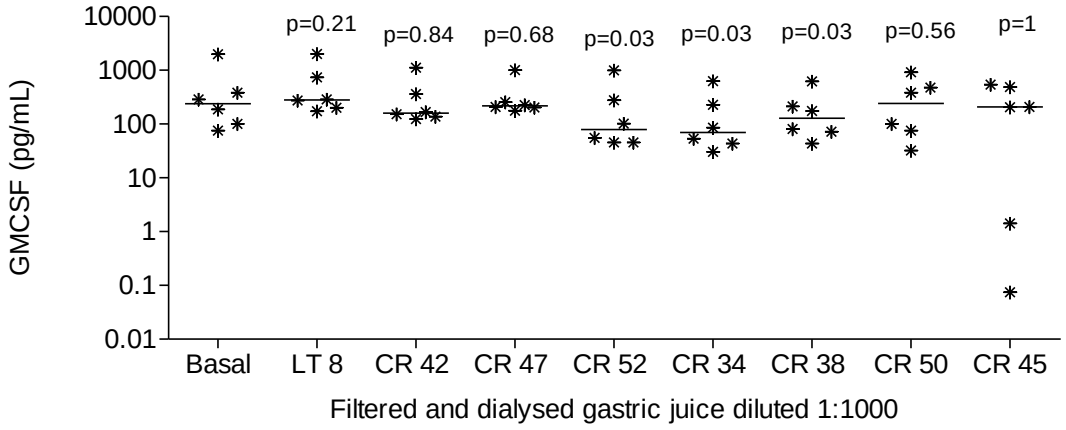

Figure 51: The effect of stimulating PBECs from explanted CF patients with filtered and dialysed human gastric juice at the same 1:1000 dilution and its effect on GMCSF release as a log plot (log 10), following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=6.

4.2.8 Comparison IL-8, IL-6 and GM-CSF levels, after cell stimulation with whole, filtered and dialyses human gastric juice at 37 ºC for 24 hours in 5% CO2.

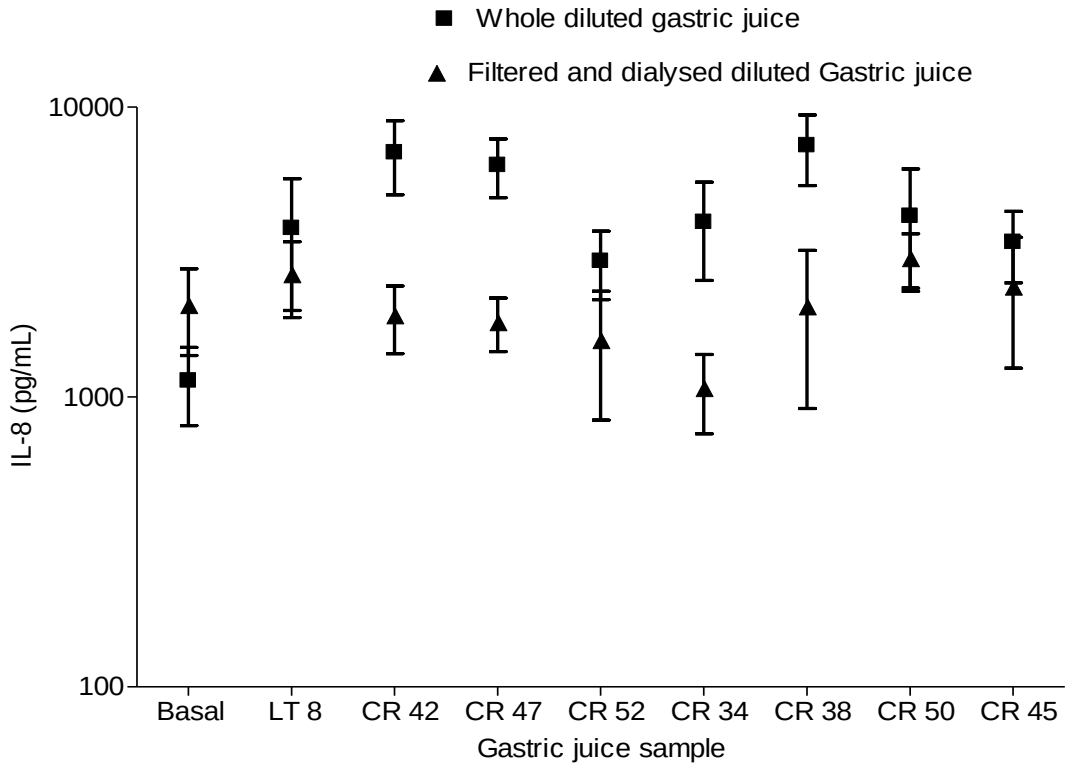

Figure 52: The effect of stimulating PBECs from explanted CF patients with whole, filtrated and dialysed gastric juice at the same 1:1000 dilution, its effect on IL-8 release as a log plot (log 10), following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. The resulte shown are mean & SEM values from experiments, n=6.

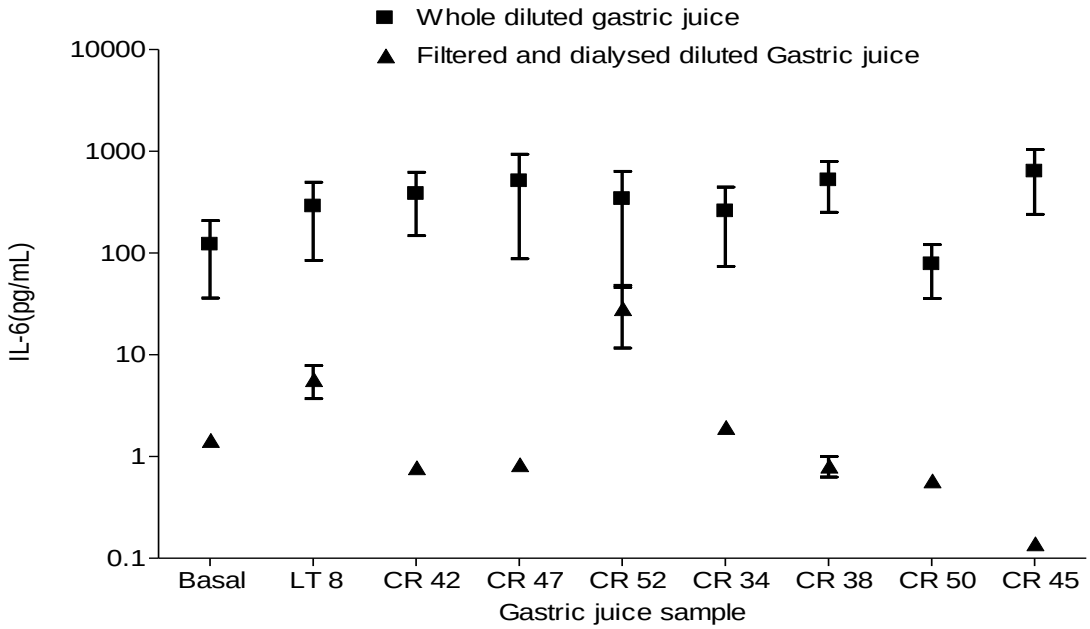

Figure 53: The effect of stimulating PBECs from explanted CF patients with whole, filtrated and dialysed gastric juice at the same 1:1000 dilution, its effect on IL-6 release as a log plot (log 10), following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. The resulte shown are mean & SEM values from experiments, n=6.

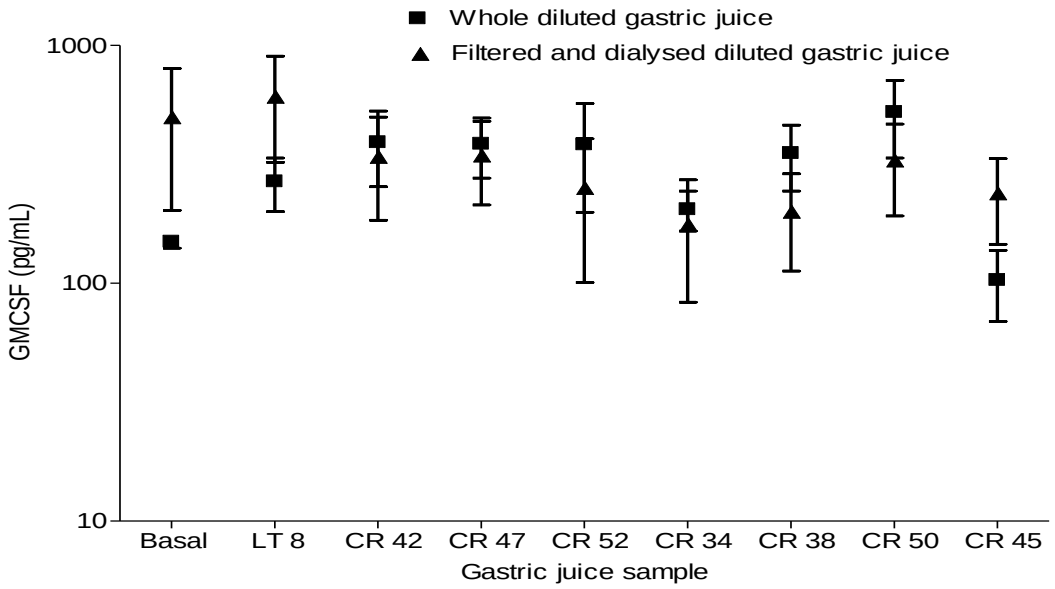

Figure 54: The effect of stimulating PBECs from explanted CF patients with whole, filtrated and dialysed gastric juice at the same 1:1000 dilution, its effect on GMCSF release as a log plot (log 10), following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. The resulte shown are mean & SEM values from experiments, n=6.

4.2.9 IL-8, IL-6 and GM-CSF levels, after cell stimulation with bile acids mixture, at 37 ºC for 48 hours in 5% CO2.

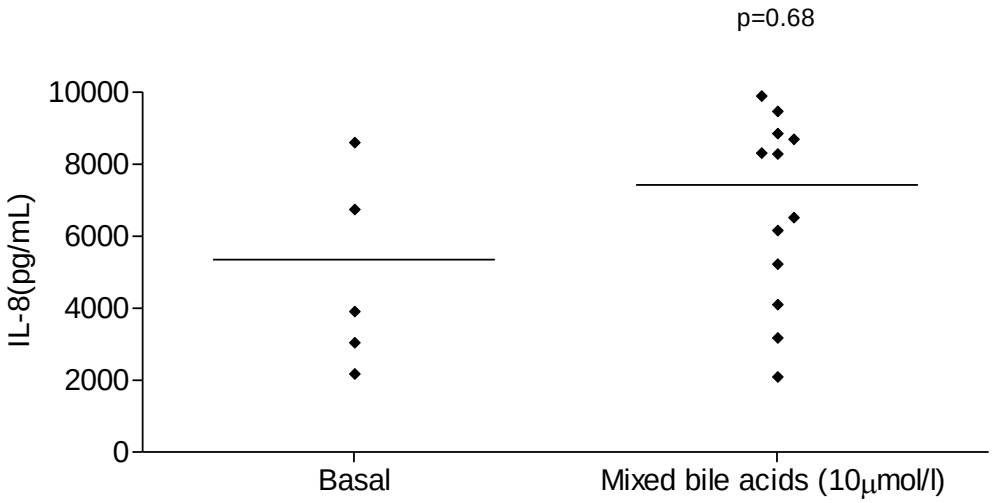

Figure 55: The effect of stimulating PBECs from explanted CF patients with bile acids mixture, its effect on IL-8 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=12.

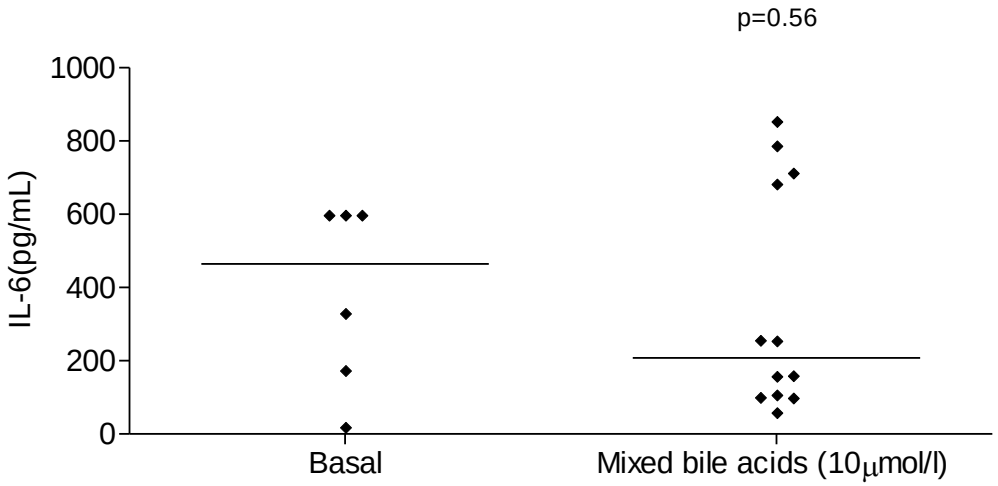

Figure 56: The effect of stimulating PBECs from explanted CF patients with bile acids mixture, its effect on IL-6release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=12.

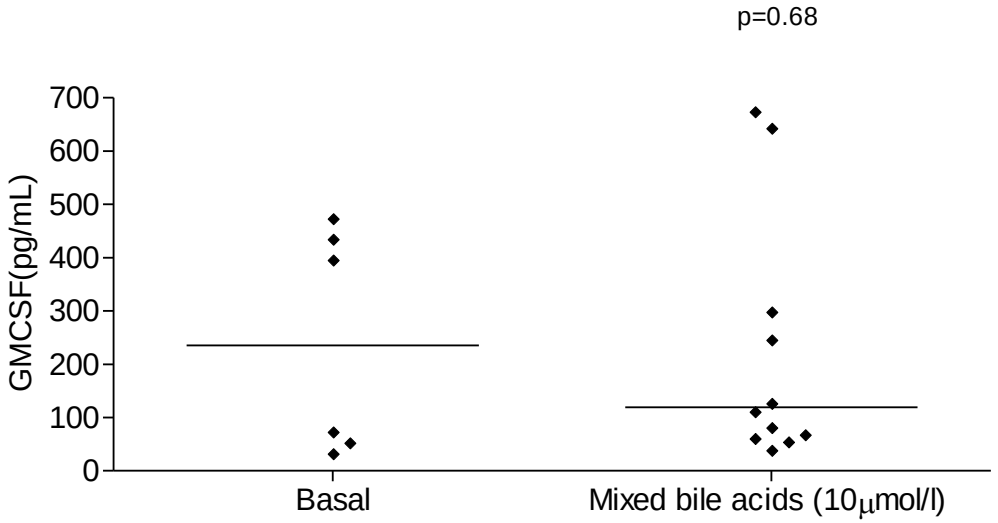

Figure 57: The effect of stimulating PBECs from explanted CF patients with bile acids mixture, its effect on GMCSF release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=12.

Experiments with primary epithelial cells from explanted lung of CF patients have demonstrated an increase in the release of the key inflammatory mediators IL-8 and IL-6 caused by physiologically achievable levels of individual primary and secondary bile acids as well as different patient derived gastric juice (whole, filtered and dialysed) diluted samples. It was of interest that porcine pepsin and an artificial bile acid mixture had no effect on cytokine production. The lack of effect by porcine pepsin may be related to the pH of the incubation (that was 7.4). Pepsin is inactive at this pH and activity may be necessary for a cytokine related effect. The lack of an effect of the bile acid mixture is more difficult to explain when considering its concentration. This mixture (10µmol/l) is made up of two conjugated bile acids, 50% taurocholic acid, 15% taurodeoxycholic acids, one primary and one secondary. In addition the mixture contains two free bile acids, one primary (30% chenodeoxycholic acid) and one secondary (5% lithocholic acid). Consequently taurocholic acid, taurodeoxycholic acid will be present at 5µmol/l, and 1.5µmol/l respectively. Chenodeoxycholic acid and lithocholic acid would be present at 3µmol/l and 0.5µmol/l.

