UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE
Clinical and Experimental Sciences Unit

The role of gastro-oesophageal reflux in airway inflammation and symptoms of asthma

by

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Thesis for Doctor in Philosophy

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Abstract

FACULTY OF MEDICINE
Clinical and Experimental Sciences Unit – Respiratory medicine
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THE ROLE OF GASTRO-OESPHAGEAL REFLUX IN AIRWAY INFLAMMATION AND SYMPTOMS OF ASTHMA

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Gastro-oesophageal acidic reflux disease (GORD) is associated with treatment-resistant asthma and recurring asthma exacerbations. Trials of acid suppressants have not shown benefit in asthma management. This could imply that: 1) GORD plays no role in asthma, 2) reflux may be relevant only in some subsets asthmatics or 3) weakly/non-acid reflux, that continues unabated by PPIs, is an important factor in asthma. I undertook a detailed assessment of clinical and pathobiological changes in patients with severe asthma in relation to GORD applying novel, objective techniques for better patient stratification to identify those in whom GOR is clinically relevant. My work was in 3 parts. In **Part 1,** I performed a retrospective analysis of clinical data and sputum samples from the UBIOPRED programme, to seek associations between GOR (established by history) and a host of novel potential biomarkers identified by state-of-the-art 'omics technologies. The study showed that severe asthmatics have a higher prevalence of GORD and suffer from worse asthma control and quality of life. This is associated with the finding of proteomic biomarkers in patients with active GORD symptoms, including increased levels of Lipocalin-1 which has role in mucosal defence, the role of which requires further elucidation.

In **part 2,** I designed a prospective, cross-sectional study, followed by an open label sequential medicine intervention study. First, I characterized GORD in detail using 24hr pH/impedance monitoring. I used a validated cough meter, full clinical and pathobiological assessments in healthy individuals and severe asthmatics with and without GORD. At baseline, all participants were assessed by standard asthma and GORD questionnaires, pulmonary function, sputum analysis for diff. cell counts, lipid-laden macrophages (LLM), and pepsin in both throat clearate and saliva (to detect GOR). These were then repeated with each trial of anti-reflux treatments. I performed bronchoscopy for stigmata of reflux, with laryngeal and bronchial biopsies and BAL for additional comparisons of pathology in patients with and without GOR. I showed a significant improvement in asthma control and symptoms with treatment of GORD. Treatment of GORD in severe asthmatics does control acid reflux but has little or no impact on the weakly acidic and non-acid reflux or the proximal extent of the refluxate as detected by 24-hour pH/impedance. I also observed that Pepsin measured in throat clearates, is a good predictor of GORD in asthma but does not respond to GORD treatment. This also suggests a role of weakly acid and non-acid reflux as well as proximal reflux, and whether this requires further treatment, remains to be elucidated. Sputum and BAL diff. cell counts and immunohistochemical analysis of laryngeal and bronchial biopsies between severe asthmatics with and without GORD did not show any differences. I concluded that these biomarkers lack the sensitivity to show any changes that may have occurred as a result of GORD.

In **part 3**, my study enabled collaboration with Dr Jeanne-Marie Perotin Collard who developed an in vitro model of GORD using differentiated BECs from healthy and severe asthmatics exposed to a combination of pepsin, low pH and bile acids using a multiple challenge protocol. The clinical data bronchial brushings, biopsies and BAL samples from part 2 were used in this study. RNA-sequencing of bronchial brushings from controls and severe asthmatics with and without GORD was done. Exposure of BECs to the refluxate (as part of the MCP) caused structural disruption, increased permeability, IL-33 expression, inflammatory mediator release and changes in gene expression. The cultures from severe asthmatics were significantly more affected than those from healthy donors. IL-33 expression was increased in bronchial mucosa in severe asthmatics with GORD. RNA-sequencing of bronchial brushings from this group identified 15 of the top 37 dysregulated genes found in MCP treated BECs, including genes involved in oxidative stress responses. These results suggest the need for research into alternative therapeutic management of GORD in severe asthma.

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1. Tariq K, Schofield JPR, … Dimitrov B, Djukanović R; U-BIOPRED Study Group. **Sputum proteomic signature of gastro-oesophageal reflux in patients with severe asthma**. Respir Med. 2019 Apr;150:66-73. doi: 10.1016/j.rmed.2019.02.008.

2. Perotin JM, Wheway G, Tariq K, Davies DE, Djukanovic R. **Vulnerability to acid reflux of the airway epithelium in severe asthma**. Eur Respir J. 2022 Jan 7:2101634. doi: 10.1183/13993003.01634-2021.

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Definitions and Abbreviations

AET	Acid exposure time
ACQ	Asthma control questionnaire
ADH	Alcohol dehydrogenase
AQLQ	Asthma quality of life questionnaire
BAL	Bronchoalveolar lavage
BiD	Twice daily
BOS	Bronchiolitis obliterans syndrome
BMI	Body-mass index
BTS	British Thoracic Society
CD	Crural diaphragm
CE	Collision energy
CFV	Contractile front velocity
CI	Contractile integral
COPD	Chronic obstructive pulmonary disease
DCI	Distal contractile integral
DL	Distal latency
DTE	Dithioerythritol
ECG	Electrocardiography
ECP	Eosinophil cationic protein
NGAL	Neutrophil gelatinase associated lipocalin
ERS	European Respiratory Society
ENO	Enolase
ESS	Epworth sleep score
EU	European union
FENO	Fractional exhaled nitric oxide
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
GABA	Gama amino-butyric acid
GMA	Glycol methacrylate
GINA	Global Initiative for Asthma
GOR	Gastroesophageal reflux
GORD	Gastro-oesophageal reflux disease
HADS	Hospital anxiety & depression score

HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HRM	High resolution manometry
ICS	Inhaled corticosteroids
Ig	Immunoglobulin
IL	Interleukin
IMI	Innovative Medicines Initiative
IMS	Ion mobility spectrometry
IRP	Integrated relaxation pressure
IQR	Inter-quartile range
LCM	Leicester cough monitor
LC	Liquid Chromatography
MS	Mass spectrometry
LABA	Long acting beta-agonist
LFD	Lateral flow device
LLM	Lipid laden macrophage
LLMI	Lipid laden macrophage index
LOS	Lower oesophageal sphincter
LPR	Laryngopharyngeal reflux
MHRA	Medicines and Healthcare products Regulatory Agency
MID	**Minimum important difference**
MLR	Multivariate logistic regression
MRI	Magnetic resonance imaging
MW-U	Mann-Whitney U analysis
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
NICE	National Institute for Health and Care Excellence
NIHR	National Institute for Health Research
NRES	National Research Ethics Service
OCS	Oral corticosteroids
OD	Once daily
OGJ	Oesophago-gastric junction
OSA	Obstructive sleep apnoea
PEF	Peak expiratory flow
PEFR	Peak expiratory flow rate
PFT	Pulmonary Function tests
pH	Negative log of hydrogen ion concentration

pMDI	Pressurised metered dose inhaler
ppb	Parts per billion
PPI	Proton pump inhibitors
PY	Pack years
RBRU	Respiratory Biomedical Research Unit
SABA	Short acting beta-agonist
SGRQ	St. Georges respiratory questionnaire
SIGN	Scottish Intercollegiate Guidelines Network
SNOT20	Sino-nasal outcome test - 20
TDS	Three times daily
TLOSR	Transitory lower oesophageal sphincter relaxation
U-BIOPRED	Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes
ULR	Univariate logistic regression
UPLC	Ultra Performance Liquid Chromatography
UOS	Upper oesophageal sphincter
VAS	Visual analogue scale

Chapter 1 Introduction

My research project has been designed with the aim to elucidate the role of gastro-oesophageal reflux disease (GORD) in severe asthma. My thesis is based on two areas of study. First, I collected and analysed data from the large, multicentre collaborative U-BIOPRED study for clinical and biological associations of GORD in severe asthma. Second, I studied well characterised severe asthmatics with and without GORD in comparison with healthy controls. Severe asthma has been defined for the purpose of this research project as per the BTS/SIGN asthma guidelines at step 4 to Step 5 [1]. My work involved objective characterisation of GORD and analysis of sputum, bronchial and laryngeal biopsy samples from the recruited patients to describe the inflammatory profiles in the context of GORD in severe asthmatics. This work is ultimately expected to help with clinical management of severe asthmatics with reflux.

1.1 Asthma

Asthma has been described in the BTS/SIGN asthma guidelines as a collection of more than one symptom from the list of wheeze, breathlessness, chest tightness and cough in combination with variable airflow obstruction. The definition includes airway hyperresponsiveness and airway inflammation, with the aim to encompass the developing understanding of the heterogeneity of asthma and increasing knowledge of various endotypes and phenotypes of the disease [1,2].

Asthma prevalence in the UK is estimated to be around 5.4 million [3]. Worldwide, asthma is estimated to affect over 330 million people with over 1000 deaths per day [4-6]. Studies have shown a significant relationship between asthma severity and high costs of healthcare and non-healthcare parameters in these patients [7]. Severe or refractory asthma constitutes approximately 5-10% of asthma patients and their disease accounts for a significant proportion of the healthcare resources directed towards asthma, making this a very important target population to manage effectively [8 9].

1.1.1 Co-morbidities and associations with asthma severity

Factors contributing to the clinical severity of asthma have been described in detail in multiple studies. They include environmental factors (e.g. pollution, allergens, smoking and occupational exposure), drug effects (e.g. aspirin, NSAIDs, beta-blockers, ACE inhibitors), psychological and psychiatric disorders, endocrine factors (e.g. gender preponderance, pregnancy, thyroid dysfunction etc.) and comorbidities such as GORD, chronic rhinitis, nasal polyposis, sinusitis, obesity, sleep apnoea and vocal cord dysfunction[8,10,11]. In their study of 136 difficult to treat asthma, ten Brinke et al [12] investigated the association of 13 clinical and environmental factors

which may have an impact on the frequency of exacerbations. They showed that gastro-oesophageal reflux was significantly associated with frequent exacerbations with an odds ratio of 4.9 (CI 1.9-17.8) (see figure below)[12].

Figure 1-1: Risk factors associated with asthma exacerbations
(taken from Ten Brinke et al [12])

TABLE 5 Odds ratios (ORs) for factors potentially associated with frequent exacerbations in difficult-to-treat asthma

	Adjusted OR# (95% CI)
Psychological dysfunctioning	10.8 (1.1–108.4)
Recurrent respiratory infections	6.9 (1.9–24.7)
Gastro-oesophageal reflux	4.9 (1.4–17.8)
Severe chronic sinus disease	3.7 (1.2–11.9)
Obstructive sleep apnoea	3.4 (1.2–10.4)
Hormonal influences	2.8 (0.5–15.8)
Hyperthyroidism	1.9 (0.2–19.6)
Occupational sensitisers	0.7 (0.2–2.1)
Poor inhaler technique	0.6 (0.1–2.9)
Food allergens	0.6 (0.1–3.5)
Ongoing allergen exposure	0.5 (0.2–1.3)
Relative immune deficiency	0.4 (0.1–1.7)
Drugs	0.2 (0.1–1.9)

CI: confidence interval. #: OR adjusted for age and asthma duration.

Management of the above factors constitute a crucial part of overall asthma management in addition to standard medical therapy.

1.2 Gastro-oesophageal reflux disease (GORD)

The Montreal definition and classification of GORD issued in the Global Consensus Group statement [13] defines GORD as *"a condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications."*. Systematic reviews of the epidemiology of GORD, the condition was defined as at least weekly symptoms of heartburn and/or acid regurgitation. The prevalence of GORD in the western world is between 10 and 20% of the population [14]. In 2006, The Montreal definition and classification consensus statement took into account all parts of the world when confirming this estimate [13].

1.2.1 GORD and extra-oesophageal co-morbidities

GORD has been associated with a host of extra-oesophageal complications, which adds to the complexity of the diagnosis and management of both GORD and the extra-oesophageal effects. The

reported extra-oesophageal co-morbidities linked to GORD include a mix of established associations and proposed associations as shown in a figure from Labenz et al[15].

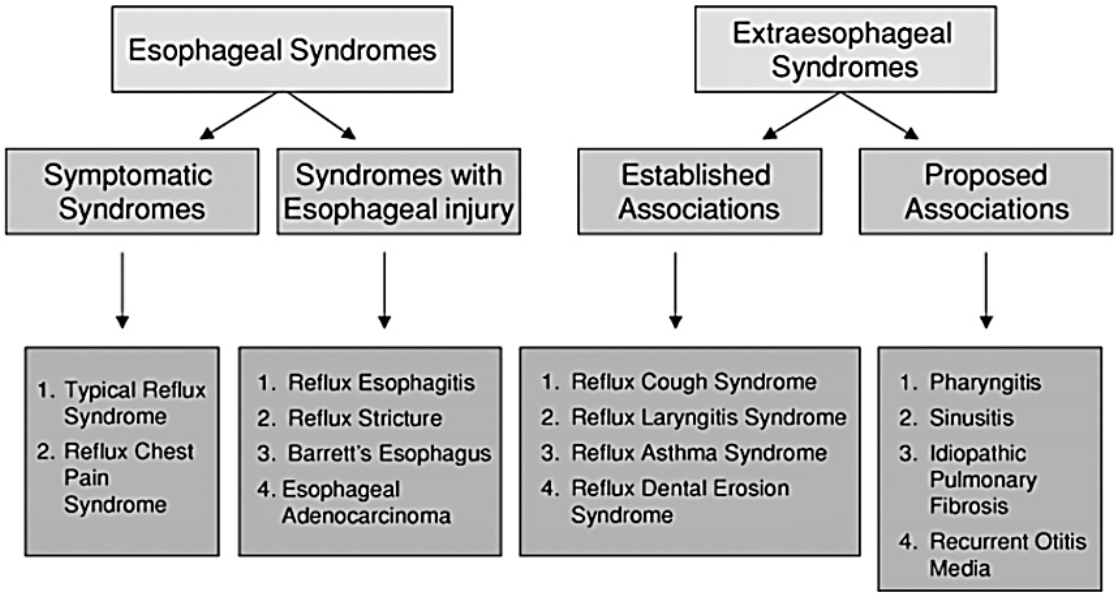

Figure1-2: Extra-oesophageal effects of GORD [15]

In the *ProGERD* study, Kulig et al observed a significant association between quality of life and GORD. They found a significant and clinically relevant improvement in general and specific quality of life after a period of treatment with PPI, even as short as 2 weeks [16,17]. GORD has been associated with upper airway pathological processes like rhinosinusitis and otitis media, particularly in the paediatric population [18-20]. Similarly, associations have been found with lower lung diseases such as asthma, chronic cough, bronchiectasis, interstitial lung disease [15,21]. Hurst et al, reporting on the ECLIPSE study, showed an independent association of GORD with frequent asthma exacerbation phenotype[22]. Similar assertions have been made in other studies in relation to frequent exacerbation in COPD [23]. Its role has been investigated with interest in cystic fibrosis. Moreover, it is considered important to control GORD effectively in transplanted lungs as GORD has been associated with Bronchiolitis obliterans (BOS) which is a significant factor for the survival of the allograft [21,24-27].

1.3 Association between GORD and asthma

GORD has been associated with asthma for a long time. In large cohort of over 100,000 US army veterans, El-Seraag et al [28] showed the association of GORD with airways diseases, with asthma being one of the major co-morbidities. More recently, in their study of over 1500 patients Emilsson et al [29] have shown an association of persistent nocturnal GORD with asthma. Additionally, they found that persistent nocturnal GORD was associated with a new diagnosis of asthma, suggesting possible causality. Several large asthma cohort studies have shown a significant relationship between severe or difficult to control asthma with GORD [9,12,30-32]. In many studies GORD has been shown to be a predictor of uncontrolled asthma [33] and GORD remains one of the risk factors for recurrent asthma exacerbations [12,34].

For many years, anti-reflux therapies such as PPI and H2 receptor blockers have been used to treat GORD, including in asthmatic patients. Even though these drugs show a remarkable effect on the symptoms of GORD, they have a modest effect, if at all, on symptoms of asthma or pulmonary function [34-37]. Littner et al studied 207 asthma patients in a randomized, double-blind placebo-controlled trail of Lansoprazole 30mg BiD and showed that this PPI decreased the overall frequency of asthma exacerbations and increased the time to first exacerbation [34]. This effect was noted particularly in patients receiving more than one asthma therapy. A large study of 412 inadequately controlled asthmatics conducted by the American Lung Association did not find any benefit of controlling GORD with esomeprazole 40mg BiD [35]. In their trial of esomeprazole 40mg OD and BiD vs placebo Kiljander et al found only a minor benefit in FEV_1 (+0.09L and +0.12L for OD and BiD dose, respectively) and asthma related quality of life with treatment versus placebo [37]. A further study by the same group could not reproduce the benefit in FEV_1 or quality of life with PPI treatment compared to fundoplication [36]. The results from these studies, treating GORD with PPI in severe asthmatics, have not been consistent and that may reflect a poor understanding of the pathophysiology of GORD affecting the airway.

1.3.1 Proposed mechanisms of GORD in lung diseases

Transient lower oesophageal sphincter relaxation (TLOSR) and LOS pressure insufficiency are the accepted mechanical defects underlying GOR. The presence of a hiatus hernia changes the position of the lower oesophageal sphincter (LOS) and, as a result, the frequency of TLOSR is increased, which, in turn, increases GOR events [15,21]. Grossi et al reported a significant association of TLOSR with distal reflux but could not find a relationship between proximal reflux and TLOSR [38]. In chronic airways diseases, including asthma, the frequent use of bronchodilators for e.g. β-agonists and theophylline is associated with increased likelihood of GORD [39,40]. While these agents act on smooth muscle to achieve bronchodilation, they also have an effect on the LOS tone, causing relaxation

and, thereby, leading to reflux [39,40]. Studies have shown that β-adrenergic stimulation leads to decreased tone in the LOS, while α-adrenergic stimulation is associated with increased LOS tone [41]. In lung transplant patients, the use of immune suppressive therapy, such as calcineurin inhibitors, e.g. tacrolimus and cyclosporin, is associated with gastroparesis, thus leading to reflux. There is evidence to suggest that airway obstruction increases TLOSR and, thereby, reflux as a result of hyperinflation of the lungs that decreases the efficiency of the crural diaphragm and/or by affecting the pressure gradient across the LOS [42,43]. Similarly, lung transplantation itself may be associated with iatrogenic injury to the vagus nerve during surgery which can also lead to reduced LOS tone [25,27,44].

The mechanism(s) whereby the airways are exposed to refluxate and the pathophysiology behind the effects that are attributed to GORD in asthma have been the subject of a number of studies [15,21]. Direct exposure of the upper airways to refluxate from the stomach may take place in the form of aspiration or micro-aspiration of stomach secretions. Alternatively, mixed gaseous-liquid refluxate or aerosol inhalation during gaseous or mixed gaseous-liquid reflux events can result in exposure of the respiratory tract to gastric contents. The refluxate may consist of acid and/or bile. The combination of acid and bile is associated with much higher incidence of mucosal injury and LOS/oesophageal dysfunction compared to acid alone and changes are uncommon with reflux of bile alone [45]. Direct exposure may lead to local inflammation in the laryngo-pharyngeal area, which may lead to bronchial hyper-responsiveness or susceptibility to infection [46,47]. Neural reflex mechanisms linking the oesophagus and the airways on exposure to the refluxate and the extra oesophageal effects of such neural stimulation have also been proposed as important mechanisms [48,49].

1.3.2 High Resolution Manometry (HRM)

Manometry of the oesophagus helps to study the main components of oesophageal function that results in transportation of the swallowed bolus to the stomach and prevention of reflux of stomach contents into the oesophagus and/or upper airway. There is a complex interplay between the upper Oesophageal sphincter (UOS), the body of the oesophagus and the lower oesophageal sphincter (LOS). Manometry measures pressure changes due to muscle contractions or relaxation in the oesophagus in relation to time [50]. High-resolution manometry (HRM) is a significant improvement on the conventional standard oesophageal manometry. There is a significantly higher number of pressure sensors placed in very close proximity in HRM catheters compared to standard manometry catheters. This increases the sensitivity of the sensors and in combination with highly advanced plotting algorithms the data are displayed in coloured topographical plots which makes it easier to ascertain the physiology and any pathological changes in the plots [51,52]. Due to the high resolution topographic plots showing dynamic images of focal areas of high pressures (UOS, peristalsis and

LOS) in the oesophagus, it is easier to visualize the LOS and UOS and to follow the peristaltic waves from the beginning to the end and the relaxation of the LOS before and after the bolus is transported into the stomach (See Figure 1-3) [53-55]. Oesophageal manometry is, thus, useful to locate the LOS for accurate pH catheter placement and provides information about defects in oesophageal peristalsis and propulsion of bolus.

The normal physiological oesophageal motility criteria are based on the following terms:

Integrated relaxation pressure (IRP) is lowest average pressure for 4 seconds within the deglutitive relaxation window (Figure 1-4) [56,57].

Distal contractile integral (DCI) is a combination of length, contractile vigour and duration of contraction of the first 2 sub-segments of the distal oesophageal segment contraction (Fig 1-5)[57].

Distal latency (DL) and **contractile front velocity (CFV)** are tools to evaluate propagation of oesophageal pressure waves where CFV is a measure of peristaltic velocity in smooth muscle oesophagus (S2 and S3) and DL is the time from opening of the UOS to the deceleration point of the peristaltic wave in the smooth muscle oesophagus [56,57].

Other important components of the oesophageal motility are oesophageal junction morphology (LOS-crural diaphragm (LOS-CD) separation), oesophago-gastric junction (OGJ) tone and OGJ contractile integral (OGJ-CI) [58-61].

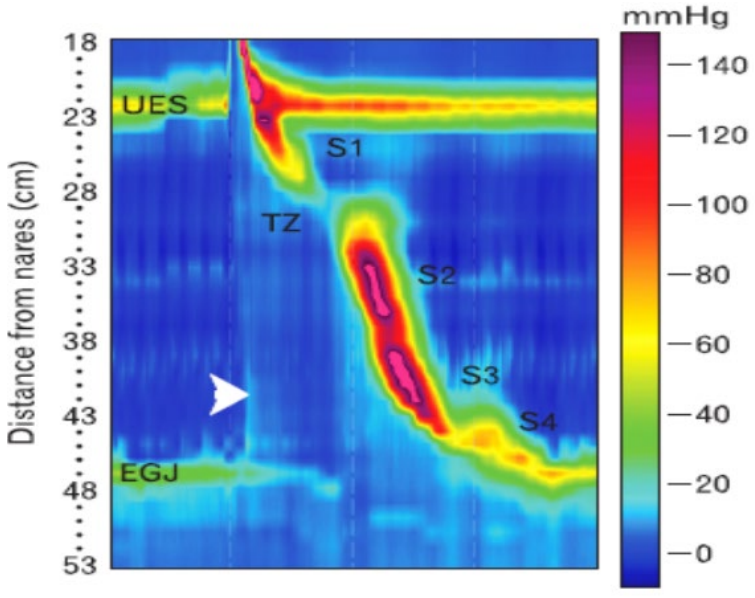

Figure1-3: Clouse plot or Oesophageal pressure topography from high resolution manometry of a normal swallow.

S1 is the striated muscle oesophagus, S2 and S3 are proximal and distal ends of the smooth muscle distal oesophagus and S4 is the LOS repositioning itself at the resting position following a swallow. Pressure in the swallowed bolus (intra-bolus pressure) is indicated by the range of colours from blue to purple indicating a rise or fall in pressure. Taken from Conklin et al. [56].

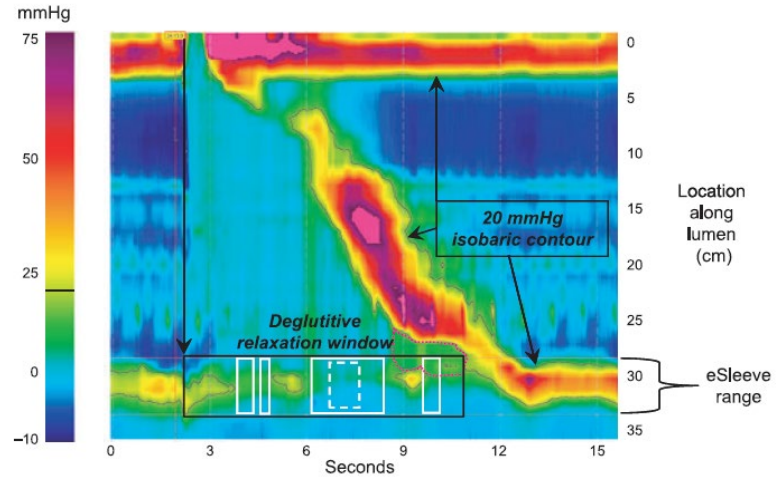

Figure 1-4: Integrated relaxation pressure (IRP) is determined within the deglutitive relaxation window by calculating the average of lowest pressures over 4 continuous or dis-continuous seconds. IRP is a measure of Oesophagogastric junction function during swallow. Taken from Pandolfino et al. [57]

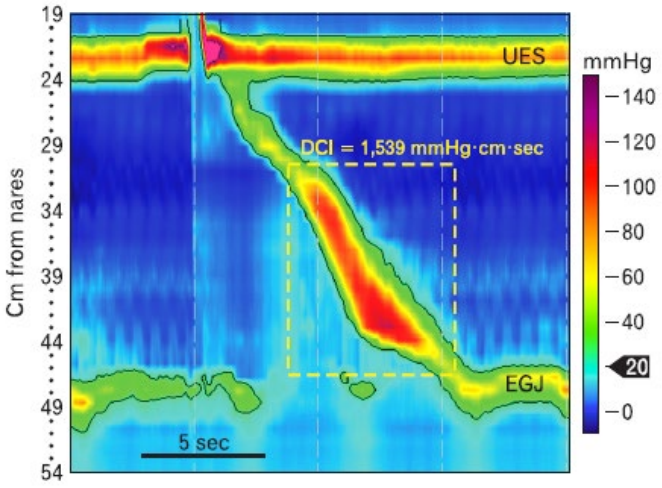

Figure 1-5: Distal contractile integral (DCI) is produced by segments S2 and S3. It determines the robustness of peristalsis in the smooth muscle oesophagus. Taken from Conklin et al. [56]

1.3.3 24-hour pH and Impedance monitoring

Although conventional pH testing is an effective method to investigate GORD in most patients, it has limitations. It measures pH in the distal oesophagus and records drops in pH to < 4.0 over a 24 hour period [62]. It uses agreed criteria to objectively measure reflux, such as total number of reflux episodes, duration of exposure and their relation to position of the body (supine or upright). Additionally, the DeMeester score is calculated to provide a summary marker for quantification of pathological reflux [55,63,64]. However, it does not provide any information about the extent of reflux reaching the proximal parts of the oesophagus, the quantity of the refluxate and any non/weakly acidic nature of the refluxate [62]. The latter measurements are now possible using impedance, a method first described in 1991 by Silny et al as a step up in physiological and pathological study of the gastro-intestinal tract. The procedure measures electrical impedance, which is a measure of resistance to alternating electrical current between closely placed electrodes in the impedance catheter placed intra-luminally in the oesophagus via the intranasal route [65]. The impedance levels measured in the oesophagus depend on the contents in the oesophagus. Thus, impedance levels are lower in the presence of liquids and higher in the presence of a gaseous mixture in the oesophagus. In case of liquids, it can be further elaborated into swallows or reflux episodes based on the patterns of impedance changes across the sensors along the length of the oesophagus [66,67].

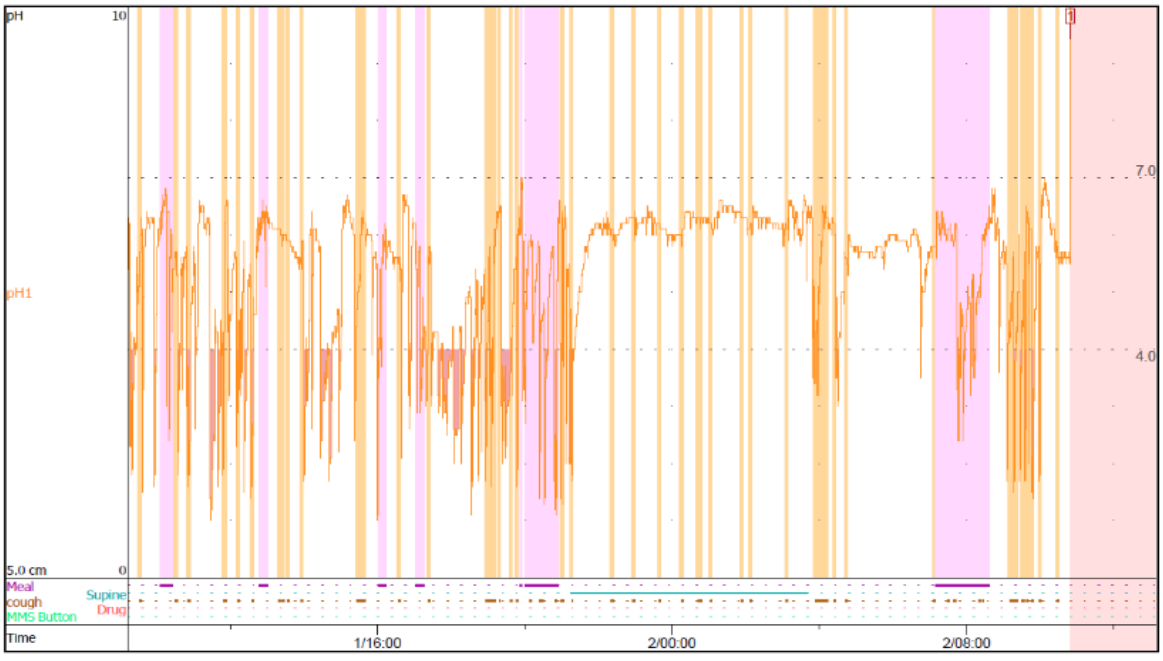

Figure 1-6: pH and Impedance trace of a participant with pathological reflux

Since the impedance changes with liquids and gas are highly sensitive irrespective of the volume of the bolus, volume quantification of the refluxate is not a reliable measurable metric for assessing

the volume of refluxate [68]. Impedance testing also provides information about the proximal extent of the reflux in the oesophagus due to the multiple sensors recording impedance data in addition to the pH sensors [69,70]. 24-hour pH and Impedance testing also gives the added benefit of recording the pH of the refluxate across the range of pH from acid reflux (pH < 4), weakly acid reflux (pH 4-6) and non-acid reflux (pH > 6) [62]. See Figure 1-6 for a sample 24-hr trace.

The proximal extent of the refluxate, diagnosed by using impedance measurement in the oesophagus, can provide evidence with regards to the risk of extra oesophageal complications of GORD, such as cough and asthma. Overall, when compared to standard pH monitoring, 24-hour pH and impedance provide significantly more insight than pH monitoring alone about how the reflux is affecting the patient and gives valuable information about silent reflux episodes as well as weakly acid and non-acid reflux episodes (and not just acid reflux) and its proximal extent.

DeMeester Score

24-hour oesophageal pH monitoring provided an opportunity to assess and observe acid reflux in the oesophagus. However, it presented a problem with regards to how to represent this information concisely and objectively. To address this a scoring system was developed which is known as DeMeester score. This score consists of 6 parameters derived from the 24 Hr pH monitoring and includes the following measures to develop a composite score[63,71]; Total reflux time pH ≤ 4

- Total reflux time in upright position - pH ≤ 4
- Total reflux time in supine position - pH ≤ 4
- Number of reflux episodes ≥ 5 mins
- Total duration of longest reflux episode pH ≤ 4
- Total number of episodes pH ≤ 4

A DeMeester score of > 14.72 is considered to be indicative of pathological reflux. The largest weightage in the DeMeester score is derived from the total reflux time in supine position as it is considered to be responsible for pathological impact. In this study I assessed 24 Hr pH and impedance and diagnosed GORD based on standard criteria, applying the DeMeester score[72] in both healthy cohort and asthmatics. Allowance was made for participants with a prior physician diagnosis of GORD who scored just below the DeMeester score of 14.72 if they had a symptomatically better than usual 24-hour period in the context of GORD symptoms.