Chenodeoxycholic acid has been show in figures 31-33 to upregulate IL-8 production at 0.25µmol/l, and also IL-6 and GMCSF at 1µmol/l, so it is present in the mixture at high enough levels to produce stimulation of all the cytokines. Lithocholic acid upregulates IL-8 production at 0.25µmol/l and also IL-6 and GMCSF at 1µmol/l (figures 37-39), so it is present at a high enough concentration to significantly increase IL-8 but not IL-6 or GMCSF levels. It is possible that the lack of stimulating effect of the mixture is because the conjugated bile acids are having an inhibitory effect. This would be something for future research to address and to determine if it is related to differences in the solubility and hydrophobicity of the bile acids (Pearson et al., 2011)

Importantly, these findings suggest that duodeno-gastro-oesophageal reflux and subsequent microaspiration may contribute to the neutrophilic inflammation that is the distinctive hallmark of CF lung disease (Hallberg et al., 2004).

IL-8 levels were significantly elevated by all the individual bile acids, and at all concentration used. Higher concentrations of bile acids were required to upregulate IL-6 and GMCSF levels suggesting that these CF airway epithelial cells have an IL-8 phenotype in terms of inflammatory cytokine production. This phenotype may well be a characteristic of most epithelial cells as human gingival epithelial cells respond to bacterial stimuli with a 7 fold greater IL-8 output composed to IL-6 (Stathopoulou et al., 2010).

Some of the whole diluted human gastric juices were able to cause an increase in IL-8 production by CF PBECs but this was dependent on the components of the juice. After dialysis the effective components were removed.

Chapter 5

The effect of refluxate components on the mucus secreting goblet cell line HT 29- MTX

5.1 Introduction

The role of the bronchial epithelium in the pathogenesis of various pulmonary disease states has been extensively researched during the last decade. As in the skin and gastrointestinal tract, epithelial cells form the lining of the entire respiratory tract and remain continually exposed to the external environment.

Traditionally the bronchial epithelium was believed to be a physical barrier to inhaled irritants and noxious substances; as such it was assumed to have a mechanical function of propelling tracheobronchial secretions toward the pharynx. However, it is well documented that the bronchial epithelium as well as acting as a physicochemical barrier may also play a crucial role in initiating and augmenting pulmonary host defence mechanisms. This defensive mechanism includes the synthesis and release of a variety of mediators that can cause inflammatory cell differentiation, chemotaxis, and activation during both health and disease. There are many types of epithelial cells within the airways, of which columnar, ciliated and goblet cells are the most prominent in the large and intermediate sized airways. Other subtypes include serous, basal, clara and neuroendocrine cells. The different characteristics of these cells contribute to the complicated functionality of the bronchial epithelium (Davies and Devalia, 1992, Mills et al., 1999). Transepithelial electrolyte transport and propelling of tracheobronchial secretions toward the pharynx is carried out by ciliated cells. Synthesis of mucin and formation of a viscoelastic blanket of mucus that covers much of the bronchial epithelium is the function of goblet and serous cells (Mills et al., 1999). The pseudostratified appearance of the epithelium is because of the presence of basal cells which are also involved in the attachment of superficial cells to the basal membrane. Basal cells have been suggested as the precursors to the other epithelial cell types within the airways.

Distal airways contain numerous clara cells which have an active role in the production of surfactant at these sites. The specific roles of the other epithelial cell types are less well understood but evidence exists that they could be involved in cell-to-cell signalling processes.

Bronchial epithelial cells have been linked with the synthesis and release of a variety of proinflammatory cytokines involved in airway inflammation and epithelial damage (Hamid et al., 1993, Mills et al., 1999). In addition, development of airflow obstruction in both asthma and chronic obstructive pulmonary disease (henceforth referred to as COPD) has been linked to endothelins, which are peptides that have been noted for causing profound vaso- and bronchoconstriction (Howarth et al., 1995). Similarly, mediators such as the cysteinyl leukotrienes, derived from metabolism of arachidonic acid, have been shown to be capable of causing smooth muscle contraction, effecting cell chemo-attraction, changes in vascular permeability, and excessive production of mucus (Howarth et al., 1995)

The relationship between the production of proinflammatory cytokines by the airway epithelium and allergic conditions such as asthma and allergic rhinitis has been widely studied and it has been established that these compounds influence the activity of inflammatory cells such as eosinophils, T lymphocytes, and mast cells, all of which are the typical infiltrating cells in these disorders (Djukanovic et al., 1990, Corrigan and Kay, 1991). More recently, interest has been steadily growing in the study of the role of airway epithelial cell-derived pro-inflammatory cytokines in the pathogenesis of nonallergic airway conditions, such as chronic bronchitis and COPD (Adachi et al., 1997, Becker et al., 1997, Khair et al., 1995, Devalia et al., 1993, Wang et al., 1996).

This chapter evaluates whether the goblet cell line MTX 29 can produce a variety of cytokines, both constitutively and after stimulation with different bile acids, porcine pepsin, different gastric juice samples and a bile acid mixture.

5.2 Method and Statistical analysis

When the HT29 MTX goblet cells were 80-90% confluent in 24 well plates, they were rested for 24 hours in serum free medium as stated in 2.2.5. The cells were then stimulated with individual primary and secondary bile acids, porcine pepsin, different human gastric juice (whole, filtered and dialysed) samples and a bile acid mixture at concentrations as shown in section 2.2.5.

The Titre-blue assay was used to assess Cell viability (2.6). IL-8, IL-6 and GMCSF were measured by using a MSD 96-well (2.7). Analysis of data was by GraphPad Prism version 4 as outlined in (2.8).

5.3 Results and Discussion

An aim of this project was to process and culture a human goblet cell line. Approximately 90% success rate for cell culture was achieved during this projecy. The cells were cultured under different conditions. Figure 58 (A) shows 24 well cell cultures of the goblet cells in complete media (DMEM) at 37°C with high CO_2 (10%).

In addition, it was possible to culture cells in a 24 well cell culture and to stimulate them with different bile acids, porcine pepsin, different gastric juice samples was well as a 'physiological' mixture of bile acids (figure 58 (B)). After cryopreservation, it was possible to re-establish these cells in culture. These cells have the typical appearance of healthy, goblet cell line HT-29 MTX in submerged culture, which is shown by the 'cobblestone' morphology. The cells are clearly adherent with minimal numbers of floating or fibroblast like cells which can be seen in figures 58(A) and 58(B).

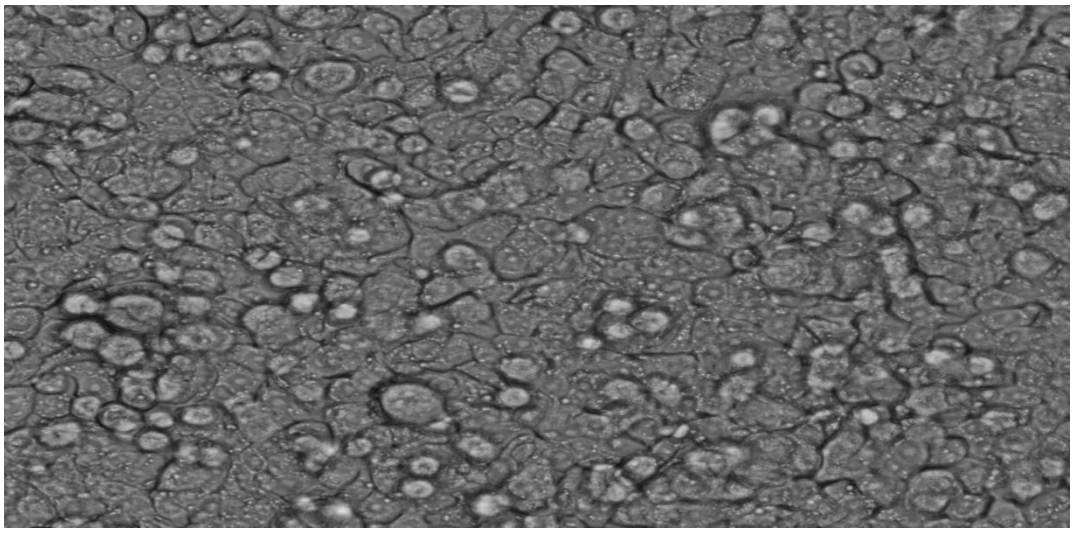

Figure 58(A): Goblet cell line cultured in complete media (DMEM) in 24 well cell cultures, at 37°C with 10% CO_2 for 96 hours (x200 magnification). These cells have the typical appearance of healthy, goblet cell line HT-29 MTX in submerged culture. This is shown by the 'cobblestone' morphology and the cells appear more rounded than PEBCs. The cells are clearly adherent, with minimal numbers of floating or fibroblast like cells

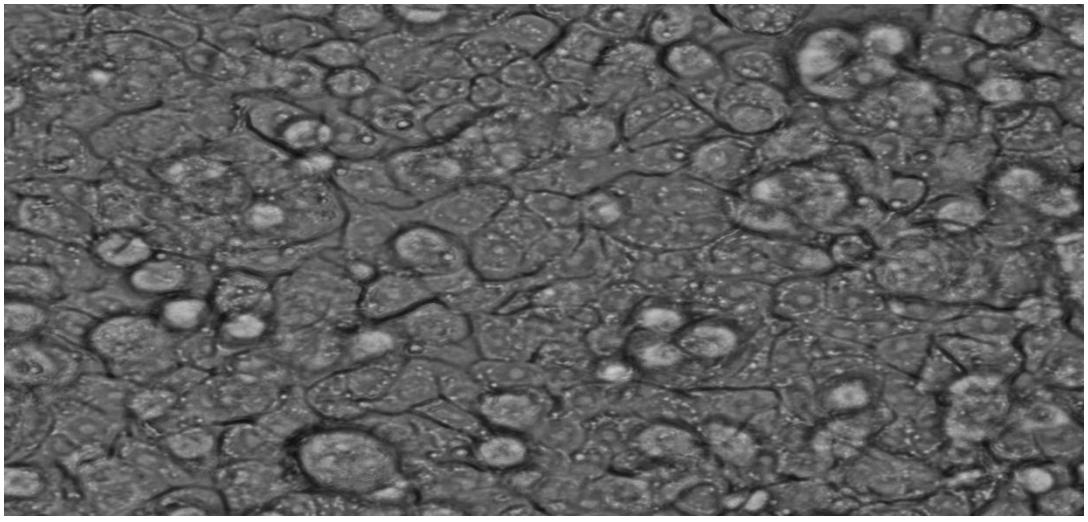

Figure 58(B): Goblet cell line cultured in 24 well cell cultures for stimulation experiments at 37°C with 10% CO2 for 96 hours (x300 magnification). These cells have the typical appearance of healthy, primary epithelial cells in submerged culture. This is shown by the 'cobblestone' morphology. The cells are clearly adherent, with minimal numbers of floating or fibroblast like cells. The difference between Figure 58(A) and 58 (B) is that the cells are clearly confluent and ready for stimulation experiments.

Another aim of this project was to establish whether the goblet cell line HT-29 MTX was able to produce significant changes in IL-8, IL-6 and GMCSF levels after exposure to bile acids, porcine pepsin, different diluted (1:1000) human gastric juice samples (whole, filtered and dialysed) and bile acid mixture (2.2.5) as seen in Table 9.

The levels of inflammatory cytokine production were measured for IL-8, IL-6 and GMCSF from the goblet cell line HT- 29 MTX after stimulation with different concentrations between 0.25μmol/l and 10μmol/l for 48 hours with the primary bile acid chenodeoxycholic acid. IL-8 basal output was similar (≈ 3000pg/ml) to PBECs. However, the IL-6 and GMCSF produced by HT-29 MTX were lower (max 10pg/ml for both) than PBECs basal GMCSF and basal IL-6 (≈ 200 pg/ml and ≈ 80 pg/ml respectively), again this is evidence of an IL-8 phenotype. Figures 59 to 61 are shown that compared to basal, IL-8, IL-6 and GMCSF levels varied after exposure to chenodeoxycholic acid with sometimes an increase and sometimes a decrease, however none of the changes were significant.

Figures 62 to 64 shown the levels for IL-8, IL-6 and GMCSF released from HT-29 MTX cells after stimulation with the secondary bile deoxycholic acid. Similarly to chenodeoxycholic acid the levels of IL-8, IL-6 and GMCSF varied after exposure to deoxycholic acid with sometimes an increase and sometimes a decrease, however none of the changes were significant. Figures 65 to 67 show HT-29 MTX cells were unable to release any significantly different levels of IL-8, IL-6 and GMCSF after treatment with different concentrations of lithocholic acid (0.25μmol/l to 10μmol/l) over a 48 hour period. Overall the goblet cell line was unresponsive in terms of cytokine release when exposed to primary and secondary bile acids at concentrations up to 10μmol/l. Figures 68 to 70 show that exposure of the goblet cell line for 48 hours to porcine pepsin (25 to 100ng/ml) caused a non significant decrease in IL-8 release and has no significant effect on IL-6 or GMCSF release.

Figures 71 to 72 and Table 16 show that the goblet cell line HT-29 MTX was able to release significantly increased levels from IL-8 after stimulation with diluted (1:1000) whole human gastric juices from certain patients (LTx8, CR47, CR52, CR34, CR38, CR50 and CR45), however CR42 did not. All of the diluted human gastric juice samples have different amounts of pepsin, total bile acid, a different pH and different pathogen microbiology (Table 9). It seems unlikely that pepsin levels or presence of pathogens are causing this increase in IL-8 levels. As CR42 which does not invoke an increase has the second highest bile acid and pepsin content and contains *Acinetobacter junii* and *Candida parapsilosis strains.* IL-6 levels are significantly increased by gastric juices CR47, CR52 and CR34, however the levels measured (shown in Figure 72) are below the limit of accurate quantification for IL-6, i.e. 0.6pg/ml, so will not be considered further.

Following dialysis and filtration only CR34 showed an increase in IL-8 levels and CR42, CR47, CR38 and CR45 now showed a significant decrease in IL-8 levels (Table 17). CR34 after dialysis and filtration contains 75 µmol/l bile acids, reduced from 1247 µmol/l and did not contain any measured pathogens. It appears that whatever was producing the stimulation is not removed by dialysis so must have molecular weight larger than 10 – 14kDa but is not as large as bacteria which would be removed by filtration (0.22µm). The significant decrease in IL-8 levels compared to basal levels is more difficult to explain. One possibility is that the juice contains stimulatory and inhibitory factors, the stimulatory factors are removed by dialysis and filtration but the inhibitory factors remain. There is also a general down regulation of IL-6 and GMCSF by the dialysed and filtered juices but as explained above the changes are too small to be considered.

The mixture of bile acids had no effect on IL-8 and GMCSF production but showed a significant increase in IL-6 production. However the median basal level of 5.4pg/ml is calculated from only two measurements, compared to the bile acid stimulation median of

6.4pg/ml calculated from 14 measurements. In addition the difference of 1.1pg/ml is close to the limit of accurate quantification so will not be considered further.

When the HT-29 MTX goblet cells were stimulated with individual primary and secondary bile acids, porcine pepsin, different gastric juices (whole, filtered and dialysed) samples and a bile acid mixture, there was no significant effect on cell viability as measured by CellTiter- Blue cell viability assay (Promega. Madison).

From these experiments it appears that the goblet cells have a similar phenotype to PBEC in that they basally secrete high levels of IL-8 and very low levels of GMCSF and IL-6 and they respond to inflammatory stimuli via an IL-8 response.

5.4.1 IL-8, IL-6 and GM-CSF levels, after goblet cell stimulation with chenodoxeycholic acid at 37 ºC for 48hours in 10% CO2.

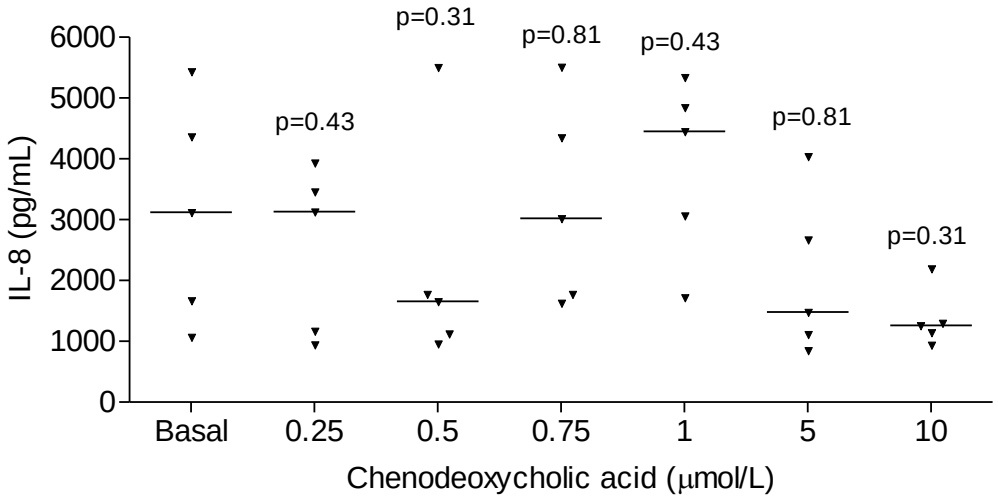

Figure 59: The effect of stimulating goblet cell HT-29 MTX with chenodeoxycholic acid and its effect on IL-8 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

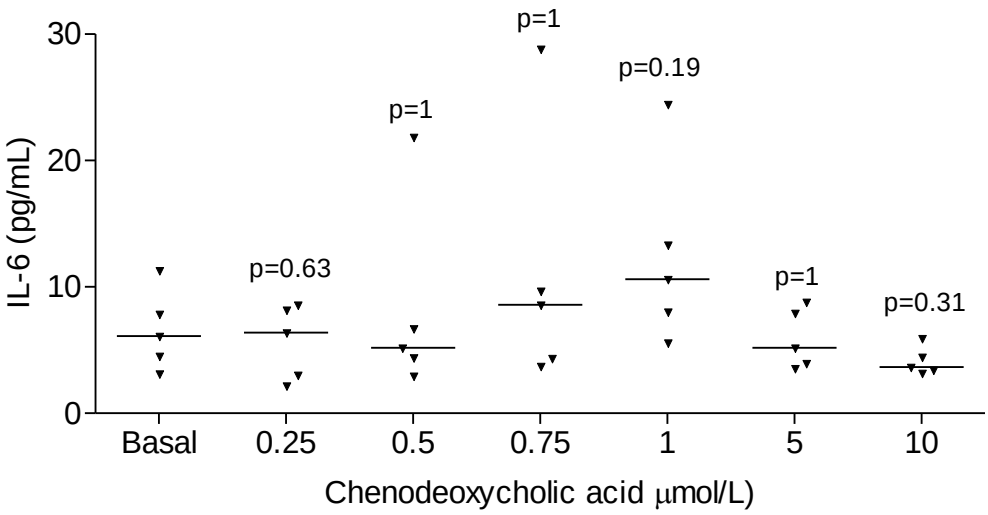

Figure 60: The effect of stimulating goblet cell HT-29 MTX with chenodeoxycholic acid and its effect on IL-6 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

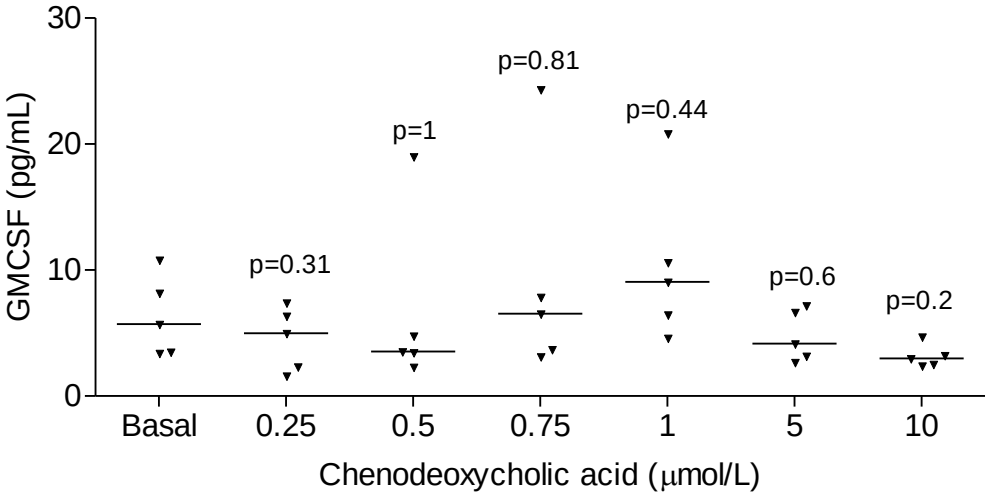

Figure 61: The effect of stimulating goblet cell HT-29 MTX with chenodeoxycholic acid and its effect on GMCSF release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

5.4.2 IL-8, IL-6 and GM-CSF levels, after goblet cell stimulation with the individual secondary bile acid (doxeycholic acid) at 37 ºC for 48hours in 10% CO2.

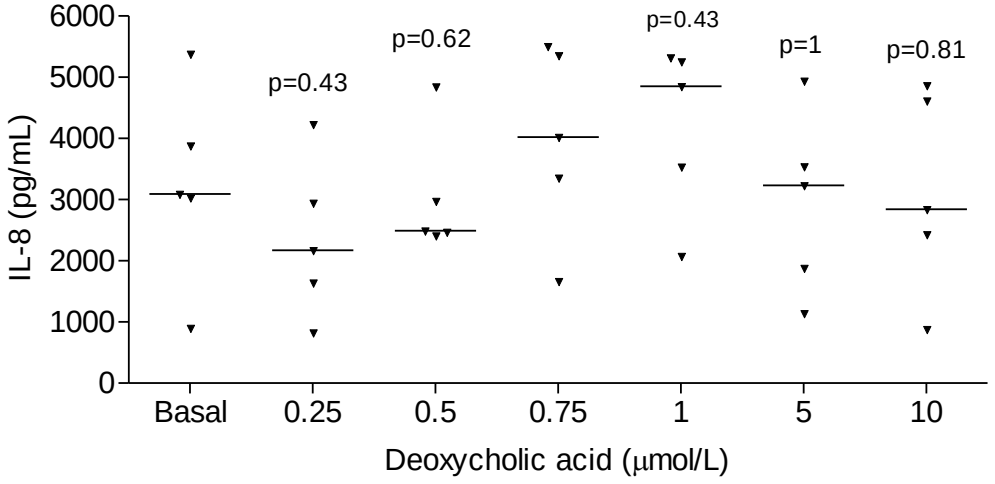

Figure 62: The effect of stimulating goblet cell HT-29 MTX with doxeycholic acid and its effect on IL-8 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

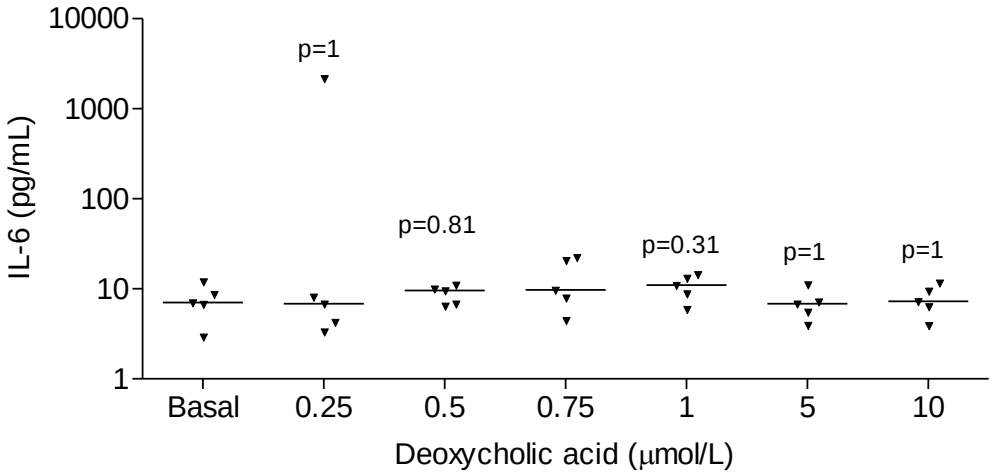

Figure 63: The effect of stimulating goblet cell HT-29 MTX with doxeycholic acid and its effect on IL-6 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

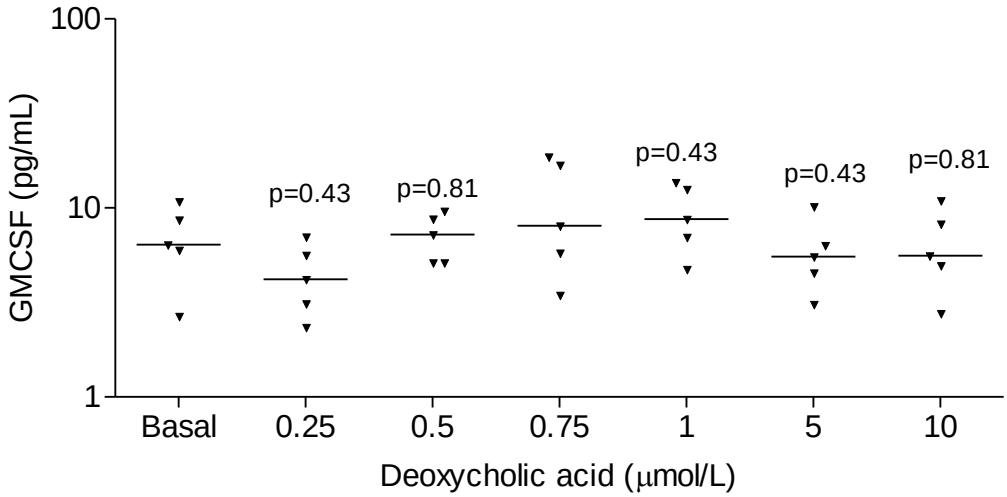

Figure 64: The effect of stimulating goblet cell HT-29 MTX with doxeycholic acid and its effect on GMCSF release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

5.4.3 IL-8, IL-6 and GM-CSF levels, after goblet cell stimulation with the individual secondary bile acid (lithocholic acid) at 37 ºC for 48hours in 10% CO2.

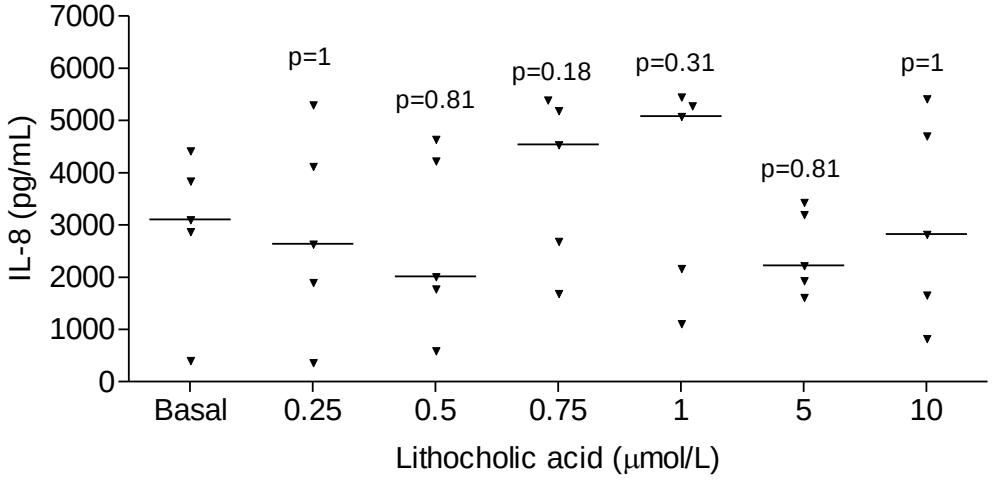

Figure 65: The effect of stimulating goblet cell HT-29 MTX with lithocholic acid and its effect on IL-8 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

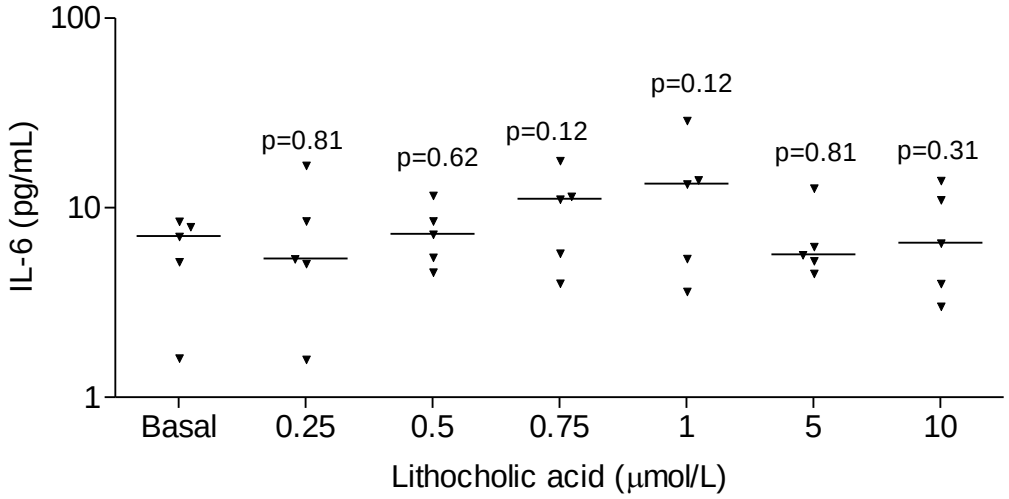

Figure 66: The effect of stimulating goblet cell HT-29 MTX with lithocholic acid and its effect on IL-6 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

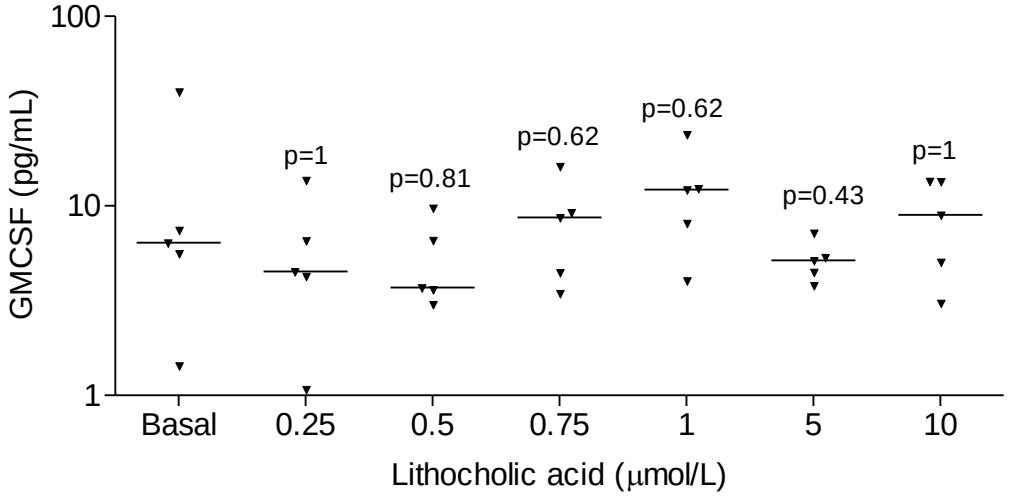

Figure 67: The effect of stimulating goblet cell HT-29 MTX with lithocholic acid and its effect on GMCSF release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=5.