1.4 Management options for controlling GORD in asthma

1.4.1 Medical therapy

Treatment of acid secretion

There is extensive evidence to suggest that acid suppression, using proton pump inhibitors and H2 receptor blockers, benefits symptoms such as heartburn but does not prevent regurgitation of weakly acidic or non-acidic stomach contents [73,74]. Various outcome measures have been used in studies to measure the impact of treating GORD on asthma. These outcomes include asthma symptoms score and/or nocturnal and daytime symptoms (using visual analogue scales), rescue inhaler use, frequency of exacerbations and asthma quality of life questionnaire (AQLQ) as measures of clinical impact of treatment, while peak expiratory flow (PEF) and forced expiratory volume in 1 second (FEV_1) has been used to judge the effects on lung functions. Some studies have shown a benefit in quality of life using the Asthma Quality of Life Questionnaire (AQLQ); however, these changes have been small and less than the clinically relevant change in AQLQ of 0.5 (table 1-2)[34,37,75]. Asthma control improvement has been reported based on visual analogue scores of changes in symptoms of dyspnoea, nocturnal and daytime symptoms [36,76,77], but little or no benefit has been noted on lung function [37,75], while Littner et al. have shown an improvement in rates of exacerbations in treatment vs placebo (8.1% vs 20.4%) [34]. Ranitidine in particular was noted to have a beneficial effect in nocturnal symptoms [76,77]. Overall, the benefit of medical therapy of reflux to improve asthma control remains unclear [36,78].The series of reviewed studies of impact of anti-reflux medication in asthma are listed in table 1-1.

Table 1-1: Selection of studies using anti-reflux medication to control GORD in asthma

Medical treatment	Demographics	Impact on clinical aspects of asthma	Impact on lung function	Reference
Ranitidine 150mg twice daily vs placebo	*N* = 48 *M/F* = 30/18 *Age* = 58.5 (28-70) *Asthma severity* = moderate to severe	Improved nocturnal symptoms, reduced rescue inhaler use	no change in PEF, FEV_1 or BHR	Ekstrom et al. 1989 [76]
Ranitidine 150mg or 300mg nocte (weight dependent) vs placebo	*N* = 37 *M/F* = 22/15 *Age* = 14 *Asthma severity* = not available	Improved nocturnal symptoms (30%) and overall asthma	Not measured	Gustafsson et al, 1992[77]

		symptoms using VAS.		
Omeprazole 40mg twice daily or placebo	*N* = 36 *M/F* = 17/19 *Age* = 52 *Asthma severity* = moderate to severe	no change in asthma symptom score	No change in FEV_1, BHR, PEF	Boeree et al. 1998. [79]
Omeprazole 20mg vs placebo	*N* = 9 *M/F* = NA *Age* = NA *Asthma severity* = NA	Improved AQLQ score including all domains	Improved PEFR, no effect on FEV_1	Levin et al. 1998 [75] *(full text not available)*
Omeprazole 40mg daily vs placebo	*N* = 107 *M/F* = 35/72 *Age* = 49 (21-75) *Asthma severity* = All	Reduction in nocturnal symptoms by 30%	Nil	Kiljander et al 1999 [80]
Lansoprazole 30mg twice daily vs placebo	*N* = 200 *M/F* = 33/67 *Age* = 47 *Asthma severity* = moderate to severe	Improved AQLQ emotional domain +0.2, reduced exacerbation frequency (8.1% vs 20.4%)	No improvement	Littner et al, 2005. (Littner, Leung et al. 2005)
Esomeprazole 40mg twice daily vs placebo	*N* = 412 *M/F %*= 28/72 (placebo) *M/F%*=36/64 (Rx group) *Age* = 42(+/-13) *Asthma severity* = moderate to severe	No improvement in QoL, asthma control, symptoms or pulmonary function (SF-36)	Nil	Matronarde et al. 2009[35]
Esomeprazole 40mg once daily and 40 mg twice daily vs. placebo	*N* = 828 *M/F %*= 23/77 (once daily) *M/F%*=25/75 (twice daily) *M/F%*=21/79 (placebo) *Age* = 45 *Asthma severity* = moderate to severe	Improved AQLQ: +0.28 and +0.41	Improvement in PEF: +3.5L/min and +5.5L/min FEV1: +0.09L and +0.12L	Kiljander et al, 2010 [37]
Esomeprazole 40mg twice daily vs fundoplication	*N*=69 (asthmatics (n)=12) *M/F*=37/32	Reduction in cough and dyspnoea from visual analogue	No improvement in FEV1, airway responsiveness or exhaled NO.	Kiljander et al 2012[36]

	Age = 46.6 (26-65) *Asthma severity* = moderate to severe	scores on medical treatment with further benefit from fundoplication	No placebo group.	
Omeprazole 20mg twice daily and Domperidone 10mg three times daily vs placebo (6 weeks)	*N*=30 (Rx group (n)=15) *M/F*= Rx group – 6/9, Placebo – 7/8 *Age* = Rx group – 34.9 (+/-19.2), Placebo – 35.6 (+/-17.4) *Asthma severity* = moderate	Not measured	Improvement in FEV_1 (9%), FVC (16.3%), PEF (14.6%)	Jiang et al 2003. [81]
Omeprazole 20mg twice daily and Domperidone 10mg three times daily vs placebo (16 weeks)	*N*=198 (asthmatics (n)=12) *M/F%* =68.7/31.3 *Age* = 51.5 (+/-9.83) *Asthma severity* = mild to moderate	Improved daytime (17.4%) and nocturnal (19.6%) asthma symptoms (VAS), decreased rescue inhaler (23.2%) use,	Improvement in PEFR (7.9%), FEV_1 (11.1%) and FVC (9.3%)	Sharma et al. 2007 [82]

Prokinetics

Prokinetics, drugs that increase LOS tone and gastric emptying, have been suggested as a way to prevent reflux of gastric contents in GORD [83]. Metoclopramide and Domperidone are commonly used prokinetics as an adjunct to treatment of GORD that act peripherally as dopamine antagonists [84-86]. Two small studies in asthmatics have looked at the effects of domperidone in combination with omeprazole and have found significant improvement in lung functions, and asthma symptoms [81,82] (also see **table 1-1**). Although effective in nausea, dyspepsia and GORD, the role of prokinetics in asthma control remains inconclusive due to lack of well-designed trials. Use of domperidone and cisapride is limited because of their extensive side-effect profile. The Medicines and Healthcare products Regulatory Agency (MHRA) issued a warning in 2013, advising doctors against using metoclopramide and domperidone for gastroparesis and GORD, limiting its use to short term (5-10 days) use for nausea and vomiting because of neurological side effects of metoclopramide and cardiovascular risks associated with domperidone use[87,88].

Baclofen

Baclofen is a $GABA_B$ (Gamma amino-butyric acid) agonist which is known to increase the tone of the LOS. This decreases the frequency of TLOSR, thereby decreasing upright reflux and symptoms of reflux such as belching and regurgitation, and the overall effect is a reduction in the DeMeester score [89]. Using HRM and magnetic resonance imaging (MRI) of the OGJ and stomach, Curcic et al showed that baclofen increased the LOS pressure, made the angle between the oesophagus and the stomach more acute and increased the length of the oesophagus which is more obtuse in patients with GORD, thereby reducing TLOSR and reflux in both healthy volunteers and GORD patients [90]. The benefit was noted in both acid and non-acid reflux, especially in post-prandial GOR [91]. Baclofen has, therefore, been recommended as a possible means of controlling GORD [92] in respiratory conditions such as chronic cough [93,94]. However, its efficacy in improving asthma control has not been studied to date.

1.4.2 Surgical treatment

The current standard of care for patients with severe GORD, as proposed by National Institute for Health and Care Excellence (NICE) guidance on GORD and dyspepsia for patients who are unable to tolerate PPI therapy or remain symptomatic despite medical treatment, is laparoscopic Nissen fundoplication which offers good control of GORD symptoms and compares effectively with medical therapy [95,96].

Fundoplication is used frequently in patients undergoing lung transplantation because of the detrimental effects of GORD leading to bronchiolitis obliterans syndrome (BOS) post lung transplantation, which significantly decreases the survival of the allograft [24-26,97,98]. Fundoplication in these patients has been shown to significantly improve allograft survival [24,25,97] (see table 1-2).

Table 1-2: Studies assessing GORD in lung transplant recipients

Surgical treatment	Impact on clinical aspects	Impact on lung function	Reference
Fundoplication	Improved BOS score, improved actuarial survival	Improved FEV_1 (24%)	Davis et al. 2003. [25]
Fundoplication	Improved BOS free period (96% vs 60%), improved actuarial survival (92% vs 76%)	Not measured	Cantu et al. 2004. [24]
Fundoplication	No data	Improved FEV_1 (84% vs 75%)	Hartwig et al. 2011. [97]

Fundoplication is effective in controlling GORD and has also been used as a therapeutic option in patients with severe asthma (see table 1-3). The results from surgical treatment have been mixed, with some studies reporting benefits in asthma control and other reporting no significant change. Kiljander et al reported improvement only in the patient reported quality of life outcome SGRQ (St Georges Respiratory questionnaire) and a small improvement in cough and dyspnoea on visual analogue scale (VAS) [36]. The study had a significant limitation that the number of asthmatics was small and therefore not clearly representative of a response to fundoplication in a larger asthmatic population. In 2003, Sontag et al reported their study of 62 patients with GORD and asthma. The group treated with fundoplication reported a sustained reduction in nocturnal symptoms of wheeze, cough and dyspnoea measured by visual analogue scales. This group also reported the highest rate of overall improvement of asthma symptoms (74.9% vs 4.2%), measured by visual analogue scales, compared to medical therapy and controls [99]. Hu et al reported similar results in their study more recently with sizeable improvement in symptoms of cough, wheeze and chest tightness as well as overall asthma symptoms. On review of studies, it is evident that whereas, fundoplication does have a beneficial effect in asthma symptoms such as cough, wheeze and dyspnoea, there is no clinically relevant effect on lung functions. Please see below the studies reviewed.

Table 1-3: Selection of studies using fundoplication as anti-reflux procedure in asthma

Surgical treatment	Demographics	Impact on clinical aspects of asthma	Impact on lung function	Reference
360^{o} Nissens fundoplication	*N*=39 *M/F*=15/24 *Age* =46(18-67) *Asthma severity* = severe	Improvement in asthma symptoms, nocturnal asthma reduced oral corticosteroid use, reduced asthma exacerbations	Not assessed	Spivak et al. [100]
Partial fundoplication in asthma and cough	*N*=24 (asthmatics – 13, Chronic cough - 11) *M/F*= Asthma – 7/6, chronic cough 2/9 *Age* = Asthma – 56(45-70), chronic cough – 55(40-72) *Asthma severity* = Not specified	No significant improvement in asthma parameters. Reduction in cough symptoms (Day-47% and night-80%) and reduced hoarseness and expectoration.	No improvement	Ekstrom et al. [101]

Nissens fundoplication	*N*=15 (asthma – 3) *M/F*=10/5 *Age* = 44 (+/-11) *Asthma severity* = moderate to severe	Non-significant post-op reduction in bronchial hyper-responsiveness in asthmatic population. Study was not designed to measure asthma symptoms	Improvement in FEV_1 (4%)	Kiljander et al. [102]
Nissens fundoplication vsRanitidine 150mg three times daily vs control	*N*=62 (Control – 24, Ranitidine – 22, Fundoplication - 16) *M/F*=10/5 *Age* = control – 52 (27-75), Ranitidine – 52(26-75), Fundoplication – 48(25-65) *Asthma severity* = moderate to severe	Surgical group showed improvement in nocturnal wheeze, coughing and dyspnoea (74.9% vs 9.1% vs 4.2%), improved asthma symptom score (43% vs >10% vs >10%) (VAS).	Determined by peak expiratory flows (PEF) which showed a non-significant decrease in PEF variation in the surgical group	Sontag et al. [99]
Nissens fundoplication vs esomeprazole 40mg twice daily	*N*=69 (asthmatics (n)=12) *M/F*=37/32 *Age* = 46.6 (26-65) *Asthma severity* = moderate to severe	Improvement in cough (VAS), improvement in SGRQ.	No improvement	Kiljander et al. [36]
Nissens fundoplication vs Stretta radiofrequency (SRF)	*N*=57 (SRF-24. Fundoplication – 33) *M/F*=18/39 *Age* = 47.3 +/-13.3 *Asthma severity* = severe	Post-op reduction in cough, wheeze and chest tightness (58.4, 53.9 and 51.9 %) and overall asthmatic symptoms (54.3%) by VAS compared to	Not measured	Wei-Hu et al. [103]

However, the possibility remains that patient selection is a key decision in such patients. It is vital that the complexity of the intervention and its unwanted sequelae have to be minimised if it is to be used in severe asthmatics and an alternative approach to augmentation of the LOS pressure needs to be considered. In the UK, various procedures for managing GORD being considered either in the research setting or clinical service are endoscopic radio frequency ablation, laparoscopic

insertion of magnetic beads, endoluminal gastroplication, endoscopic augmentation of the LOS using hydrogel implants, other endoscopically delivered bulking agents for the LOS and electrical stimulation of the LOS [104]. In all circumstances, a reconstruction or augmentation of the LOS is considered important in decreasing the reflux / regurgitation of the stomach contents.

1.5 Hypothesis

GOR triggers inflammation in the upper and lower respiratory tract. The inflammation is a response to the acid as well as weakly and non-acid components of the refluxate

Specific Aims

My project has the following specific aims:

1. Assess the prevalence of symptomatic and "silent" GORD in severe asthmatics and compare this to healthy subjects:
    a. The prevalence of symptoms of GORD
    b. The prevalence of objectively demonstrated reflux, i.e. the prevalence of the 5 types of reflux:
        i. acid reflux
        ii. weakly acid reflux
        iii. non-acid reflux
        iv. gas reflux
        v. mixed gas and liquid reflux

2. Identify biomarkers in asthmatic patients that are associated with GORD symptoms and reflux (and with the different types of reflux):
    a. Non-invasive biomarkers that can help with the diagnosis of GORD and inform the management of the disease in the clinic
    b. Invasive biomarkers that would be useful in research and proof of concept clinical trials of medical and surgical treatments of GORD

3. Analyse the impact of the 5 types of reflux on the pathophysiology of the respiratory tract.

    a. Upper airways pathology

        i. Macroscopic appearance of the larynx

        ii. Laryngeal mucosal pathology

    b. Lower airways pathology

        i. Inflammatory cell profile

        ii. Macroscopic appearance of the bronchi

        iii. Inflammatory mediators

        iv. Lung function

4. Assess the role of current medical therapy being used to control GORD and its effect on the respiratory extra-oesophageal manifestations.

1.6 Summary and objectives of the thesis

There is ample evidence to suggest that GORD is an important co-morbidity which is related to severe asthma, resulting in an asthma phenotype characterised by more frequent exacerbations. Conventional treatment of GORD with PPI and H2 blockers has produced mixed results in controlling symptoms of asthma in whom the asthma may continue to be affected by weakly acid and non-acid GOR. Understanding the pathophysiology of the effects of acid, weakly acid and non-acid GOR on the airways is, therefore, crucial, possibly leading to more effective management strategies for GORD in asthma.

This raises the following questions which I have addressed in my thesis

1. How does untreated GORD in asthma effect clinical parameters (e.g. asthma control), lung physiology and lung function?

2. Are there any clinical markers or pathobiological biomarkers to suggest that GORD directly affects asthma?
3. What type of reflux (acidic, non-acid) is present in severe asthma?
4. How does GORD affect the upper and lower airways pathobiology?
5. Does medical treatment for GORD have any bearing on clinical symptoms of asthma and/or markers of inflammation?
6. What are the molecular mechanisms whereby GORD impacts on the bronchial mucosa?

To investigate the role of gastro-oesophageal reflux in asthma I divided my research into 3 parts:

1. Analysis of the large U-BIOPRED study cohort based on diagnosis of GORD as a patient reported outcome and description of clinical and biological features associated with GORD in severe asthmatics.
2. Study of a well characterised group of severe asthmatics with and without GORD that I recruited and assessed objectively using invasive and non-invasive sampling to achieve detailed profiling of inflammatory features associated with GORD.
3. A study of the mechanisms that underlie the effects of GORD on the bronchial mucosa. This study was done in collaboration with a European Respiratory Society Fellow, Dr Jeanne-Marie Perotin-Collard visiting from the University of Reims, France, and jointly supervised by Professor Ratko Djukanovic and Professor Donna Davies.

After the methods section, the six studies that I have conducted for the purpose of this thesis are presented:

Study 1: Sputum proteomic signature of GOR in patients with severe asthma
Study 2: Physiological assessment of GORD in severe asthmatic
Study 3: Clinical assessment of GORD in severe asthmatics
Study 4: Assessment of cough in severe asthma
Study 5: Pathobiological characteristics in the airways of patients with GORD
Study 6: Vulnerability to acid reflux of the airway epithelium in severe asthma

Chapter 2 Methods

2.1 Study design

The work in this thesis was conducted in three phases. The initial work was conducted on study participants from the U-BIOPRED cohort which enabled the assessment of the prevalence and clinical associations of GORD in patients with severe asthma. I followed this up by recruiting a further cohort of healthy participants and severe asthmatic participants with and without GORD.

In the third phase, in collaboration with Dr Jeanne-Marie Perotin-Collard, *in vitro* and *ex vivo* approaches were used to study damage and inflammation patterns in the airway caused by GOR using microscopy, immunohistochemical analysis and analysis of gene expression. The methods for this study are described in Chapter 8 – Vulnerability to acid reflux of the airway epithelium in Severe asthma.

For the purpose of the U-BIOPRED, the detailed raw data were made available to me to analyse myself. I was also directly involved in running the study, sample collection and data collection for the U-BIOPRED study as the BRU/BRC research fellow.

In this chapter, I wish to describe in detail all the key methods that I have used in my thesis. As appropriate, I will briefly summarise them again within the chapters that describe the individual studies.

2.2 U-BIOPRED (Unbiased BIOmarkers for the Prediction of REspiratory Disease Outcomes) project – assessment of GORD and its impact on the airways

2.2.1 Introduction

U-BIOPRED (Unbiased Biomarkers Predictive of Respiratory Disease Outcomes) is a large European Union (EU) project funded by the Innovative Medicines Initiative (IMI) set up to improve the understanding of asthma and its mechanisms. The aim of this study was to identify phenotypes of severe asthma with the help of cutting edge 'omics technologies (genomics, transcriptomics,

proteomics, lipidomics, and metabolomics) and to find new treatment targets using a systems biology approach [105].

An initial analysis of the clinical characteristics of participants in the U-BIOPRED project confirmed GORD as a significant co-morbidity in asthma, its prevalence being particularly high in severe asthma [105]. This is in keeping with other studies showing an association between GORD and asthma severity [106,107].

Sputum supernatant of more than 240 participants, was collected and processed through an unbiased state of the art quantitative liquid chromatography with untargeted mass spectrometry (LC/MS^E) that can detect with confidence more than 250 proteins in at least 60% of individuals studied. Using multiple regression, I then identified the proteins that are predictive of GORD in severe asthma.

2.2.2 Methods

As part of the study team running the U-BIOPRED programme at the Southampton - NIHR Respiratory Biomedical Research Unit (RBRU) at University Hospital Southampton NHS Foundation Trust, I was directly involved, as the Respiratory BRU/BRC research fellow in the recruitment, screening and study procedures, such as collection of clinical data, bronchoscopy and sample collection and helped with sputum sample collection and processing. Mass spectrometry for proteomics analysis was performed by Dr. Dominic Burg and Dr. James Schofield who guided me in respect of understanding the processes involved.

2.2.3 Study design and clinical assessment

U-BIOPRED was a prospective, multicentre cohort study involving 16 clinical centres in 11 European Union countries recruiting healthy and asthmatic participants according to pre-set criteria for clinical stratification as previously published in detail [105]. For the purpose of this study, the following clinical data in adults (patient reported and laboratory derived) were specifically evaluated: history of GORD and activity, oral corticosteroid use [OCS], frequency of exacerbations in the past year, spirometry and standardised disease activity questionnaires (short and full version of the Asthma Control Questionnaire [ACQ5 and ACQ7], Asthma Quality of Life Questionnaire [AQLQ], Hospital Anxiety and Depression Scores [HADS], Sino-nasal Outcome Test [SNOT-20], and the Epworth sleep score [ESS]. All participants were asked to provide sputum induced by standard protocol, usually on the day of clinical assessment; for those in whom sputum samples failed the quality criteria or sputum induction was unsuccessful, a repeat induction was performed within the week.

The study received ethics approval in all the countries involved and all participants provided written informed consent. It is registered on the Clinical Trials.gov (identifier: NCT01982162).

2.2.4 Cohort description

A total of 610 adult participants were stratified into 4 groups: Group A (n=311) - poorly controlled severe asthmatics on high-dose inhaled corticosteroids (ICS) ≥1000 µg fluticasone propionate (or equivalent), with no smoking in the past year and <5 pack-year smoking history, Group B (n=110) - severe asthmatics defined as for Group A but with either current smoking history or ex-smokers with >5 pack-year smoking history, Group C (n=88) - mild/moderate asthmatics with controlled or partially controlled asthma (as defined by GINA) using <500 µg fluticasone propionate (or equivalent) ICS with no smoking in the past year and <5 pack-year smoking history and Group D (n=101) - healthy individuals with no history of chronic respiratory disease, pre-bronchodilator FEV_1 ≥80% of predicted and non-smoking for ≥1 year or ex-smokers with a smoking history of <5 pack years.

The groups were further stratified by presence/absence of previous physician-made diagnosis of GORD (ALL GORD subgroup), i.e. all participants with a diagnosis of GORD and/or on treatment with anti-reflux medication), participants with a diagnosis of GORD who, at the time of assessment, had typical symptoms of GORD regardless of treatment (Active GORD subgroup), and those without a history of GORD and not on medication for GORD (NO GORD).

2.2.5 Sputum sample collection and analysis

Induced sputum was acquired and processed using the guidelines of the ERS Task Force for sputum induction and processing [108]. Using dithioerythritol (DTE) in HEPES buffered saline added in a 4:1 w/v ratio as a mucolytic for selected mucoid portions of the induced sputum sample, a cell pellet was obtained after centrifugation at 400*g*. The resultant supernatant was centrifuged at 12000*g* to remove the cell debris and stored at -80^{o}C for downstream mass spectrometric analysis. The cell pellet was dissolved in phosphate buffered saline and processed for dead/live cell quantification and finally inflammatory cell counts done using Diff-quick Rapid Romanowsky stain [109].

2.2.6 Mass spectrometry

Peptide extracts were re-suspended in buffer A; 3% ACN, 0.1% Formic acid (v/v) and the concentration measured using a Direct Detect System (Millipore). An internal standard mixture of *E. coli* ClpB Hi3 standard (Waters), yeast enolase (ENO) and yeast alcohol dehydrogenase (ADH) was added to a final concentration of 250ng/µl sputum peptide in 20µl, 12.5 fmol/µl of ClpB, 12.5 fmol/µl of ENO, and 8.75 fmol/µl of ADH. Samples were analysed in duplicate via LC-IMS-MS^{E} on a

Waters G2S high-definition mass spectrometer coupled to a nanoAcquity UPLC system. 4 µl of peptide extract were injected onto a C18 BEH trapping column (Waters) and washed with buffer A for 5 min at 5 µl/min. Peptides were eluted using a 25 cm T3 HSS C18 analytical column (Waters), with a gradient of 3-50% ACN + 0.1% formic acid over 50 min. at a flow rate of 0.3µl/min. Eluted samples were sprayed directly into the mass spectrometer operating in MS^E mode. Data were acquired from 50 to 2000 *m/z* using alternate low and high collision energy (CE) scans. Low CE was 5V and elevated CE ramp from 15 to 40V. Ion mobility separation was implemented prior to fragmentation using a wave velocity of 650 m/s and wave height of 40V. The lock mass Glu-fibrinopeptide, $(M+2H)^{2+}$, m/z = 785.8426) was infused at a concentration of 100 fmol/µl with a flow rate of 250 nl/min and acquired every 60 sec.

Data curation and searching

Raw data were processed using a custom package (Regression tester) based upon executable files from ProteinLynx Global Server 3.0 (Waters). The optimal setting for peak detection across the dataset was determined using Threshold Inspector (Waters) and these thresholds were chosen: low energy = 100 counts; high energy = 30 and a total energy count threshold of 750. Database searches were performed using regression tester and searched against the Uniprot human reference database (20/11/2014) with added sequence information for internal standards. Quantity was estimated in absolute amounts using the Hi 3 method [110,111]. The ion accounting output files [112] were compiled and summary information generated from search log files using custom Python scripts. Information contained in ion accounting files were collated into a single .csv document using a custom Python script.

Data filtering and normalisation

Protein identifications collated from the ion accounting files were further quality filtered by allowing only identifications with the following criteria: identification in at least two separate samples (not including duplicates), a process that required at least three high quality unmodified peptides using the Hi3 method, and 2 peptides with at least 4 fragment ions for each protein. All other protein identities were removed. Proteins were ranked according to coverage across the samples and samples were ranked according to the order in which they were run. QC information was added for each sample (batch information, protein concentration, ion counts).

Inforsense software (ID Business Solutions, Guildford, UK) was applied to generated heat maps for the top 150 proteins using both 'top 3 peptide intensity sum' (a proxy for concentration) and peptide concentrations (expressed in fmol) on column calculated from internal standards. Sample-wise correlation plots were created using Inferno RDN (http://omics.pnl.gov/software/infernordn)[113]. Heat maps and correlation plots were inspected for

poor samples or injections; those with very low or no ID's and/or poor correlation were removed from the dataset.

Samples were analysed in duplicate and the intensity values from the injections was averaged. Replicate injections were inspected for consistency in quantitation, to enable this an average of the two injections 'top 3 peptide intensity sum' was calculated and a distance matrix was calculated by taking the Euclidian distance between the two injections as a function of the average of the injections. The resulting values were visualised in heat map, enabling rapid inspection of duplicates with high variance, which likely indicated a technical issue between injections (e.g. sprayer dropout, or failure to inject the correct volume). To uniformly remove suspect injections from the dataset we created the following universal rule: For samples with >2 fold between-injection difference in average intensity of proteins, the following rule (Rule 1) was applied: "report injection one intensity values for proteins, unless protein was only quantified in injection two, then include this value for increasing coverage".

While the above method was useful in identifying whole samples with poor repeatability between injections, there were cases where the concentrations of individual proteins were highly variable. To assess these cases, a log was created using a custom script, which highlighted those proteins where the ratio between injections was >1.5. Proteins with high frequency of poor measurement stability across all samples were processed according to 'Rule 2: "if the variation between injections is greater than 1.5-fold, take the quantity measured using injection one". The rationale behind taking the injection one values was that these are likely the cleanest: following on from an injection blank and extended equilibration, and less influenced by column carry over.

Mean values were derived from replicate sample injections except for those cases where rule 1 and rule 2 were applied, and those cases where the protein was quantified in only one sample; then the intensity value was taken for the single sample injection.

Differences in run-to-run intensity (loading) were adjusted by normalising each run to the sum of top 3 intensities of the proteins up to the point where the sample set reached 10% missing data (we refer to this as 'top-90 normalisation').

Statistical analysis of clinical data

Comparisons of protein profiles defined by MS^E analysis were restricted to the severe asthma groups A and B in order to avoid the confounding effect of disease severity. The comparison between protein profiles in Active GORD and NO GORD was the primary endpoint while that between ALL GORD and NO GORD participants was the secondary endpoint. Clinical and demographic data were analysed by parametric and non-parametric tests following assessment of

distribution using the Shapiro-Wilk normality test by GraphPad Prism (version 6.0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) and SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA) [114]. Protein concentrations in severe asthmatics (groups A and B) with presence of ≥60% were analysed by non-parametric tests and all those with significance at p≤0.1 were further assessed by univariate logistic regression (ULR). The proteins identified as possible predictors of GORD subsets by univariate logistic regression (with p≤0.05) were selected for multivariate logistic regression (MLR), which was conducted with stepwise backward selection (adjusted for smoking) to rule out weak associations and to select the best predictors of GORD. All regression analyses were conducted in SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA) [114].

Statistical analysis of proteomic data

The measurement of proteins by untargeted mass spectrometry is advantageous because of the inherent lack of bias in the proteins measured. However, untargeted mass spectrometry results in missing values for many of the proteins measured due to amongst other things, the peptide being at too low a level in the sample for detection, the peptide is not present in the sample, variability in the behaviour of the peptide in the mass spectrometer resulting in stochastic measurement [115]. Regression analysis is affected by missing values or incorrect imputation. To minimise the probabilities of identifying false positives in this study, we studied proteins measured in at least 60% of samples and imputed median values. The median value is based on a large sample size (at least 60% of samples) and therefore reflects the true average measurement of the protein. Imputation of median values increases the chance of false negatives, as group variances are reduced, however, the reduction of false positive identifications justifies this reduced probability of identifying false negatives.

2.3 Methods used in phase 2 - The study of the role of gastro-oesophageal reflux in asthma – an in-depth analysis of the impact of GORD and its medical treatment on clinical and pathobiological features of asthma

2.3.1 Introduction

This study forms the second part of my thesis. It builds on the analysis of the large U-BIOPRED cohort data of severe asthmatics with GORD as patient reported outcome by performing a detailed characterisation of GORD. I performed an analysis of healthy and severe asthmatics as a pilot study to further assess the role of gastro-oesophageal reflux in airway inflammation. The study was

approved by NRES Committee South Central – Hampshire A (13/SC/0182). Study objectives and purpose

I investigated the impact of GORD on the respiratory system and elucidate whether and how this contributes to asthma symptoms. This included an assessment of acid, weakly acid and non-acid gastro-oesophageal reflux and its effect on the respiratory system comparing healthy individuals and asthmatic participants on Step 4/5 of asthma BTS / SIGN guidelines, using established as well as novel laboratory techniques and biomarkers that enable more precise quantification of reflux and its impact on the airways. Participants were selected on the basis of presence of symptoms and diagnosis of asthma and the level of control of asthma symptoms as well as a history or diagnosis of GORD. After an objective assessment of gastro-oesophageal reflux, all the participants with GORD were asked to undertake stepwise medical therapy in the form of treatment for GORD such as acid suppressants and prokinetics.

2.3.2 Study design

This was a study comparing healthy participants and patients with severe asthma combining cross-sectional observation and intervention with drugs that have the potential to improve control of GOR and to alter the pathobiology in the airways if the hypothesis is correct. As part of the study, proton pump inhibitors (PPI), H2 receptor blockers (Ranitidine) prokinetics e.g. Metoclopramide and Domperidone and GABA agonist (Baclofen) were added to standard asthma treatment in participants found to have objective evidence of GORD using 24-hour pH and Impedance monitoring as recommended in the BTS Chronic cough guidelines for adults where GORD is suspected to be the trigger [93]. The aim of this intervention is to assess the effects of inhibiting acid production (using PPI (omeprazole) and H2-blockers (ranitidine)), increasing oesophageal motility and gastric emptying (using prokinetics metoclopramide and domperidone) and improve lower oesophageal sphincter tone (using Baclofen) i.e. treatment of GORD, on the quantitative and qualitative degree of gastro-oesophageal reflux and the resultant change in inflammatory biomarkers in the upper and lower airway.

PPIs and ranitidine are standard care and licensed treatments for GORD and are very widely used [116]. Prokinetics such as metoclopramide and domperidone have been widely used as anti-emetics and also in treatment of GORD. They function by increasing the rate of gastric emptying which, in turn, reduces the chance of stomach contents regurgitating back into the oesophagus. Baclofen is a $GABA_B$ receptor agonist known to increase the tone of the lower oesophageal sphincter, thereby reducing the reflux of stomach contents into the oesophagus [81,83,89,117].

This sequence of therapeutic options with trial of high dose PPI and prokinetics and further evidence for Baclofen, a GABA agonist, is recommended as standard of care in the BTS chronic

cough guidelines (2006) in adults with GORD. Since the BTS cough guidelines (2006) presented a thorough and step-wise approach to dealing with GORD based on clinical evidence covering the clinical scenarios of micro aspiration into the tracheobronchial tree and vagally mediated oesophageal reflex due to volume reflux into the oesophagus, I decided to use the same algorithm on the assumption that the aetiology and impact of reflux may be similar in asthma.

2.3.3 Study details

I recruited 20 healthy participants and 24 severe asthmatics into the study: 9 participants were severe asthmatics with GORD (SA-GORD) group, while 6 were severe asthmatics without GORD group (SA-no GORD) as judged by 24-hour pH/Impedance. The healthy cohort included 15 participants of whom 5 were found to have GORD (HC-GORD) on 24-hour pH/impedance while 10 had no evidence of GORD (HC-no GORD). Participants with severe asthma were designated ID code GA3 followed by the participant number while healthy cohort were designated ID code GA1 followed by the participant number.