5.4.4 IL-8, IL-6 and GM-CSF levels, after goblet cell stimulation with Pepsin at 37 ºC pH7.4 for 48hours in 10% CO2.

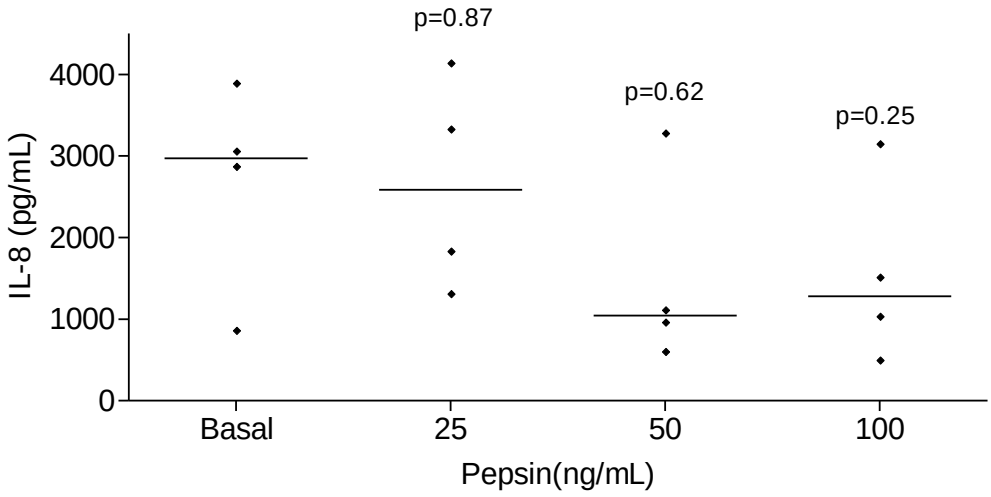

Figure 68: The effect of stimulating goblet cell HT-29 MTX with porcine pepsin and its effect on IL-8 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=4.

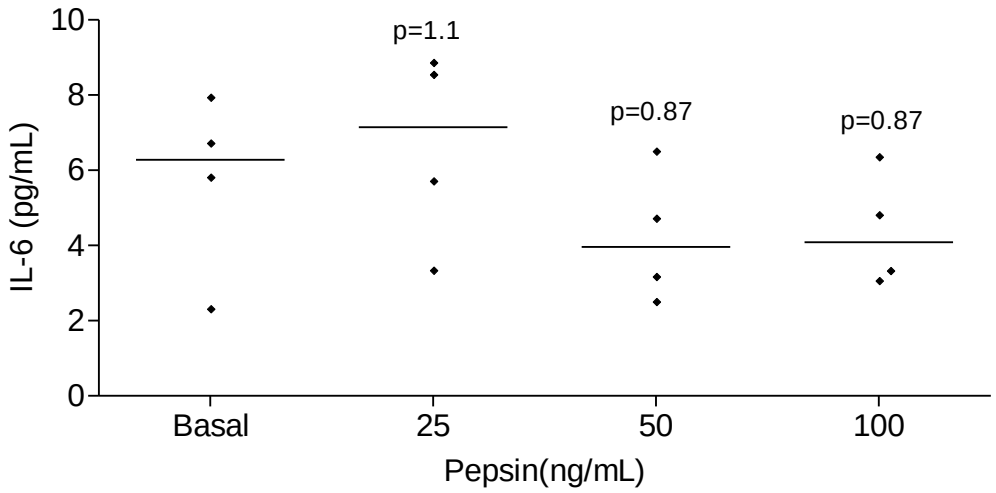

Figure 69: The effect of stimulating goblet cell HT-29 MTX with porcine pepsin and its effect on IL-6 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=4.

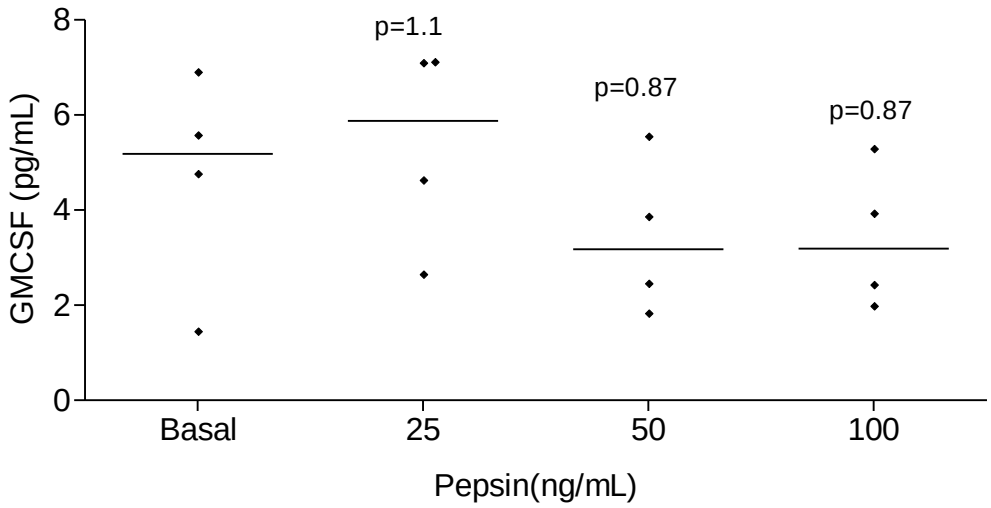

Figure 70: The effect of stimulating goblet cell HT-29 MTX with porcine pepsin and its effect on GMCSF release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n=4.

5.4.5 IL-8, IL-6 and GM-CSF levels, after goblet cell stimulation with whole human gastric juice diluted 1:1000 at 37 ºC for 24 hours in 10% CO2.

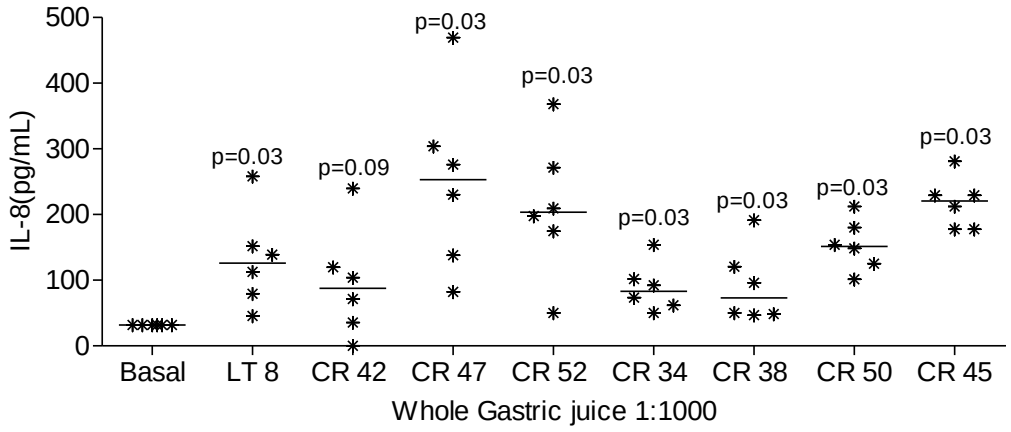

Figure 71: The effect of stimulating the goblet cell line HT-29 MTX with whole gastric juice and its effect on IL-8 release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n= 6.

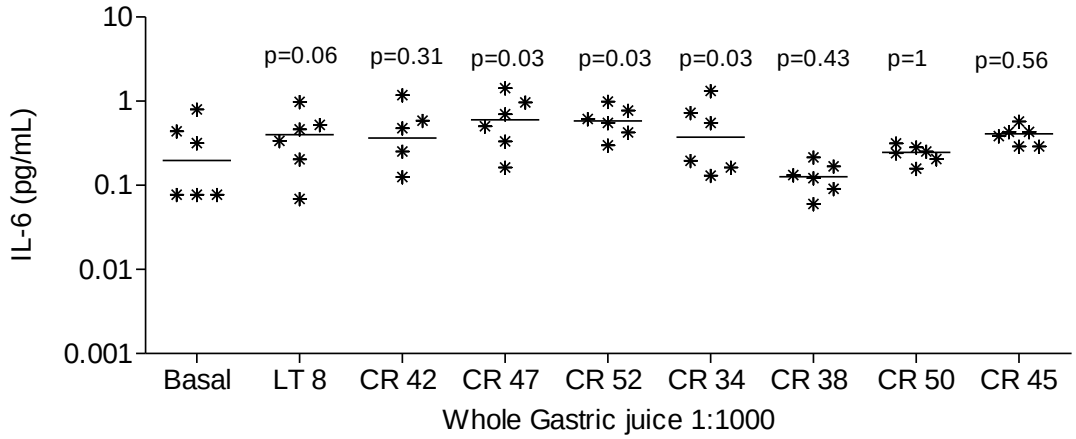

Figure 72: The effect of stimulating goblet cell HT-29 MTX with whole gastric juice and its effect on IL-6 release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n= 6

Table 16: The whole patient's gastric juice that demonstrated a significant effect of cytokine release.

	whole gastric juice patients 1:1000							
	LTx8	CR42	CR47	CR52	CR34	CR38	CR50	CR45
IL-8	↑*	___	↑*	↑*	↑*	↑*	↑*	↑*
IL-6	___	___	___	___	___	___	___	___
GMCSF	___	___	___	___	___	___	___	___

Table 18: The whole gastric juice patients challenge for the goblet cell line HT-29 MTX, which demonstrated significant stimulation of cytokine release.
↑ Increase
↓ Decrease

5.4.6 IL-8, IL-6 and GM-CSF levels, after cell stimulation with filtered and dialysed human gastric juice at 37 ºC for 24 hours in 10% CO2.

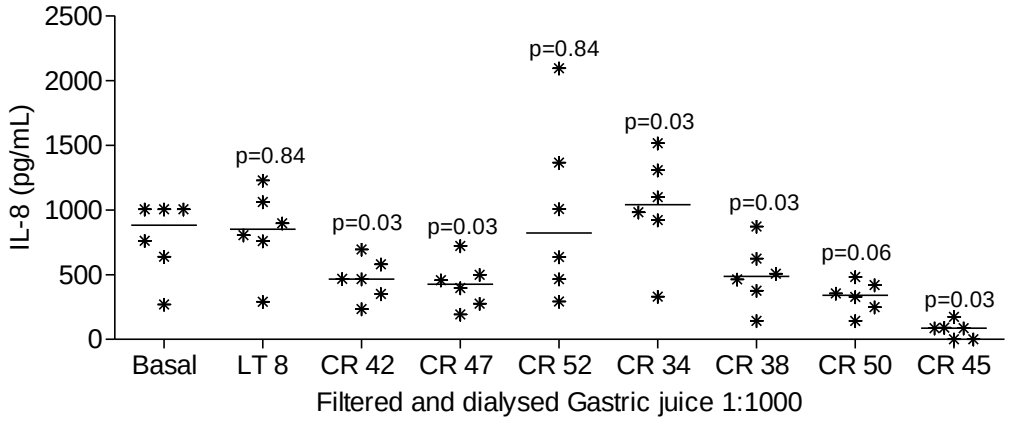

Figure 73: The effect of stimulating goblet cell HT-29 MTX with filtered and dialysed sed gastric juice and its effect on IL-8 release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n= 6.

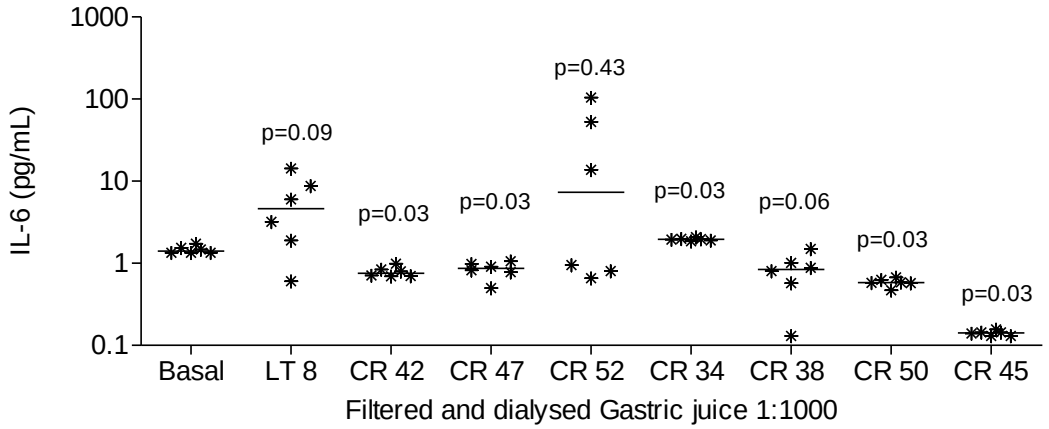

Figure 74: The effect of stimulating goblet cell HT-29 MTX with filtered and dialysed gastric juice and its effect on IL-6 release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n= 6.

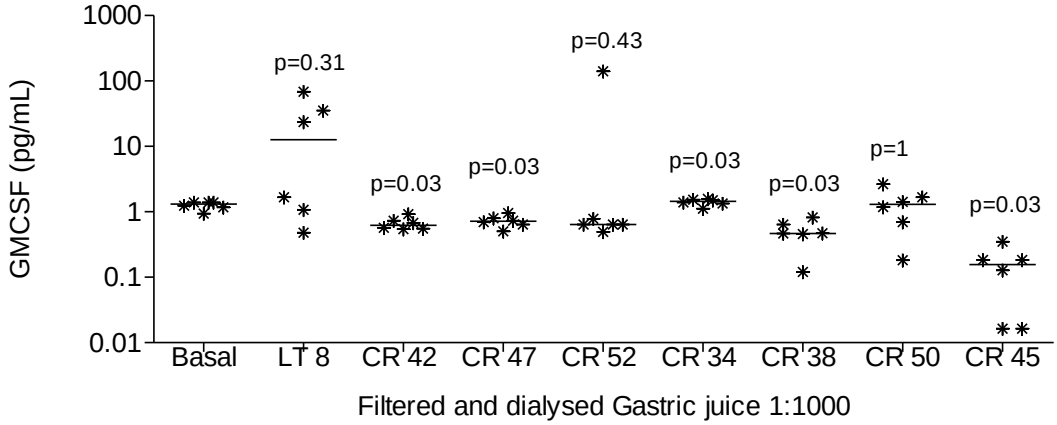

Figure 75: The effect of stimulating goblet cell HT-29 MTX with filtered and dialysed gastric juice and its effect on GMCSF release, following stimulation for 24 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant. The results shown are median values from experiments, n= 6.

Table 17: the diluted whole patient gastric juice filtered and dialised which demonstrated a significant effect on cytokine release.

	whole gastric juice patients 1:1000							
	LTx8	CR42	CR47	CR52	CR34	CR38	CR50	CR45
IL-8	___	↓*	↓*	___	↑*	↓*	___	↓*
IL-6	___	↓*	↓*	___	↑*	___	↓*	↓*
GMCSF	___	↓*	↓*	___	↑*	↓*	___	↓*

Table 19: The whole gastric juice (1:1000) challenge for goblet cell HT-29 MTX, which demonstrated significant changes to cytokine release.
↑ Increase
↓ Decrease

5.4.7 Comparison IL-8, IL-6 and GM-CSF levels, after cell stimulation with whole, filtered and dialysed human gastric juice at 37 ºC for 24 hours in 10% CO2.