In the severe asthma group, 1 participant had to withdraw early from the study due to a medical complication unrelated to the study. This participant progressed till visit 9. 1 participant withdrew early due to work commitments and did not progress beyond visit 5, 1 failed to attend further appointments after the initial assessments for GOR were completed and therefore did not proceed beyond visit 3, 3 participants failed to attend after initial consent, 2 withdrew from the study to enrol into a study of biologics treatment, and 4 were unable to tolerate the pH/impedance procedure and were, therefore, withdrawn from further participation.

In the healthy cohort, 4 participants were unable to tolerate the pH/Impedance testing and 2 chose not to attend after initial consent.

The severe asthmatics were divided into 2 sub-groups based on their symptoms as those with reflux and without reflux. The general plan of the study was as follows and consisted of 12 visits (See Figure 2-1):

- Visit 0 – Screening visit to assess suitability for all participants: assessment by clinical questionnaires

SA participants with symptoms of GORD and already on anti-GOR treatment were asked to stop all GOR treatment for 2 weeks and attend visits 2 and 3.

- Visits 2 and 3 – All participants (severe asthmatics and healthy controls) came on visit to undergo baseline assessment of reflux using 24-hr pH and impedance monitoring (first visit

to place the probe and a second visit 24 later to remove it) in combination with a 24-hour cough monitor (Leicester cough monitor-LCM) to assess any cough associated with reflux.

- Visit 4 - Sputum induction visit
- Visit 5 - Endoscopic airway examination by naso-endoscopy and bronchoscopy

From this point, only SA-GORD participants were followed up.

For SA-GORD participants (pH/impedance test positive for reflux), 8-week treatment with omeprazole and ranitidine was started followed by:

- Visit 6 - Clinical data collection and repeat 24-hour pH and impedance monitoring and cough monitoring with LCM.
- Visit 7 – Removal of catheter
- Visit 8 – Assessment of the effect of PPI and H2 antagonists on asthma control and PFT. Start of metoclopramide for participants with poorly controlled asthma symptoms (ACQ>1.5); start of a 4-week course of metoclopramide whilst continuing acid suppressant treatment to additionally control reflux.
    - Visit 8a: Repeat sputum induction visit, and start of a 4-week treatment with metoclopramide
    - Visit 8b – 2 weeks after starting metoclopramide to review any drug-associated side-effects.
- Visit 9 – Assessment of effect of metoclopramide and sputum induction. Cessation of metoclopramide and start of 4 weeks of domperidone in participants with poorly controlled asthma symptoms (ACQ>1.5) whilst continuing acid suppressant treatment to additionally control reflux.
    - Visit 9a: Assessment of effect of metoclopramide and sputum induction
    - Visit 9b – 2 weeks after starting domperidone to review any drug-associated side-effects. This included an ECG to review for any drug associated side-effects.
- Visit 10 – Assessment of effect of domperidone, sputum induction. Cessation of domperidone in participants with poorly controlled asthma symptoms (ACQ>1.5) and start of 4 weeks of baclofen whilst continuing acid suppressant treatment to additionally control reflux.
- Visit 11 – Sputum induction and end of study assessment

Figure 2-2 shows a CONSORT diagram to explain the study groups and visits within their investigative domains and the number of participants at each stage.

General Inclusion Criteria for all participants

- Male or female, aged ≥18 but ≤65
- Able to comprehend the information sheet and provide written informed consent.
- Absence of any significant co-morbidity
- Motivation to complete all of the study visits and procedures, and ability to communicate well with the investigator and be capable of understanding the nature of the research and its treatment including risks and benefits.
- Able to comprehend and complete various disease related symptom questionnaires
- Presence or absence of atopy was not an inclusion criterion for any participant group.
- Non-smoker or ex-smoker for at least 12 months and <10 pack/years history of smoking.

Specific inclusion criteria for non-asthmatic participant:

- Normal lung function tests (spirometry and DLCO)
- Absence of change in FEV_1 ≥ 12% or 200ml predicted after inhalation of 400-800 mcg salbutamol checked at the Visit 1.

Inclusion Criteria for Asthmatic Participants on Step 4 / 5 of BTS/SIGN Guidelines:

A clinical diagnosis of asthma as follows;

- Improvement in FEV_1 ≥ 12% or 200ml predicted after inhalation of 400-800 mcg salbutamol **OR**
- Airway hyper-responsiveness (PD_{20} <8mg/ml) **OR**
- Diurnal variation in PEF: amplitude % mean of twice daily PEF > 8% **OR**
- Decrease in FEV_1 >12% and >200mls within 4 weeks after tapering treatment with one or more of the following drugs: ICS, OCS, LABA, SABA

OR

- A history of wheeze occurring spontaneously or on exertion.

- On Step 4 or Step 5 of asthma management as per BTS / SIGN asthma guidelines
- Historical evidence for variation of $FEV_1 \geq 12\%$, be it spontaneous or with treatment
- Asthma features (measured by sACQ as more than 2.5) poorly controlled with inhaled steroids and/or maintenance oral steroids.

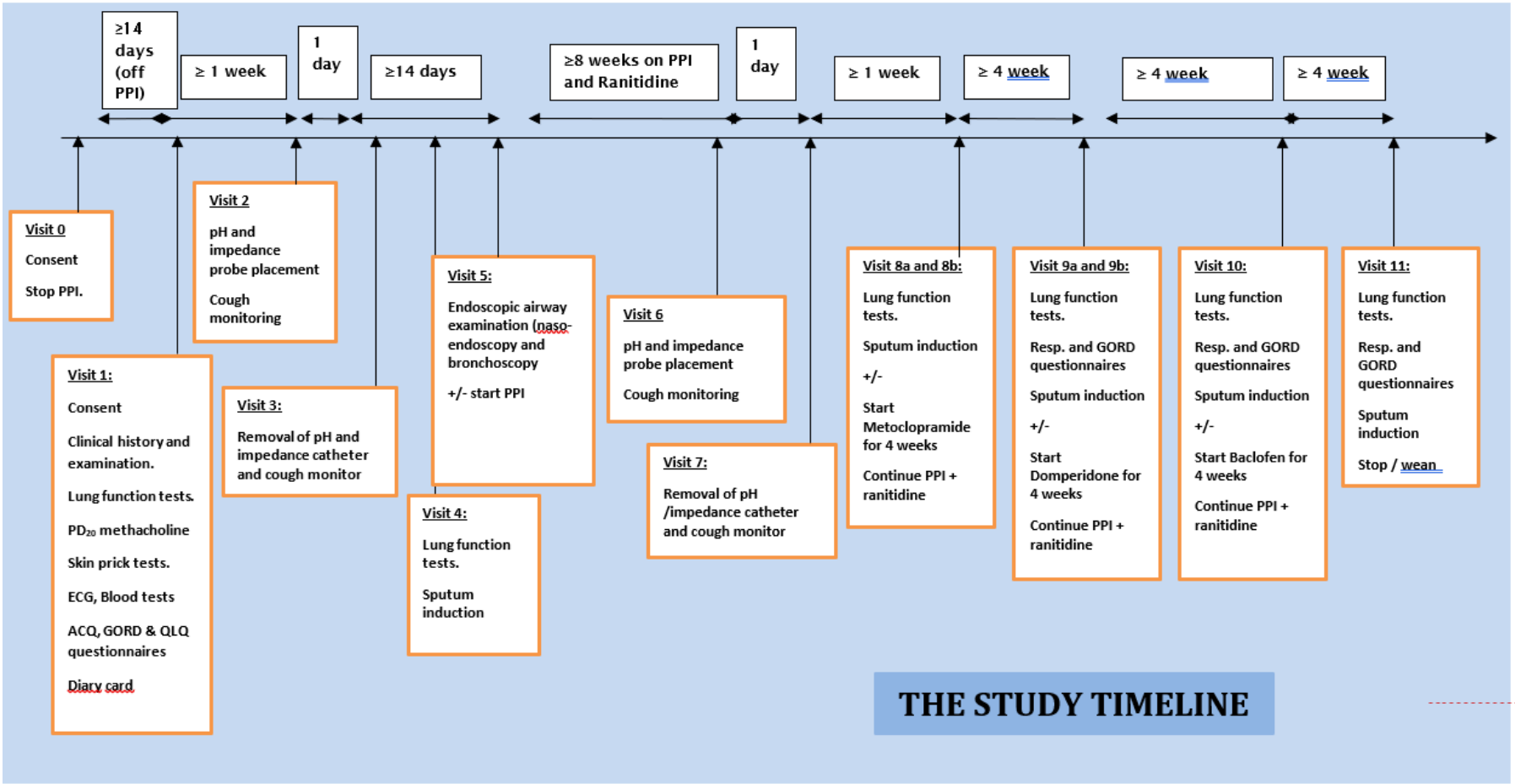

Figure 2-1: The study timeline for the phase 2 study

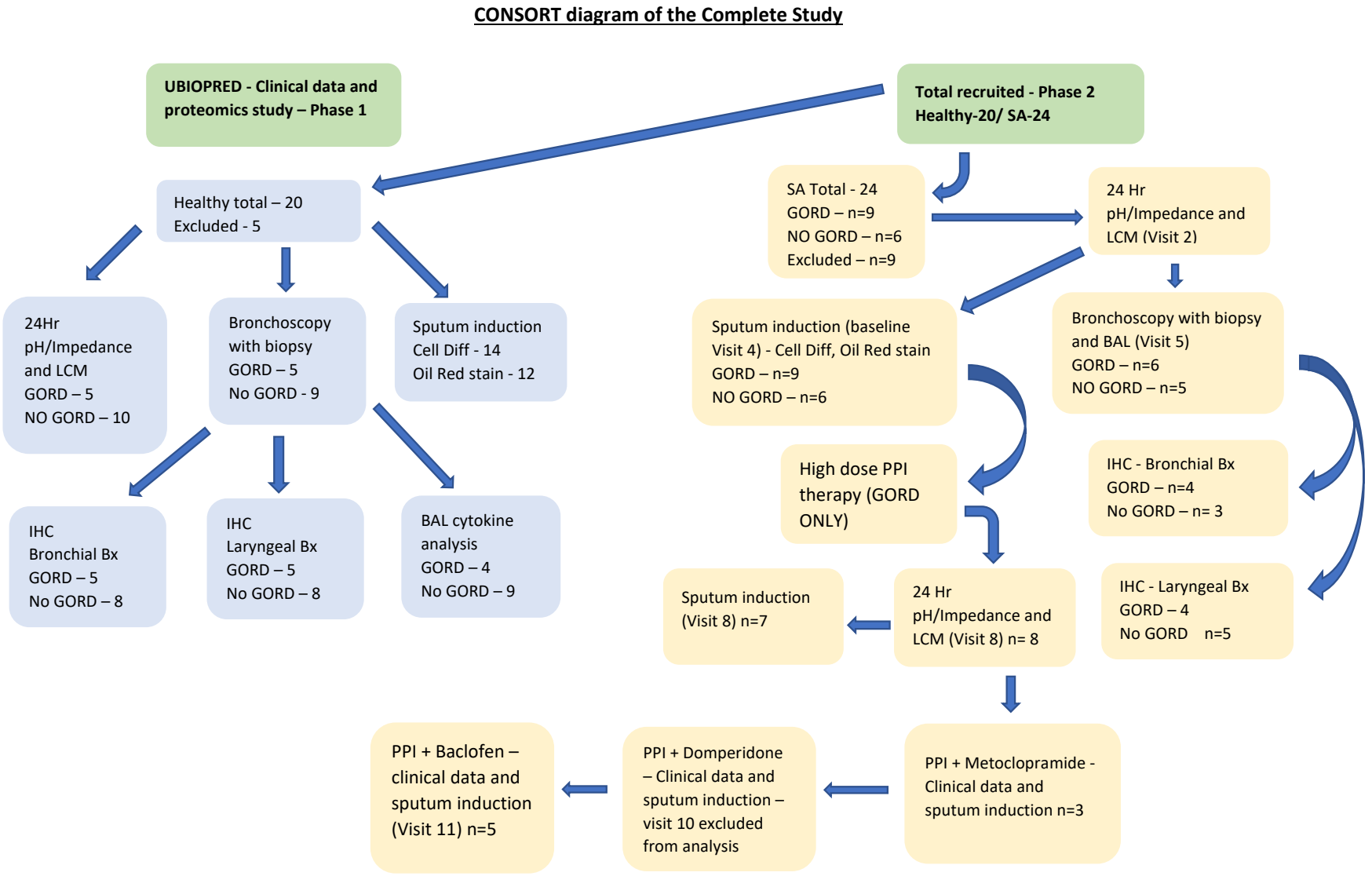

Figure 2-2. CONSORT diagram of the study visits with participant numbers and investigative steps

Exclusion criteria

General:

• Unable to provide written informed consent.

• Pregnancy either current or planned over the duration of the study.

• Breastfeeding.

• Significant medical (cardiac or otherwise) or surgical co-morbidity or medication which may interfere with the study drugs

• History of significant psychiatric or surgical disorders which may interfere with the study.

• History of cancers in the last 5 years.

• Prisoners.

• Children under age 18**.**

2.3.4 Methods

Pulmonary function tests

Spirometry and reversibility

All participants had pulmonary function tests pre- and post-salbutamol inhalation at the screening visit and subsequent visits as defined by the protocol and study design. The CareFusion Jaeger™ Masterscreen V5.22 is used for spirometry using reference values as per the ECCS data [118].

Reversibility was tested using Salbutamol 400-800 mcg pMDI (pressurised metered dose inhaler) inhaled via Volumatic™ spacer device (Allan and Hanbury's, UK). Reversibility was defined according to the British Thoracic society Guidelines, 2008 as (post bronchodilator FEV_1 – Pre bronchodilator FEV_1)/Pre bronchodilator FEV_1 x 100) an increase in FEV_1 of 12% or more was considered clinically significant [1].

Peak flow

Peak flow rates were measured twice daily using a European Standard Mini-Wright peak flow meter (Clement Clarke, Harlow, UK), recording the best of three attempts at each time-point into a diary

card. Peak flow variability is defined as ≥20% variability in peak flow over a period of 2 weeks. The peak flows were measured standing up twice daily and calculated using - max PEFR – min PEFR)/max PEFR x 100, expressed as a percentage according to British Thoracic Society (BTS) guidelines (2008).

Methacholine challenge

Methacholine challenge was performed on individuals where there was no clear diagnosis of asthma or absence of a physician's diagnosis of asthma. Historical results of methacholine challenge, reversibility and/or physician diagnosis were accepted for recruitment.

Exhaled nitric oxide (FENO)

Exhaled Nitric oxide was measured with single breath repeated 3 times at a flow-rate of 50 mL/s using NIOX MINO (Aerocrine Inc., Princeton, NJ, USA) or NObreath (Bedfont Scientific Ltd, Maidstone, UK). There is evidence to show that both devices have good reproducibility with little inter-device difference [119]. Upper limit of normal was considered to be exhaled Nitric oxide levels of >25 ppb.

Phlebotomy

Serum

Samples were collected into 10 ml BD Vacutainer® serum tubes (367895, BD) using the 21 gauge BD Vacutainer® Safety-Lok™ blood collection set (367282, BD, Plymouth, UK). The tubes were stored upright at room temperature for 30-60 mins to clot. The tubes were centrifuged for 15 min at 1500G (300rpm LabFuge 400R) at 4^{o}C to separate the serum. Serum was transferred from the blood tube to a clean 15 ml polypropylene tube and gently inverted 5 times. Serum samples were stored as aliquots of 0.5 ml till later use.

Full blood count, Liver functions, urea, electrolytes and coagulation

Samples were collected using 21-gauge BD Vacutainer® Safety-Lok™ blood collection set (367282, BD, Plymouth, UK) and sent to the University Hospital Southampton NHS Foundation Trust (UHSFT) laboratory for full Blood Count (using BD Vacutainer® K3E, 368857), liver function tests, urea and electrolytes (using BD Vacutainer® SST II *Advance*). Additionally, the coagulation profile (Prothrombin time (PT) and INR) was assessed (using BD Vacutainer® 9NC, 363095) to ensure safety of biopsy during bronchoscopy.

2.3.5 Sputum induction

Sputum induction was performed according to the ERS Taskforce for sputum guidelines [108] and departmental protocols.

Sputum processing

Sputum was processed within 2 hours of induction. Mucoid portions and plugs from the expectorated sputum were selected using forceps and transferred into a separate petri dish. The sample was mixed thoroughly and transferred into a pre-weighed polypropylene tube. The sample was re-weighed and diluted with 4 volumes of reducing agent [48mg of 1,4 dithioerythritol (DTE) in a 50mL polypropylene centrifuge tube mixed with 2.5 ml of 1M Hepes buffer and 1.44 ml of 5M NaCl made up to 50 ml with distilled water]. The sample was placed on bench roller for 30 minutes at room temperature with the tube inverted every 5 minutes. The tube contents were filtered through a 100µm sterile cell strainer (Fischer Scientific) into a pre-weighed 50mL polypropylene centrifuge tube to remove un-solubilised mucus. The tube was reweighed to measure the recovered filtrate which was transferred into a 15 ml Falcon tube and centrifuged at 400*g* (LabFuge 400R) for 10 mins at 4^{o}C to pellet the cells for cytospins. The supernatant was separated carefully and transferred to 1.5ml micro-centrifuge tubes for ultracentrifugation at 12000*g* for 10 mins at 4^{o}C. The supernatant was carefully retrieved without disturbing the debris pellet at the bottom and snap-frozen with isopropanol (pre-chilled on dry ice) in aliquots (250µl) into 2 mL cryotubes and stored in -80^{o}C freezers for further 'Omics analysis.

Sputum cytospin processing

The cell pellet from the sputum processing was re-suspended in 0.5 mls phosphate buffered saline (PBS) and transferred to a 2mL labelled cryotube. 15µl of the cell suspension is mixed with 15µl of trypan blue and incubated on ice for 5 mins to stain cells for a live/dead stain. 10µl of the stained cells were transferred to each side of the Neubauer haemocytometer to calculate cell viability under the microscope.

Labelled slides were placed into a Shandon cytospin clamped in place with a filter card and funnel. 70µl of cell suspension was then added to each slide. The slides were spun on centrifuge at 450 rpm for 6 mins. Quality of slides was checked to attain a balanced density of cells with the aim to achieve 4-6 slides of good quality. Slides were air-dried overnight and stored at -80^{o}C.

Lipid laden macrophage in cytospins

Cytospins from induced sputum were stained with Oil Red O stain to high-light lipid contents of the macrophages and calculate the lipid-laden macrophage index (LLMI) according to Gibeon et al's

modified technique [120]. Cells were scored from 0-4 where 0= no opacification, 1=0 to ¼ , 2= ¼ to ½ opacification, 3= ½ to ¾ opacification, 4=totally opacified as per the method described by Gibeon et al (see Figure 2-3). Scoring was done by me by analysing 100 alveolar macrophages per cytospin.

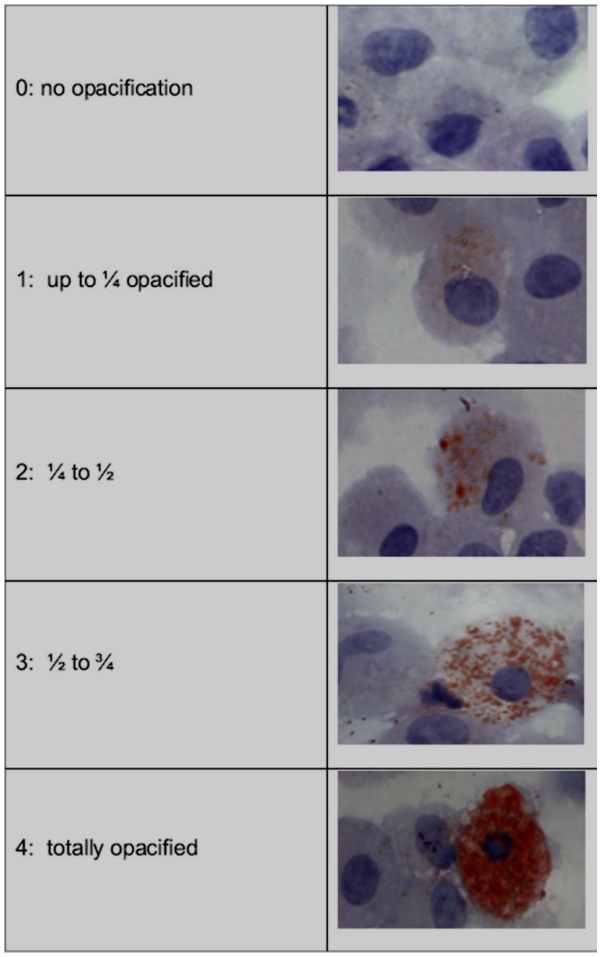

Figure 2-3: Criteria used by Gibeon et al to calculate LLMI from alveolar macrophages. Macrophages are graded according to the quantity of the red stained cytoplasm with the oil-Red-O stain and scored from 0 to 4 as shown.[120]

Pepsin test (PepTest™)

Pepsin level was checked in the saliva and the unselected portion of the induced sputum using the Peptest™ Kit (RD Biomed Limited, Hull, UK). The Peptest™ is a colorimetric assay that contains 2 monoclonal antibodies against pepsin A. The test is conducted using a lateral flow device and an optical lateral flow device reader.

At the time of sputum induction, at least 1 ml of saliva sample cleared from the back of the throat (throat clearate) was collected prior to starting the sputum induction protocol. This sample was transferred to a pre-weighed polypropylene tube and weighed again followed by addition of 0.1M citric acid in a ratio of 1 in 10. The sample was then mixed on the vortex mixer for 5 seconds. This sample is then centrifuged at 4000 rpm (micro-centrifuge) for 5 mins to attain a clear supernatant. If the supernatant was not clear in the first attempt the sample was centrifuged again as per

protocol from the manufacturer. Using an automated pipette 80µl of clear surface layer fluid was drawn out from the centrifuged sample and transfer to a clear screw top micro-tube containing 240 µl of migration buffer solution provided by the manufacturer in the Peptest™ Kit. This sample was mixed on the vortex mixer for 10 seconds. The Peptest™ lateral flow device (see Figure 2-4) is then removed from the packaging and placed on a level surface. 80µl of the sample was applied in the circular well of the Peptest™ device. After a few minutes a blue line will appear under the letter 'C' on the viewing window of the Peptest™ device. This is the control line which confirms that the test is working correctly. If the pepsin is detectable in the sample, then a second blue line will appear under the letter 'T' (Test). The results take between 5-15 minutes for a visual assessment of a positive or negative answer to the presence of pepsin, however, for an objective value the Peptest™ lateral flow device is placed into the Peptest™ LFD reader after resting for 15 mins. The Peptest™ LFD reader is switched on and after selecting the appropriate options the Peptest™ LFD is then read. The resulting reading is then compared against an appropriate LFD batch-matched conversion table to convert the reader values to pepsin levels.

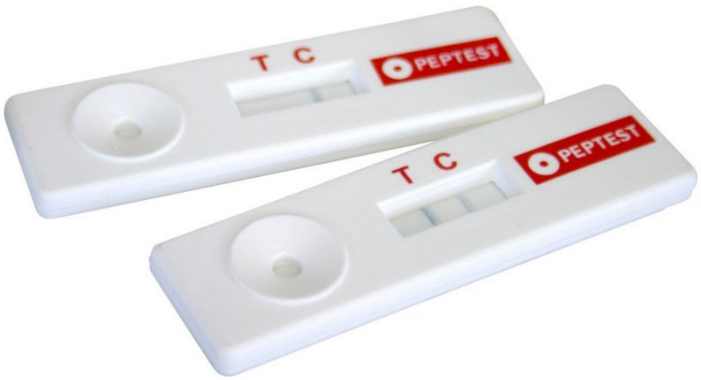

Figure 2-4: PEPTEST™ pepsin testing, lateral flow device.

Sample is placed in the circular well on the left side of the device and a functioning device will show a line under C (control) within a few minutes. Presence of pepsin will be indicated by a line under "T" which is then read by the lateral flow device reader giving a numerical value for the pepsin.

The procedure was repeated on the unselected portion of the induced sputum. After the sputum plugs were selected for sputum processing, the left-over unselected portion of the sputum sample was collected in a pre-weighed polypropylene tube and weighed. The rest of the procedure was repeated exactly as for the throat clearate to find the pepsin levels in the unselected sputum.

2.3.6 Bronchoscopy

Bronchoscopy was performed using a Pentax colour CCD flexible bronchoscope (Pentax UK, Slough, UK) in a purpose built bronchoscopy suite adhering to the British Thoracic Guidelines for Diagnostic fibre-optic bronchoscopy, 2013 and local SOPs [121]. Participants had been fasted for at least 4 hours prior to the procedure. After physical examination and pre-treatment with nebulised salbutamol 2.5mg, intravenous access was gained using BD Venflon™ 20GA in the right or left cubital fossa as appropriate. Local anaesthesia is given in the nose using Instillagel™ (CliniMed, High Wycombe, UK) and/or Lignocaine 10% spray. Oral-pharyngeal local anaesthesia is achieved with Lignocaine 10% spray orally. Scope was inserted via nasal route or oral route if no satisfactory nasal access. Further local anaesthesia was achieved with Lignocaine 2% at laryngeal (vocal cords) level and Lignocaine 1% in the trachea and bronchial tree as appropriate, titrated as per patient requirements. Mild sedation and analgesia is provided using IV Midazolam (0-5mg) and Alfentanil (0-1000mcg) and titrated as per patient needs.

Bronchoalveolar lavage (BAL) was performed in the right upper lobe. The scope is wedged in the sub-segmental bronchus and 6 X 20 mls of warmed sterile normal 0.9% saline was injected and aspirated via the suction channel into a sterile lavage trap. The sample was stored on ice immediately for processing.

4-5 bronchial biopsies were taken from the 2^{nd} – 4^{th} carinae of the right lower lobe of the lung using 1.8 mm alligator cupped biopsy forceps (CONMED™ Precisor, 100503). 2-4 laryngeal biopsy were done using the same biopsy forceps on the way out of the trachea towards the end of the bronchoscopy. The samples were collected from the arytenoid processes in the post-cricoid area after further local anaesthesia is given with lignocaine 2% in the laryngeal area. The biological samples were collected by lab technicians and stored for processing.

Broncho-alveolar lavage (BAL) processing

BAL processing was carried out within 2 hours from collection. The BAL sample was filtered through a 100-µm sterile cell strainer (Fischer Scientific) to remove debris. The filtrate was collected into a pre-weighed 50mL centrifuge tube to assess the filtrate weight. The sample was centrifuged at 400*g* (LabFuge 400R) for 10 minutes at 4^{o}C. The supernatant was removed into labelled tubes and stored at -80^{o}C after snap-freezing in isopropanol for 5 mins, pre-chilled on dry ice. The cell pellet was re-suspended in PBS. Cell viability and cell count was then calculated using the Neubauer haemocytometer and trypan blue exclusion method to bring the cell count to 0.5 x 10^6 cells/ml. Labelled slides were placed into a Shandon cytospin clamped in place with a filter card and funnel. 70 µl of cell suspension was added to each slide. The slides were spun on centrifuge at 450 rpm for

6 min. Quality of slides was checked to attain a balanced density of cells with the aim to achieve 4-6 slides of good quality. Slides were air-dried overnight and stored at -80^{o}C.

BAL cytokine analysis

The supernatant from BAL filtrate were processed with the MSD® Multi-Spot Assay System (MSD, Maryland, USA) – Vplex® Proinflammatory Panel 1 (human) Kits as per methods described by in the manufacturer specifications. The inflammatory markers tested included IFN-gamma, IL-1beta, IL-10, IL-12p70, IL-13, IL-2, IL-4, IL-6, IL-8 and TNF-a.

Bronchial biopsy processing

Biopsy is removed from the forceps with a fine gauge needle and placed in the appropriate fixative.

Processing of biopsies into paraffin wax

1-2 bronchial biopsy samples were used for processing in paraffin wax. The biopsy samples were completely immersed into 10% neutral buffered formalin for 12-24 hours. Approximately 5 ml of the fixative is recommended for the bronchial biopsies. The biopsy samples were then cut and processed as per departmental protocols.

Processing of biopsies into Glycol Methacrylate (GMA):

2-4 bronchial biopsies were used for processing in GMA in the following order

1. Biopsy is immediately placed in ice cold acetone containing 2mM phenyl methyl sulphonyl fluoride (35mg/100ml, 175mg/500ml) and 20mM iodoacetamide (370mg/100ml, 1.85/500ml).
2. Tissue is fixed overnight at -20^{o}C.
3. Fixative is replaced with acetone at room temperature, 15mins.
4. Then with Methyl benzoate at room temperature, 15 mins
5. Biopsy tissue is then infiltrated with processing solution: 5% methyl benzoate in GMA solution A at 4^{o}C. This stage is repeated 3 times every 2 hours.
6. Embedding solution is prepared as follows:
    - GMA solution A – 10mls
    - Benzoyl peroxide – 70 mg

Benzoyl peroxide is dissolved in GMA solution A by gentle shaking, when dissolved add

- GMA solution B 250ul

7. Specimen is embedded in freshly prepared embedding solution in Taab flat bottomed capsules placing biopsy in the bottom of the capsules and filling to the brim with resin and closing the lid to exclude any air bubbles
8. Tissue is polymerised at 4^{o}C for 48 hours.
9. Resin blocks are stored in air-tight boxes at -20^{o}C – for further analysis

Toludine blue for tissue morphology

GMA embedded specimens were cut as per departmental protocols. Toludine blue staining was done to look at tissue morphology and to check the suitability of the biopsy sample for staining for immunohistochemistry (IHC).

1. Cut sections were air dried until slide is completely dry or cut sections are dried on hot plate for at least 10 minutes
2. Toluidine blue staining solution was applied for 1-2 minutes until green halo appears around stain
3. Slides were washed in running tap water
4. Blot dried
5. Mounted in pertex

Immune-histochemistry (IHC) staining

IHC staining was done as per departmental protocols. Suitable biopsy sample identified after Toludine blue staining were cut and air dried until slide is completely dry or cut sections are dried on hot plate for at least 10 minutes. The slides are then treated as below to stain for mast cells (AA1), eosinophils (EG2), neutrophils (neutrophil elastase-NOE), macrophages (CD68), CD3+ T cells, CD4+ T cells, CD8+ T cells in both the sub-mucosa and epithelium.

1. Inhibit endogenous peroxidase using a solution of 0.1% sodium azide and 0.3% hydrogen peroxide in ROW, 30 mins.
2. Wash with TBS, 3 x 5 mins.
3. Drain slides and apply culture medium, 30 mins.

4. Drain slides and apply primary antibodies at appropriate dilutions, cover with coverslips and incubate overnight at room temperature.
5. Wash with TBS, 3 x 5 mins.
6. Drain slides and apply biotinylated second stage antibodies at appropriate dilutions, 2 hours.
7. Wash with TBS, 3 x 5 mins.
8. Drain slides and apply avidin biotin-peroxidase complexes at appropriate dilution, 2 hours.
9. Wash with TBS, 3 x 5 mins.
10. Drain slides and apply
    a. a: AEC substrate – 20 mins at room temperature
    b. b: DAB substrate – 10 mins at room temperature
11. Rinse with TBS and wash in running tap water, 5 mins.
12. Counterstain sections with Mayer's haematoxylin and blue in running tap water.
13. Drain slides and apply aqueous mounting medium and bake at 80^{o}C for 15 mins.
14. Allow slides to cool and mount in pertex.

Laryngeal biopsy processing

2-4 laryngeal biopsy samples were processed as per the processing method specified for bronchial biopsy processing in GMA. The slides are then treated as below to stain for mast cells (AA1), eosinophils (EG2), neutrophils (elastase), macrophages (CD68) and CD3+ T cells in both the sub-mucosa and epithelium. No paraffin processing is done for laryngeal biopsy samples.

2.3.7 High Resolution Oesophageal manometry (HRM)

High resolution oesophageal manometry (HRM) was performed using a customized single-use oesophageal 24 channel water perfused catheter (12 F (AHC-HR2412, Mui Scientific, Ontario, Canada) and Solar GI-HRM machine (Labori MMS). The catheter is attached to the transducer ports on the Solar GI-HRM machine. After entering the patient details the transducers were opened to perfuse the catheter. Once all 24 channels were perfused (visible water droplets from the tip of the catheter) the catheter was raised from a zero level to a marked level equivalent to 50 mmHg. Pressure across all channels is displayed on the manometry screen as a check to see that all channels register a pressure of 50 mmHg. The machine is then re-zeroed prior to insertion with the catheter at the height of the chest with the patient in supine position.

Patients were given lignocaine 10% spray nasally prior to insertion of the catheter. The catheter was inserted via the nasal route with the aim to capture the oesophageal pressure signal generated

by the upper oesophageal sphincter (UOS) and the lower oesophageal sphincter (LOS) on the screen. This is likely with an initial insertion depth of approximately 55cm from the nose, with additional adjustments where necessary, (depending on patient height) and then adjusted accordingly to capture both the upper and lower oesophageal sphincter pressure on the screen. Input the catheter depth from the nares before beginning the study.

The procedure commenced by allowing the patient to acclimatise to the presence of the catheter for a recommended time of 3 minutes. Once the patient was comfortable with the catheter, their physical position was adjusted so they are laying at the same level as the transducers. The patient may rest their head on a pillow for comfort.

A recording of the resting lower oesophageal sphincter pressures for approximately 30 sec was made during a representative period where the patient is calm and is not swallowing (see Figure 2-5). The proximal and distal sphincter margins were adjusted on the screen and oesophageal sphincter position / depth was recorded. The following provocations were undertaken:

- 10 Liquid bolus of 5 mls of water each
- 5 liquid bolus of 2 mls of water at 2 second intervals
- Wait to see a total clearance trace on HRM screen
- 5 Solid bolus swallows (piece of bread) one every 30 seconds or longer if more time is require to clear the bolus.
- 200mls of water within 30 seconds of the last solid bolus swallow to achieve a full clearance trace

The HRM catheter was removed, and the necessary preparation were performed for the insertion of pH/impedance catheter.

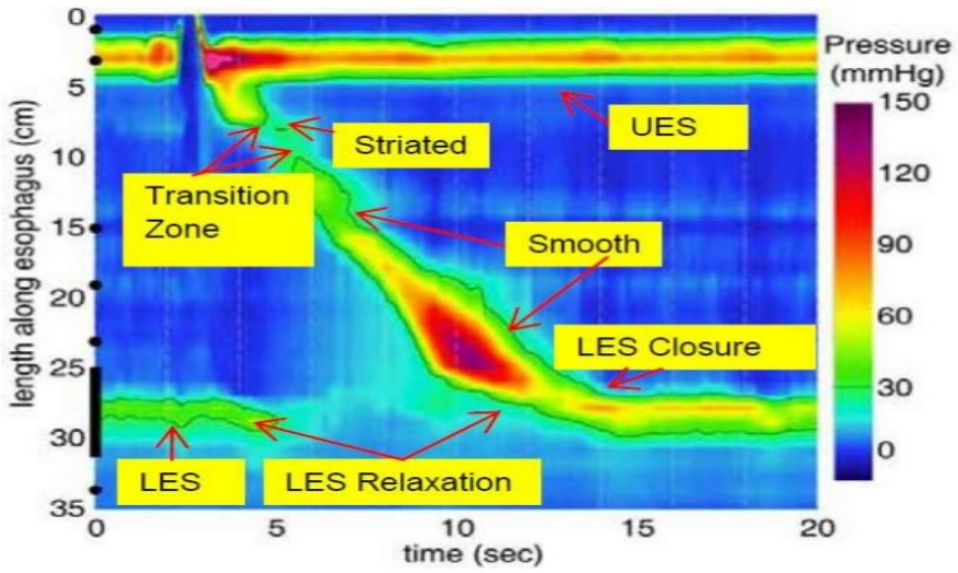

Figure 2-5: A normal high resolution manometry trace on a wet swallow.

2.3.8 24 hour pH and Impedance monitoring

The impedance catheter was calibrated in pH 4 and pH 7 buffer solution and saline as per the manufacturer's instructions to prepare for recording in accordance with the departmental standard operating procedures.

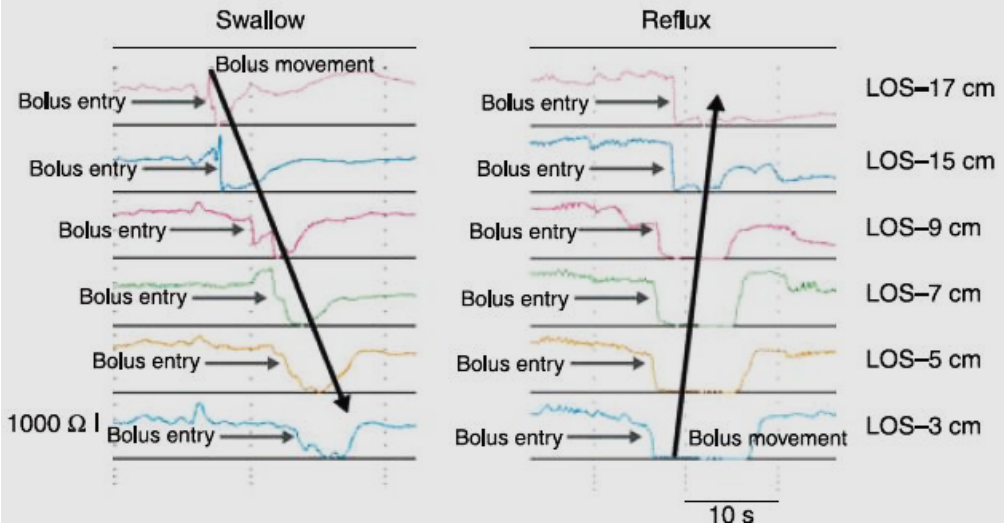

Figure 2-6: Intra-oesophageal impedance trace of a normal swallow in comparison with a reflux event.

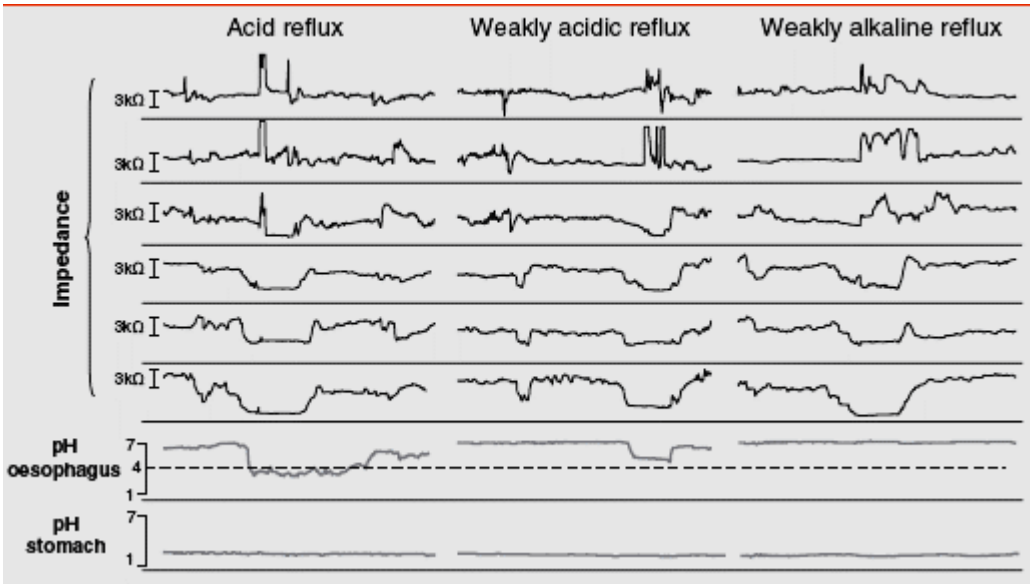

Figure 2-7: Examples of acid, weakly acid and non-acid/weakly alkaline reflux with the help of a pH and impedance trace.

The HRM gives an accurate measurement of the position of the LOS. The pH and impedance catheter (pHTip™, K6011-El-0632, Unisensor AG, Attikon, Switzerland) was inserted via the nares and positioned at 5cm above the proximal border of the LOS and secured at the nose, face and neck.

The connector was passed through the patient's shirt / top and attached to the recording device (Ohmega™ Labori MMS). The device was then turned on, and the start time from the device was recorded.

The patient was asked to swallow air and burp if possible and cough so that input is recorded on both the impedance recording as well as the cough monitor. The normal swallow of the bolus is observed (see Figure 2-6). Figure 2-7 shows the difference between a swallow and episodes of reflux of acidic, weakly acidic and non-acid/weakly alkaline types comparing the impedance trace with the oesophageal pH trace. All patients were sent home with instruction to use the buttons on the recording device (Ohmega™ Labori MMS) to record symptoms, start and end of mealtimes and start and end of bedtime. They were also provided with a symptoms sheet so that they can record the same data on hard copy as well.

Patients returned the following day to remove the pH/ impedance catheter and the recording device. The data was then uploaded onto the main database for analysis. The whole procedure was done as per the departmental guidelines in the Gastro-intestinal physiology department.

2.3.9 Cough monitoring (Leicester Cough Monitor)

Cough monitoring was done using the Leicester cough monitor – LCM [122]. The voice recorder was attached to the patients at the same time as the 24 hr pH and impedance (Ohmega™) device. The clock on the voice recorder was matched with the clock on the Ohmega™ device with the aim that all cough episodes can be potentially matched with any impedance or pH episodes. The voice recorder is turned off and taken off at the same time as the pH and impedance catheter removal at the end of the study and the time recorded as per the Ohmega™ device clock.

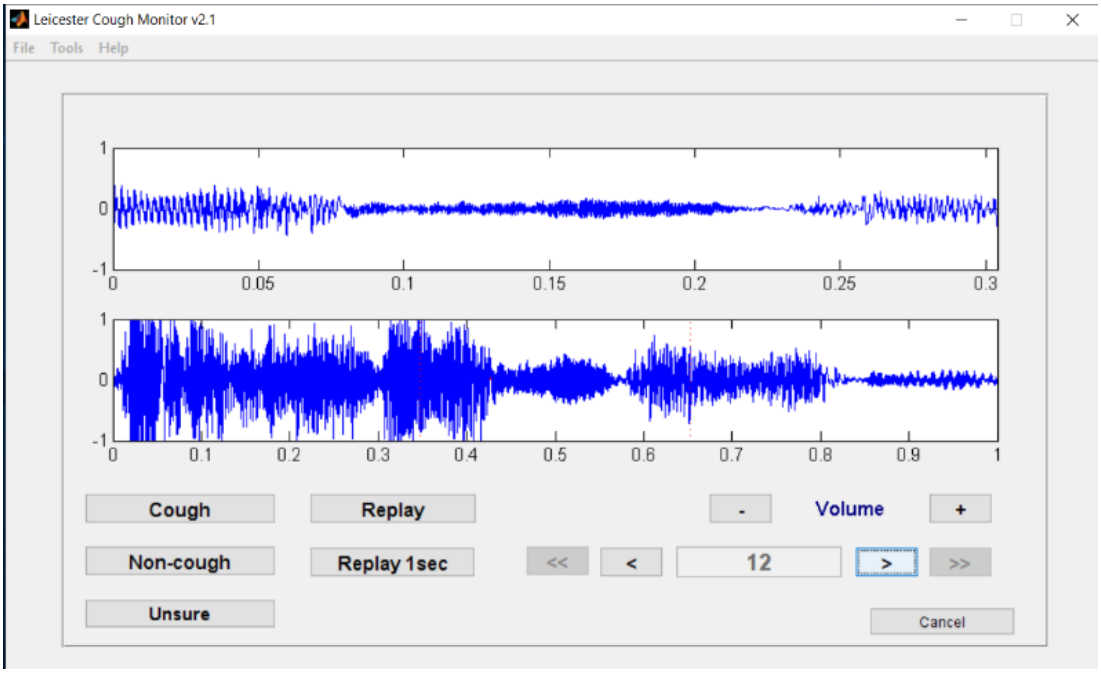

Figure 2-8: The LCM software interface. Sounds selected by software are then further refined by the operator.

The voice data is then loaded onto the LCM software and sounds filtered as per the LCM software protocol with the operator selecting whether the algorithm selected sounds are cough, non-cough or unsure. The software then re-assesses the sounds during the process, re-assessing and re-selecting further sounds from the remaining recording based on earlier selections until the complete recording is analysed (see figure 2-8). The results are saved as per the protocol.

Chapter 3 Sputum proteomic signature of gastro-oesophageal reflux in patients with severe asthma – Study 1

3.1 Introduction

Asthma is a disease of varying severity with complex underlying mechanisms. Its pathological features have been studied extensively, including in patients with severe disease, [11] but the roles of known and suspected triggers of asthma remain poorly understood. Amongst these is gastro-oesophageal reflux disease (GORD), a co-morbidity widely associated with asthma. Based on history, its prevalence is estimated as high as 80%,[123] which is significantly higher than in the general population, while between 32 and 84% of asthmatics have abnormal acid reflux demonstrated by pH studies,[124-126] although a substantial proportion do not have typical symptoms.[124] The higher prevalence of GORD in asthmatics has long been viewed as a risk factor, with evidence of a two-fold increase in risk of new diagnosis of asthma and respiratory symptoms in patients with persistent nocturnal reflux,[29] and a five-fold increased risk of exacerbations.[12] Our recent assessment of participants in the U-BIOPRED (**U**nbiased **BIO**markers for the **P**rediction of **RE**spiratory **D**isease Outcomes) project confirmed GORD as a significant co-morbidity in severe asthma,[105] in keeping with other studies.[106,107] However, such associations do not necessarily imply that reflux is the cause of severe asthma; alternatively, GORD could be the result of severe asthma due to altered lung mechanics, such as hyperinflation, complicated by increased weight, obesity and asthma drugs, which are all associated with GORD.[127]

Proton pump inhibitors (PPI) are effective at controlling GORD symptoms like heartburn[128] but are variably effective at improving asthma control; the same is true for fundoplication which physically blocks reflux.[36] This is the case even when acid reflux is confirmed by pH monitoring.[35] Such variability in response could be due to sub-optimal patient selection. Effectiveness could be improved if biomarkers were available to demonstrate that some of the gastric refluxate is inhaled and that this impacts on the underlying asthma pathobiology. Using this argument as the rationale for the current study, we hypothesised that the airways of severe asthmatics with active GORD are exposed to oropharyngeal refluxate by inhalation into the lower airways where it causes measurable biological effects. To test this hypothesis, we studied more than 240 participants in the U-BIOPRED project with good quality induced sputum samples and applied to their sputum supernatant a state of the art, quantitative liquid chromatography and untargeted mass

spectrometry analysis, LC/MS^E. Using multiple logistic regression, we then identified the proteins associated with GORD in severe asthma.

3.2 Methods

Study design

U-BIOPRED is a prospective, multicentre cohort study involving sixteen clinical centres in eleven European Union countries, recruiting healthy and asthmatic participants according to pre-set criteria for clinical stratification as previously published.[105] For the purpose of this study, the following clinical data were evaluated: history of GORD, smoking history, atopy, oral corticosteroid (OCS) treatment, exacerbation frequency in the past year, standardised disease activity questionnaires (short and full version of the Asthma Control Questionnaire [ACQ5 and ACQ7), Asthma Quality of Life Questionnaire [AQLQ], Hospital Anxiety and Depression Scores [HADS], Sino-Nasal Outcome Test [SNOT-20], Epworth sleep score [ESS]), spirometry, and exhaled nitric oxide. All participants were asked to provide sputum, usually on the day of clinical assessment; if samples failed the quality criteria or induction was unsuccessful, sampling was repeated within one week.

The study received ethics approval in all the countries involved and all participants provided written informed consent.

Cohort description

The recruitment criteria have been reported previously.[105] A total of 610 adult participants were stratified into four groups: Group A (n=311) - severe asthmatics on high-dose inhaled corticosteroids (ICS) ≥1000 µg fluticasone propionate (or equivalent), with no smoking in the past year and <5 pack-year smoking history, Group B (n=110) - severe asthmatics defined as for Group A but with either current or past (at least 5 pack-year) smoking history, Group C (n=88) – mild-moderate asthmatics with controlled or partially controlled asthma (defined by GINA) using <500 µg fluticasone propionate ICS (or equivalent) with no smoking in the past year and <5 pack-year smoking history, and Group D (n=101) - healthy individuals with no chronic respiratory disease, pre-bronchodilator FEV_1≥80% of predicted and non-smoking for ≥1 year or ex-smokers with a smoking history of <5 pack years.

The groups were further stratified into subgroups by previous physician-made diagnosis of GORD (ALL-GORD subgroup), i.e. all participants with a diagnosis of GORD and/or on treatment with anti-reflux medication) and participants who, at the time of assessment, had symptoms of GORD (ACTIVE-GORD), and those without a history of GORD and not on medication for GORD (NO-GORD).

Sample collection and analysis

Induced sputum was acquired and processed using approved U-BIOPRED standard operating procedures[108], using dithioerythritol (DTE) as a mucolytic to obtain supernatant for mass spectrometric analysis and cytospins for inflammatory cell counts[109].

Mass spectrometry

For full details of the mass spectrometric analysis, data curation, protein identity searching, data filtering and normalisation see the online supplement. Samples were analysed in duplicate via LC-IMS-MS^E on a Waters G2S high-definition mass spectrometer coupled to a nanoAcquity UPLC system. Database searches were performed using a custom package (Regression tester) based upon executable files from ProteinLynx Global Server 3·0 (Waters) and searched against the Uniprot human reference database (20/11/2014) with added sequence information for internal standards. Quantity was estimated in absolute amounts using the Hi 3 method.

Statistical analysis

Clinical and demographic data were analysed by parametric and non-parametric tests following assessment of distribution using the Shapiro-Wilk normality test by GraphPad Prism (version 6·0 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com) and SPSS (IBM SPSS Statistics for Windows, Version 22·0. Armonk, NY, USA). Comparisons of protein profiles defined by MS^E analysis were restricted to the severe asthma groups A and B in order to avoid confounding effects of disease severity. Comparison between protein profiles in ACTIVE-GORD and NO-GORD was the primary endpoint while that between ALL-GORD and NO-GORD participants was secondary. Feature selection for univariate logistic regression (ULR) of proteins predictive of ACTIVE-GORD and ALL-GORD in severe asthma (groups A and B) was performed by selecting proteins with differential concentrations in ACTIVE-GORD and ALL-GORD compared with NO-GORD subgroups (Mann–Whitney U test, $p \leq 0{\cdot}1$, without adjustment for multiple testing and with p-values raw and unadjusted). Analysis was limited to proteins present in ≥60% of participants to counter factors such as missingness which can adversely affect mass spectrometry analysis. The proteins identified as being associated with GORD by ULR (with $p \leq 0{\cdot}05$) were selected for multiple logistic regression (MLR) conducted with stepwise backward selection (adjusted for smoking and oral corticosteroid use) to rule out weak associations and select an efficient model of GORD. All regression analyses were conducted in SPSS.

3.3 Results

3.3.1 Clinical characteristics and associations with GORD in the complete U-BIOPRED cohort

The prevalence of GORD was higher in both severe asthma groups (54% and 66%, respectively) compared to mild/moderate asthmatic (18%) and healthy participants (5%) (Table 3-1). Similarly, active GORD was more prevalent in both severe asthma groups (33% and 46%) (Table 3-1). Regardless of asthma severity, BMI in the asthmatics was raised at the nominal unadjusted significance level ($p<0{\cdot}05$) in the ALL-GORD and ACTIVE-GORD subgroups, as compared to NO-GORD, except in smoking severe asthmatics where BMI in the ACTIVE-GORD and NO-GORD subgroups was not different. Age, atopy, smoking, asthma exacerbation rates, spirometry, exhaled nitric oxide concentrations were not related to GORD; however, OCS use was more prevalent in GORD subgroups in non-smoking severe asthmatics. Sputum neutrophil counts were significantly higher in mild/moderate asthmatics with active GORD when compared to those without GORD. Sputum eosinophil counts were lower in smoking severe asthmatics (ALL-GORD and ACTIVE-GORD). A number of patients reported outcomes were associated with GORD (table 3-2): in smoking severe asthmatics, ACQ5, ACQ7, AQLQ, HADS and SNOT-20 scores were raised in patients with GORD, while in the non-smoking severe asthmatics GORD was associated with higher HADS, SNOT-20 and ESS. GORD was also associated with AQLQ and HADS in mild/moderate asthma.

Table 3-1: Demographic and clinical features of U-BIOPRED cohort groups A, B, C and D.

	Severe asthma (Group A, non-smokers)				Severe asthma (Group B, smokers)				Mild/Moderate asthma (Group C)				Healthy (Group D)			
	All in group	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**	**All in group**	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**	**All in group**	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**	**All in group**	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**
N (%)	311 (50·98%)	142 (45·7%)	169 (54·3%)	103 (33·1%)	110 (18·03%)	37 (33·6%)	73 (66·4%)	51 (46·4%)	88 (14·42%)	72 (81·8%)	16 (18·2%)	10 (11·4%)	101 (16·55%)	96 (95%)	5 (5%)	3 (2·97%)
Age (Yrs)	53 (43-62)	51 (41-60)	55 (43·5-62)	53 (42-62)	55 (48-61·3)	53 (47-63)	55 (49-61)	54 (48-63)	42·5 (28-52·8)	38·5 (26·3-52)	46 (35·5-61)	46·5 (34·5-52)	34 (27-49)	34 (26·3-48·8)	54 (45-61·5)	51 (41-54)**
SEX -M/F (%)	106/205 (34/66)	58/84	48/121*	32/ 71	54/56 (49/51)	23/14	31/42	22/29	44/44 (50/50)	38/34	6/10	4/6	62/39 (61/39)	58/38	4/1	3/0
Atopy POS/NEG (%POS of ALL in group)	213 /59 (78·3%)	104/24 (38·2%)	109/35 (40·1%)	68/21 (25%)	62/25 (71·3%)	23/8 (26·4%)	39/17 (44·8%)	25/12 (28·7%)	72 /6 (92·3%)	59/4 (75·6%)	13/2 (16·7%)	8/1 (10·3%)	36/42 (46·2%)	34/41 (43·6%)	2/1 (2·6%)	1/0 (1·3%)
BMI	27·7 (24·5-33·6)	26 (23·8-31·3)	29·7 (25·2-34·5)**	28·93 (24·6-34·4)*	28·88 (25·2-32·6)	27·3 (24·3-31·3)	29·56 (25·9-33·4)*	29·24 (25-33·7)	24·85 (23-28·9)	24·55 (21·9-27·8)	28·52 (24·6-32·8)**	28·52 (25·9-34·2)**	24·69 (22·7-27·5)	25·12 (22·8-27·5)	23·9 (20-26·6)	23·9 (20·1-28·9)
Smoking (Pack-years)	0	0	0	0	17·38 (10-26)	20 (10-23·6)	16·5 (10·5-30)	16·4 (10-25)	0	0	0	0	0	0	0	0
On oral steroids (%)	122 (n=311) 39·2%	43 (35·2%)	79 (64·8%) **	44 (36·1%)*	36 (n=110) 32·7%	11 (30·5%)	25 (69·4%)	16 (44·4%)	0	0	0	0	0	0	0	0
Exacerbations in last 12 months	2 (2-4)	2 (2-3)	3 (2-4)	3 (1-4)	2 (1-4)	2 (2-4.3)	2 (1-4)	N/A	0	0	0	0	0	0	0	0
FEV_1 (% pred)	67·49 (50·7-84·9)	66·4 (47·7-85·7)	67·7 (52·5-84·1)	67·36 (54·9-81·5)	65·86 (51·8-77·8)	65·68 (52·8-82·1)	67·68 (51·7-76·3)	71·56 (51·3-78·8)	91·74 (75·9-101·7)	92·38 (74·5-99·7)	89·35 (83·9-105·3)	102·8 (82·3-106·1)	102·14 (93·6-110·7)	102·4 (94-111)	99 (86·8-107·5)	105·3 (99-109·8)
FVC (% pred)	87·22 (19·6)	86·1 (19·4)	88·2 (19·8)	87·45 (19·4)	89·72 (18·2)	91·04 (19)	89·05 (17·8)	88·72 (16·8)	104·4 (18·9)	103·4 (19·8)	109·7 (13·2)	108·7 (15·7)	107·8 (13·4)	107·9 (13·7)	104·8 (100·9-112·6)	104·8 (100·7-112·4)
Exhaled NO	26·5 (15·5-47·6)	26 (16-46)	27 (15-48·5)	31 (18·3-48·5)	23·5 (12-43·5)	29·5 (14·1-41·3)	21·5 (11·9-51)	17·75 (10·3-51)	25 (18-55)	27·5 (18-60)	20·25 (13·5-37)	22·5 (9·5-35)	19·25 (13·1-29)	19 (13·5-29)	24 (11·8-41·5)	24 (12·5-56)

Sputum-Macrophage %	26·24 (10·4-48·7)	24·6 (9·8-38·6)	29·7 (12-52·1)	39·86 (11·5-52·6)	33·73 (15·1-48·8)	30·25 (15·3-44·4)	34·76 (13·6-50·9)	34·32 (17·5-49·7)	44·01 (35·8-68·1)	44·58 (36-68·8)	36·16 (18·2-40·4)	29·28 (18·2-40·4)	58·92 (37·5-76·7)	58·1 (37·4-77·1)	68·79 (62·2-75·4)	75·42 (75·4-75·4)
Sputum-Eosinophil %	2·75 (0·4-20·5)	2·6 (0·4-25·5)	2·9 (0·37-17·5)	3·44 (0·4-14·7)	3·81 (0·7-13·7)	4·89 (1·6-19·5)	2 (0·4-12·3)	1.9 (0·4-6·2)	0·78 (0·2-3·4)	0·79 (0·2-3·4)	0·21 (0-3·4)	1·68 (0-3·4)	0 (0-0·3)	0 (0-0·4)	0	0
Sputum-Neutrophil %	53·69 (32·5-76·4)	56·6 (37·2-78·9)	52·3 (30·6-73·5)	50·65 (34·2-74·4)	55·1 (35-65·9)	55·52 (34·8-66·8)	54 (35-65·3)	57·3 (37·4-74·6)	41·71 (23·7-63·3)	38·78 (22·9-59·3)	76·4 (63·6-76·7)*	76·56 (76·4-76·7)*	39·56 (20·9-61·5)	39·81 (21·2-62·2)	28·6 (20·8-36·5)	20·76 (20·7-20·7)

Continued table 3-1.

Note (Table 3-1): Data from each group are shown for the whole group (All in group), for the subgroup of participants with no history of GORD (NO-GORD), for the subgroup with a history/diagnosis/current treatment of GORD (ALL-GORD) and for those with symptoms of GORD at the time of assessment (ACTIVE-GORD).

*= p<0·05 and **= p<0·01 for the comparison between participants in the NO-GORD category as compared with ACTIVE-GORD or ALL-GORD. All values are shown as median (range) or mean (SD) depending on distribution.

BMI = Body mass index (Kg/m^2). FEV_1 = Forced expiratory volume in 1 second, FVC = Forced vital capacity. Atopy results were available for 515 individuals based on skin prick tests and/or RAST. Group A - 272, Group B - 87, Group C – 78 and Group D - 78. Pos = positive for atopy, Neg = negative for atopy. %POS = percentage of participants positive for atopy out of the All in group. Exhaled NO = exhaled nitric oxide levels in parts per million. % = percentage.

Table 3-2: Patient reported outcomes in the U-BIOPRED cohort groups A, B, C and D.

	Severe asthma (Group A, non-smokers)				Severe asthma (Group B, Smokers)				Mild/Moderate asthma (Group C)				Healthy (Group D)			
	All in group	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**	**All in group**	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**	**All in group**	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**	**All in group**	**NO-GORD**	**ALL-GORD**	**ACTIVE-GORD**
ACQ5	2·2 (1·4-3·2)	2·2 (1·2-3)	2·4 (1·5-3·3)	2·4 (1·8-3·2)	2·2 (1·4-3)	1·9 (0·6-2·8)	2·5 (1·8-3·2)**	2·8 (1·8-3·4)**	0·8 (0·3-1·4)	0·8 (0·3-1·4)	1 (0·3-1·6)	0·8 (0-1·7)	N/A	N/A	N/A	N/A
ACQ7	2·71 (1·7-3·6)	2·57 (1·6-3·4)	2·8 (1·7-3·7)	2·86 (2-3·7)	2·57 (1·7-3·4)	2·29 (1.2-3)	2·79 (1·8-3·9)*	2·93 (1·9-3·9)*	1·0 (0·4-1·6)	1·0 (0·4-1·5)	1·14 (0·4-1·7)	0·93 (0-1·7)	N/A	N/A	N/A	N/A
AQLQ	4·51 (3·6-5·4)	4·59 (3·8-5·5)	4·31 (3·2-5·4)	4.31 (3.5-5.3)	4·44 (3·5-5·3)	5·03 (4·3-6·1)	4·03 (3·2-5)**	4·06 (3·1-5·1)**	6·13 (5·4-6·5)	6·25 (5·6-6·6)	5·41 (4·9-6·3)*	5·41 (5-6·1)*	N/A	N/A	N/A	N/A
HADS	12 (6-18)	10 (5-16)	14 (6·5-19)*	14 (7-20)*	13 (7-19·8)	9 (4·8-14·3)	14·5 (10·8-20·3)**	14 (4·8-20)*	5 (2-11)	1 (0-4)	4·5 (2·8-12·8)**	6·5 (2·8-15·8)**	4 (1-8·5)	4 (1·3-9·3)	NA	NA
SNOT-20	31 (19-43)	28 (16-39·3)	34 (23-45)**	35 (23·8-44·3)**	30 (17-48)	22·5 (10·8-42·3)	32 (20-49)*	34·5 (21·3-49·8)*	13 (5-21)	13 (5-19)	14·5 (8·25-29·8)	21 (9·5-31·3)	2 (0-8)	2 (0-7·8)	N/A	N/A
ESS	7 (4-10·5)	6 (3-10)	8 (4-11)**	8 (4-12)	8 (4-11)	8 (4-10·3)	7 (4-11)	7 (4-11)	5 (3-8)	5 (2-8)	5·5 (3-8)	6 (3·8-8·8)	5 (1-7)	4·5 (1-7)	N/A	N/A

Note (Table 3-2): Data from each group are shown for the whole group (All), for the subgroup of participants with no history of GORD (NO-GORD), for the subgroup with a history/ diagnosis/ current treatment of GORD (ALL-GORD) and for those in whom symptoms of GORD were present at the time of assessment (ACTIVE-GORD). *= p<0·05 and ** =p<0·01 for the comparison between participants in the NO-GORD category as compared with ACTIVE-GORD or ALL-GORD. Data are shown as median (IQR) or mean (SD) depending on distribution. Abbreviations: ACQ = Asthma Quality Questionnaire; AQLQ = Asthma Quality of Life Questionnaire; HADS = Hospital Anxiety and Depression Scale; SNOT-20 = Sino-Nasal Outcomes Test; ESS = Epworth Sleepiness Score. N/A in group D implies that the questionnaire is not relevant for group or the sample size is too small for analysis.

3.3.2 Clinical characteristics and associations with GORD in the severe asthma sub-set analysed for proteins predictive of GORD

Analysis of the clinical and demographic characteristics of the severe asthma subset (Group A and B) that was further analysed for proteins predictive of GORD showed that Group A and Group B had very similar prevalence of GORD in ALL-GORD as well as ACTIVE-GORD groups (please see table 3-3). The OCS dose in ACTIVE GORD group in the Proteomics analysis subset was significantly lower than the full cohort [Cohort A - 0(0-7.5) and 10(5.3-14.4) p=<0.0001, Cohort B – 0(0-10) and 11.25(8.3-23.8) p=0.0016). No other significant differences were noted in the two data sets.

Table 3-3 Questionnaire and clinical characteristics of the severe asthma subset analysed for proteins predictive of GORD.