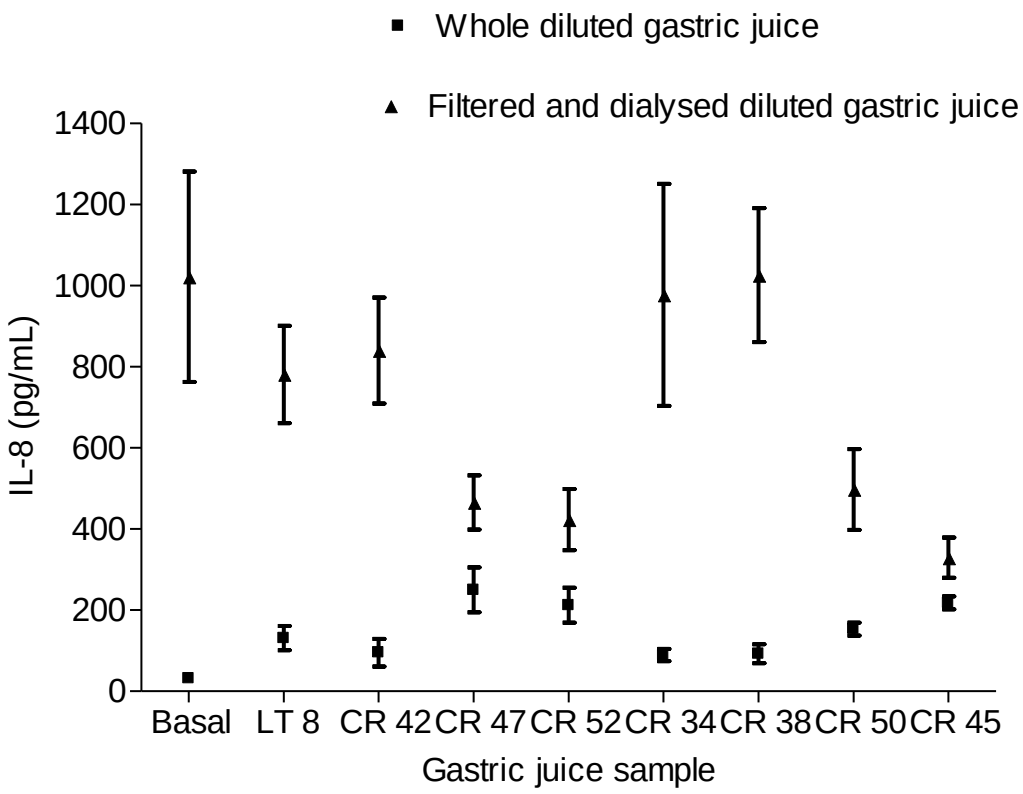

Figure 76: The effect of stimulating goblet cell HT-29 MTX with whole, filtrated and dialysed human gastric juice at the same 1:1000 dilution, its effect on IL-8 release, and cytokine secretion in cell supernatants was measured by MSD ELISA. The results shown are mean & SEM values from experiments, n= 6.

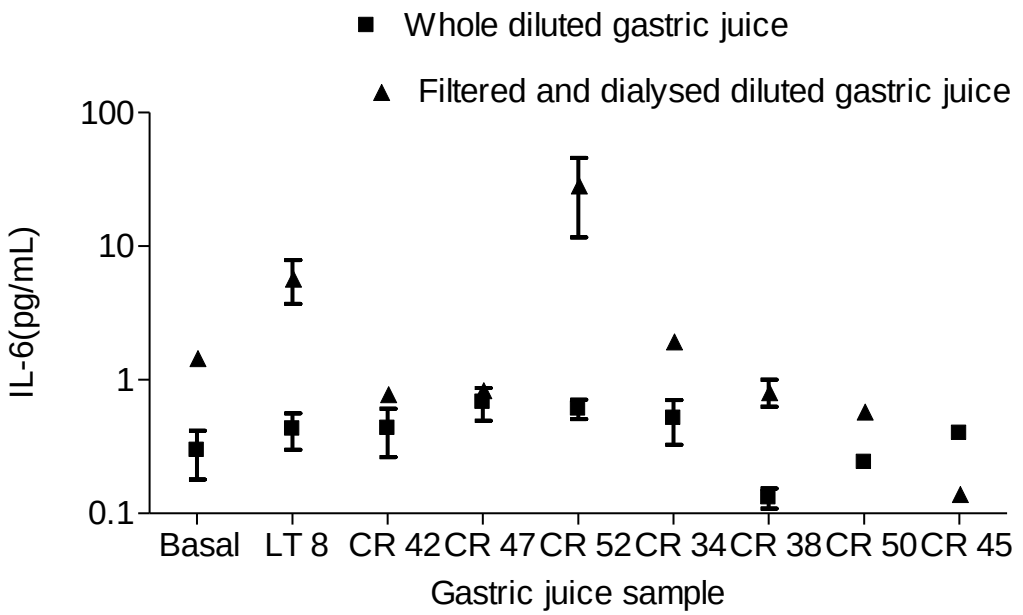

Figure 77: The effect of stimulating goblet cell HT-29 MTX with whole, filtrated and dialysed human gastric juice at the same 1:1000 dilution, its effect on IL-6 release, and cytokine secretion in cell supernatants was measured by MSD ELISA. The results shown are mean & SEM values from experiments, n= 6.

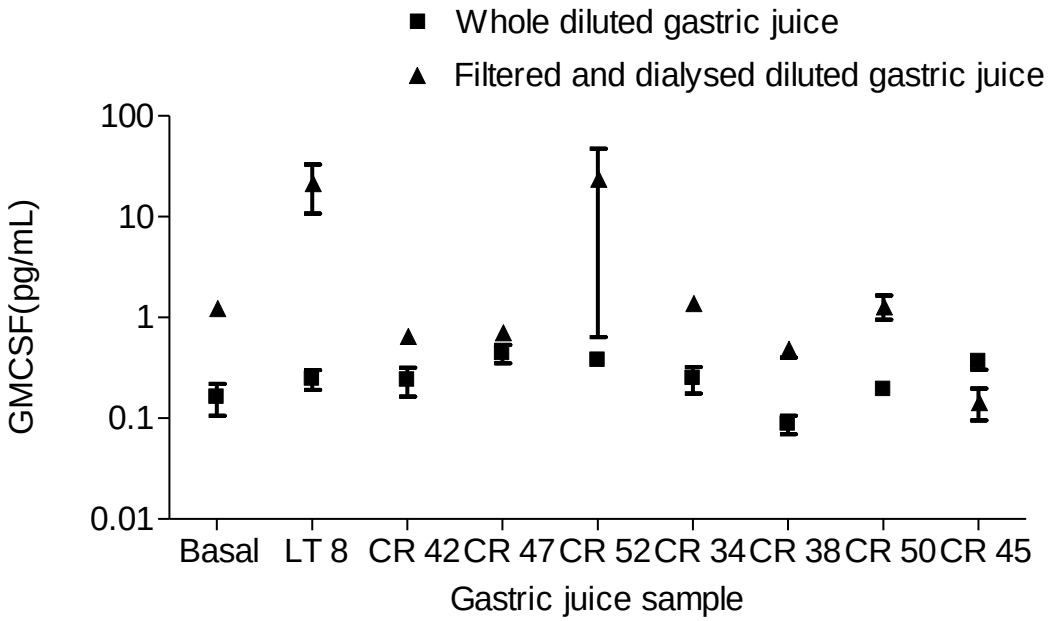

Figure 78: The effect of stimulating goblet cell HT-29 MTX with whole, filtrated and dialyses human gastric juice at the same 1:1000 dilution, its effect on GMCSF release, and cytokine secretion in cell supernatants was measured by MSD ELISA. The results shown are mean & SEM values from experiments, n= 6.

5.4.8 IL-8, IL-6 and GM-CSF levels, after goblet cell stimulation with a bile acid mixture, at 37 ºC for 48hours in 10% CO2.

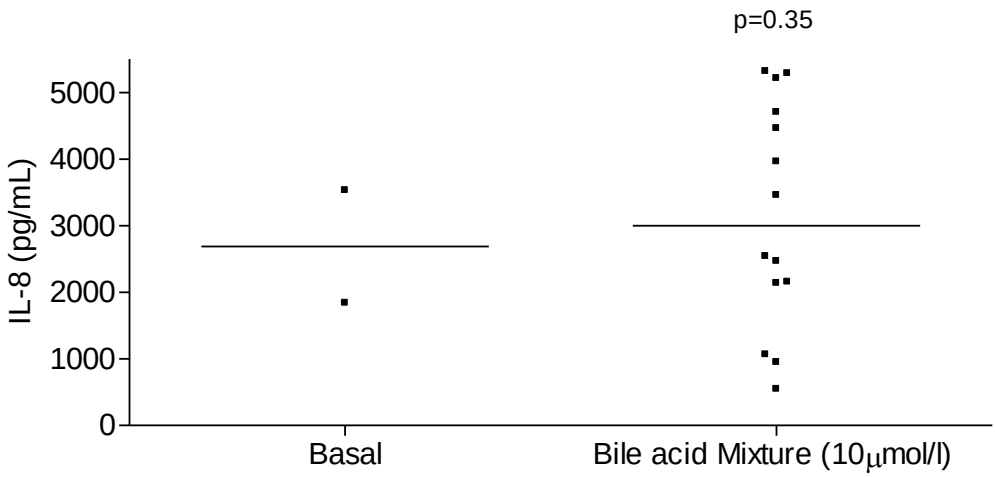

Figure 79: The effect of stimulating goblet cells HT-29 MTX with bile acids mixture, its effect on IL-8 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant.The results shown are median values from experiments, n= 14.

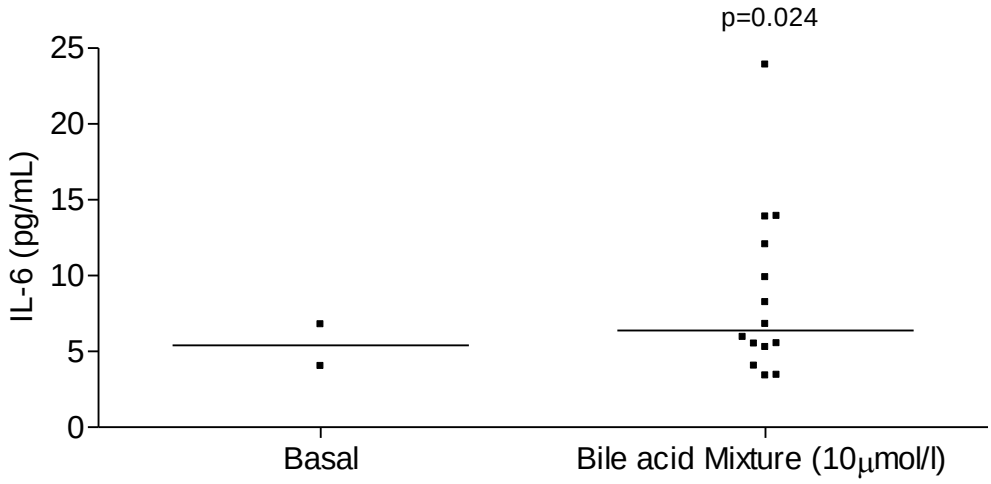

Figure 80: The effect of stimulating goblet cells HT-29 MTX with bile acids mixture, its effect on IL-6 release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant.The results shown are median values from experiments, n= 14.

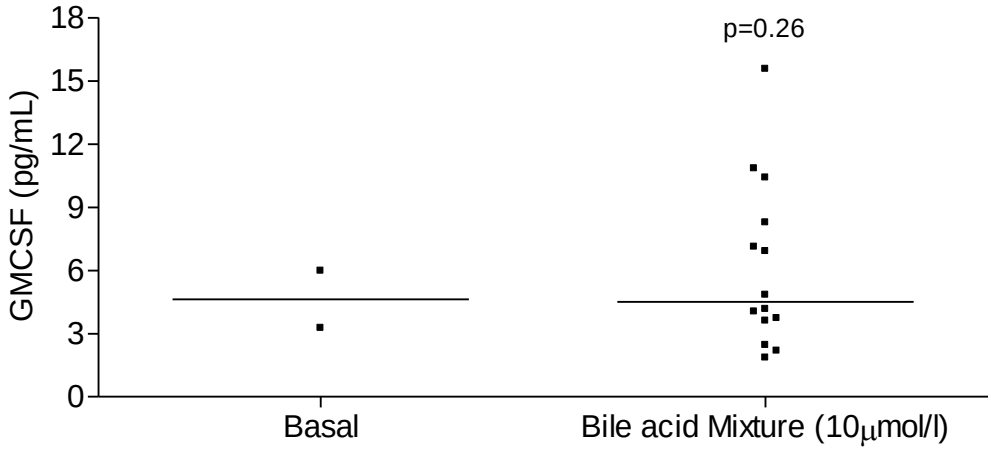

Figure 81: The effect of stimulating goblet cells HT-29 MTX with bile acids mixture, its effect on GMCSF release, following stimulation for 48 hours. Cytokine secretion in cell supernatants was measured by MSD ELISA. P values of 0.05 or less were regarded as significant.The results shown are median values from experiments, n= 14.

Goblet cells are important cells of the airway in health and disease. However the source of the HT29-MTX cells used in my experiments must be considered, as these goblet cells are an immortalised cell line derived from those of the human colon. Unlike goblet cells of the colon, which biosynthesise and secrete MUC2 these goblet cells act like those of the airways in their secretion of MUC5AC and MUC5B (Smirnova et al., 2002). The fact that these cells are acting atypically may suggest that the stimulation data derived from them is non-physiological and can only provide an indication of *in vivo* situation. It could also be considered that these cells, being derived from an environment where bile and other gastric contents are common place, are innately resistant to damage or stimulation by such components. This is confirmed by the lack of any changes in cell viability. It has previously been shown that IL-8 regulates mucin gene expression in lung epithelial tissues *in vitro* (Bautista et al., 2009*).*In the HT29-MTX cell linethe Newcastle group has previously shown that IL-8 upregulated the secretion of MUC5AC and MUC5B mucins in a concentration-dependent manner, with a maximum response at an IL-8 concentration of 20 ng/ml, with the response persisting for up to 5 days(Smirnova et al., 2002).

Chapter 6

General Discussion

6.1 General Discussion

For patients with a variety of end stage lung diseases, lung transplantation has become an effective therapy, however, the development of post transplant bronchiolitis obliterans remains a major limitation to the long-term success of this process. Bronchiolitis obliterans (i.e. a process of obliteration of the small airways) and its clinical correlate the bronchiolitis obliterans syndrome (BOS, identified as a persistent drop in the forced expiratory volume in 1 second post transplantation) can rapidly develop and has a poor response to augmented immunosuppression. This accounts for more than 30% of all deaths occurring after the third post operative year (Burke et al., 1984, Boehler et al., 1998). Associations between BOS severity and time to first appearance of BOS and bile acid aspiration, alveolar neutrophilia and IL-8 have also been reported (D'Ovidio et al., 2005b). The bronchoalveolar lavage fluid obtained from patients with BOS contains bile acids that would appear to be a marker of retrograde aspiration (D'Ovidio et al., 2005b). The underlying cause of bronchiolitis obliterans are poorly understood; although alloimmune-mediated injury directed against endothelial and epithelial structures are thought to be the major culprit, non-alloimmunologic inflammation, including viral infections or ischemic injury have also been implicated to have a major role (Estenne et al., 2002). Amongst other possible inflammatory conditions, it is thought that aspiration secondary to gastroesophageal reflux (GOR) can contribute to lung allograft dysfunction and lead to possible development of bronchiolitis obliterans (Berkowitz et al., 1995, Davis et al., 2003b). Consequently whether or not GOR and aspiration in lung transplant patients predicts the development of BOS needs further investigatation. GOR after lung transplantation may be promoted due to the potential for vagal nerve injury during the

course of transplantation and later on because of the effects of immunosuppressive drugs such as calcineurin inhibitors (cyclosporine and tacrolimus) that prolong gastric emptying (Berkowitz et al., 1995, Young et al., 2003). The association between lung disease and GOR has been suspected for a considerable time (Pearson and Wilson, 1971, Belsey, 1979, D'Ovidio et al., 2005b). In fact, GOR prevalence within patients with a variety of lung diseases has been documented in many instances, for example, patients with asthma, cystic fibrosis (CF), and idiopathic pulmonary fibrosis (Feigelson et al., 1987, Tobin et al., 1998) have been shown to have abnormal levels of GOR. In addition, GOR has also been associated with the development of bronchiolitis obliterans and pneumonia (Sadoun et al., 1988). Pathophysiological studies of chronic respiratory diseases such as chronic cough (Irwin et al., 1981, Sifrim et al., 2005) , cystic fibrosis (Feigelson and Sauvegrain, 1975, Blondeau et al., 2008a), and asthma (Rosztoczy et al., 2008, Harding, 2001) have found gastroesophageal reflux GOR to be a potential common instigator. Increased GOR after lung transplantation (LTx) can also contribute to the development of chronic rejection or bronchiolitis obliterans syndrome (BOS) (Blondeau et al., 2008b, D'Ovidio et al., 2006a, Palmer et al., 2000, Young et al., 2003). Ciliated and nonciliated airway epithelial cells can be damaged by exposure to gastric contents (Ohrui et al., 1997, Wynne et al., 1981) (Harding, 2001, Blondeau et al., 2008b, D'Ovidio et al., 2006a). Aspirated gastric contents cause impairment of mucociliary transport and are associated with the colonization of microorganisms in airways (Zeiher and Hornick, 1996, D'Ovidio et al., 2006a). Besides the lung injury, acid aspiration also induces the production of an inflammatory cytokine, IL-8 as measured in alveolar and bronchoalveolar lavage fluid (Zeiher and Hornick, 1996). It is known that children and adults suffering with CF have abnormally high levels of reflux (Ledson et al., 1998, Cucchiara et al., 1991, Blondeau et al., 2008a). Furthermore, there is increasing acceptance that clinically hidden, non-acidic reflux and aspiration could be an important source of lung injury (Blondeau et al., 2008a).