	Severe asthma (Group A non-smokers)				Severe asthma (Group B Smokers)				Severe asthma (Non-Smokers and smokers combined)			
	All in group	**NO GORD**	**All GORD**	**Active GORD**	**All in group**	**NO GORD**	**All GORD**	**Active GORD**	**All in group**	**NO GORD**	**All GORD**	**Active GORD**
N(%)	108 (70.12%)	48 (44.4%)	60 (55.55%)	34 (31.48%)	46 (29.87%)	16 (34.78%)	30 (65.22%)	21 (45.65%)	154 (100%)	64 (41.56%)	90 (58.4%)	55 (35.7%)
Age *(Yrs)*	55.5 (44.3-62)	54.5 (39.3-63.3)	56.5 (47.5-62)	57.5 (49.3-62)	55 (46.8-62.3)	55.5 (46.3-64.8)	55 (49.8-61.3)	55 (48-62)	55 (46-62)	55 (41.8-64)	55.5 (48.5-62)	55 (50-62)
Gender-M/F (%)	39/69 (36/64)	21/27	18/42	12/22	18/28 (39/61)	8/8	10/20	7/14	57/97 (37/63)	29/35	28/62	19/36
BMI	27.75 (23.9-33.3)	27.3 (23.7-31.9)	28.3 (24.2-33.9)	29.1 (23.1-33.8)	29 (25.4-33.5)	27.3 (25.4-32.5)	29.88 (25.5-36.7)	29.4 (25.1-37.6)	27.83 (24.6-33.3)	27.3 (24.1-32.1)	29.39 (25-34.1)	29.3 (24-34.4)
Smoking (PY)	0	0	0	0	20 (12.8-26.7)	21.25 (13.3-27.8)	26.25 (12.8-26.9)	15 (12.8-20.7)	0	0	0	0
On oral steroids - N (%)	42 (38.9%)	14 (33.3%)	28 (66.7%)	15 (18.3%)	21 (45.7%)	6 (28.6%)	15 (71.4%)	9 (24.3%)	63 (40.9%)	20 (13%)	43 (27.9%)*	24 (20.2%)
Exac. in last 12 months	2 (1-3)	2 (1-3)	2 (1-4)	2 (1-4)	2 (1-4)	2 (0-3.5)	2 (1-4.3)	2 (1-4)	2 (1-3)	2 (1-3)	2 (1-4)*	2 (1-4)
Exhaled NO	23.5 (15-41.3)	25.5 (14-44.8)	25.5 (14-44.8)	23.8 (19.3-38.3)	20 (10.5-44.8)	24.5 (10.3-33.8)	18.8 (10.4-71.8)	20 (10.8-55)	23 (14-41)	25.5 (12.3-36.4)	22.5 (14.5-45)	23 (14.5-41.8)
Atopy POS/NEG (% Pos)	69/21 (76.7%)	34/7 (37.8%)	35/14 (38.9%)	21/8 (23.3%)	24/12 (66.7%)	11/2 (30.6%)	13/10 (36.1%)	8/8 (22.2%)	93/33 (73.8%)	45/9 (35.7%)	48/24 * (38.1%)	29/16 * (23%)
FEV1 *% pred*	64.03 (21.4)	64.31 (22.2)	63.82 (20.9)	63.6 (20.8)	65.18 (17.7)	62.9 (20.1)	66.39 (16.5)	67.67 (18.6)	64.38 (20.3)	63.95 (21.6)	64.68 (19.5)	65.16 (19.9)
FVC *% pred*	87.1 (18.6)	88.62 (17.4)	85.9 (19.5)	85.1 (20.1)	89.9 (17.5)	91.25 (18.6)	89.18 (17.1)	89.6 (19.7)	87.94 (18.2)	89.28 (17.6)	86.9 (18.7)	86.82 (19.9)
Sputum-Macrophage *%*	26.98 (9.7-50.2)	26.12 (9.5-39.1)	27.52 (10.1-55.2)	37.31 (9.5-56.2)	34.08 (15.3-49.3)	30.25 (16.9-43.6)	34.84 (13.5-54.2)	34.91 (15.4-56.8)	27.83 (12.6-49.3)	27.52 (12.8-40.9)	30.04 (12.5-53.5)	36.77 (11.9-56)

	Severe asthma (Group A non-smokers)				Severe asthma (Group B Smokers)				Severe asthma (Non-Smokers and smokers combined)			
	All in group	**NO GORD**	**All GORD**	**Active GORD**	**All in group**	**NO GORD**	**All GORD**	**Active GORD**	**All in group**	**NO GORD**	**All GORD**	**Active GORD**
Sputum-Eosinophil %	2.59 (0.3-17.9)	2.08 (0.2-20.6)	3.04 (0.4-17.9)	3.47 (0.4-15.2)	3.55 (0.4-13.6)	4.89 (1.9-23.1)	1.89 (0.4-11.3)	1.79 (0.3-5.9) *	2.75 (0.4-16)	3.41 (0.4-21.1)	2.64 (0.4-14.9)	2.8 (0.4-8)
Sputum-Neutrophil %	56.69 (32.1-78.8)	62 (37.3-82)	54.47 (29.8-75.1)	51.5 (33.7-77.4)	55.55 (34.8-65.6)	55.52 (34.4-65.8)	56.52 (34.7-66.3)	59.44 (34.4-73.1)	55.1 (33.5-74.4)	58.17 (36.8-77.7)	52.48 (31.7-73.1)	52.67 (34.2-75.4)
ACQ5	2 (1-3)	2 (1-3)	3 (2-3)*	3 (2-4)*	2 (1-3)	2 (1-3)	2 (2-3)	3 (2-3.5)*	2 (1-3)	2 (1-3)	2 (2-3)**	3 (2-3.75)**
ACQ7	3 (2-3.3)	2 (1-3)	3 (2-4)	3 (2-4)	2 (2-4)	2 (1.3-3)	3 (2-4)*	3 (2-4)*	2 (2-3.5)	2 (1-3)	3 (2-4)*	3 (2-4)**
AQLQ-Total	5 (4-6)	5 (4-6)	4 (3-5)**	4 (4-5)*	4.5 (4-5.8))	5 (4-6)	4 (3.5-5)	4 (3-5)	5 (4-6)	5 (4-6)	4 (3-5)**	4 (4-5)*
AQLQ-Activity	4 (3-6)	5 (4-6)	4 (3-5)*	4 (3-5)	4 (4-6)	5 (4-6)	4 (3-5.5)	4 (3-6)	4 (4-6)	5 (4-6)	4 (3-5)**	4 (3-5)*
AQLQ-Symptom	5 (4-6)	5 (4-6)	4 (3-5.5)*	4 (3.3-5)*	4.5 (3.3-5)	5 (4-6)	4 (3-5)*	4 (3-5)	5 (4-6)	5 (4-6)	4 (3-5)**	4 (3-5)**
AQLQ-Emotional	5 (4-6)	5 (4-6)	4 (3-6)**	4 (3-6)*	5 (4-6)	5 (4-6)	5 (3.5-5.5)	5 (3-5)	5 (4-6)	5 (4-6)	5 (3-6)**	5 (3-6)**
AQLQ-Environment	5 (4-6)	5 (4-7)	5 (3-6)*	5 (4-6)	5 (3.25-6)	5 (4-7)	4 (3-5.5)	4 (3-6)	5 (4-6)	5 (4-7)	5 (3-6)**	5 (4-6)*
HADS	12.5 (6-18)	11 (6-16.5)	14 (6-18.3)	17 (7-20)	12 (7-17.3)	8 (4-11.3)	13 (10.3-20)**	14 (12-22.5)***	12 (6.8-18)	9.5 (6-14.8)	13.5 (7-19.3)*	15 (7.5-20)*
SNOT20	31 (23-43)	30 (16-38)	33.5 (24-47.5)	36.5 (24.8-46)	24 (12-42)	16.5 (5.3-25.8)	32 (18-47)**	35 (20-48.5)**	29 (18-43)	25.5 (14-37)	32 (24-47)**	36 (24-47)**
ESS	7.5 (5-10.8)	6 (3.5-10)	9 (6-11)	9 (5.5-12)	8.8 (4-11.3)	9 (5.5-10.8)	7 (3.8-12)	8 (4.3-11.8)	8 (4.8-11)	7 (4-10)	9 (5.5-11.5)	9 (5-12)

Continued Table 3-3

Note (Table 3-3): Data from each group are shown for the whole group (All), for the subgroup of participants with no history of GORD (NO GORD), for the subgroup with a history of GORD (ALL GORD) and for those in whom symptoms of GORD are present at the time of assessment (Active GORD). In the comparison of NO GORD and Active GORD or ALL GORD * represents $p<0.05$, ** represents $p<0.01$ and *** represents $p<0.001$. Atopy data excluded data points where the result of combined atopy was "uncertain". ACQ (Asthma control questionnaire), AQLQ (Asthma quality of life questionnaire), HADS (Hospital anxiety and depression score), SNOT (Sino-nasal outcome test), ESS (Epworth sleep score)

Proteins associated with GORD

In order to remove any confounding effect of disease severity and because of low prevalence of GORD in healthy participants, proteomics data from mild/moderate asthmatics and healthy participants were excluded from the analysis. A total of 154 samples from severe asthmatics which passed QC were assessed (108 non-smokers and 46 smokers): of these, 90 had a diagnosis of GORD (ALL-GORD) and 55 also had active symptoms and/or were taking PPI (ACTIVE-GORD). This sub-cohort was slightly different from the complete U-BIOPRED cohort (Table 3-3): when compared with the NO-GORD subgroup, the GORD (All and Active) subgroups were not different in age, BMI, or lung function but more severe asthmatics were on OCS in the ALL-GORD compared to NO-GORD subgroups.

Exacerbation frequency, ACQ5, ACQ7, AQLQ, HADS and SNOT-20 scores were higher in both ALL-GORD and ACTIVE-GORD.

The primary comparison of sputum protein profiles between ACTIVE-GORD (n=55) and NO-GORD (n=64) subgroups identified 152 proteins detectable in ≥60% of participants, with 5 proteins being differentially abundant at p<0·05: Ig lambda-2 chain C regions was raised in ACTIVE-GORD, while alpha-1-antichymotrypsin, plasma protease C1 inhibitor, immunoglobulin lambda variable 1-47 and alpha-1-acid glycoprotein 1 were reduced (Table 3-3). These proteins were analysed by ULR together with another 6 proteins with significance at p≤0·1: lactotransferrin, lipocalin-1, serotransferrin, keratin type II cytoskeletal 6B, keratin type I cytoskeletal 10 and heat shock cognate 71 kDa protein (Appendix A - Table A-3). Subsequent MLR, adjusted for smoking history and OCS treatment, identified four proteins associated with ACTIVE-GORD (Table 3-5): immunoglobulin lambda variable 1-47, plasma protease C1 inhibitor, lipocalin-1, and Ig lambda-2 chain C region. The first three proteins were retained in a further multiple regression analysis with backward selection (Figure 3-1).

The backward selection with adjustment for OCS dose and smoking was necessary to minimize the impact from these factors on the proteomic profile to identify the most statistically robust proteomic predictors. As part of the logistic regression analysis with backward selection the Goodness of fit for each step is checked with Hosmer and Lemeshow Test (Figure 3-2). This test was not significant at any stage which meant that the regression model had a high Goodness to fit suggesting that the observed values match the expected observations without any significant differences. Additionally, I calculated the effect size using the R^2 *value* from the Logistic regression model (Figure 3-2) and calculating the Cohens F^2 from an online calculator[129]. This showed a F^2 value of 0.28 which is keeping with a medium size effect (Table 3-6). This value decreased with each step

of the logistic regression model suggesting that as the backward selection ruled out the weak associations the effect size was also affected until it reached its strongest associations.

A further Mann-Whitney U analysis, comparing sputum proteomes from the ALL-GORD and NO-GORD subgroups, yielded 10 differentially abundant proteins for ULR analysis (Appendix A, Table A-2 and Figure A-1): 3 (immunoglobulin lambda variable 1-47, Alpha-1-antichymotrypsin and heat shock cognate 71 kDa protein) were related to the diagnosis of GORD ($p \leq 0{\cdot}05$) and were associated with ALL-GORD when adjusted for smoking and OCS use (see Table 3-5).

Table 3-4: Comparison of protein abundance between ACTIVE-GORD and NO-GORD in the Severe asthmatics (Cohort A and B).

Mann-Whitney U comparison of proteins between ACTIVE-GORD and NO-GORD					
Protein name	**Uniprot ID**	**ACTIVE-GORD Protein Abundance (IU)**	**NO-GORD Protein Abundance (IU)**	**Z**	**p value**
Immunoglobulin lambda variable 1-47	P01700	26781 (15624-37298)	38693 (19555-48680)	-2·788	0·005
Alpha-1-antichymotrypsin	P01011	110332 86807-140619)	130569 (102641-165315)	-2·708	0·007
Alpha-1-acid glycoprotein 1	P02763	28638 (15646-38783)	39145 (28541-46295)	-2·510	0·012
Ig lambda-2 chain C regions	P0CG05	460018 (282052-564478)	397731 (238317-481500)	-2·191	0·028
Plasma protease C1 inhibitor	P05155	21081 (13898-37249)	30850 (15568-43890)	-2·111	0·035
Lactotransferrin	P02788	327432 (285341-387855)	385396 (293012-436902)	-1·903	0·057
Lipocalin-1	P31025	41899 (28461-88086)	39573 (20233-49739)	-1·812	0·070
Serotransferrin	P02787	184384 (153424-278884)	178905 (131771-227614)	-1·764	0·078
Keratin, type II cytoskeletal 6B	P04259	16914 (5055-41084)	36110 (7661-43661)	-1·743	0·081
Keratin, type I cytoskeletal 10	P13645	35580 (17494-48261)	44056 (25742-72086)	-1·695	0·090
Heat shock cognate 71 kDa protein	P11142	21238 (13041-32211)	23376 (16362-38133)	-1·690	0·091

Note: Protein abundance (IU = international units) is shown as Median (IQR). Z test shows the standard deviation from the mean for the given value and help determine the significance of set of data. A Z-score of +1.96 to -1.96 would be in line with a p value less than or equal to 0.05, hence rejecting the Null hypothesis and confirming the statistically significant difference between the two groups.

Table 3-5: Proteins identified as best predictors of ACTIVE-GORD and ALL-GORD vs NO-GORD by multiple logistic regression analysis adjusted for smoking and OCS use with backward selection

Proteins Identified as predictors of ACTIVE-GORD *vs* NO-GORD using MLR with backward selection		
Protein name	**Uniprot ID**	**P value**
Immunoglobulin lambda variable 1-47	P01700	0·017
Plasma protease C1 inhibitor	P05155	0.043
Lipocalin-1	P31025	0·034
Proteins Identified as predictors of ALL-GORD *vs* NO-GORD on MLR with backward selection		
Immunoglobulin lambda variable 1-47	P01700	0·011
Alpha-1-antichymotrypsin	P01011	0·015
Heat shock cognate 71 kDa protein	P11142	0·02

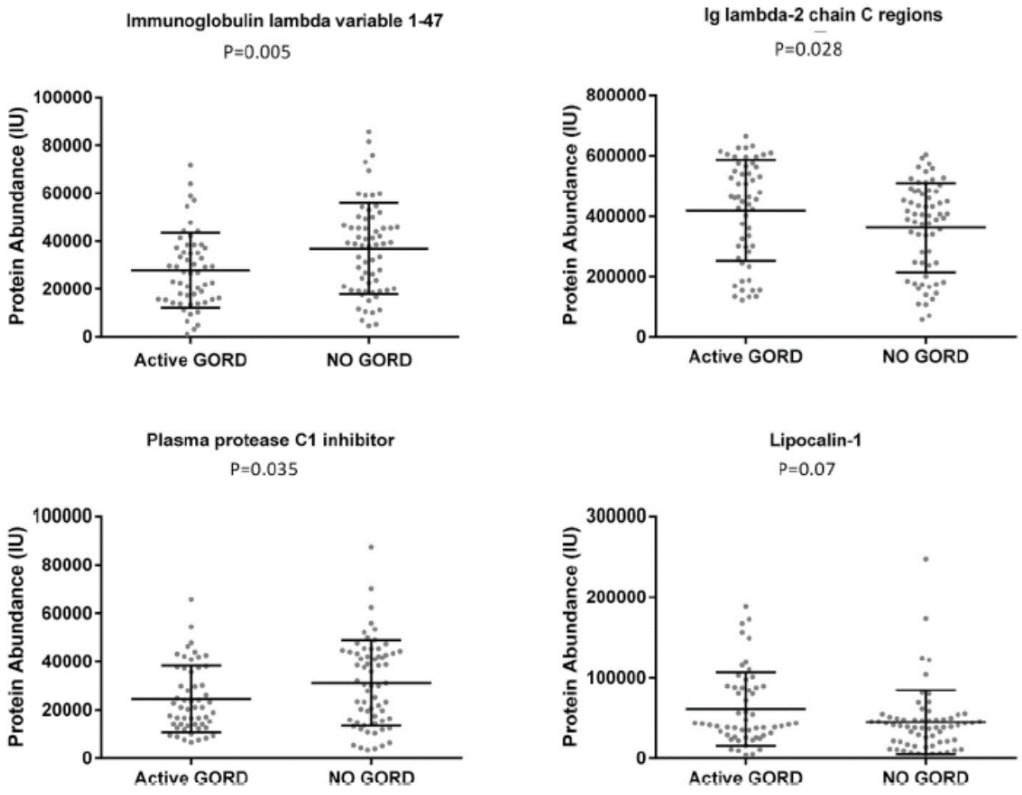

Figure 3-1: Proteins identified as best predictors of ACTIVE GORD. P values denote significance from initial Mann-Whitney U tests.

Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	130.324[a]	.248	.332
2	130.326[a]	.248	.332
3	131.899[b]	.238	.318
4	134.926[b]	.219	.292

Hosmer and Lemeshow Test

Step	Chi-square	df	Sig.
1	2.582	8	.958
2	2.491	8	.962
3	2.786	8	.947
4	6.170	8	.628

Figure 3-2. shows the R^2 values for the MLR analysis with backward selection adjusted for OCS use and smoking and the goodness to fit for the model with the Hosmer and Lemeshow test. (Taken from SPSS)

Table 3-6. Effect size measure (Cohen's F^2) based on the R^2 values given by the MLR model in SPSS

Note – Cohen's F^2 effect size interpretation 0.02=small, 0.15=medium, 0.35=large

	R^2 value (Cox and Snell)	**Cohen's F^2**	**Nagelkerke R^2**	**Cohen's F^2**
Step 1	0.248	0.329	0.332	0.497
Step 2	0.248	0.329	0.332	0.497
Step 3	0.238	0.312	0.318	0.466
Step 4	0.219	0.28	0.292	0.412

3.4 Discussion

To our knowledge, this study provides the first evidence that suggests a biological effect of GORD within the lungs and the first evidence of that effect in severe asthma. A study by Parameswaran et al. suggested that lipid-laden macrophages (LLMs) are markers of oropharyngeal reflux,[130] although it did not report that numbers were increased in asthmatics. Similar to our study, there were no differences in inflammatory cell counts between participants with and without reflux. A further study by Gibeon et al. [120] of 21 severe and 17 mild/moderate asthmatics confirmed that LLMs were more frequent in patients with GORD but did not show any relationship with asthma severity even though the prevalence of GORD was three-fold higher in severe asthmatics, possibly because it was a small study and all participants were on treatment for reflux.

The observed low prevalence of GORD in mild/moderate asthmatics and healthy participants in our study, and the absence of its impact on clinical outcomes in these groups, makes it unlikely that GORD has much impact on the lungs in milder disease or health. For this reason, detailed sputum proteomic analysis was limited to severe asthmatics in U-BIOPRED cohorts A and B, thereby removing disease severity as a bias. The 154 severe asthmatics whose sputum samples were assessed by mass spectrometry were representative of the wider cohort, with similar gender distribution, BMI, prevalence of GORD and ACTIVE-GORD, lung function, quality of life measures and sputum inflammatory cells. Asthma control, quality of life, and HADS scores were worse in ALL-GORD and ACTIVE-GORD subgroups. The higher SNOT-20 scores suggested a moderate effect of GORD on symptoms of rhinosinusitis. In contrast to the wider cohort, in the restricted group of severe asthmatics who provided sputum, those with GORD had more exacerbations and a higher proportion were on OCS than those without.

Eleven proteins were differentially abundant in severe asthmatics with active GORD compared to those without a GORD diagnosis at a significance level of $p<0{\cdot}1$; multiple logistic regression analysis with adjustment for smoking history and OCS use showed that three of these were associated with ACTIVE-GORD. While the concentrations of immunoglobulin lambda variable 1-47 and plasma protease C1 inhibitor were lower in ACTIVE-GORD when compared to participants with no history of GORD, the concentration of lipocalin-1 was higher. Analysis also identified three proteins associated with the diagnosis of GORD: immunoglobulin lambda variable 1-47, alpha-1-antichymotrypsin and the heat shock cognate 71 kDa protein, all of which were at lower concentrations in patients with GORD. The majority of proteins associated with GORD in this study have predominantly protective functions.[131] Some (heat shock cognate 71 kDa, alpha-1-acid glycoprotein, Ig light chains and

serotransferrin) are transported into the epithelial lining fluid by transcytosis or by transudation from the circulation. Others, like lipocalin-1 and lactoferrin, are derived from mucosal glands and exert anti-microbial properties, while alpha-1-antichymotrypsin is an acute phase protein mainly produced in the liver with anti-inflammatory and proteolytic properties.[132]

Lactoferrin plays a role in innate immunity. It is produced by exocrine glands and is detected in all mucosal secretions. It is also stored in neutrophil secondary granules from which it can be released by allergen stimulation. Its concentrations are increased in bronchoalveolar lavage of asthmatics [133], so our finding of slightly lower concentrations in GORD suggests a different mechanism of regulation in the presence of reflux. In contrast, lipocalin-1, the archetypal member of the lipocalin superfamily, which also includes retinol binding proteins, apolipoprotein D and lactoglobulins[134], was increased in patients with GORD. In view of structural similarities to known antimicrobial peptides in the same superfamily and widespread distribution in the bronchial epithelium, its primary role is thought to be epithelial defence. We have previously shown reduced levels in COPD but not in mild-moderate asthmatics.[109] The finding in the current study that levels are higher in severe asthmatics with GORD points to a distinct phenotypic feature of severe asthma which could underlie the increased risk of exacerbations found in our study. Lipocalin-1 sequesters siderophores produced by bacteria, thereby inhibiting bacterial growth through competition for iron reserves.[134] Lipocalins carry hydrophobic ligands such as lipids, steroids, hormones and other substances. When loaded with ligands, they induce regulatory T cells, leading to non-allergenic inflammation, whereas in empty state, they promote Th2 responses and inflammation.[135] Whether or not lipocalin-1 is bound to its ligands and whether this results in additional inflammation or tolerance in GORD requires further research.

Two subtypes of keratin were reduced in patients with GORD: keratin type-1 cytoskeletal 10 is widely distributed, while keratin type-2 cytoskeletal 6B is specific of distinct types of epithelia in the mouth and esophagus. As these are intracellular proteins, we speculate that their reduction reflects metaplasia where keratin isoforms could be altered, although there is at present no evidence of metaplastic epithelial changes in asthmatics with GORD. Complement C1 inhibitor, alpha-1-antichymotrypsin and alpha-1-acid glycoprotein are positive and serotransferrin is a negative acute phase marker. The finding that positive acute phase markers were lower in ACTIVE-GORD suggests that ACTIVE-GORD is associated with lower systemic inflammation. Elevated serotransferrin, the only negative acute phase marker, was elevated in ACTIVE-GORD, supporting this explanation.

Excess light chains synthesised during antibody synthesis are normally cleared rapidly by the kidney, while high levels of polyclonal Ig light chains are observed in a number of inflammatory conditions, including asthma. The conflicting patterns depending on the specific isoform seen in this study are

possibly due to differential secretion of light chain isoforms from the endoplasmic reticulum during chronic inflammation or differential proteolytic cleavage events post-secretion. Binding of free light chains to neutrophils elicits IL-8 secretion *in vitro* and inhibits neutrophil apoptosis.[136,137]

The current study confirms the reported association between asthma and GORD,[9,12] with more than half severe asthmatics having a diagnosis of GORD and one third ACTIVE-GORD, and the smoking group having an even higher prevalence than non-smokers. As previously reported,[127,138] raised BMI was associated with GORD. Furthermore, GORD diagnosis and symptoms were associated with anxiety and depression, in both severe asthma groups and in mild/moderate asthmatics. Patients with severe asthma had more symptoms of rhinitis and sinusitis than mild/moderate asthmatics and healthy individuals and these were associated with GORD as previously reported.[106,107] While the association between SNOT-20 scores and GORD has been reported previously,[18,19] to our knowledge, this association has not, until this study, been extended to asthmatics.

This study has a number of limitations. The GORD diagnosis was based on history supplemented by therapy records at the time of recruitment. The complexity of the U-BIOPRED programme, including participants' time and cost, precluded assessment of GORD by pH/impedance monitoring. Thus, it is likely that asymptomatic reflux was not picked up. Furthermore, we did not assess separately, participants on anti-reflux treatment, some of whom could have silent, weakly acid or non-acid reflux. Our analysis treated as a single group current and ex-smokers with significant smoking history. As this group had a higher prevalence of GORD in keeping with reported effects of smoking on GORD,[139] additional research is needed to assess the impact of cigarette smoking. The results for keratins and immunoglobulins will need confirmation because both are large families of highly homologous proteins with an increased probability of incorrect identification by MS that results in false positive results.

In summary, this study suggests that severe asthmatics with reflux have a distinct airways phenotype characterised by elevated anti-microbial proteins and reduced proteins that could be linked to systemic inflammatory responses and epithelial integrity, associated with poor asthma control, quality of life and additional co-morbidities. Further studies are required to confirm our findings, elucidate the roles of the differentially abundant proteins, and show whether the protein levels can be modulated by aggressive therapy of reflux.

Chapter 4 Physiological assessment of GORD in severe asthmatics – Study 2

4.1 Introduction

Gastro-oesophageal reflux disease (GORD) is a common chronic disease and affects up 27% of population in North America and Europe. Its prevalence is higher in the developed world which may be explained, to an extent, by the greater availability of diagnostic tests which have significantly improved our understanding of the nature and characteristics of the refluxate[140]. Application of pH monitoring provides information about the presence of refluxate in the oesophagus and it does this by picking up events where the pH is less than 4. However, factors like drinking or swallowing food can result in a drop in pH and thereby confound the results. In contrast, pH and Impedance monitoring overcomes this limitation very effectively by giving information about the nature of the reflux episode, e.g. movement of liquid, gas or a combination of the two, in addition to the direction of travel of the bolus, i.e. regurgitation (upwards movement) or swallow (downwards movement)[65,66]. It also gives the pH of the refluxate from a range of +7 to -7, providing a much wider scope to detect acid/non-acid nature of the refluxate and duration of exposure. This can provide further insight into the pathobiological effects of the refluxate in the context of its acidity. For this study I took advantage of monitoring of pH and impedance to assess whether there is any role for non-acid or weakly-acidic reflux in severe asthma. Indeed, duodeno-gastric reflux which is weakly acidic or non-acidic has been implicated as a risk for injury to the mucosa both in the GI tract as well as the respiratory system[27,98,141-146]. Furthermore, treatment of acid reflux has been associated with an increase in weakly and non-acid reflux which may be related to the acid suppression from PPI and H2 antagonists. This complicates the management of GORD where despite treatment with PPI and H2 antagonists symptoms may continue either due to treatment resistant acid reflux or increased prevalence of weakly acid and non-acid reflux after acid suppression [147-149].

Multiple questionnaire-based diagnostic tools for GORD have been used in various studies to date. However, these questionnaires do not provide the information describing the nature of the refluxate. I chose to use the Reflux Disease Questionnaire (RDQ) [150] at all the study time points because it has been used in previous studies of assessment and management of chronic cough related to GOR [37,151-153]. To use this questionnaire, I sought permission from AstraZeneca, the pharmaceutical company that had developed and validated it for the needs of their studies of the PPI, esomeprazole in chronic cough-associated GORD[150].

Physiological assessment of GORD in severe asthmatics was carried out in all participants at baseline (pre-treatment). In those severe asthmatics with a history of GORD, confirmed by 24-hour pH/impedance manometry, I repeated the monitoring 2 months after the patients received current standard care treatment with anti-reflux treatment with PPI (Omeprazole 40mg BD) and H2 Receptor blocker (Ranitidine 300mg OD nocte). In view of the unpleasant nature of the procedure, I chose not to repeat this physiological assessment after the additional three interventions (Metoclopramide, Domperidone and Baclofen) where I chose to limit my study to only questionnaires. For this study I took advantage of the DeMeester score and acid exposure time (AET) as two key assessments. While the DeMeester score is a composite score made up of multiple clinical parameters and allows a measure of GORD by using acid exposure time, the AET is measured as a percentage of time during the reflux duration that the oesophagus is exposed to liquid or gas at pH ≤4. Impedance events were measured along the full length of the pH/Impedance probe. Proximal extent of reflux as well as its nature (acidic and weakly acidic) in upright and supine position were also noted.

4.2 Hypotheses and aims

I hypothesised that

1. GORD is associated with acid, weakly acid and non-acid components and treatment of GORD reduces levels of acid and weakly-acid. I also speculated that acid reflux could convert to non-acid reflux, resulting in a treatment-related augmentation of non-acid and weakly acid reflux.

   To address this hypothesis, my aims were to:

   1. Assess the levels of GOR and its characteristics in severe asthmatics and healthy individuals.
   2. Quantify levels of acidic, weakly acidic and non-acidic reflux before and after treatment with acid-secretion blockers (PPI, H2 receptor);
   3. Compare pH/impedance analysis with the RDQ questionnaire to test the robustness of the RDQ questionnaire.

4.3 Methods

High resolution manometry (HRM) was performed in all participants (asthmatics and healthy participants) at visit 1 to locate the lower oesophageal sphincter (LOS) and, thus, position the

pH/Impedance catheter 5cm above the proximal border of the LOS. The catheter was then secured and connected to the event recording device which the participants carried attached via a belt to their waist. The catheter was removed 24 hours later and data from the recording device uploaded to the main database (in the hospital GI physiology department) in keeping with the departmental guidelines. Participants were asked to record episodes of reflux and cough by pressing a symptom button on the recorder (which doesn't differentiate between the two symptoms). They also pressed appropriate buttons to record the time of meals and going to bed. Additionally, the following day, when the catheter was removed, they were asked if they had had a standard reflux day or a better than normal reflux day.

In severe asthmatics, a screening HRM with pH and impedance was measured at visit 2, i.e. after 2 weeks of suspension of anti-reflux treatment (PPI, H2 Receptor blockers), and if there was evidence of GORD on the screening investigations, a second pH and Impedance monitoring was carried out after 8 weeks of treatment ((visit 8) with Omeprazole 40mg twice daily and Ranitidine 300mg once daily to assess the impact of high dose anti-reflux therapy on GORD diagnostics and its impact on the airways.

GORD was assessed and diagnosed based on standard criteria, applying the DeMeester score[72] in both healthy cohort and asthmatics. DeMeester score is a composite score based on duration, frequency and the position in which reflux occurs to give a risk score (see Figure 4-1). Allowance was made for participants with a prior physician diagnosis of GORD who scored just below the DeMeester score of 14.72 if they had a symptomatically better than usual 24-hour period in the context of GORD symptoms.

In addition to the DeMeester score, the following were analysed: Acid Exposure Time (AET) in upright and supine positions, total number of impedance events and impedance events with acid and weakly-acid exposure, proximal reflux events (acid and weakly acid) and proximal reflux events in the upright and supine position.

The full details of the methods are provided in the **Methods section 2.3.7 and 2.3.8.**

4.4 Results

The SA-no GORD participants were older than the HC-no GORD participants (table 4-1). Female sex was predominant (83.3%, 85.7% and 100% in SA-GORD, SA-no GORD and HC-no GORD, respectively), other than in the HC-no GORD group (33.3%). There was no significant difference in FEV1, FVC and PEF between the four groups (HC- no GORD, HC-GORD, SA-no GORD and SA-GORD) see table 4-1. However, there was a significant difference in MEF 25-75 %pred between HC-no GORD and SA-no GORD (p=0.002). Although there was a statistically higher degree of smoking pack/years in HC-GORD compared to HC-no GORD, albeit the median pack years in the HC-GORD group was only 0.7.

15 healthy participants underwent HRM and 24-hour pH and impedance testing. Of these, 5 were noted to have GORD while 10 did not have evidence of reflux. Of the 15 severe asthmatic participants who were assessed, 9 met the pH/impedance criteria for GORD while 6 had normal results. Two participants in the severe asthma group did not fully meet the DeMeester score but they were included in the SA-GORD group because their symptoms were a better than usual and their DeMeester score was borderline (at or just below the cut-off of 14.72). There is some evidence noted on online DeMeester score calculators regarding DeMeester score range relating to severity of reflux (DeMeester >14.7 – 50 = mild reflux, 51 – 100 = moderate reflux and >100 = severe reflux), however, for the purpose of this study I did not limit reflux into these categories. On assessment of the 15 severe asthmatic participants that were assessed, 6 would be in the mild reflux category while 3 would be in the moderate reflux category.