A duodeno-gastro-oesophageal refluxate is more likely to be non-acidic due to the neutralising effects of duodenal contents and potentially contains bile acids which have a wide range of biological actions.

Primarily bile acids are involved in the digestion of fat (Kullak-Ublick et al., 2004, Lefebvre et al., 2009). Produced from cholesterol by the liver, primary bile acids consist of cholate and chenodeoxycholate. Water-soluble conjugates are formed when these primary bile acids couple with the amino acids taurine or glycine. Bile acids are actively reabsorbed during movement through the ileum and efficiently recycled to the liver. Formation of secondary bile acids results from the enzymatic modification of remaining primary bile acids by gastrointestinal bacteria. Hence, chenodeoxycholic acid leads to lithocholic acid and cholic acid is converted to deoxycholic acid due to 7α-dehydroxylase activity possessed by intestinal bacteria (Kullak-Ublick et al., 2004, Lefebvre et al., 2009, Setchell et al., 1985).

My studies have shown that IL-8, IL-6 and GMCSF release is significantly increased by primary (chenodeoxycholate) and secondary (deoxycholate and lithocholate) bile acids when PBECs from lung transplantation recipients and CF PBECs are challenged. The levels at which bile acids have the effect an these cytokines is within the range that the bile acids have been measured in the circulation of children with CF i.e. 0.2 to 0.8 µmol/l (Setchell et al., 1985).

In terms of constitutive production of cytokines there was no difference between non-CF PBECs and the CF PBECs. This results differs from the report of Sutanto et al, where they reported that CF airway cells from children produced greater amounts of IL-6 than airway cells from from healthy children with mean levels of 6000 pg/ml/10^6 cells and 250 pg/ml/10^6 cells respectively (Sutanto et al., 2011). In the studies presented in this thesis CF PBECs and non CF PBECs produced mean basal levels for IL-6 of 260 pg/ml/4x10^5 and 150 pg/ml/4x10^5 cells. Normalising the data to 10^6 cells gives 650 pg/ml and 375

pg/ml respectively. This is higher than the normal pediatric airway epithelial cells (AEC) but much lower than the CF pediatric cells. This high level of constitutive IL-6 production is also a characteristic of AECs from children with asthma (McNamara et al., 2008). Some of the differences could be related to the cells used in this thesis, because they are adult. In addition the CF cells are from end stage lungs at the time of transplantation, where the patient has been subject to life long infections and anti microbial treatments. This compares with studies in paediatric patients with a median age of 4.1 years and no evidence of *P. aeruginosa* infections and consequently a much lower exposure to infection / antibiotic treatment. Both Non CF PBECs and CF PBECs have high levels of constitutive IL-8 production with mean basal levels of 3000 pg/ml/4x10^5 cells and 2500 pg/ml/4x10^5 cells respectively, showing IL-8 production is about 10 times higher than IL-6 and GMCSF. In terms of the cytokines measured both these types of PBECs have an IL-8 phenotype unlike the pediatric AECs which have an IL-8/IL-6 secreting phenotype (Sutanto et al., 2011).

My study using CF lung derived cells and brushings from lung transplant patients has shown that IL-8 release from CF PBECs was stimulated by low levels of all the individual bile acids used, 0.25 µmol/l and above. Non-CF PBECs do not respond until 1µmol/l. As tables 18 and 19 shows CF PBECs respond to bile acid challenge with a lower threshold than PBECs from lung transplant recipients. This increased responsiveness may be explained by the fact that these PBEC samples are brushings from transplanted (healthy) lungs where patients use immunosuppressant drugs, however CF PBECs samples are produced by enzymatic dysaggregation and scraping from end stage cystic fibrosis explant lungs, which are exposed to long term infection and therapeutic interventions.

CF PBECs were not stimulated to produce IL-6 or GMCSF until 1 µmol/l bile acids. In the non-CF PBECs IL-6 productions was not stimulated until 5µmol/l with GMCSF stimulated at similar levels as the CF cells.

Bile acids	Chenodeoxycholic acid						Deoxycholic acid						Lithocholic acid					
Bile acids conc µmol/l	**0.25**	**0.5**	**0.75**	**1**	**5**	**10**	**0.25**	**0.5**	**0.75**	**1**	**5**	**10**	**0.25**	**0.5**	**0.75**	**1**	**5**	**10**
IL-8	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*
IL-6	—	—	—	↑*	↑*	—	—	—	—	↑*	↑*	↑*	—	—	—	↑*	↑*	↑*
GMCSF	—	—	—	↑*	↑*	↑*	—	—	—	↑*	↑*	↑*	—	—	—	↑*	↑*	↑*

Table 20: The effect of different levels of bile acids on PBECs from explanted **Cystic Fibrosis** patients 48 hours, which demonstrated significant stimulation of cytokines release.

↑ Increase

↓ Decrease

Bile acids	Chenodeoxycholic acid						Deoxycholic acid						Lithocholic acid					
Bile acids conc µmol/l	**0.25**	**0.5**	**0.75**	**1**	**5**	**10**	**0.25**	**0.5**	**0.75**	**1**	**5**	**10**	**0.25**	**0.5**	**0.75**	**1**	**5**	**10**
IL-8	—	—	—	↑*	↑*	↑*	—	—	—	↑*	–	↑*	—	—	—	↑*	↑*	↑*
IL-6	—	—	—	—	↑*	—	—	—	—	–	–	–	—	—	—	—	↑*	—
GMCSF	—	—	↑*		↑*	↑*	↑*	↑*	—	↑*	↑*	↑*	—	—	—	↑*	↑*	↑*

Table 21: The effect of different levels of bile acids on PBECs from **lung transplant recipient** 48 hours, which demonstrated significant stimulation of cytokines release.

↑ Increase

↓ Decrease

There is limited research relating to the characterisation of bile salts in the lung and in particular in advanced CF lung disease. Recently a detailed study of aspiration injury in patients with ventilator-associated pneumonia has reported the detection of lithocholic acid in the lung at a mean (SEM) concentration of 2.8 ± 0.6 µmol/l (Wu et al., 2009). The elevated levels of bile acids were linked with lung injury and were higher when compared to patients ventilated but without pneumonia. The study by Wu *et al*. is comparable and supportive of the approach used in my research. The research by Wu *et al.* evaluated the effect of bile acid challenge on cultured alveolar epithelial cells and concluded that chenodeoxycholic acid challenge led to IL-8 generation at the message and protein level. The increase in IL-8 production was demonstrated to be via activation by phosphorylation

of a p38 MAP kinase pathway, as the IL-8 up regulation was inhibited by dexamethasone and the selective p38 inhibitor SB203580 (Wu et al., 2009). The fact that the bile acids act via a phosphorylation pathway is not surprising, in that bile acids have been shown to be regulating molecules. Bile acids activate cell signalling pathways, c-jun N terminal kinase 1/2, AKT and ERK 1/2 in epithelial cells of the gut (Hylemon et al., 2009)

The formation, secretion and re-absorption of bile acids take place under precise control (Raufman et al., 2003). Retention of bile acids through homeostatic mechanisms includes active, receptor-mediated uptake in the duodenum and ileum through the apical sodium dependent bile acid transporter, coupled with the enterohepatic circulation. Therefore, in the normal situation less than 5% of bile acids are excreted (Kullak-Ublick et al., 2004, Lefebvre et al., 2009). However, in CF extraction of bile acids can be impaired at both intestinal absorption and hepatic levels because of, dysregulated lipid metabolism (Brodlie et al.) and overall compromised liver function (O'Sullivan and Freedman, 2009, Ratjen, 2009). Children with CF have higher faecal excretion of bile acids; CF patients with pancreatic insufficiency have been reported with bile acid malabsorption (Setchell et al., 1985). It is possible that the complexity of bile acid profiles in CF may occur due to lifelong antibiotic treatment. This may have the result of disturbing the normal homeostatic gastrointestinal bacterial populations, responsible for producing secondary bile acids (Kullak-Ublick et al., 2004, Lefebvre et al., 2009). Due to the fact that CF involves (O'Sullivan and Freedman, 2009) disordered bile homeostasis, (Setchell et al., 1985) duodeno-gastro-oesophageal reflux, (Sweet et al., 2009, Fathi et al., 2009) and aspiration; it may best be regarded as a disease that involves both the respiratory and gastrointestinal tract (Blondeau et al., 2008a, D'Ovidio et al., 2006a). Within this broader paradigm, aspiration of refluxate may be an under recognised contributor to airway injury in CF (Appel et al., 2007). Epithelial injury has long been suspected to have a connection with bile acids. For example, Barrets' oesophagus is considered to be a potentially pre-

cancerous lesion, denoted by epithelial injury linked to bile acid reflux (Sital et al., 2006). Similarly, post-lung transplantation lung injury could be caused by bile acid aspiration (Blondeau et al., 2008a, D'Ovidio et al., 2006a) with potential mechanisms involving disruption of surfactant and overall innate immunity (D'Ovidio et al., 2006a). Work associated with my research has identified both primary and secondary bile acids at levels similar to, or greater than those shown in ventilator-associated aspiration pneumonia. The range of chenodeoxycholic acid concentration was 1.5 to 5.3µmol/l and lithocholic acid was 2.8 ± 0.6µmol/l (Wu et al., 2009). I have therefore evaluated the effect of levels of chenodeoxycholic acid, deoxycholic acid and lithocholic acid on PBECs at levels which can occur in CF lung disease. Cell viability for PBECs and CF PBECs are affected by low concentrations of lithocholic acid 5µmol/l but not with the other bile acids used at this concentration.

Bile acid molecules are predominantly ampipathic structures, having a wedge like molecular shape (Resarcher, 2008). They have a steroid part which is hydrophobic in character, and a hydrophilic side represented by the hydroxyl group (Hofmann, 1999a). The intrinsic toxicity profile of the bile acids may well be linked to their hydrophobicity, as the more hydrophobic bile acids have been found to be more toxic. On the other hand, the hydrophobicity has an inverse relationship with the number of OH groups. Therefore, the highly hydrophobic and highly toxic lithocholic acid has only one OH group, whereas, decreasing hydrophobicity and decreasing toxicity can be observed in deoxycholic acid and chenodeoxycholic acid with two OH groups (Resarcher, 2008). Mucosal cell membranes may be damaged by the cytotoxic effects of bile acids. In fact, many tissues may be adversely affected, both intra- and extra cellularly. Lithocholic acid is a highly toxic substance produced in the body, and is known for its carcinogenic properties. Lithocholic acid enters the liver where it is either sulphated or esterified to glucuronic acid and then excreted (Resarcher, 2008). The toxic effect of lithocholic acid

on PBECs is shown in Figures 6 (E) and 6 (D) with 200µmol/l and 50µmol/l concentrations for lithocholic acid. This is in agreement with Wu et al., (2009) where human alveolar epithelial cells become detached and floated with chenodeoxycholate levels of over 40 µmol/l (Wu et al., 2009). Concentrations of chenodeoxycholate above 10µmol/l cause cell death with 25µmol/l causing 50% cell death. Concentrations of 1µmol/l or above for deoxycholate and lithocholate affect cell viability of PBECs, however the effect is variable, for example 25µmol/l of chenodeoxycholic acid causes 83% cell death, but with lithocholic acid the effect on cell viability starts at concentration above 5µmol/l lithocholic acid where 45 % of PBECs cells are dead and at 25 µmol/l 80% of the cells are dead.

Throughout this study the production of cytokines and growth factors by PBECs from lung transplantation recipients and CF PBECs was evaluated, concentrating on cytokines known to be implicated in airway inflammation and remodelling common to CF and BOS. Pathophysiological studies of CF have implicated IL-8 and IL-6 (Dufresne et al., 2009). IL-8 is a neutrophil chemokine and mediator of vascular remodelling (Walters et al., 2008). Increased levels of IL-8 and neutrophils are a distinctive presentation of the CF airway. This inflammation is part of the early stage of CF lung disease, with research showing increased levels can occur before bacterial colonisation and infection (Balough et al., 1995). It is relevant to point out that IL-8 is a mucin secretagogue (Smirnova et al., 2002, Smirnova et al., 2001) given that accumulation of mucus in neutrophilic inflammation is also a key element in CF lung pathophysiology. GMCSF, a growth factor that can regulate both the accumulation and the activity of neutrophils was also produced as a direct outcome of bile acid stimulation of PBECs. Based on my data, neutrophilic inflammation, tissue remodelling (Walters et al., 2008) and mucus hyper-secretion (Smirnova et al., 2002, Smirnova et al., 2001) can be attributed as potential consequences of lung injury following bile acid aspiration. Due to the fact that reflux and potential

aspiration has previously been documented in paediatric and adult populations, it would be plausible to treat it as a chronic contributor to lung damage in CF (Blondeau et al., 2008a, Brodzicki et al., 2002, Button et al., 2005, Gustafsson et al., 1991, Heine et al., 1998, Ledson et al., 1998, Malfroot and Dab, 1991, Scott et al., 1985, Vic et al., 1995). In work connected with this study my research group has demonstrated that the lungs of people with advanced CF lung disease removed at the time of transplantation contained significant levels of bile acids, lithocholic acid concentrations ranged between 0.6-25.2µmol/l. These levels detected were used to inform the challenge of cultured airway epithelial cells from explanted CF patients. The results of these experiments have indicated that an increase in the release of the key inflammatory mediators IL-8 and IL-6 has been caused by physiologically achievable levels of individual primary and secondary bile acids. With the non-CF PBECs if one compares the maximal response for IL-8 in terms of fold increase above basal, the chenodeoxycholate (CD) deoxycholate (DC) and lithocholate (LC) produce 3.75, 7.6, and 8.9 fold increase in output (by comparing the basal median to the highest median output at any bile acid concentration). Demonstrating lithocholate has the biggest inflammatory effect. Doing the same calculation for IL-6 and GMCSF shows IL-6 levels are stimulated by 4 (CD), 2.9 (DC) and 3.4 (LC) fold and GMCSF by 2.3 (CD), 2.3 (DC), and 54 (LC) fold. Again showing that lithocholate is a powerful inflammatory stimulant particularly for GMCSF.

In the case of CF PBECs, the fold increase for IL-8 were 4 (CD), 5 (DC), and 8 (LC), for IL-6 1.32 (CD), 5.3 (DC), and 2.9 (LC), for GMCSF 4 (CD), 2 (DC), and 14 (LC). Demonstrating that the CF cells respond most to lithocholate.

When PBECs and CF PBECs were challenged with porcine pepsin at pH 7.4 there was no effect on PBECs and CF PBECs *in vitro* levels of IL-8 IL-6 and GMCSF. This conclusion was also supported by another study which showed no association between pepsin concentration and IL-8 production (Mertens et al., 2010) . At a low pH pepsin may well

be able to cause some damage to the connective tissues and extracellular matrix of the lung tissues *in vivo* pepsin will be denatured at pH 7.2 consequently the data presented here are for inactive pepsin.