DeMeester scoring results (Score according to DeMeester normal values)

Score component	Patient	Score	Mean	SD	
Total reflux time (Total)	12.9	9.41	1.51	1.36	Total %
Total reflux time (Upright)	16.8	7.16	2.34	2.34	Upright %
Total reflux time (Supine)	1.3	1.68	0.63	1.00	Supine %
Nr of reflux periods	115.3	8.55	19.00	12.76	in 24 hours
Nr of long reflux periods > 5 min.	7.5	6.64	0.84	1.18	in 24 hours
Longest reflux	15.8	2.16	6.74	7.85	min

DeMeester score: 35.60 (14.72 is upper limit of 95.0 percentile of normal)

Impedance results

Impedance event overview

Total impedance events	103
Impedance events during meal	18
Swallows	7
Unknown directions	1
Refluxes	77

Figure 4-1: Example of the DeMeester score from a study participant

Table 4-1: Cohort characteristics as in table 5-1

	Severe asthma – GORD (A)	Severe asthma – No GORD (B)	Healthy control – GORD (C)	Healthy control – No GORD (D)	P value
N	*9*	*6*	*5*	*10*	n/a
Age	53 (44-60.5)	54 (48.5-62)	44.4 (19-59)	36.9 (22.0-57.0)	B-D-p= 0.0231
Sex M/F	*1/8*	*1/5*	*0/5*	*4/6*	ns
BMI	35 [26.1-39]	31.5 [26.1-38.2]	25.0 [23.8-31.4]	24.2 [22.2-26.4]	
Atopy Y/N	6/2	2/4	2/5	4/6	ns
Smoking (Pack Years)	0 [0-0]	0 [0-1.8]	0.7 [0.3-4]	0 [0-0]	C-D-p=0.029
Age of onset	12 [3.0,47]	11.5 [3.3,48.8]	n/a	n/a	ns
PreBD FEV1%pred	103.5 [79.8-126.1]	91.4 [72.3-116.4]	110.4 [105.3-124.1]	103.3 [93.6-116.2]	ns
PreBD FVC%pred	112.9 [100.1-139.5]	110.5 [94.5-142.7]	118 [112.5-147.6]	114 [105.9-122.4]	ns
PreBD MEF25-75%pred	52.4 [40.5-66.7]	23.2 [21.3-24.9]	63.7 [41-83.8]	72.6 [61.2-94.5]	B-D- p=0.0026
PreBD PEF%pred	84.1 [65.7-121.0]	109.5 [73.2-119.6]	106.4 [98.4-118.7]	106.6 [89-112.4]	ns

Note : Table shows general demographics and clinical characteristics of the full cohort in the phase 2 study. n/a = not applicable, ns = no significant difference. PreBD – Pre-bronchodilator, FEV1 – Forced expiratory volume in 1 second, FVC – Forced vital capacity, MEF 25-75% - Mean expiratory flow between 25% and 75% of the FVC representing small airways. PEF%pred – Peak expiratory flow percentage predicted.

Comparison of the DeMeester scores within all 4 groups showed a significant difference between SA-GORD and SA-no GORD (p=0.004). Similarly, there was a significant difference between SA-GORD and HC-no GORD (p=0.001). HC-GORD versus HC-no GORD showed a trend towards significance (p=0.067). In the SA-GORD participants, the baseline DeMeester score (pre-treatment with PPI and H2 receptor blockers) was 40.2 (16.7-62) and reduced significantly after treatment (4.2 (2.5-15.5), p=0.007). Please see Table 4-2 which covers the various measurements from the 24-Hr pH/Impedance.

Comparison of AET in the 4 groups showed a significant difference between the four groups (table 4-2) in line with the DeMeester score. Median (IQR) AET in SA-GORD reduced significantly from 13.2 (5-16.9) at baseline to 1.5 (0.57-4.9) after treatment(p=0.007). This significant response to treatment was maintained in both the upright [Pre - 10.5 (5.6-16.7) Vs Post - 1.9 (0.8-2.3) p=0.007] and supine [Pre - 8.5 (1.3-18.2) Vs Post - 0 (0-1.3) p=0.04] positions.

Analysis of Total impedance events (acidic, weakly acidic, and non-acidic) showed no significant difference in any of the 4 groups and in severe asthmatics before treatment. With treatment, the Acid Impedance events reduced significantly from 53.8 (38.4) to 13.6 (15.5) (p=0.04]. In contrast, there was no significant change in weakly acid impedance events in the 4 groups with treatment.

Proximal impedance events whether acidic or weakly acidic, reduced somewhat but this did not reach significance. Although there was provision to measure non-acid impedance events and proximal impedance events in the supine position, there were not enough data points above zero to undergo any statistical analysis.

pH and Impedance measurements

	SA-GORD (a)	SA-no GORD (b)	HC-GORD (c)	HC-no GORD (d)	significance (p<0.05)
DeMeester-PrePPI	40.2 (16.7-62)	4.2 (0.7-9.4)	18.8 (16.5-51.3)	4.1(3.5-19.5)	a-d**, a-b*
DeMeester-PostPPI	4.2 (2.5-15.5)##				
TotAET-prePPI %	13.2 (5-16.9)	1.9 (0.5-3.2)	5.8 (4.1-10.7)	1.4 (1-2.4)	a-d**, a-b**
TotAET--postPPI %	1.5 (0.57-4.9)##				
Total AET Upright-prePPI %	10.5 (5.6-6.7)	3.2 (0.9-5.3)	3.4 (1.8-6.5)	1.5 (1.1-2.7)	a-d**, a-c*, a-b*
Total AET Upright-postPPI %	1.9 (0.8-2.3)##				
Total AET Supine-prePPI%	8.5 (1.3-18.2)	0 (0-0.05)	6.3 (1.8-28.4)	0 (0-1)	
Total AET Supine-postPPI%	0 (0-1.3)##				
Total Impedance events-prePPI	72.1 (44)	37 (27-53.5)	53 (25.5-87.5)	54 (40-58.3)	
Total Impedance events-postPPI	49.3 (20.4)				
Total Impedance events - Acid-prePPI	53.8 (38.4)	24 (5.3-35.3)	20 (10-39)	22 (8.7-33.8)	
Total Impedance events - Acid-postPPI	13.6 (15.5)#				
Total Impedance events - Weak acid-prePPI	16.9 (12)	15 (10.8-19.5)	26 (8-52.5)	22.5 (10-31.3)	
Total Impedance events - Weak acid-postPPI	31 (25.8)				
Proximal reflux events-prePPI	24.8 (18.5)	7 (3-17.5)	5 (2.5-23.5)	8.5 (3-13.3)	
Proximal reflux events - postPPI	14.9 (9.1)				
Proximal reflux events - Acid-prePPI %	41.8 (17.9)	28.5 (18.3-55.8)	15 (11.5-49.5)	18 (8.3-26.5)	a-d*
Proximal reflux events - Acid - postPPI %	33 (28)				
Proximal reflux events - Weak acid - prePPI %	25.4 (16.1)	6.5 (0-30)	4 (0-26.5)	13 (0-16.3)	
Proximal reflux events - Weak acid - postPPI %	23.5 (10.7)				
Proximal reflux events - Upright - prePPI	23 (22.3)	7 (3.3-16.3)	4 (4-37)	7.5 (3-13.3)	
Proximal reflux events - Upright - postPPI	14.9 (9.3)				

Table 4-2: Data from 24-hour pH and Impedance monitoring for all the 4 study groups.* = p≤0.01, **=p≤0.001 for comparison between cohort groups; # = p≤0.01, ##=p≤0.001 for comparison of same group before and after treatment with acid-suppressants (PPI and H2 receptor blocker, ranitidine).

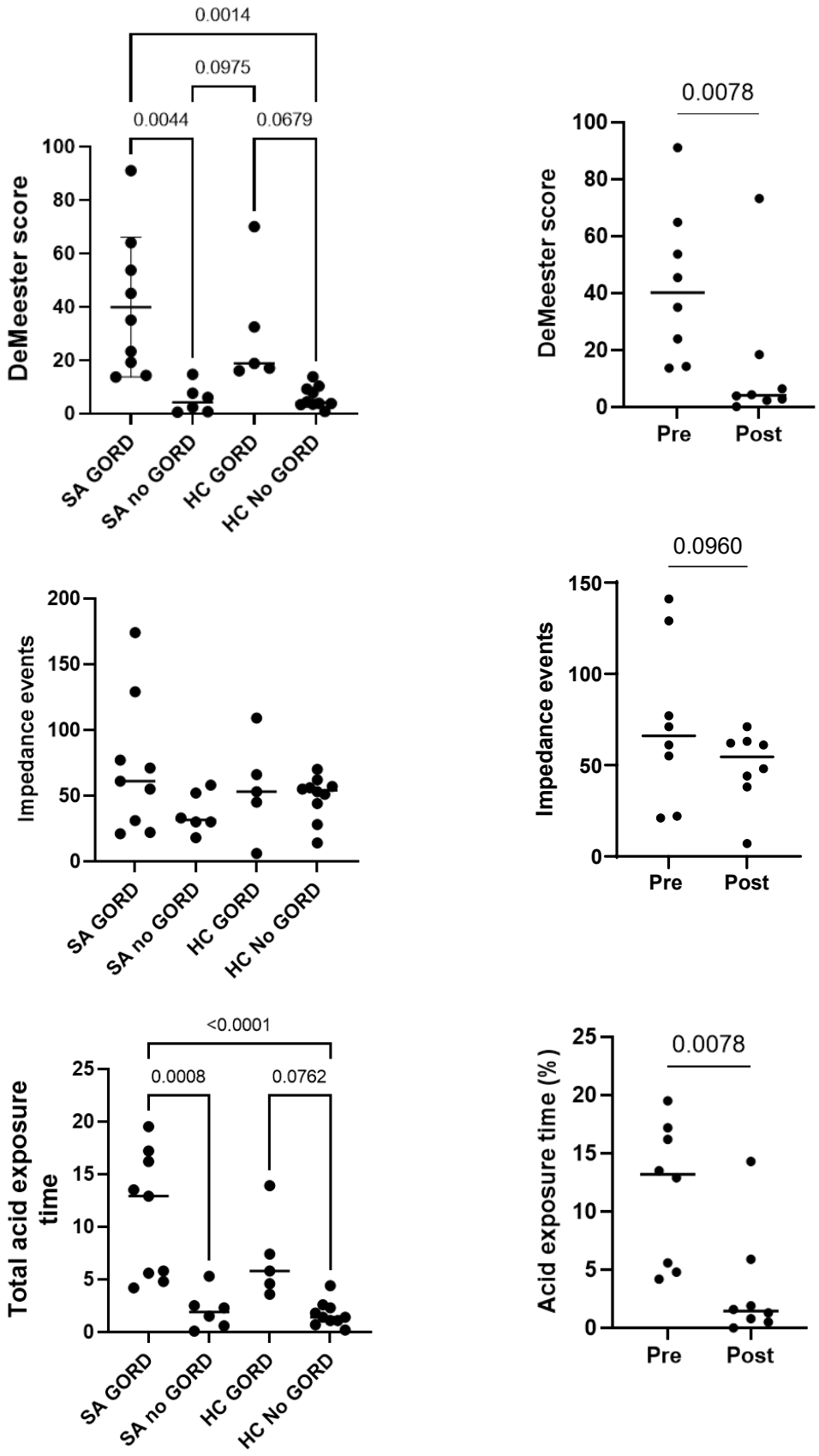

Figure 4-2: Graphical presentation of data from table 4-2. DeMeester score and impedance events across the 4 groups and pre- and post-treatment for reflux with PPI and H2 receptor blocker.

4.5 Discussion

The DeMeester score and pH and Impedance studies have been used extensively to assess the extent of GORD in clinical practice. More recently Acid exposure time (AET) has also been utilised. Studies have shown the usefulness of both measurements in the context of GORD [72,154]. As part of this study the aim was also to make an objective assessment of GORD in severe asthmatics in comparison with healthy individuals. Further, the assessment included a quantitative and qualitative measurement which would help explain the relationship of acid reflux and weakly or non-acid reflux in severe asthma. Whilst a few studies assessing reflux formally have been conducted in asthma [37,155-157] my search of the literature has identified a small number studies where these physiological tools were used in patients with severe clinical asthma [78,80]. Here I show that 60% of severe asthmatic have GORD documented formally and not just based on clinical history of GORD symptoms. My intention was also to breakdown the analysis into acidic, weakly-acidic and non-acidic, something that has not been described before. Finally, I wanted to assess the impact of intensive treatment with a high-dose of PPI and Ranitidine, an effective H2-antagonist, to see to what extent patients' GORD may be resistant to treatment.

The DeMeester score compared across the 4 groups showed a significant difference between SA-GORD and SA-no-GORD (p=0.004), confirming that the groups had been classified correctly. Similarly, there was a significant difference between SA-GORD and HC-no-GORD (p=0.001). HC-GORD versus HC-no-GORD showed a trend towards significance (p=0.067) possibly failing to reach significance because of insufficient number of participants. The lack difference between the HC-GORD and SA-GORD groups and HC-no GORD and SA-no GORD groups in respect of measures of GOR, suggests that the intensity of reflux per se is not an important factor, i.e., that severe asthmatics do not have more reflux than healthy individuals.

The study has shown significant reduction in both AET and DeMeester scores after treatment with PPI and H2 antagonist medication, as expected based on the recognised effectiveness of this treatment. Although, total Impedance events were reduced post treatment, this failed to reach significance (p=0.09), while the impedance events involving acid contents showed significant reduction post treatment, this statistical significance was not present in the weakly acid impedance events. It has been suggested that treatment with anti-reflux medication results in a larger degree of reflux / impedance events consisting of weakly acid and non-acid contents from the stomach [158,159]. My study could support that explanation because I observed a drop in DeMeester score, which is largely dependent on acid reflux events, while post treatment total impedance events, did not reduce to a significant extent. Further evidence in support of this explanation comes from the significant drop in acid impedance events post treatment whereas weakly acid impedance events showed an increase post-treatment though statistical significance was not achieved. In summary,

my study suggests that the treatment predominantly affects the acid component of reflux but does not markedly reduce the weakly or non-acidic components, hence the lack of statistically significant difference in total reflux.

Of note, 2 out of 8 patients who completed treatment with PPI and H2-antagonsist maintained a higher than normal reflux as judged by the DeMeester score. Normal AET is <4% and in both of these two SA-GORD patients failed to drop the value to below 4%, indicating lack of a good response. This suggests that a substantial proportion of severe asthmatics with GORD fail to respond to standard GORD treatment. While this study does not have sufficient numbers to provide firm conclusions, it does suggest that treatment resistant GORD may be a clinically relevant issue. One could speculate that such patients may benefit from surgical treatment. Further studies to explore this are needed.

Finally, I took the opportunity to assess the value of two additional means of quantifying reflux. First, there is some evidence to suggest that supine reflux/impedance events may lead to more symptoms such as cough[29,160,161], but in my analysis a significant suppression of AET was seen in both the supine and upright positions after anti-reflux treatments. Similarly, some studies have suggested that proximal reflux events are particularly relevant for symptoms like cough [162,163]. Although proximal impedance events showed a reduction post treatment, it did not reach significance. Data points for proximal reflux/impedance in supine position were too few for it give any reasonable statistical suggestion.

In conclusion, the study confirmed that severe asthmatics with a clinical diagnosis of GORD have pathological GORD as shown by high DeMeester score and higher AET as well as higher frequency of upright and supine reflux and impedance events compared to severe asthmatics without GORD and healthy participants with GORD. This also shows that although the anti-reflux treatment suppresses the acid contents of the refluxate leading to reduced AET and reduced acid impedance events, it does not have a statistically significant impact on weak acid impedance events which increased after treatment with PPI and H2 antagonists suggesting ongoing weak acid and non-acid reflux.

I hypothesised that GORD in severe asthmatics has an impact on symptoms of asthma and airway inflammation. To assess this hypothesis, I undertook a further assessment shown in Chapter 5 (Study 3) combining the data from this chapter with questionnaire-based assessments severity of asthma using ACQ and respiratory disease quality of life measures with questionnaires (AQLQ. HCHQ, SGRQ and RDQ) which would shed a light on any differences which can be attributed to the 4 groups (SA-GORD, SA-no GORD, HC-GORD and HC-no GORD) based on presence or absence of GOR.

Chapter 5 Clinical assessment of GORD in Severe asthmatics – Study 3

5.1 Introduction

In routine clinical asthma management validated questionnaires are used to assess and identify various clinical aspects which need to be optimised to achieve control of asthma. These questionnaires have been validated in multiple studies, including ACQ, AQLQ, LCQ, and SGRQ. The questionnaires focussing on GORD include the HCHQ and RDQ.

In this study (study 3), my aim was to use standard, validate asthma, GOR and Cough questionnaires to assess whether there are clear differences between severe asthmatics with and without reflux and, if there are differences whether these are due to acidic, weakly acidic or non-acidic reflux. Furthermore, my aim was to assess whether response to treatment of GORD in severe asthmatics is associated with a benefit in quality-of-life measures. Finally, I sought to address the clinical utility of questionnaires as a means of identifying patients who benefit from GORD treatment to control their severe asthma without requiring repeat invasive investigations, i.e. post-treatment pH/impedance assessment.

ACQ and AQLQ are both well-established questionnaires with a defined minimum important difference (MID) of 0.5. The ACQ score provides a reliable measure of the severity and control where an increase in score implies increasing loss of control. The AQLQ provides a measure of the quality-of-life burden in asthmatics and gives insight into symptoms, activity, emotional and environmental impact of the disease. A higher AQLQ score implies better disease related health measures. Previous studies have shown a higher disease burden in severe asthmatics with GORD [105,107,164], and the aim of this study was to observe if controlling GORD can improve the asthma control and asthma related quality of life measures to support this hypothesis.

Similarly, LCQ is a validated questionnaire which is used in the measurement of cough related quality of life and symptoms in chronic cough. A higher score implies better symptoms control and quality of life. The defined MID for LCQ is 1.3. Its domains cover physical, social and psychological aspects all of which are significantly impacted in patients with chronic cough. The study's aim was to observe any differences in cough related quality of life in severe asthmatics once GOR was brought under control.

The SGRQ has been extensively used in management of COPD and due to its robustness in severe asthma [165,166]. It allows an extensive overview of the respiratory disease related quality of life

through its symptom, activity and impact domains. The purpose of using SGRQ in this study was to see its suitability for assessing severe asthmatics before and after treatment for GORD.

The Hull cough hypersensitivity questionnaire has been used in the management of cough associated with GORD and a score ≥24 is considered to be predictive of GOR as a likely aetiology.

Finally, the Reflux disease questionnaire (RDQ) was developed with the purpose of providing an easy and non-invasive tool for physicians to identify and manage GORD. I used the RDQ in this study in combination with other questionnaires to identify severe asthmatics with GORD with the aim that patients may not necessarily have to be re-investigated invasively.

5.2 Methods

Subjects

16 patients with severe asthma (9 SA-GORD and 7 SA-no GORD) were compared at baseline with 15 healthy participants (5 HC-GORD and 10 HC-no GORD). The effect of GORD treatment was assessed in 8 SA-GORD patients but not in healthy controls.

The clinical characteristics of the cohort are given in table 5-1.

The full method details have been provided in the Methods chapter.

All the above-mentioned questionnaires were used in the severe asthma cohort. In the healthy cohort, all questionnaires except the asthma related questionnaires were used. Since cough was a measure being looked at in the context of GORD, I assessed the outcomes from cough monitoring with the help of questionnaire (LCQ) as well as the cough monitor (LCM).

Patients were first assessed by 24-hour pH/impedance studies for evidence of GOR. Based on these results they were classified as follows: healthy participants without evidence of GORD (HC- no GORD), healthy participants with evidence of GORD (HC-GORD), severe asthmatics without evidence of GORD (SA-no GORD) and severe asthmatics with evidence of GORD (SA-GORD) (see Table 3-1). The questionnaires were provided to all participants at the first visit – Visit 0. Visit 0 was the baseline visit for all participants. Participants in the SA-GORD group were asked to re-do the questionnaires at Visit 1 after having stopped any anti-reflux treatment they were on to capture data that were not influenced by their anti-GOR treatment. None of the HC-GORD participants were on any anti-reflux treatment prior to the study and their diagnosis was considered tentative (based on history alone) until formal confirmation of GORD by 24-hour pH/impedance study. Questionnaire data were collected after each subsequent intervention stage in the SA-GORD group. After baseline assessment and washout, the severe asthmatics were started on treatment

Only the SA participants were enrolled into a follow-up study during which respiratory questionnaires as well as the reflux related questionnaires were used at each stage of therapeutic intervention to check the response to intervention. The study consisted of 11 visits in total, but only the following visits were used for assessment of GORD:

Visit 0 - baseline visit where all participants were assessed as they presented.

Visit 1 – assessment of severe asthmatics with history of GORD who had discontinued their anti-reflux treatment for 2 weeks.

Visit 8 – Severe asthmatics with GORD diagnosed on 24 Hr pH/impedance treated with 2 months of intense anti-reflux treatment (Omeprazole 40mg BD and Ranitidine 300mg OD nocte). Participants were then invited to add metoclopramide to this treatment.

Visit 9 – Participants from visit 8 on anti-reflux treatment plus Metoclopramide 10mg TDS. Participants were then asked to stop their Metoclopramide and start Domperidone.

Visit 10 – Visit 9 participants on anti-reflux treatment as visit 8 plus Domperidone 10mg TDS (Metoclopramide discontinued). Patients were then asked to stop Domperidone and start Baclofen.

Visit 11 – Visit 10 or 9 on anti-reflux treatment as at visit 8 **plus Baclofen 5mg TDS (Metoclopramide or domperidone discontinued).**

During the study, the MHRA issued new information about the risks associated with use of Domperidone of sudden cardiac death. Even though the study had the approval from the ethics committee, I decided to discuss this in detail with each participant starting the study as well as those already in the study to offer them the option of declining visit 10 intervention if they wished so. This led to a sizeable number opting out of this visit and moving straight to Visit 11 from Visit 9. One participant had a prior history of side-effect caused by metoclopramide and developed side-effects during the study and, therefore, stopped taking it.

5.2.1 Statistical analysis

Comparisons were made between all 4 groups and, additionally, within the SA-GORD group as the GORD treatment progressed. GraphPad Prism 9.2.0 was used for analysis. For assessment of data distribution (normality), the Shapiro-Wilk and Kolmogorov-Smirnov tests were used. The Chi-squared test was used to assess categorical data (e.g. sex). Parametric data were analysed using paired and unpaired t-tests and 1-way ANOVA or Mixed-effects analysis with Tukey's test as appropriate. For unpaired data Kruskal-Wallis test was used. For non-parametric data, the Mann-Whitney test and Friedman's test with Bonferroni correction was applied using SPSS.

5.3 Results

The SA-no GORD participants were older that the HC-no GORD participants. The participants were mainly female (83.3%, 85.7% and 100% in SA-GORD, SA-no GORD and HC-GORD, respectively), other than the HC-no GORD group (33.3%). There was no significant difference in FEV1, FVC and PEF between the four groups (HC- no GORD, HC-GORD, SA-no GORD and SA-GORD) see table 5-1. There was a significant difference in MEF 25-75 %pred between HC-no GORD and SA-no GORD ($p=0.002$). Although there was a statistically higher degree of smoking pack/years in HC-GORD compared to HC-no GORD, the median pack years in the HC-GORD group was only 0.7 and, therefore, not a clinically significant quantity.

Table 5-1: Cohort characteristics

n **Mean (range)$** **Median [Q1,Q4]t**	**Severe asthma - GORD (A)**	**Severe asthma - No GORD (B)**	**Healthy control - GORD (C)**	**Healthy control - No GORD (D)**	**P value**
N	9	6	*5*	*10*	n/a
Age	53 (44-60.5)	54 (48.5-62)	44.4 (19-59)	36.9 (22.0-57.0)	B-D-p= 0.0231
Sex M/F	1/8	1/5	*0/5*	*4/6*	ns
BMI	35 [26.1-39]	31.5 [26.1-38.2]	25.0 [23.8-31.4]	24.2 [22.2-26.4]	
Atopy Y/N	6/2	2/4	2/5	4/6	ns
Smoking (Pack Years)	0 [0-0]	0 [0-1.8]	0.7 [0.3-4]	0 [0-0]	C-D-p=0.029
Age of onset	12 [3.0,47]	11.5 [3.3,48.8]	n/a	n/a	ns
PreBD FEV1%pred	103.5 [79.8-126.1]	91.4 [72.3-116.4]	110.4 [105.3-124.1]	103.3 [93.6-116.2]	ns
PreBD FVC%pred	112.9 [100.1-139.5]	110.5 [94.5-142.7]	118 [112.5-147.6]	114 [105.9-122.4]	ns
PreBD MEF25-75%pred	52.4 [40.5-66.7]	23.2 [21.3-24.9]	63.7 [41-83.8]	72.6 [61.2-94.5]	B-D- p=0.0026
PreBD PEF%pred	84.1 [65.7-121.0]	109.5 [73.2-119.6]	106.4 [98.4-118.7]	106.6 [89-112.4]	ns

Note : Table shows general demographics and clinical characteristics of the full cohort in the phase 2 study. n/a = not applicable, ns = no significant difference. PreBD – Pre-bronchodilator, FEV1 – Forced expiratory volume in 1 second, FVC – Forced vital capacity, MEF 25-75% - Mean expiratory flow between 25% and 75% of the FVC representing small airways. PEF%pred – Peak expiratory flow percentage predicted.

5.3.1 ACQ scores

ACQ6 scores were significantly different between SA-GORD and SA-no GORD (p=0.02). Within the SA-GORD group, the ACQ6 score increased off anti-reflux treatment (Visit 0 to Visit 1), suggesting some loss of control, although this did not reach significance, but the score dropped significantly after 2 months treatment with anti-reflux treatment at visit 8 (p=0.01), dropping further at visit 11 but without this change reaching significance (see Table 5-2, Figure 5-1).

Correlation analysis was done between the ACQ6 and DeMeester score at Visit 1 and Visit 8 to see if there was relationship between the ACQ6 score and DeMeester score and AET. There was very weak correlation between ACQ6 and DeMeester score and ACQ6 and AET. The correlation was very poor between the difference in pre and post PPI ACQ6 and the DeMeester score and AET (table 5-3).

Table 5-2: ACQ6 scores in severe asthma with and without GORD during treatment

	SA-no GORD	SA-GORD V0	SA-GORD V1	SA-GORD V8	SA-GORD V9	SA-GORD V11
ACQ6	1.7(1.1)	2.6(1.1)	2.8(0.7)	1.9(1)	2.1(1)	1.5(1)

Table 5-3: Correlation between ACQ6 and DeMeester score and AET at Visit 1 and Visit 8

	SA-GORD V1 ACQ6 Vs DMs	SA-GORD V1 ACQ6 Vs AET	SA-GORD V8 ACQ6 Vs DMs	SA-GORD V8 ACQ6 Vs AET
Spearman's r	0.23	0.29	0.08	0.22
P value	0.59	0.47	0.85	0.60

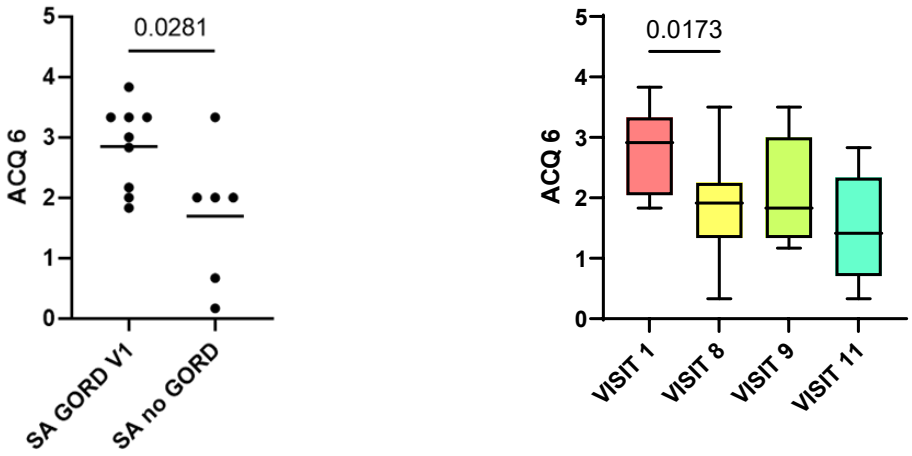

Figure 5-1: Comparison of ACQ6 score in SA-GORD and SA-no GORD group and in SA-GORD with anti-reflux treatments

5.3.2 AQLQ scores

AQLQ scores for the symptom domain showed a significant difference between SA-no GORD and SA-GORD. Within the SA-GORD group a trend to improvement in symptom domain scores was noted when Visit 1 was compared with Visit 8 and Visit 11, and an improvement in mean AQLQ score of 1.5 points. The activity, emotion and environment domain in the SA- GORD group did not reach significance (see table 5-4, Figure 5-2).

Table 5-4: AQLQ domains in SA-no GORD and SA – GORD visits

	AQLQ-symptoms	AQLQ-activity	AQLQ-emotion	AQLQ-environment	Total
SA-no GORD	5.14(1.4)	4.6(1.2)	5.7(1.5)	4.7(2)	5(1.3)
SA-GORD – V0	4.3(1.3)	3.8(1.2)	5(1.3)	4(1.7)	4.1(1.4)
SA-GORD – V1	4.1(0.5)	4.4(1)	5(1.3)	4.1(0.9)	3.8(1.2)
SA-GORD – V8	5.4(1)	4.5(1.2)	5.9(1)	4.4(1.1)	5.1(0.9)
SA-GORD – V9	5.4(0.9)	4.1(0.3)	5.9(0.5)	4.8(1.4)	5.1(0.7)
SA-GORD – V11	5.5(1)	4.7(1.1)	6.2(1)	4.7(0.5)	5.4(5-5.6)

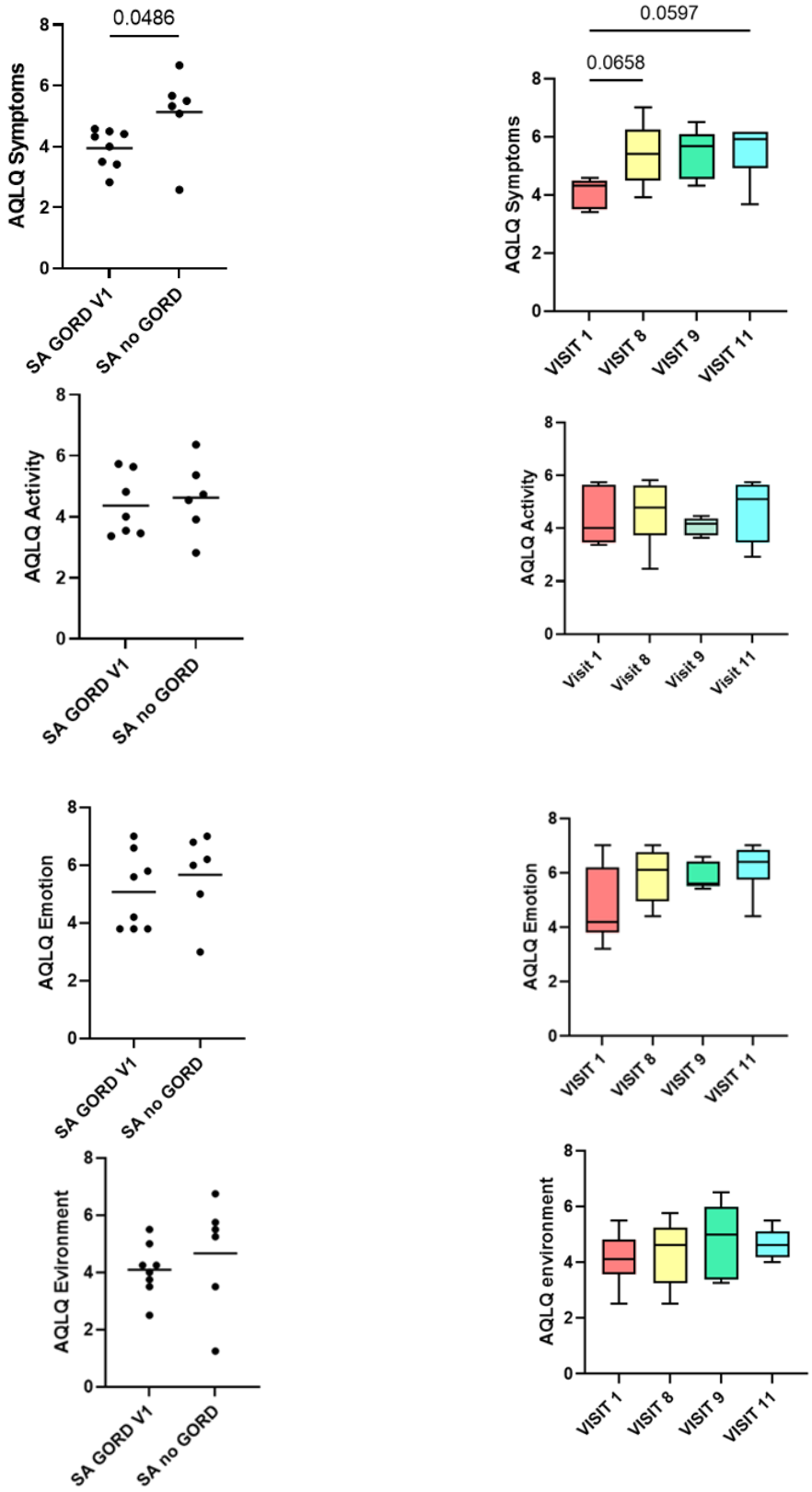

Figure 5-2: All domains of AQLQ, its difference in SA-GORD and SA-no GORD (left) as well as domain scores with anti-reflux interventions (right).