In addition a bile acid mixture had no effect on the PBECs and CF PBECs in terms of release of any significant levels of cytokines. The bile acid mixture was made up in composition and concentrations of bile salts as close as possible to those present in human bile (50% taurocholic acid, 15% taurodeoxycholic acid, 30% chenodeoxycholic acid and 5% lithocholic acid) so 65% are conjugated. Conjugation may be important in the effect of produced (Hofmann, 2004), in that there will be an association between structure of the bile acid and effects on transepithelial secretory responses. It is difficult to explain why the bile acid mixture does not cause up regulation of cytokine production, as the mixture contains the free bile acid chenodeoxycholate at a concentration of 3µmol/l. This concentration would also stimulate IL-8, IL-6 and GMCSF production from PEBCs. The mixture is 65% (65µmol/l) conjugated bile acids which are much less hydrophobic compared to the free bile acids. Wu et al., reported that conjugated bile acids do not stimulate IL-8 production at concentration of 10 µmol/l from human alveolar cells (Wu et al., 2009). In addition chenodeoxycholate was five times more potent in up regulating prostaglandin synthesis in oesophageal squamous cells (Zhang et al., 2001). It has not been reported how PBECs react to bile acid mixtures containing both conjugated and non-conjugated bile acids but one could speculate, based on large differences in effect that they may antagonise each other leading to no increase in cytokine production.

As with the bile acid stimulation experiments the CF PBECs were more responsive to gastric juice samples than the PBECs. With only two out of eight juices stimulating production of IL-8 from non CF PBECs compared to four out of eight with the CF PBECs. In terms of IL-6 up regulation only one juice was effective with the PBECs compared to three out of eight with the CF PBECs. Interestingly the gastric juice has little

effect on GMCSF levels. In addition there was marked variability in the levels of the cells response to the gastric juice.

When the gastric juice was filtered, the stimulatory effect was abolished with both CF and non-CF cells, suggesting that the major inflammatory signals had been removed. In the same cases the molecules responsible would appear to be bile acids and in others pathogens or a mixture of the two. Pepsin did not seem to have a direct role. The variation in response could be pathogen species related as demonstrated by Stathopoulou et al., (2010) who showed that IL-6 levels produced by human gingival epithelial cells was significantly higher with *F. nucleatum* compared to S. gordonii (Stathopoulou et al., 2010). Also Sutanto et al., (2011) showed that CF airway cells responded to human rhinovirus with a marked increase in IL-8 compared to non-CF cells (Sutanto et al., 2011). So it seems pathogen type and cells type may play a role in the cytokine response.

The goblet cells line produced a similar basal output of IL-8 as the PBECs i.e. ~3000 pg/ml, but the output of IL-6 and GMCSF was less than 10 pg/ml. These levels for IL-6 and GMCSF are approximately 20 times lower than the PBECs basal output. The goblet cell line has a constitutive IL-8 phenotype in agreement with Smirnova et al (Smirnova et al., 2002). These cells have a resistance to bile acids unlike the PBECs.

The goblet cell line did not release significantly increased levels of IL-8 when exposed to seven out of eight of the gastric juice samples. The stimulation effects of the gastric juice on goblet cells needs to be considered with some caution as the basal output of IL-8 in the whole gastric juice was only 25 pg/ml and the highest stimulated levels were around 300pg/ml, whereas the basal output in the experiments with filtered and dialysed juices was 975pg/ml. Both these basal levels are much lower than the basal level for the bile acid stimulation experiments. Suggesting the cells was behaving abnormally although there were no overt signs of cell death or infection. Taken overall the results with the goblet cells, assuming we can consider them as representative of airway goblet cells,

would imply that IL-6, GMCSF driven inflammatory process instigated by reflux and aspiration, will not be added to by the goblet cells.

The data presented in this thesis showing the potential for reflux/aspiration containing bile acids to cause damage to the CF lung pre and post transplantation, produces further evidence supporting the need for for clinical and surgical interventions to prevent lung damage associated with reflux

The macrolide antibiotic Azithromycin has been documented as having positive effects on gastric motility (Larson et al., 2010), which may be beneficial in reflux associated injury. Recent reports have suggested that azithromycin treatment can prevent damage to transplanted lungs and in some cases reverse the development of BOS (Yates et al., 2005, Gerhardt et al., 2003a). In addition a single centre trial (double-blind randomised controlled) showed that over a two year period patients receiving azithromycin had a significantly lower incidence of BOS compared to placebo, 12.5% versus 44.2% and that patients on azithromycin had a better BOS-free survival time (Vos et al., 2011). However, overall survival time was not different between the groups. This suggests that whatever is damaging the lungs is not completely dealt with by azthromycin treatment.

It is therefore possible that some level of reflux/aspiration persists on azithromycin treatment even though azthromycin should reduce reflux/aspiration because of its pro-motile effect on the gut (Larson et al., 2010). In fact Mertens et al 2011 present evidence that transplant patients that have measurable bile acids in their BAL had reduced survival time and faster decline in FEV_1 and progression of BOS compared to those without bile acids, even when undergoing azithromycin treatment.

Consequently it would seem that further methods to stop reflux need to be employed such as fundoplication even when azthromycin therapy is applied. Although this dual therapy would only need to be applied when evidence of bile acid aspiration is available and can be reliably measured.

6.2 Overall Conclusion

To conclude the work done in this project, a success rate of 70% in culturing primary bronchial epithelial cells, from both lung transplantation recipients and explanted lungs for cystic fibrosis patients was achieved. However the success rate was 85% with the goblet cell line HT-29 MTX.

PBECs from lung transplant patients had different responses to stimulation shown by IL-8, IL-6 and GMCSF production after treatment by individual primary and secondary bile acids, using concentrations for the bile acids 1µmol/l and above.

CF PBECs were able to release significantly stimulated levels of IL-8 after treatment by all the bile acid concentrations including the lowest concentration 0.25µmol/l. However, the CF PBECs are similar to transplant PBECs in terms of IL-6 and GMCSF stimulation, in that bile acid concentrations of 1µmol/l and above were required.

The cells used in this study did not increase the levels of IL-8, IL-6 and GMCSF released after treatment by porcine pepsin and a bile acid mixture as shown in section (2.2.5).

The different human gastric juice preparations evaluated (whole, filtered and dialysed) samples regulated the cells to release significant levels (increased / decreased) depending, on the gastric juice content as (Table 9).

The goblet cell line HT-29 MTX are resistant to bile acids, and did not release any significantly increased levels of IL-8, IL-6 and GMCSF after exposure to porcine pepsin and a bile acid mixture at concentrations as shown in section (2.2.5) but were stimulated with gastric juice.

As a result from this work I recommend that bile acid assessments should be made in lung samples as a regular test for the patients who have had lung transplantation and especially those transplanted for cystic fibrosis disease. CF patients may well go on to have elevated

levels of bile acids in the new lungs considering the CFTR related defects in bile acid transport. My data indicate that high concentrations of bile acids are toxic for the transplant PBECs and CF PBECs, but lithochlic acid at 5µmol/l and above is the most toxic as shown by cell viability.

6.3 Future Directions for Research

As we can see in this study 30% of lung transplantations in our local hospital are for cystic fibrosis and the results show that significant stimulated levels of IL-8, IL-6 and GMCSF are released from cystic fibrosis primary bronchial epithelial cell CF PBECs after stimulation by bile acids and gastric juice.

- In future studies we could use more cystic fibrosis patient samples for additional studies; we can use CF PBECs samples from explants and lung transplantations, studying the same patients' pre and post lung transplantation. In particular it would be useful to carry out a longitudinal study of bile acid levels with time in the newly transplantation lung.
- For the gastric juice studies, future studies could take samples from the same patients and analyse these for pathogens, pepsin activity, pH and total bile acid levels in the same time the patients have bronchoscopy, six times in the first year after transplant.
- Bile acids should be measured by sensitive methods such as tandem mass spectrometry. This is because the lowest level of detection with the colourimetric assay is around 5µmol/l.
- In this study we stimulated the samples with gastric juice for 24 hours so future studies could evaluate the effect of 48 hours stimulation with bile acids.
- For cell viability we can look for more accurate and high quality methods such as the methylthiazolyldiphenyl-tetrazolium bromide) assay.
- As we know it is hard to find enough patients from our local hospital population for study. We could in future contact other lung transplantation centres to organise multi centre larger studies.

- A study should be set up to investigate the possibility that bile acids level in the lung is a biomarker which may predict for chronic rejection (using the enhanced bile acid measurement technology).
- Future studies should identify whether bile acids are present in people with CF before and after transplantation in the lung.

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Appendices

From gastric aspiration to airway inflammation

I.A. Brownlee[1], A. Aseeri[1], C. Ward[2], J.P. Pearson[1]

ABSTRACT: *From gastric aspiration to airway inflammation. I.A. Brownlee, A. Aseeri, C. Ward, J.P. Pearson.*

The airways are poorly protected from potentially damaging agents contained within gastric contents. While digestive factors are obvious damaging agents, gastric aspiration may also deliver microbial agents, cytokines or food antigens to airway tissues. Direct damage or the triggering of the inflammatory cascade by gastric aspiration is believed to drive airways disease onset and/or progression.

Evidence exists from experimental models demonstrating direct instillation of damaging factors to a range of airways epithelia causes damage and/or an inflammatory response. Clinical longitudinal studies have also noted an association between the presence of biomarkers of reflux in airways samples and disease progression. A shared pathophysiology of many chronic airways diseases is a more negative intrathoracic pressure. Such changes would drive an increased abdominothoracic pressure gradient. These changes in respiratory mechanics mean that chronic lung disease patients may be predisposed to reflux and subsequent aspiration. Therefore, it appears that gastric aspiration and airways disease progression may be linked not solely as cause and effect, but seemingly within a vicious cycle.

A range of physiological factors govern both occurrence of gastric reflux into the pharynx/larynx and could also increase the susceptibility of certain individuals to disease progression. A range of long-term surgical and pharmacological intervention studies are necessary to test the benefit of such therapies in reducing disease progression or driving symptom improvement. Such studies may be hampered by the reliability of such therapies in halting gastric aspiration and the difficulty in the clinical or biochemical assessment of gastric aspiration.

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Introduction

The term aspiration refers either to the inhalation of food within the oropharynx or gastric contents that have moved up the oesophagus in retrograde fashion [1]. An association between aspiration and inflammatory airways disease was first suggested during the 1960s and 1970s (e.g. [2-5]). Aspiration by either route will lead to the presence of increased amounts of potentially damaging agents into the airways, which is hypothesised to drive the onset and/or progression of a wide spectrum of pathological conditions that affect the lungs and airways [6, 7]. During gastric aspiration, potentially damaging digestive factors from the stomach (via the oesophagus) pass into the relatively unprotected airways. Gastric aspiration is therefore hypothesised as being a prime causative candidate for almost every chronic upper airways disease. This hypothesis has proved difficult to test, due to both the difficulties of assessing the presence of reflux clinically or diagnostically [8-10], alongside the fact that pharmacological and surgical interventions for reflux can only reduce, but not abolish, the occurrence of gastric content reaching the oesophagus/airways.

The symptomology of airways disease is as divergent as the potential aetiologies. One common feature is the presence of an inflammatory response within the affected mucosal surfaces [11-15]. The process of inflammation involves a complex cascade of cellular, molecular and systemic events that are aimed at benefitting the clearance of noxious agents from the mucosal surface. In most pathophysiological cases, the inflammatory response appears to be in excess of the normal state, and is believed to play a role in disease progression. The inflammatory response is not necessarily in proportion to the damaging potential of the initiating agent, and can drive further damage to the surrounding tissues [16].

Previous studies would suggest a high incidence of gastric aspiration in chronic diseases of the lungs and upper airways [17-21]. In some cases, association have been made between measured occurrence of biomarkers of reflux and disease progression [22] or proxy measures of the inflammatory response [23, 24]. A number of observational studies have also noted an increased co-morbidity between oesophagitis and various airways diseases [25-27]. Perhaps the most commonly held beliefs are a) aspiration (particularly the reflux of

gastric contents) is a potential initial drive for disease progression and b) symptomatic individuals may have more frequent/injurious aspiration events than non-symptomatic individuals. While these hypotheses appear sound, they both neglect key issues within the association of aspiration and airway inflammation, and their impact on disease progression. The remainder of this review will discuss the interplay between gastric aspiration and airways inflammation in greater detail.

Aspirate content and lung inflammation

While direct aspiration of ingested food can present antigens or bacterial load into the airways, gastric aspiration has further potential modes of antagonism of the airways' mucosa. Firstly, the presence of digestive factors, including digestive enzymes, bile acids and other detergents (e.g. lecitihins) and gastric acid have all previously been shown to have the potential to damage airways mucosa directly [28-31]. The main digestive protagonists from gastric juice that have previously been suggested to drive mucosal damage are gastric acid, pepsin and bile acids.

Gastric acid and pepsin are secreted by the parietal cells and chief cells respectively in the stomach. These gastric secretions are important both in the early stages of protein digestion, but may also act as an important innate barrier to microbes entering the body orally [32-35]. Bile acids are produced by the liver conjugated to glycine or taurine and secreted into the small intestine where they act to emulsify dietary lipids to aid fat digestion and absorption [36, 37]. Small intestinal contents are believed to frequently reflux from the duodenum into the stomach, with well over 50% of GORD patients reported to experience the movement of mixed gastric and duodenal contents up the oesophagus [38]. A range of small intestinal enzymes could also end up in the stomach by this route. If they are not degraded or denatured during the retrograde passage from duodenum to stomach to the aerodigestive tract (e.g. trypsin is unaltered by exposure to pepsin at pH 4.0, but inactivated by exposure to pepsin at pH 2.0), they also have the potential to cause mucosal damage.

Aspiration of digestive products may also lead to an indirect drive for mucosal inflammation. Firstly, homogenised and partially hydrolysed foods may act as a more amenable substrate to bacterial species already occurring within the airways. Secondly, the hydrolysis and denaturation of dietary proteins during normal digestion could lead to the appearance of previously sequestered antigen. The potential mechanisms for damage by gastric aspirate are summarised in table 1.

Pharmacological therapies for acid suppression have been shown to greatly reduce oesophageal exposure to low pH [39, 40]. Such therapies will act to reduce the total volume of the gastric juice [41-43], but may act to effectively increase the concentration of pepsin, bile acids and other putatively damaging digestive factors due to the lower volume of gastric secretion. Further from this, recent studies have demonstrated an increased incidence of intestinal infection and communicable diseases following acid suppression [44-48]. This is due to an overgrowth of oral-type bacteria within the stomach [49] as a result of the decreased innate immunity with the removal of the acid barrier. Subsequent aspiration events under such therapies may therefore be of lower total volume and higher pH, but may have a higher concentration of other putatively damaging endogenous and microbial factors.

Direct damage of the airways mucosa by gastric aspiration is the most obvious trigger for an inflammatory response. However, a number of key routes for gastric aspiration-driven inflammation are also hypothesised. The majority of the airways epithelium is lined by a functional mucus barrier that acts to reduce mucosal exposure of damaging inhaled agents, as well as entrapping such agents and facilitating their removal through the process of mucociliary clearance [50, 51]. As such, efficient mucus barrier function plays a vital role in the innate defence of the airways [50]. Many airways diseases are characterised by mucus hypersecretion [52, 53]. This can be a result of increased fluid output, or increased release of mucin granules by the epithelial goblet cells and/or increased gland-based secretion (driven by secretagogues, such as IL-8). If the mucus layer becomes too rheologically thick or thin, mucociliary clearance is greatly reduced, leaving the underlying tissues more susceptible to damage and infection [50].

Inflammatory responses to damaging agents may also be partly driven by the presence of specific receptors within the epithelium. In the case of factors like bacterial lipopolysaccharides, such receptor-mediated pathways are fairly well researched [54, 55] and appear to be mediated by toll-like receptor activation and a subsequent inflammatory cascade The triggering of nociceptors such as the capsaicin receptor in the airways by low pH is also well documented [56, 57]. Evidence would also suggest that recurrent cough could also cause airways damage/act to mediate local inflammatory pathways. In a group of patients with chronic non-productive cough, a subset of individuals who did not have asthma or acidic reflux ("idiopathic" non-productive coughers) had elevated levels of Mast cells within their bronchoalveolar lavage fluid in comparison to non-smoking, healthy controls [58]. Recent preliminary data have also suggested that there may be specific receptors that are triggered by the presence of gastric juice factors, such as pepsin and bile [59, 60].

A summary of these pathways is suggested in figure 1 below.

Recent interest in the field has noted that retrograde movement of gas boluses from the stomach may also be an important route of gastric aspiration [61, 62]. While this would not deliver the same volume of gastric contents to the airways, it is believed that aerosolised vapour could act to coat airways mucosa. The aerosolised content would be expected to contain similar damaging agents to those outlined in table 1.

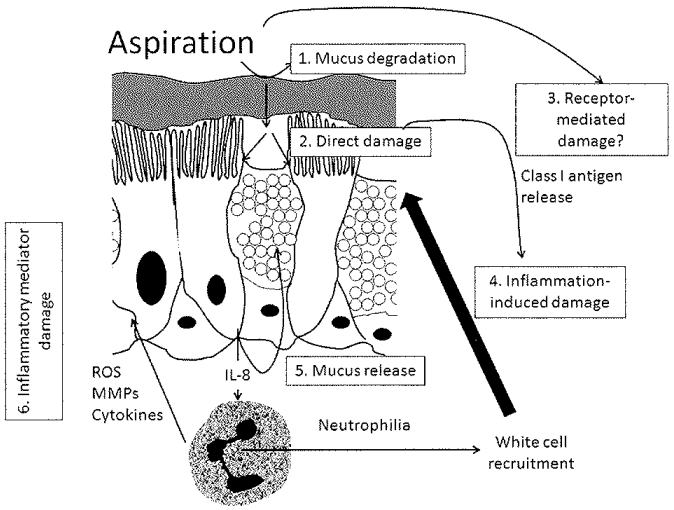

Fig. 1. - A summary of the hypothetical routes through which gastric aspiration could drive mucosal inflammatory responses within the airways. 1. Mucus degradation will potentially expose the underlying mucosa leading either to 2. Direct mucosal injury/insult from gastric aspirate, or indirect mucosal injury/insult from other damaging agents inhaled into the airways. As a result of putative receptor-mediated response, or a consequence of mucosal damage, an increased drive for white cell recruitment occurs, resulting in further 4. Inflammation-induced damage. Further from this, the airways epithelial cells respond to damage by releasing a range of local inflammatory mediators, including IL-8. These mediators drive neutrophil recruitment, as well as 5. Mucus release from goblet cells, and 6. Inflammatory-mediator damage driven by factors released by the recruited neutrophils. Mucus hypersecretion can result in loss of mucociliary clearance, leading to further exposure of the mucosa to damaging agents. Reactive oxygen species (ROS) and matrix metalloproteinase release will lead to further mucosal and epithelial damage from within.

Table 1. - Potential mediators of airways inflammation and damage within gastric contents

Directly damaging agents	**Agents released by digestion**
Digestive factors (i.e. enzymes, bile, acid)	Digested macronutrients
Ingested, salivary or gastric microbes	Antigen formed by dietary protein hydrolysis
Food particles	Microbe release from gastric bolus
Food antigen	

Reflux pathophysiology

Reflux of gastric contents is an episodic event in both the physiological and pathophysiological state [63]. 24-h ambulatory impedance monitoring of 72 healthy adults [64] demonstrated that the median number of gastro-oesophageal reflux events to occur in a day was 44, with a great deal of inter-individual variation (the 25th and 75th percentiles were 25 and 58 events respectively). These episodes are characterised by increased gastric motility, a transient reduction in lower oesophageal sphincter tone and increased intragastric pressure. Recent literature has focussed on a central role for transient lower oesophageal sphincter relaxations (tLOSRs) [65-69]. While evidence would suggest that tLOSRs are no more frequent in GORD patients than asymptomatic volunteers, the likelihood that GORD patients will reflux during the period of sphincter relaxation is almost twice as high [69]. While significantly higher occurrences of reduced lower oesophageal pressure are noted in GORD patients complicated with severe hiatal hernia [70, 71], it must be noted that this particular patient group is not indicative of GORD patients *per se* [63]. Functionally, reflux from the stomach to the oesophagus has the potential to occur whenever the intragastric pressure exceeds lower oesophageal sphincter pressure. Manometry studies have noted a higher pressure gradient across the lower oesophageal sphincter in GORD patients than non-symptomatic controls, with the difference in gradi-

ent owing to the a higher gastric pressure [72]. Recent assessments of gastrointestinal motility have also suggested that the position of the "acid pocket" (i.e. secreted gastric juice that sits above the meal bolus) in relation to the diaphragm [73] may also drive reflux. Further functional studies have also suggested that the occurrence of retrograde waves of peristalsis up the oesophagus appears to be propagated by the occurrence of tLOSRs [65], which could act to further increase aspiration events. A previous study in non-symptomatic volunteers noted that barostat distension of the stomach resulted in increased number of tLOSRs than distension to the same degree by an ingested meal [68]. This would suggest that the drive for gastric motility given by postprandial luminal content may reduce the frequencies of tLOSRs.

Intragastric pressure is controlled by a complex array of neurohumoral pathways that govern lower oesophageal and pyloric sphincter tone, gastric compliance, gastric secretion volume and gastric motility. Alongside vagal innervation, three main hormonal drives may govern all of these factors [74]. Cholecystokinin (CCK) release from the duodenal I cells is a major drive for reduced gastric emptying, while gastrin release from gastric G cells increases gastric mixing. Both of these factors could drive an increased intragastric pressure. At the same time, motilin release from intestinal enteroendocrine cells acts to increase the rates of gastric emptying, and thus would be expected to decrease intragastric pressure. Previous review have suggested the potential of these agents and their receptors as targets for reflux therapy [75, 76].

Reflux symptoms are prevalent in the majority of airways diseases. For instance, previous reports would suggest that around 80% of adult asthmatics have symptoms of reflux [77, 78]. It must be noted that the association between gastric aspiration and airways pathophysiology is often hypothesised to be a result of aspiration driving the disease process. However, the association between the two processes in not fully defined, and a number of researchers have suggested that the negative intrathoracic pressure caused by a result of airways obstruction and/or respiratory distress may act to drive reflux by increasing the likelihood of gastric contents refluxing into the oesophagus [78-80]. A preliminary temporal association study was carried out between episodes of coughing/wheezing in asthmatics and reflux occurrence, as assessed by pH-metry in 2001 [81]. Within this population group, reflux events preceded cough events by less than 2 minutes 40% of total cough events per patient, with only 6% of reflux events being preceded by cough events over the same time-course. These figures were elevated to 50% and 12% respectively when a five minutes inter-event time cut-off was applied. In more recent studies where impedance monitoring has been used to assess reflux in paediatric asthma [82], cystic fibrosis [83] and chronic cough [84] patients. In all cases within these studies, it must be noted that an appreciable number of cough events were associated with reflux events within two of these studies (26.6% total events within a 5-minute window [82] in paediatric asthmatics and 30.6% within a two-minute window in chronic cough patients [84]). Such temporal studies may not best represent the interplay between intrathoracic pressure and reflux/aspiration. In terms of intrathoracic pressure mediating reflux, cough represents a short-term negative intrathoracic pressure change, as opposed to a more uniform, long-term change towards lower pressure seen in disease processes where there is chronic lung obstruction. In terms of aspiration driving airways symptoms, cough is an immediate response, believed to be triggered by a range of cough receptors [85]. As there are previous reports of refluxers having reduced laryngeal sensitivity [86-89], it is perhaps unsurprising that the cough reflex is not always elicited in response to gastric aspiration. Over a short time scale, gastric aspirate may not be damaging enough to the airways mucosa to drive a relevant clearance response such as cough. As previously discussed, aspiration of aerosolised gastric contents may also lead to damaging material entering the airways, that would be considerably less likely to elicit an immediate cough reflex than a large volume reflux event. One previous study used a canine model to assess the impact of balloon-catheter-induced upper airways obstruction on reflux occurrence (assessed by pH-metry). Within this study, there was a strong and significant positive correlation ($R = 0.928$, $P = 0.023$) between the change towards a more negative intrathoracic pressure and percentage of time proximal pH was below 4 in five dogs [90]. While this study is low in numbers, it may model how long-term pressure changes affect gastric aspiration.

From the above, there is evidence that the development of an abdominothoracic pressure gradient may drive further reflux and gastric aspiration, with consequent worsening of disease symptoms. While there is no conclusive proof as to whether airways symptoms drive reflux or vice versa, it is perhaps more likely to consider that these events could conspire to worsen chronic disease progression through a vicious cycle [91]. Preliminary longitudinal data from our own group would suggest that reflux is a common occurrence post lung transplantation, even at time-points when lung mechanics should be close to normal [92].

Our group has consistently shown that pepsin, as a marker of gastric aspiration, is elevated in lung transplant recipients [21, 22] and has been associated with neutrophilic airway inflammation and pathologist graded acute rejection [22]. Acute airway rejection is known to be associated with airway inflammation and to constitute a risk factor for chronic allograft dysfunction, recognised physiologically by fixed airflow limitation (Bronchiolitis Obliterans Syndrome: (BOS) [93]. The pathology underlying BOS involves inflammation and airway remodelling and fibrosis [93-95]. This pattern of airway damage, as in the case of reflux and aspiration, is implicated in the progression of a number of airways diseases including asthma and COPD [96]. In lung allografts, this can be very aggressive and is the main reason for the chronic loss

of lung allografts [93]. In other more common lung diseases such as COPD the pattern of airway injury develops over a longer time frame.

One potential link between aspiration and airways remodelling is perturbation of TGFβ homeostasis. TGF is a pleiotropic growth factor (reviewed elsewhere [16, 97]) that is implicated in both airway repair and pathophysiology, and which is elevated in airway disease including BOS post transplantation [98].

We have shown that TGFβ can initiate epithelial mesenchymal transition (EMT) in epithelial cells from lung allografts [99]. In EMT, epithelial cells lose epithelial characteristics adopting a mesenchymal, fibroblast phenotype which may cause airway fibrosis through the production of collagen. The process of EMT has been documented in lung development, metastatic disease and a range of other settings involving organ fibrosis [100]. Consistently TGFβ is recognised to be a prototypical drive for EMT and it is therefore of interest that bile acid challenge of human airway epithelial cells has been shown to lead to the release both of inflammatory cytokines and TGFβ [101]. Most TGFβ is held in an in-active latent form. This may be activated through protease release from inflammatory cells, as well as directly by stomach acid. Type IV collagenases such as MMP-9 are also released in the inflammation with which aspiration is associated. Damage of airway epithelial basement membranes, which contain type IV collagen, is also known to promote EMT [100].

In addition to *ex vivo* studies on primary airway epithelial cells suggesting the potential for EMT in lung allografts, we have also shown that airway biopsies from both stable lung transplant recipients, express markers of the EMT proteome [99, 102]. Overall aspiration may be linked both to airways inflammation and remodelling/fibrosis. This potential linkage requires further appropriate translational research [103].

Increased susceptibility to aspiration-induced airways damage

Previous studies in non-symptomatic individuals would suggest that reflux into the oesophagus and even the pharynx is a frequent occurrence within normal life [104-107]. It might therefore be expected that gastric aspiration is also an occurrence that most individuals will have experienced. Assessment of bronchoalveolar lavage fluid in individuals asymptomatic of upper airways disease suggests that biomarkers of gastric aspiration can occur at low but detectable levels in 2 of 4 healthy individuals [22]. Some background levels of pepsin may be present in the broncho-alveolar lavage fluid of healthy individuals, as pepsinogen C (a precursor of human pepsin 5 and 6) has previously been reported to occur in type 2 alveolar cells [108]. While this data would suggest that gastric aspirate does not occur as frequently or to the same degree in a healthy population, it i is important to consider that there may be other factors that predispose some individuals to airways injury as a result of gastric aspiration, either as a result of increased volume of aspirate reaching the airways, or due to a lack of defence mechanism against aspirate-induced inflammation or injury. Data from the authors' laboratories would suggest that primary cell cultures of lung epithelia from different individuals react differently to insults from putatively damaging agents in gastric aspirate, such as the secondary bile acid lithocholic acid. While all cultures appear to be close to completely unviable at the highest concentration of lithocholate used (i.e. 50 μM), the drop-off of viable cells numbers follows a different gradient between individuals.

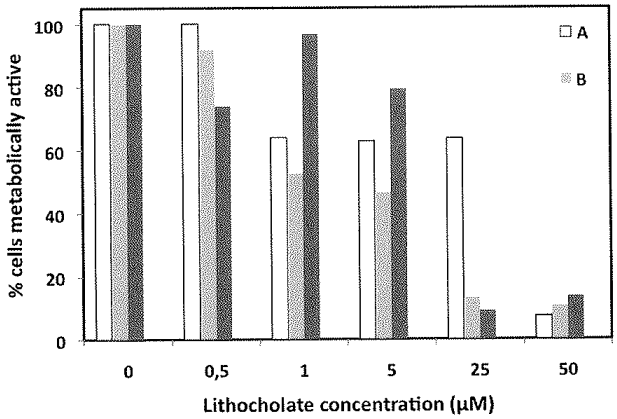

Fig. 2. - The effect of varying levels of lithocholic acid on cell viability in primary lung epithelial cultures from 4 separate lung transplant recipients (here labelled A - C). 6 - 15 months post-transplantation. Cell viability (i.e. % cells metabolically active) was assessed by CellTiter-Blue cell viability assay (Promega, Madison, WI) [115, 116].

The data above are suggestive of genetic variability in the inflammatory response caused by such mediators of damage. Previous studies have also suggested that certain patients groups who are more or less susceptible to reflux-mediated disease tend to show specific genotype [17], or differential expression of specific genes involved in airway protection [28, 109, 110]. While such studies would give credence to genetic predisposition as an important factor in aspiration-driven disease progression, it is important to consider that "susceptibility" may also come about as a result of temporal, anatomical or physiological changes in airways functionality.

In general, increased airways damage or inflammation as a result of aspiration is likely to be as a result of decreased defence against aspiration, or reduced clearance rates of damaging agents that are delivered to the mucosa. As described above, previous studies have suggested an elevated threshold for laryngeal sensitivity in refluxers [86-89], which is higher than in asymptomatic individuals [111]. A less sensitive larynx would not perform its respiratory defence mechanisms [112, 113] as well as normal upon exposure to reflux. As a result, if the larynx were to insensitive to contact with gastric contents, gastric aspiration would be more likely to occur. Factors such as vagal damage (e.g. following lung transplant surgery [114]) may also drive an increased incidence of gastric aspiration by affecting aerodigestive tract motility, and may also reduce cough and mucociliary clearance. Previous events resulting in damage or an inflammatory response in the airways could lead to an increased likelihood of subsequent damage as a result of gastric aspiration insult. This could be as a result of a loss of the innate barrier function of airways mucus, or because the underlying mucosa has become easier to penetrate. In a similar fashion, loss of effective mucociliary clearance will result in increased mucosal exposure to the damaging agents in gastric aspirate.

Future work

Current evidence is indicative of an association between airways disease progression, inflammatory processes and gastric aspiration. The next step from a clinical perspective may be the development and implementation of pharmacological or surgical interventions in the relevant patient groups, targeted at reducing gastric aspiration rates with objective measures of airways disease as the primary outcomes in adequately powered, controlled studies. In conditions where improvement of disease symptoms is unlikely (e.g. idiopathic pulmonary fibrosis), a reduction in disease progression rates may be the most relevant outcomes to measure. Such studies will be demanding and necessitate the collaboration of both gastroenterology and pulmonary specialists, and may be hampered by the difficulties in assessing occurrence of gastric aspiration/reflux clinically. Biochemical analysis of biomarkers of gastric aspiration may be a useful predictive surrogate of gastric aspiration, but further work is necessary to characterise how long such biomarkers occur within the airways following an aspiration event. Methodological standardisation is an important requirement.

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British Lung Foundation

Mr Ali Aseeri
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Dear Mr Aseeri,

I am delighted to inform you that you have been awarded a British Lung Foundation / Allen & Hanburys Travel Fellowship to attend the European Respiratory Society Annual Conference in Barcelona, Spain, 18-22 September 2010.

This is subject to confirmation that you still plan to attend the conference to present your work and you are able to accept the award.

Your cheque for £750, which should contribute towards your registration, accommodation and travel will be presented to you at a drinks reception sponsored by Allen & Hanburys in Barcelona.

With best wishes.
Yours sincerely,

Ian Jarrold
Research Manager