5.3.3 HCHQ scores

HCHQ scores showed a significant difference between all HC and all SA as expected due to the nature of asthma. Within the healthy cohort there was no significant difference in HCHQ scores. HCHQ scores showed higher baseline score between SA-GORD (Visit 1) and SA-no GORD (p=0.03)

On analysis of HCHQ scores over the visits post anti-reflux treatment in the SA-GORD group, a significant improvement of HCHQ score was seen with anti-reflux treatment (please see table 5-4, Figure 5-3) between visits 1 and visit 8 (p=0.04). The most significant improvement in HCHQ scores was seen in SA-GORD when comparing Visit 1 (off anti-reflux treatment) and Visit 11 (on PPI, H2 receptor blocker and baclofen) (p=0.006).

Table 5-5: HCHQ scores across the 4 groups and intervention visits.

Cohort	Visit 0	Visit1	Visit 8	Visit9	Visit 11
SA-GORD	37.6(20)	45.2(14)	28.8(18.8)	24.6(20)	21.6(15.4)
SA-no GORD	28.3(14)	n/a	n/a	n/a	n/a
HC-GORD	0(0-3)	n/a	n/a	n/a	n/a
HC-no GORD	0(0-1)	n/a	n/a	n/a	n/a

n/a- not analysed

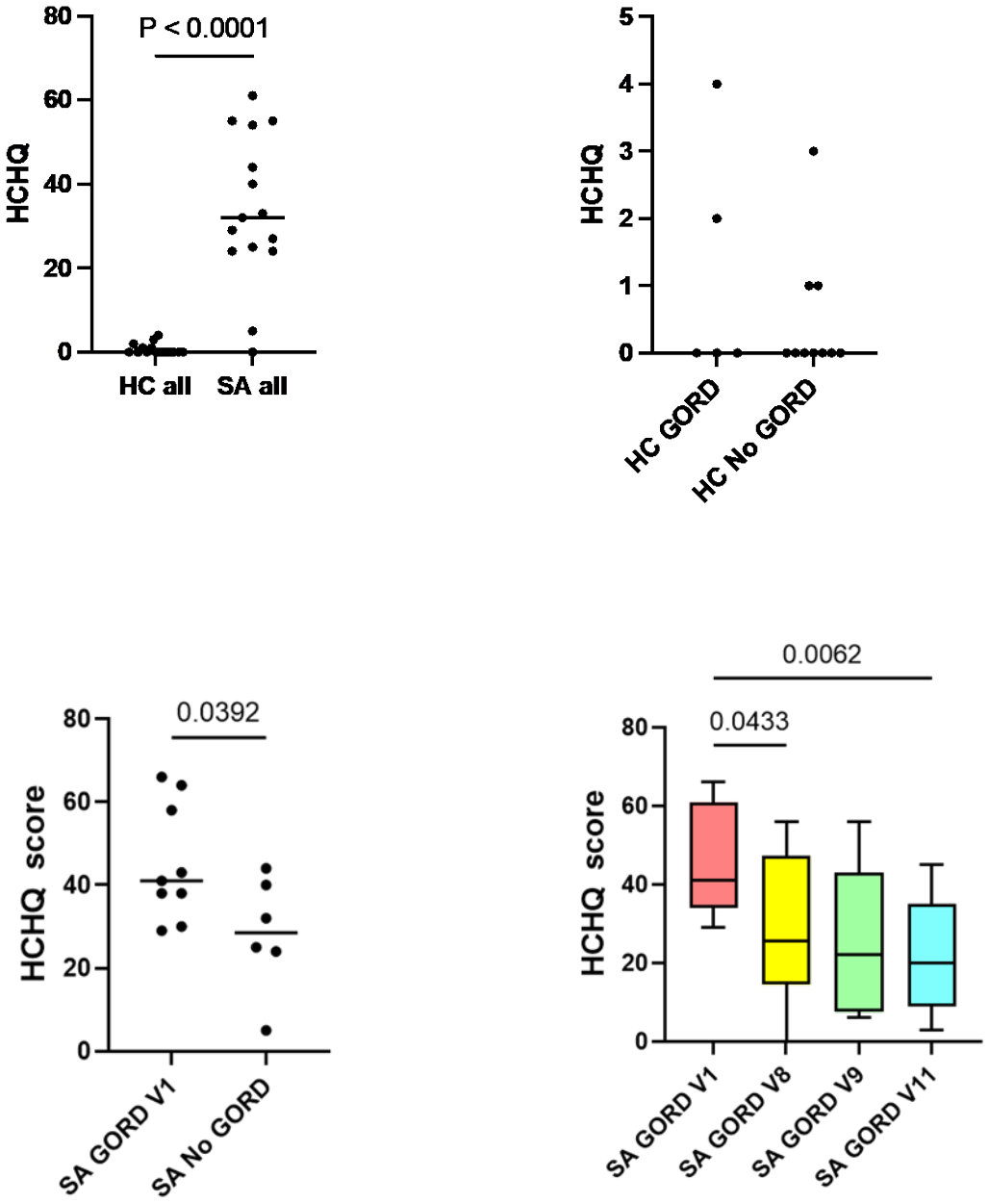

Figure 5-3: Comparison of all HC and all SA cohorts. It also shows comparison between SA-GORD visit 1 and SA-no GORD (left) and HCHQ scores within SA-GORD group at each intervention stage (right).

5.3.4 LCQ scores

Compared to SA-no GORD, the LCQ scores in SA-GORD for physical and social domains and total score were lower, indicating poorer quality of life measures caused by GORD, but this did not reach statistical significance in any domains or the total score. An analysis of LCQ scores between Visit 0

(Pre-washout for anti-reflux medication) and Visit 1 (Post-washout for anti-reflux medication) did not show any significant change in any domain (see table 5-6 and 5-7). A separate comparison of SA-GORD V1 with the SA-no GORD group with *unpaired t-test* showed a trend towards significance (Physical p=0.08, Psychological p=0.07, Social p=0.07 and Total score p=0.1), suggesting that stopping anti-GOR treatment leads to worsening of quality of life related to cough. This test was preferred over the non-parametric multiple-comparisons because the HC-GORD and HC-no GORD groups had no cough so that on clinical grounds this comparison was not deemed appropriate in a multiple comparison method. Thus, when compared to both healthy cohorts, SA-GORD and SA-no GORD scores were significantly lower in all domains suggesting poor cough related quality of life (see Figure 5-4 and 5-5).

Within the SA-GORD there was a general tendency for the scores to improve with each anti-reflux intervention, but this change was neither statistically significant nor reached the MID for LCQ (1.3)[167].

Table 5-6: LCQ domains across all cohorts

	LCQ-Physical	**LCQ-Psych**	**LCQ-Social**	**LCQ-Total**
SA-GORD V1	3.9(3.3-4.9)	5(3.9-6.6)	4.3(1.3)	4.7(1)
SA-no GORD	5(4-6.3)	6.7(5-7)	5.7(1.3)	5.7(1.1)
HC-GORD	6.8(6.8-6.9)	7(6.9-7)	7(7-7)	6.9(6.9-7)
HC-no GORD	6.9(6.8-7)	7(7-7)	7(7-7)	7(6.9-7)

Table 5-7: LCQ domains across SA-GORD with each anti-reflux intervention. For significant and relevant p values for analyses please see the figures 5-4 and 5-5.

	Visit 0	**Visit 1**	**Visit 8**	**Visit9**	**Visit 11**
LCQ-Physical	4.3(1.4)	4.2(0.9)	4.8(1.3)	5.3(1.4)	5.2(1.2)
LCQ-Psych	4.6(1.9)	5.3(1.2)	5.8(1.4)	7(3.1-3.9)	6(1.3)
LCQ-Social	4.2(2.0)	4.5(1.2)	5.2(1.5)	5.7(1.6)	5.5(1.5)
LCQ-Total	4.4(1.7)	4.7(1)	5.3(1.3)	5.7(1.5)	5.6(1.3)

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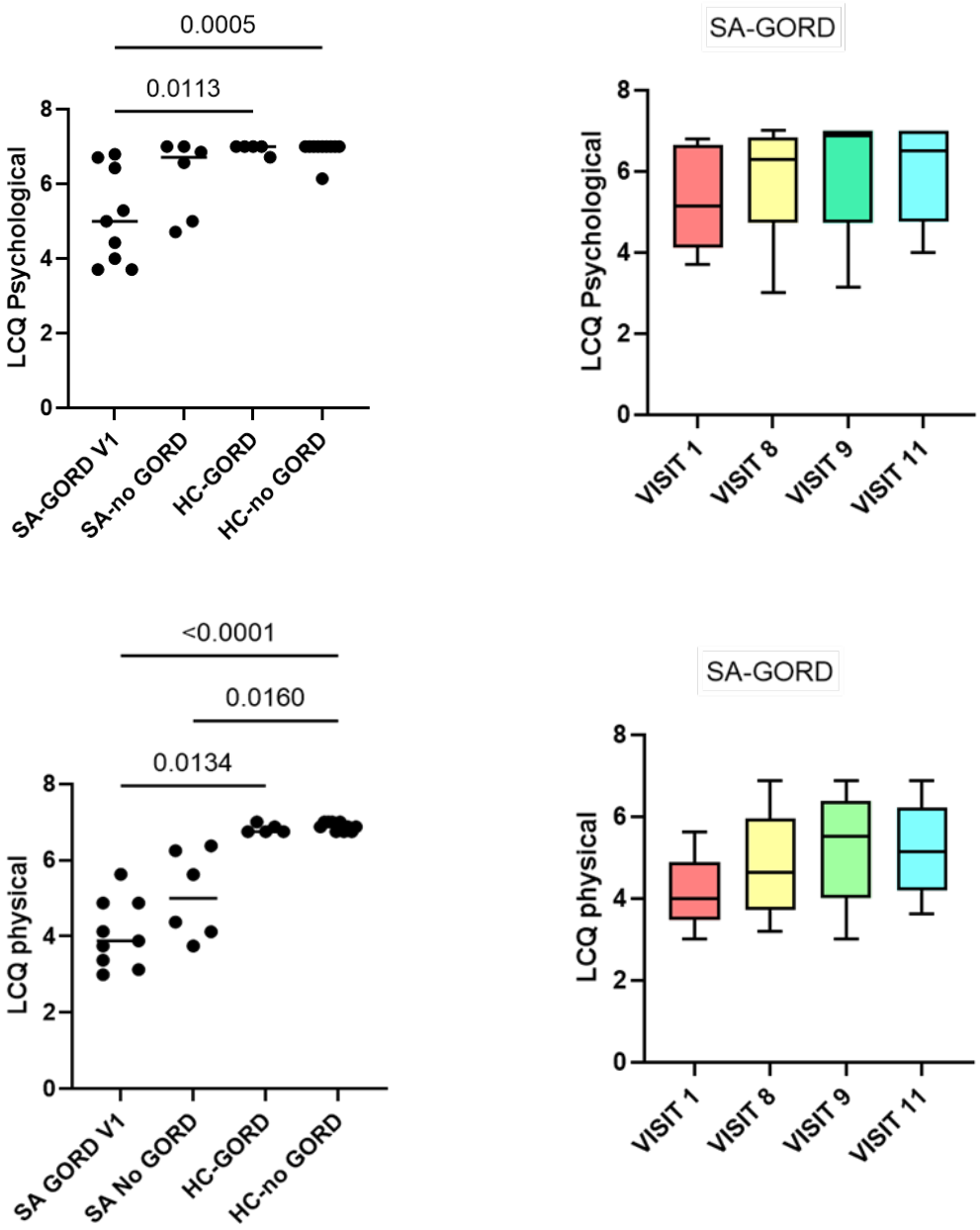

Figure 5-4: LCQ psychological domains (top) and physical domain (bottom)for all 4 groups and SA-GORD group with each anti-reflux intervention visit (right).

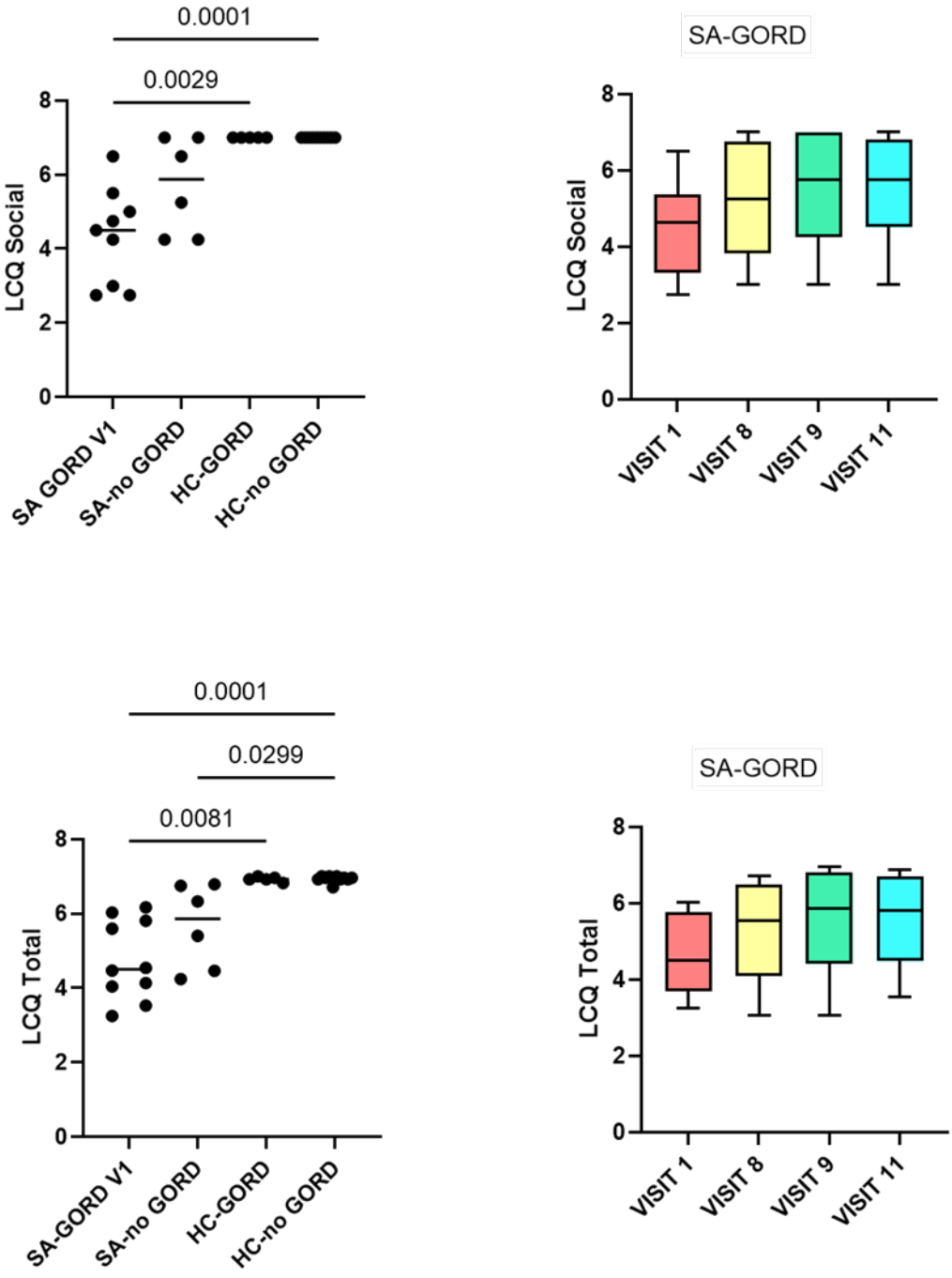

Figure 5-5: LCQ social domain (top) and total (bottom) scores for the 4 patient groups (left) and SA-GORD group with each anti-reflux intervention visit (right).

5.3.5 RDQ score

Reflux disease questionnaire (RDQ) comprises 4 domains based on symptoms. These data were not normally distributed so this required the use of non-parametric tests.

Assessment of individual domains showed differences: e.g. the Heartburn domain within the RDQ showed a significant increase in SA-GORD compared to the healthy cohort, without GORD (p=0.016) and a trend to significance in healthy cohort with GORD (p=0.06) but not SA-no GORD. Within the SA-GORD group, there was a reduction in symptoms after treatments with PPI and H2 receptor blockers (at visit 8) p=0.05, metoclopramide (at visit 9) p=0.02, and baclofen (visit 11) p=0.02 at the end of the treatment, but none of these improvements were statistically significant after Bonferroni correction for multiple comparisons (p=0.33, p=0.10, and p=0.12, respectively). Similarly, the Regurgitation domain did not show any significant differences between the groups. Please see Table 5-8, Figure 5-6 and 5-7.

The RDQ-GORD dimension domain also showed improvement on treatment in SA-GORD but it did not reach significance. There was a trend towards significance between Visits 1 and 9 (p=0.08). RDQ-Dyspepsia domain showed a trend towards significance with improvement in scores following PPI and H2 rec blockers plus metoclopramide (p=0.08) but not at other visits once adjusted for multiple comparisons. Please see table 5-6 for details.

Table 5-8: RDQ scores for Severe asthma with intervention visits and healthy cohorts

	Heartburn	Regurgitation	GORD dimension	Dyspepsia
SA-no GORD	1.1(1)	2.3(1.5)	1.7(1.2)	1.7(1)
SA-GORD-V0	1.3(1.1)	2.1(1.8)	1.7(1.3)	1.3(1.4)
SA-GORD-V1	2.3(1.6)	3.8(2.5-4.6)	2.7(1.5)	3(1)
SA-GORD-V8	1.25(0-1.3)	1.3(1.6)	1.7(2.6)	0(0-0.6)
SA-GORD-V9	0(0)	0.6(0.6)	0.1(0-4.8)	0(0-0.4)
SA-GORD-V11	0(0-03)	0.9(0.5)	0.5(0.4-1.4)	0.3(0-0.6)
HC-GORD	0(0)	0(0-0.4)	0(0-0.2)	0(0)
HC-no GORD	0(0)	0(0-0.4)	0(0-0.2)	0(0)

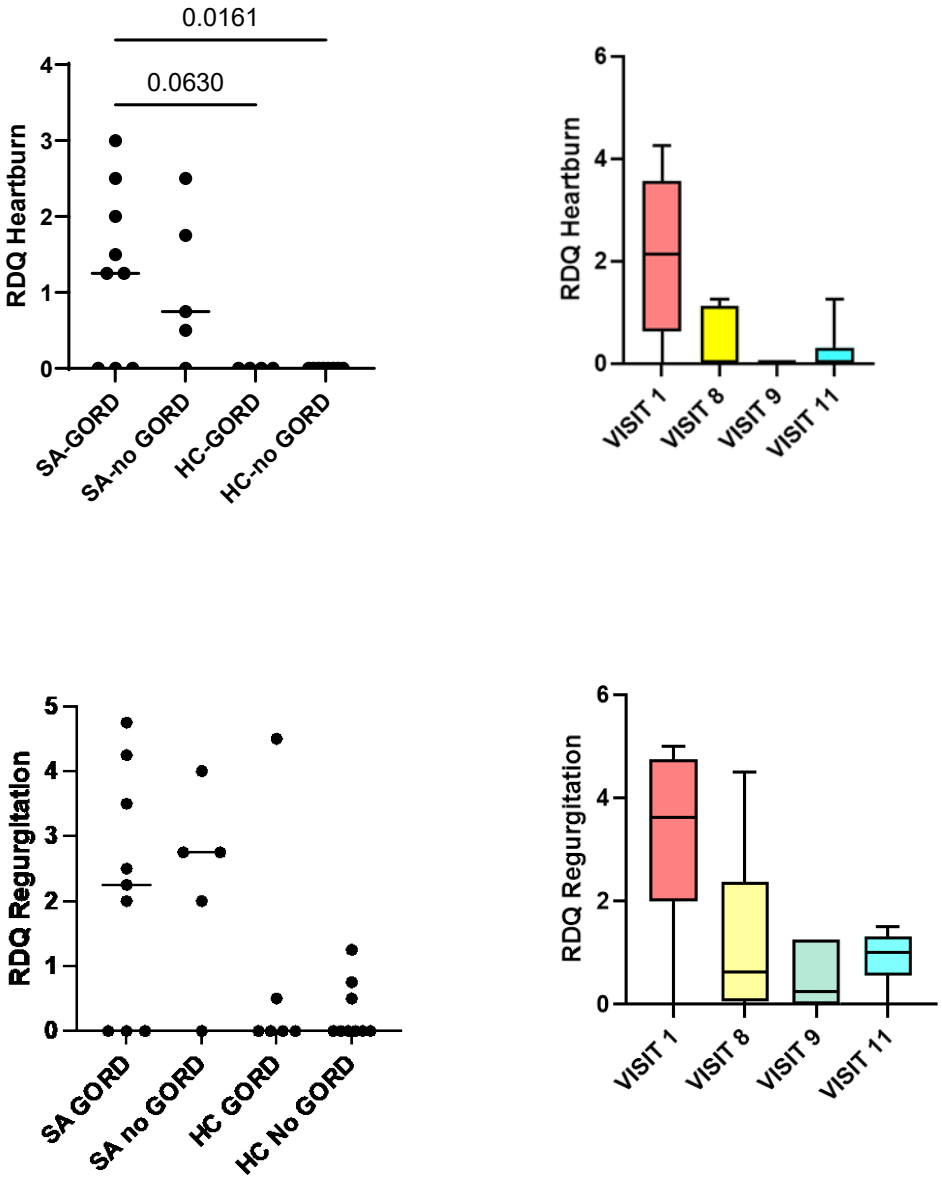

Figure 5-6: Comparison of RDQ heartburn and regurgitation domains across the 4 groups on the left and RDQ scores after anti-reflux treatment in SA-GORD group on the right side.

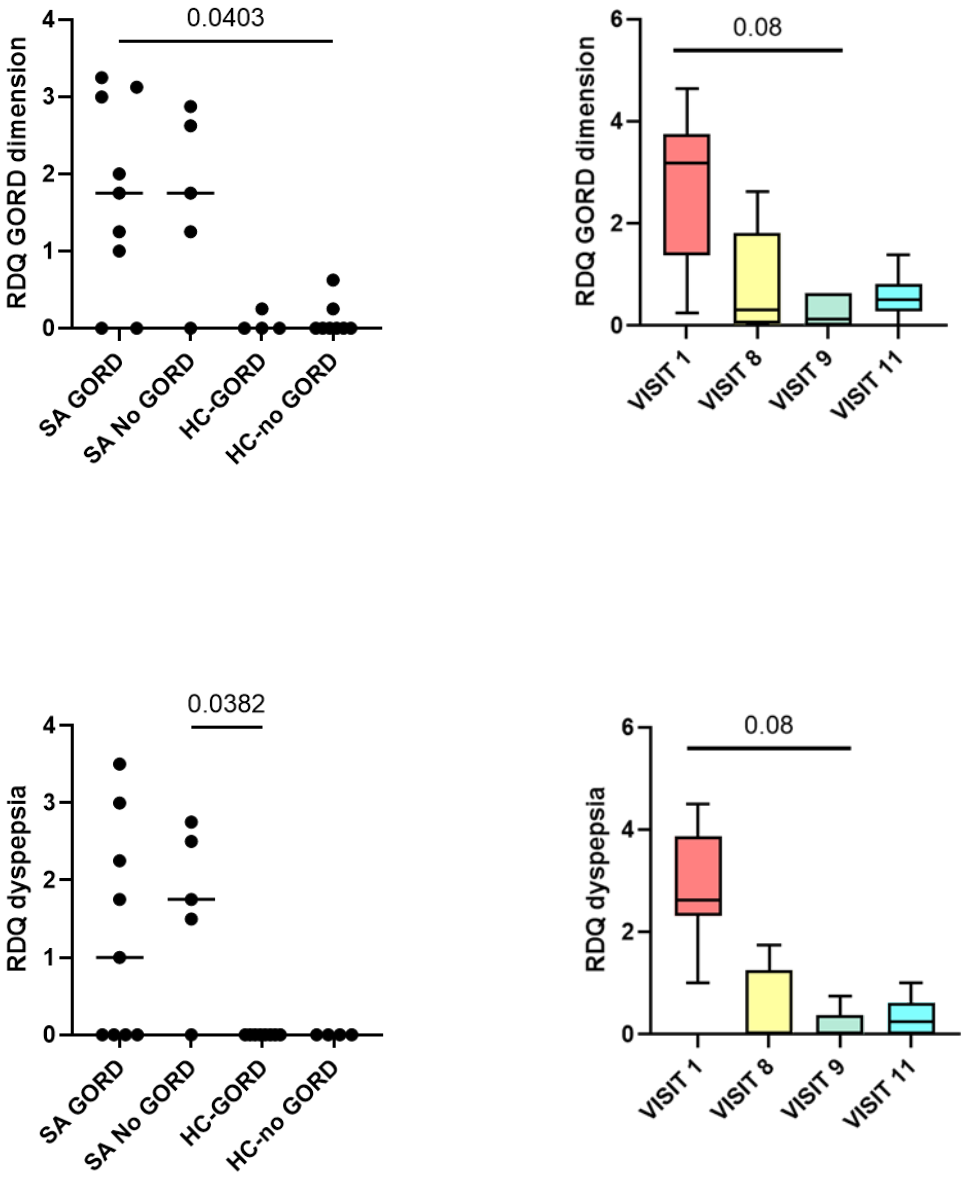

Figure 5-7: RDQ dyspepsia and GORD dimension domains across the 4 groups on the left and scores after anti-reflux treatment visits in SA-GORD group on the right side.

5.3.6 SGRQ scores

SGRQ assessments of participants in the severe asthmatics with GORD showed a significantly increased score in the symptoms domain (p=0.017). The Impact domain and total score showed a trend towards significance (p=0.07 and 0.08) respectively. Although the difference in mean score in the Impact domain was higher than the MID for SGRQ (7.5), this did not achieve significance. There was no significant response to anti-reflux treatments in any of the domains in the SGRQ within the SA-GORD group (see table 5-9 and Figure 5-8 and 5-9) despite the mean difference being more than MID for SGRQ. This is very likely to be a reflection of the limited power of the study.

Table 5-9: SGRQ domains in severe asthma groups and scores from anti-reflux treatment visits in SA-GORD

	SGRQ-Symptoms	SGRQ-Activity	SGRQ-Impact	SGRQ-Total
SA-no GORD	49.5(14.7)	56.7(33)	32.5(16.5)	42.3(19.1)
SA-GORD V0	65.1(22.5)	60.5(29.6)	45.3(20)	52.9(19)
SA-GORD V1	68(13)	69.8(24.4)	69.8(24.4)	58.6(14.7)
SA-GORD V8	50(33.3)	60.2(25)	60.2(25.1)	44.7(20.6)
SA-GORD V9	50(33.2)	67(22.9)	67.1(22.9)	47.7(25.8)
SA-GORD V11	49.2(30.9)	51(24.1)	51(24.1)	42.5(21)

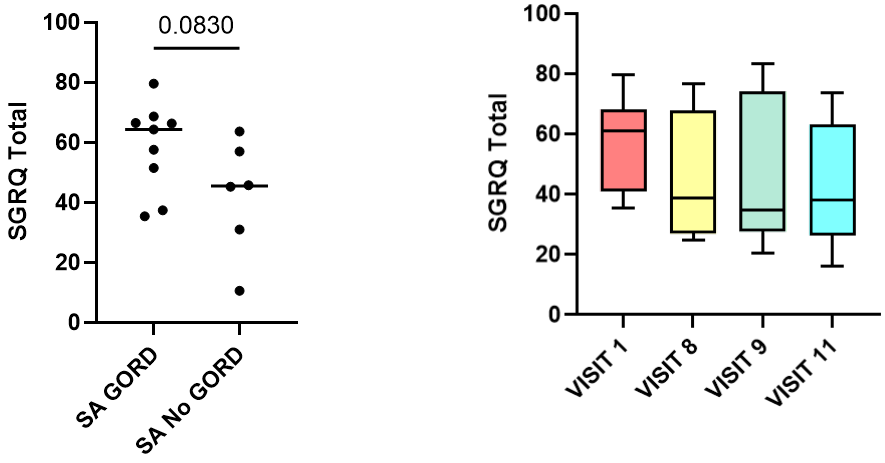

Figure 5-8: SGRQ total scores in severe asthma and after anti-reflux treatments in SA-GORD

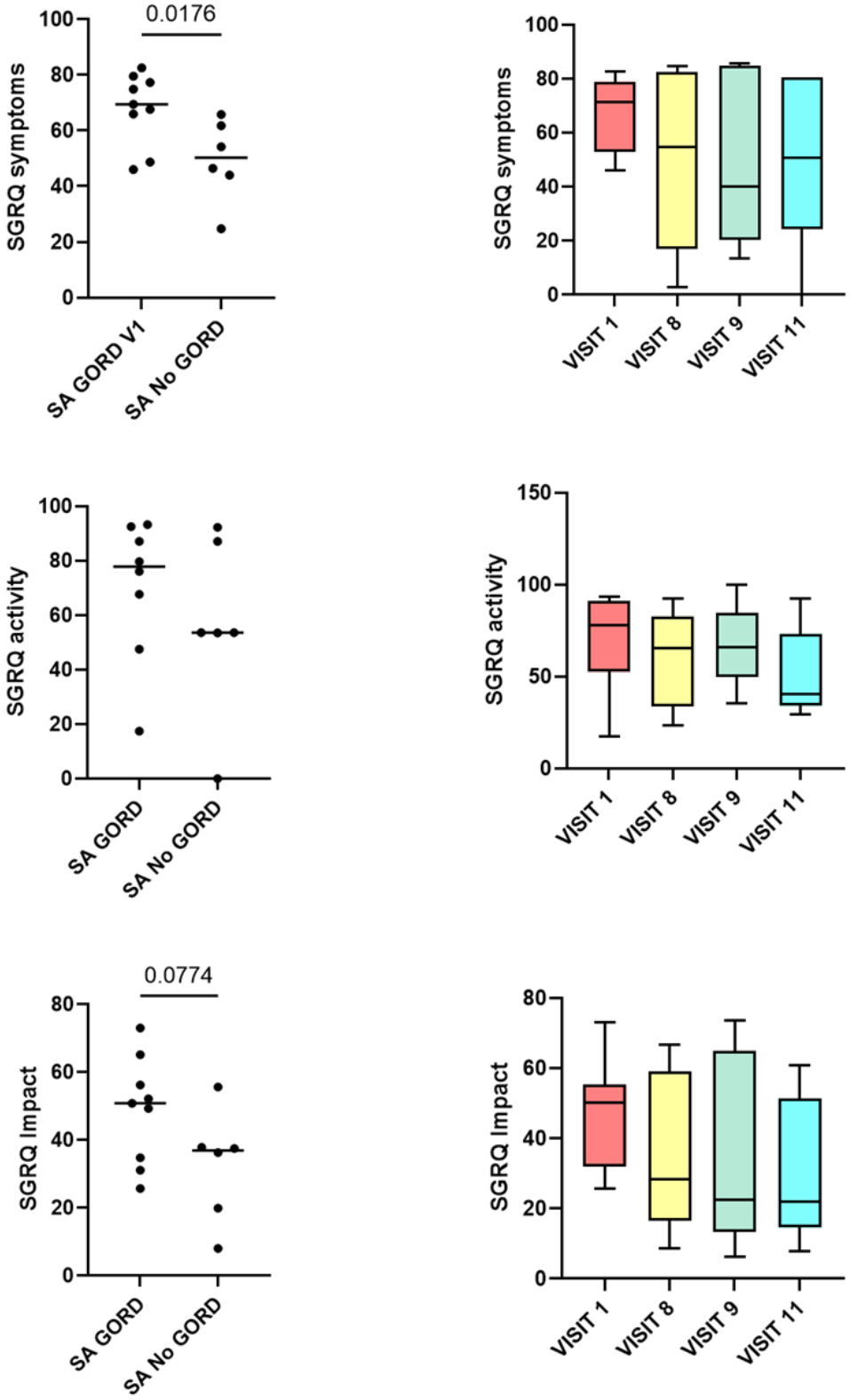

Figure 5-9: SGRQ domains in severe asthma and after anti-reflux treatment visits in SA-GORD

Amongst all the SA-GORD patients, only one patient's ACQ decreased by 1.7 after completing 8 weeks of treatment with PPI and H2-antagonist. According to the study design, this was a criterion for patients to not to progress with their treatment and start taking metoclopramide and baclofen, to treatment with anti-reflux treatment. This change in ACQ was accompanied by similarly important improvements in all questionnaire data as well as physiological assessment of GOR (pH/Impedance) and LCM measurements. The cough monitor-based response was not significant because the pre-treatment cough rate was already well below the pathological range (Cough rate Pre/Post: 0.6/0.5, Day cough rate pre/post: 1/1, night cough rate pre/post: 0.1/0.2). Please see results for this patient in tables 5.9 and 5.10.

Table 5-10: Questionnaire data pre and post anti-reflux treatment for GA3-021

	ACQ6	AQAL Total	LCQ Total	HCHQ	RDQ*	SGRQ total
Pre-Treatment (V1)	2	4.75	5.6	38	0/3.5/1.75/4.5	64.34
Post-treatment (V8)	0.3	5.71	6.7	0	0/0/0/0	25.6

Note: * - the individual RDQ domains are show as Heartburn/Regurgitation/GORD dimension/Dyspepsia

Table 5-11: pH/Impedance data for GA3-021

pH/ impedance	DM score	AET	AET Up/Sup*	Imp events total	Imp events ≠ A/WA/NA	Proximal Imp	Proximal Imp (%) A/WA β	Proximal Impedance Up/Sup
Pre - treatment	53.7	16.2	21.2/1.9	129	95/29/5	65	52/45	65/20
Post-Treatment	2.9	0.8	10.3/0	62	8/54/0	20	38/31	0/0

Note: * - Impedance events are shown as Upright/Supine, ≠ - the Impedance events are shown as Acid/Weakly acid/Non-acid, β – Acid/Weakly acid

Finally, I sought to link the questionnaire data to the physiological data, focusing on the two patients whose reflux (as shown in the previous chapter) failed to improve completely on PPI and H2-antagonis treatment. One of these had a modest clinical response to anti-reflux treatment as judged by ACQ, AQLQ, LCQ, HCHQ and SGRQ (table 5-11) while the second patient did not improve at all based on questionnaires. The first patient had a modest drop in total impedance events from 71 to 38 and proximal impedance events from 26 to 18. There was no change in this participant's acid impedance events in particular (table 5-12). In the second patient, the RDQ score showed a very good treatment response as judged by questionnaires even though their pH/Impedance continued to show ongoing reflux, with a DeMeester score of 18.46 and AET of 5.9 (Table 5-12). Additionally, in this patient there was no response to upright proximal reflux measures post-treatment with PPI and H2 antagonists.

The LCM data from the second patient showed some interesting changes (table 6-2 and table 6-3) i.e. a 171% increase in cough rate, potentially pointing to weakly acid reflux contributing to the cough, with only a minor drop or no change when it came to impedance events and their nature (acidic or weakly acid) or position.

Table 5-12: Questionnaire data from anti-reflux treatment resistant patients

ID	Treatment status	ACQ6	AQAL Total	LCQ Total	HCHQ	RDQ*	SGRQ total
GA3-009	Pre	2.17	4.83	4.5	29	1/1.5/1.25/1	37.4
	Post	2.33	5.75	4.94	25	1.25/2/1.63/1.25	36.7
GA3-010	Pre	3.33	4.01	5.8	41	0.5/0/0.25/2.5	57.6
	Post	2	6.1	6.7	11	0/0/0/0	30.5

Note: * - RDQ domains presented as Heartburn/Regurgitation/GORD dimension/Dyspepsia

Table 5-13: pH/impedance data from 2 anti-reflux treatment resistant patients

ID	Treatment status	DM score	AET	AET Up/Sup*	Imp events total	Imp events ≠ A/WA/NA	Proximal Imp	Proximal Imp (%) A/WA β	Proximal Impedance Up/Sup
GA3-009	Pre	91	19.5	2.5/42.5	71	30/40/1	26	53/23	11.2/22.3
	Post	73.2	14.3	2.3/42.5	38	30/7/1	18	57/14	14.9/25.8
GA3-010	Pre	45.42	13.5	16.5/10.6	55	44/11/0	25	45/45	22/0
	Post	18.46	5.9	11.9/0	61	44/15/2	28	55/27	24/4

Note: * - Impedance events Upright/Supine, ≠ - Impedance events Acid/Weakly acid/Non-acid,

β – Acid/Weakly acid

5.4 Discussion

This study has used a number of standard, well validated questionnaires, which either assessed asthma severity (ACQ) or cough (HCHQ) and related quality of life measures (SGRQ and LCQ). To my knowledge, this is the first study to assess the impact of standard anti-GOR treatment in people with asthma. Although this study was not a placebo-controlled trial, so its findings have to be taken with caution, its results would suggest that anti-GOR treatment can impact on a number of clinical and physiological parameters in people with severe asthma and GORD.

The rationale behind capturing questionnaire data in this study was three-fold. First, by comparing severe asthmatics with and those without GORD, I sought to identify whether any respiratory symptoms in severe asthmatics may be due to the diagnosis of GORD. Second, I looked for any changes in symptoms and quality of life as a result of anti-reflux treatment in individuals with a clearly defined GORD diagnosis. Finally, I wanted to identify which, if any, questionnaires could be of value in clinical practice to manage asthma patients where GORD is a significant co-morbidity. Based on my analyses of GORD outcomes in the UBIOPRED study (Study 1), pulmonary function tests were not expected to show any significant differences in the current study that could be due to the presence of GORD. The only observed significant difference was in respect of small airways flow, as measured by MEF 25-75 (% pred), which was significantly lower in severe asthmatics without GORD compared lower than in the healthy participant without GORD. This suggests that lung function in severe asthma is primarily determined by the airways pathology rather than the presence of GORD.

In contrast to lung function, measures of quality of life were related to the presence of GORD. Thus, the mean (SD) ACQ6 score in SA-GORD patients (2.5(0.9)) was higher when compared to SA-no GORD (1.7(1.1)). The ACQ6 score in the SA-GORD group was higher than the Minimum important difference (MID) of 0.5 and, therefore, can be considered clinically relevant. Similar observations pointing to the clinical relevance of GORD were also seen when SA-GORD participants showed a drop in ACQ6 score >0.5 (p=0.017) after two months of treatment with PPI and H2-antagonists. Additional treatment with the prokinetic, Metoclopramide, did not result in any sizeable drop in ACQ6 score at visit 9, at Visit 11(on baclofen and PPI/H2 antagonist).

Assessment of the composite AQLQ did not show any differences between patients with and without GORD. However, analysis of individual domains showed interesting significant differences between SA-GORD and SA-no GORD in the symptoms domain (p=0.048), suggesting that this quality of life measure is affected by the presence of GORD. Assessment of treatment effects showed that treatment with PPI and H2 antagonists resulted in a trend towards improvement in the symptoms

domain (Visit 1 to Visit 8 p=0.065 and Visit 1 to Visit 11 p=0.059) which suggests a possible role for PPI/H2 antagonist and baclofen).

HCHQ is a validated cough severity questionnaire and is widely used in the management of cough hypersensitivity related to GORD. In this study highly different scores were seen between healthy individuals and severe asthmatics (p<0.0001), and, additionally in the SA-GORD group compared to SA-no GORD (p=0.039). With treatment, there was a sizeable drop from V1 to V8 due to standard PPI and H2 antagonist treatment (p=0.04). The significant differences were maintained at visit 11 (after baclofen). This suggests that cough is an important component of severe asthma, which is worsened by the presence of GORD. The study does also show the value of treatment with standard drugs for GORD because this results in a significant reduction in cough intensity.

LCQ is a validated tool to assess quality of life in relation to cough. It has been used extensively to measure responses to treatment in studies investigating cough of various aetiologies. A low score in LCQ domains suggests poor cough-related quality of life. The lower LCQ scores for SA-no GORD (compared to healthy participants) are very likely a reflection of severe asthma alone as physiological assessment in this group did not show objective evidence of GORD. LCQ scores across all 4 domains (physical, psychological, social and total score) were lower in the SA-GORD group compared to healthy individuals (with or without GORD). However, the quality of life was no different within the SA group when comparing patient with and those without GORD. Of note, across all domains, SA-GORD scores were significantly different compared to healthy cohorts, whereas SA-no GORD patients were significantly different from healthy participants only in the physical domain and total scores. Taken together, quality of life related to cough is poorer in severe asthma, especially in those patients with GORD. This suggests that patients with severe asthma need to be fully assessed for cough and treated appropriately because this will result in better quality of life.

SGRQ is a validated tool in management of severe COPD and severe asthma and an MID of 7.5 in the scores is considered to be a clinically relevant change [168]. Total SGRQ mean scores were higher for all domains in SA-GORD compared to SA-no GORD but this did not reach significance (p=0.08). However, focus on the symptoms domain showed a significantly worse score in SA-GORD (p=0.017). The SGRQ scores dropped somewhat after anti-reflux treatment, but this did not reach statistical significance. These results suggest that SGRQ is not a very sensitive questionnaire for the assessment of GORD in people with severe asthma.

The Reflux disease questionnaire (RDQ) was developed as a tool to improve the diagnostic and follow up capability in an outpatient and primary care setting to help clinicians managing GORD without significant invasive interventions, with a higher score corresponding to higher symptom intensity. In this study RDQ was used in tandem with 24-hour pH and impedance to assess its utility

in a clinical setting where an invasive procedure such as pH and/or impedance may not be a practical solution. Each of the 4 domains of RDQ (heartburn, regurgitation, GORD dimension and dyspepsia) are evaluated individually. In my study, the SA-GORD group had significantly higher scores compared to both healthy groups (HC-no GORD and HC-GORD) in the heartburn domain ($p=0.01$) but, interestingly this score did not differentiate between severe asthmatics with and those without GORD. The SA-no GORD group had higher scores for the dyspepsia domain than the HC-GORD group ($p=0.038$), with no differences when compared to other groups. SA-GORD showed a trend towards significance in Dyspepsia, regurgitation and GORD dimension domains between visit 1 and visit 9 ($p=0.08$). These results suggest that the RDQ is of limited value in the assessment of patients with severe asthma and that it cannot substitute for a proper assessment by 24-hour pH/impedance studies.

My study shows that in some patients with severe asthma (in this study 2 out of 9) the degree of reflux does not improve to normal levels after standard treatment with PPI and H2-antagonists. However, because some improvement in acid reflux was observed in these patients, one could argue that no further intervention is needed, but in these patients, treatment did not result in improvement in total impedance events and in weakly acid measures that may have bearing on symptoms. Such patients could be considered for fundoplication or other surgical interventions. Given the small number of patients, these observations clearly need further study.

In conclusion, the assessed questionnaires, except for RDQ, are a useful adjunct in management of patients where severe asthma and GORD co-exists. If invasive investigations are not an acceptable option for the patient, potentially treatment with anti-reflux medicines can be commenced under monitoring of the relevant questionnaires and if there is no clinical and/or quality of life benefit after 8 weeks then review of treatment is indicated. This would be in line with the guidelines for management of chronic cough where GORD is suspected to be a potential cause of the symptoms. The clinical data also suggests that GORD in severe asthma presents a more symptomatic and challenging phenotype of asthma with a poorer quality of life and identification and management of GORD in these patients does improve the symptoms and quality of life measures.

Chapter 6 Assessment of cough in severe asthma and its relationship to GORD – Study 4

6.1 Introduction

Cough is considered as one of the defining criteria in the diagnosis and management of asthma. Additionally, cough is also known to be a complication of GORD. It can have a significant impact on the quality of life and its relevance in severe asthmatics cannot be ignored. There is a growing interest in the importance of cough in patients with asthma, but there have been few studies that have assessed this relationship in detail. For example, one study of 7125 patients with either asthma or chronic obstructive pulmonary disease (COPD) alone or in combination, found that around a third had a frequent productive cough. It was more common in patients with a joint asthma and COPD diagnosis (38.8%) and COPD alone (38.1%) than in asthma alone (25.0%)[169]. Cough was shown to increase with asthma severity as assessed by the physician and was more prevalent in current smokers as compared with former and never smokers. Patient-reported symptomatic worsening was more common in patients with a frequent productive cough. Finally, reduced post-bronchodilator FEV1 and history of pollutant exposure at home/work were also associated with frequent productive cough in all diagnoses. Of note, patients with baseline frequent productive cough were more likely to have ≥1 exacerbation over the subsequent 12 months with an OR of 1.71, including exacerbations requiring hospital admission and those treated with oral corticosteroids.

Chronic cough guidelines have long noted the importance of assessing GORD as a possible cause of cough and, as I have discussed in previous chapters, many studies have shown that GORD is a significant co-morbidity in severe asthmatics. In this chapter, the aim is to assess in depth the relationship of GORD with cough in patients with severe asthma. As mentioned earlier, there is very weak evidence to show that treating reflux can have a positive impact on cough in general, but it is not clear whether there is any benefit in severe asthmatics.

Cough measurement has advanced significantly over the last decade. In this study I used the Leicester cough monitor (LCM), a validated tool for objectively quantifying cough episodes to help management of cough. In order to assess whether cough episodes are related to reflux episodes, I chose to measure them in parallel, timing the cough measurements to coincide with each GORD episode recorded by 24 Hr pH/impedance.

In order to see whether the relationship between GORD and cough is a non-specific phenomenon regardless of any underlying condition, the same analyses were done in healthy control participants.

6.2 Methods

Subjects

For detailed methods with regards to LCM, please see chapter 2 section 2.3.9. Briefly, cough frequency was measured in all participants who underwent 24 Hr pH/impedance and the LCM was timed to start within 1 minute of starting the pH/Impedance recording so that all episodes of cough could be related to the data from the 24 Hr pH/impedance.

LCM data were collected at Visit 4 and Visit 8. Visit 4 is the baseline visit for all participants in the absence of any anti-reflux treatment in both the severe asthma and healthy cohorts. The LCM data at Visit 8 were acquired only from SA-GORD patients after 2 months of treatment with high dose anti-reflux treatment (PPI and H2-antagonists).

15 patients with severe asthma (9 SA-GORD and 6 SA-no GORD) were compared at baseline with 15 healthy participants (5 HC-GORD and 10 HC-no GORD). The effect of GORD treatment was assessed in 8 SA-GORD patients but not in healthy controls.

Statistical analysis

In order to avoid multiple comparisons that would require correction or use of ANOVA, the following analyses were pre-defined as being of key importance: comparison of all LCM scores from SA-GORD patients at baseline (before treatment) and from HC-GORD and comparison of pre- and post-treatment scores in the SA-GORD group. The aim was to assess whether cough is augmented in people with asthma and to see whether treatment ameliorates cough.

6.3 Results

LCM analysis showed that patients with severe asthma and GORD as a co-morbidity have incidence of a more severe cough than non-asthmatic individuals with GORD, as shown by significant differences between the two groups in respect of total cough count as well as day or night total counts ($p<0.05$ for all). Furthermore, there were differences in cough rates between these two groups, especially at night ($p=0.029$) (Table 6-1)

Analysis of the effects of treatment with PPI and H2-antagonists showed trends towards significance in total cough count and total cough rates ($p=0.088$ and 0.089, respectively) (Table 6-1).

There was significant variability in the extent of reduction. An assessment of change in cough rate and bout rate in the SA-GORD group showed that 50% of the participants had a reduction in cough by more than 50% whereas 2 further had a reduction in cough rate of ≥15%. A further two showed an increase in cough rate after anti-reflux treatment. Of note, in these patients the DeMeester score reduced, just as it did for all participants, but the total impedance augmented, meaning that there had been a shift from acid to non-acid reflux. This is shown in Table 6-2, 6-3 and 6-4. The trends are shown in Figure 6-1.

Table 6-1: Baseline (pre-treatment) and visit 8 (post-treatment) LCM data for severe asthmatics (with and without GORD) and baseline data for healthy control participants (with and without GORD).

	SA-GORD Pre-treatment	SA-GORD Post-treatment (p value)*	SA-no GORD	HC-GORD (p value)#	HC-no GORD
Total cough count	131(19.5-210)	37.5(24-107) (p=0.4)	54(11.8-143)	18.6(14.4) **(p=0.03)**	33.2(19.4)
Total cough count – day	127.5(17.5-180.3)	35(20.3-96.5) (p=0.8)	54(7.5-122.3)	16.2(10.3) **(p=0.059)**	29.8(18)
Total cough count night	16.3(20.4)	5(4.6) (p=0.15)	0(0-12.3)	0(0-6) **(p=0.03)**	1(0-15.3)
Total cough rate (cphr)	5.6(1-8.8)	1.7(1.2-4.6) (p=0.4)	3.7(4.4)	0.84(0.7) (p=0.06)	1.6(1.2)
Total cough rate – day	8.7(1.4-10.3)	2.6(1.6-7) (p=0.42)	3.8(0.5-8.2)	1.1(0.8) (p=0.03)	1.9(1.2)
Total cough rate-night	0.4(0.2-4.8)	0.5(0.2-1.1) (p=0.38)	0(0-1.6)	0(0-0.7) **(p=0.029)**	0.2(0-2.1)
Total bouts	25(3.5-66.5)	10(5-27.3) (p=0.7)	17.8(16.1)	6(5.6) (p=0.1)	8.4(8)
Total bouts – day	24.5(3.3-56.5)	9.5(3.3-25) (P=0.6)	16(16.3)	5(3.7) (p=0.095)	6.4(5.5)
Total bouts – night	4.6(6)	1.3(1.3) (p=0.15)	0(0-3.8)	0(0-2.5) (p=0.14)	0(0-4.5)

Note - For normally distributed data, paired t-test was used and for non-parametric data Wilcoxon test was used. Data presented as Mean (SD) and Median (IQR). *: p value for comparison of pre- and post-treatment with PPI+H2-antagonists in SA-GORD participants; # p value for comparison of baseline values in SA-GORD patients with HC-GORD patients.

Table 6-2: Changes in cough rate and bout rate in severe asthma pre- and post-treatment

	Cough rate Pre	Cough rate Post	Diff %	Bout rate Pre	Bout rate post	Diff %
SA-002	8	3.3	58.8%	2	0.4	80%
SA-003	9	1.4	84.4%	3	0.4	86.7%
SA-009	5	1.4	72%	0.9	0.6	33.3%
SA-010*	0.7	1.9	- 171%	0	0.2	NA
SA-018	1.6	1.1	31.2%	0.4	0.3	25%
SA-021	0.6	0.5	16.7%	0.1	0.1	0%
SA-022	6.2	5	19.4%	1.2	1.4	- 16.7%
SA-023*	19.5	26.1	- 33.8%	4.9	6.7	- 36.7%

Note - *:participants in whom an increase in cough parameters was noted after anti-reflux treatment

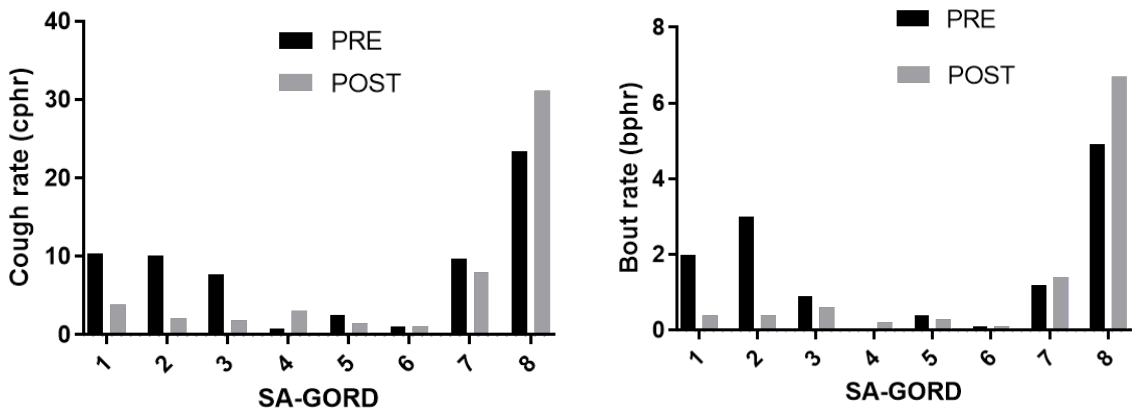

Figure 6-1: Changes in cough rate and bout rate in severe asthma pre-and post-treatment. The percent reduction in cough rate ranged from 17% to 84% but in two participants the cough rate actually increased by 171% and 33%, respectively. Please see Table 6-3 where the impedance test results are shown.

Table 6-3: Impedance data in SA-GORD group before and after anti-reflux treatment

	DeMeester score		Total Impedance events		Acid Impedance events		WA impedance events		NA impedance events		Proximal Impedance Acid (%)		Proximal Impedance WA (%)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
SA-002	64.86	0.2	141	71	119	0	20	71	2	0	39	0	20	11
SA-003	35	2.38	77	48	65	12	12	36	0	0	40	25	8	19
SA-009	91	73.2	71	38	30	30	40	7	1	1	53	57	23	14
SA-010*	45.42	18.46	55	61	44	44	11	15	0	2	45	55	45	27
SA-018	14.26	4.38	22	7	13	0	9	4	0	3	15	0	33	25
SA-021	53.7	2.9	129	62	95	8	29	54	5	0	52	38	45	31
SA-022	13.7	3.93	21	44	10	8	7	9	4	27	70	75	29	44
SA-023*	23.97	6.46	61	63	54	7	7	52	0	4	20	14	0	17

Note - *participants where an increase in cough parameters was noted post anti-reflux treatment.

Table 6-4: Measurement of reflux by 24 Hr pH/impedance monitoring in severe asthmatic (before and after treatment) and healthy controls – extract from Figure 4-1.

	SA-GORD (a)	SA-no GORD (b)	HC-GORD (c)	HC-no GORD (d)	significance (p<0.05)
DeMeester-PrePPI	40.2 (16.7-62)	4.2 (0.7-9.4)	18.8 (16.5-51.3)	4.1(3.5-19.5)	a-d**, a-b*
DeMeester-PostPPI	4.2 (2.5-15.5)##				
TotAET-prePPI %	13.2 (5-16.9)	1.9 (0.5-3.2)	5.8 (4.1-10.7)	1.4 (1-2.4)	a-d**, a-b**
TotAET--postPPI %	1.5 (0.57-4.9)##				
Total AET Upright-prePPI %	10.5 (5.6-6.7)	3.2 (0.9-5.3)	3.4 (1.8-6.5)	1.5 (1.1-2.7)	a-d**, a-c*, a-b*

Note - * denotes p <0.05, ** denotes p<0.001. ## denotes p=0.007 for both DeMeester and AET Pre Vs Post Treatment. Values are Median(IQR). AET – Acid exposure time.

6.4 Discussion

The LCM is a digital ambulatory cough monitor that records sound continuously from a free-field microphone necklace. The study, conducted by Birring et al [122,170], which validated the use of LCM, recommended the cough rate (coughs per hour), i.e. the cough frequency, as the most reliable measure for use in the assessment and management of cough. In their validation study they observed mean (SEM) automated cough counts of 48±9 in patients with chronic cough and 2±1 in the control group. This recommendation is in line with recommendations for other cough recording devices like the VitaloJAK. The guide for LCM states that, when measured for 24 hours, the normal cough frequency should be <5 coughs/hour for females and <2 cough/hour for males. An average for chronic cough diagnosis is between 10 and 20 coughs/hour [122,171]. The developers of LCM also recommended that the MID for acute cough should be a reduction in the cough rate of 54%[172]. A more recent trial of a cough suppressant in development, *gefapixant*, applying the VitaloJAK monitor, used cough frequency as its primary endpoint and a 30% reduction in cough frequency as its key secondary endpoint [173].

In my study, all the 4 study groups underwent a full cough analysis using LCM. In addition, I also assessed separate day and night-time cough counts, cough rates, cough bouts, frequency of bouts and coughs per bout. The most commonly used method of diagnosing and measuring response to treatment remains cough frequency, but day and night frequency are also used.

My assessment of cough showed statistically significant differences between healthy participants with and without GORD. As shown in Table 6-4, this was associated with a similar difference in the scores for reflux, i.e, the DeMeester score and the Acid Exposure Time (AET). This would suggest that GORD is a factor driving the cough in people with severe and GORD, although we cannot exclude entirely a role for severity of the asthma itself as a determinant of the intensity of cough.

The mean (SEM) rate of cough in severe asthmatics with GORD 5.6(1-8.8) was higher than that in severe asthmatics without GORD (3.7(4.4), suggesting a clinically relevant difference but statistical analysis did not show this to be significantly different. After treatment with anti-reflux treatment (PPI and H2 antagonists) for 8 weeks, the SA-GORD cough frequency decreased to 1.7(1.2-4.6) in SA-no GORD suggesting a clinically relevant improvement of almost 70%. Thus, the observations in this study suggest clinically relevant reflux and similarly relevant reductions with treatment.

Similar improvements were noted in all other measures of cough with largest improvements in cough rate and bout rate during the day and night. After commencement of anti-reflux treatment, total cough frequency and daytime cough frequency of SA-GORD fell below that of SA-no GORD. This would be in line with the drop in the DeMeester score and AET in SA-GORD after treatment

with anti-reflux treatment. This further suggests a role for GORD in increasing cough symptoms in severe asthmatics. However, this needs confirmation in much larger studies.

In conclusion, LCM based analysis of cough in my study showed that in severe asthmatics with GORD, treatment of GORD potentially reduces cough frequency but other factors such as weakly acid reflux and non-acid reflux or even GORD that is resistant to standard care can have an impact on the cough symptoms. Additionally, asthma itself, by definition, has cough as one of its symptoms. It is also well known that GORD, breathing pattern disorder, inducible laryngeal obstruction and psychological factors can all lead to cough hypersensitivity which can have additional impact on cough frequency. The number of participants in this pilot study were low and confirmation of the findings would require larger studies.

Chapter 7 Pathobiological characteristics in the airways of patients with GORD – Study 5

7.1 Introduction

There is abundant evidence from numerous studies that pathobiological markers in asthma can be used to identify phenotypes to support the management of asthma in the clinical setting. There has, however, been limited research to explore whether there are similar markers in samples such as sputum, bronchoalveolar lavage and bronchial biopsies that can differentiate severe asthmatics with and without GORD and where they have been assessed the results have been inconclusive [174-181]. My first study (Chapter 3) is the first detailed study of potential biomarkers but its conclusions cannot be accepted as definitive because of the lack of confirmation of GORD by physiological assessment in the U-BIOPRED study. As part of the study in this chapter, the main aim was to identify if the presence of GORD had any impact on standard inflammatory cells that have been the subject of extensive research. For my thesis, I have not only assessed the impact of GORD on the bronchial mucosa but, to my knowledge, conducted the first ever study of the laryngeal mucosa in this setting. Laryngopharyngeal reflux (LPR) is associated with upper airway symptoms such as cough, and my hypothesis was that reflux results in inflammatory changes in the larynx. Signs of LPR may be visible in the laryngopharyngeal area as erythema and inflammation is often visualised with naso-endoscopic examination[182]. The areas affected by LPR, the arytenoid processes and the aryepiglottic folds, can be visualised and assessed by flexible bronchoscopy which made it possible, in this study, for me to undertake biopsies of both the laryngeal and bronchial mucosa in one session.

7.2 Methods

Subjects

For this study the following numbers of patient were included:

1. For sputum samples and Pepsin test: 9 SA-GORD, 6 SA-no GORD; 4 HC-GORD, and 8 HC-no GORD
2. For bronchial biopsy and BAL: 7 SA-GORD, 4 SA-no GORD; 5 HC-GORD, and 9 HC-no GORD
3. For laryngeal biopsies: 7 SA-GORD, 4 SA-no GORD; 5 HC-GORD, and 9 HC-no GORD

Sputum samples were collected by induction with hypertonic saline from all participants at baseline (Visit 4), and also during visits 8, 9, 10 and 11 in severe asthmatics with GORD where the intention was to explore any effect of treatment. The samples were analysed using differential cell counts, Oil red O staining and a test for pepsin (Peptest™). I was supported in processing of some sputum samples by Dr Clair Barber as some of the patients had their study visits while I was not in the department or was back in clinical training. Additionally, although I did report some of the cytospins, majority of the cytospin reporting was done by my colleague Dr Clair Barber in the interest of getting the best and consistent results due to her extensive experience in this field.

Bronchial biopsies were performed in all participants who agreed to undergo endoscopic bronchoscopy and were only done at baseline. Bronchial and laryngeal biopsies were taken and BAL samples were collected from the right middle lobe and right lower lobe of the lung with a view that in case of aspiration these areas are most likely to be exposed to the aspirate anatomically. The bronchial biopsies were processed in GMA and later stained for immune-histochemical markers for mast cells, neutrophils, macrophages, CD3+ (total) T cells, CD8+ T cells, eosinophils, and epithelial cells. Bronchoalveolar lavage was analysed for differential cell counts and cytokine analysis and additionally for Oil Red O stain and pepsin.

Laryngeal biopsies were taken during the bronchoscopy and samples processed into GMA for immunohistochemical analysis in an identical manner to the samples taken at bronchoscopy.

I collected and processed the biopsies (laryngeal and bronchial) including GMA staining; however, the reporting of cell counts and immunohistochemical markers was done by Mr Jon Ward in the interest of quality of reporting and consistency. Bronchial lavage was collected during bronchoscopy by me and immediately taken to lab for processing which was done by my colleague Dr Laurie Lau.

Details of these methods are given in **Chapter 2-Methods** previously.

Statistical analysis

In order to limit the number of analyses, thereby avoiding correction for multiple comparisons which inevitably reduces statistical power, the following comparisons were pre-defined as primary outcomes for all biomarkers measured: SA-GORD vs SA-no GORD, and (in SA-GORD patients) pre- vs post-treatment.

7.3 Results

Sputum

There were no significant differences in the differential cell counts when comparing all groups. SA-GORD had a slightly higher % neutrophil median (IQR) value of 61.8(24.7) compared to SA-no GORD [50.8(15.8)], HC-GORD [35.6(28.5)] and HC-no GORD [42.2(31.6)] but this did not reach significance. Sputum eosinophil counts were also slightly higher in SA-no GORD compared to all other groups in keeping with possible T2 inflammation driving the inflammatory process, but this too did not reach significance. Visit-wise trends are shown in table 7-1 for macrophages, Table 7-2 for neutrophils, Table 7-3 for eosinophils, and table 7-4 for epithelial cells and Figures 7-1 and 7-2.

Table 7-1: Sputum differential counts (%) – macrophage

	SA-GORD	SA-no GORD (p value)	HC-GORD	HC-no GORD
Visit 4	32(22.2)	43.5(15.4) (p=0.7)	37.6(29.6)	51.3(29)
Visit 8 (p value)	28.5(20.3) (p=0.92)	n/a	n/a	n/a
Visit 9	39.6(20.4)	n/a	n/a	n/a
Visit 11	34.7(23.6)	n/a	n/a	n/a

n/a – not applicable as participants did not have post treatment visits.

Table 7-2: Sputum differential count - neutrophils (%)

	SA-GORD	SA-no GORD (p value)	HC-GORD	HC-no GORD
Visit 4	61.8(24.7)	50.8(15.8) (p=0.35)	35.6(28.5)	42.2(31.6)
Visit 8 (p value)	56.1(27.1) (p=0.0.96)	n/a	n/a	n/a
Visit 9	55.3(20.8)	n/a	n/a	n/a
Visit 11	58.8(22)	n/a	n/a	n/a

n/a – not applicable as participants did not have post treatment visits.

Table 7-3: Sputum differential count - eosinophils (%)

	SA-GORD	SA-no GORD	HC-GORD	HC-no GORD
Visit 4	0.95(0.3-3)	1.5(0.2-3.9) (p=0.54)	0.19(0-0.5)	0(0-0.4)
Visit 8	0.8(0.1-1.7) (p=0.71)	n/a	n/a	n/a
Visit 9	0.13(0.1-0.25)	n/a	n/a	n/a
Visit 11	0.5(0.3-6.3)	n/a	n/a	n/a

n/a – not applicable as participants did not have post treatment visits.

Table 7-4: Sputum differential count - epithelial cells (%)

	SA-GORD	SA-no GORD	HC-GORD	HC-no GORD
Visit 4	2.7(0.6-7.4)	3.5(2-4.8) (p=0.63)	1.7(0.3-2)	1.9(0.6-11.3)
Visit 8	2.9(1.3-4.8) (p=0.84)	n/a	n/a	n/a
Visit 9	5.6(3.3-5.9)	n/a	n/a	n/a
Visit 11	5(1-5.6)	n/a	n/a	n/a

n/a – not applicable as participants did not have post treatment visits.

Sputum differential counts

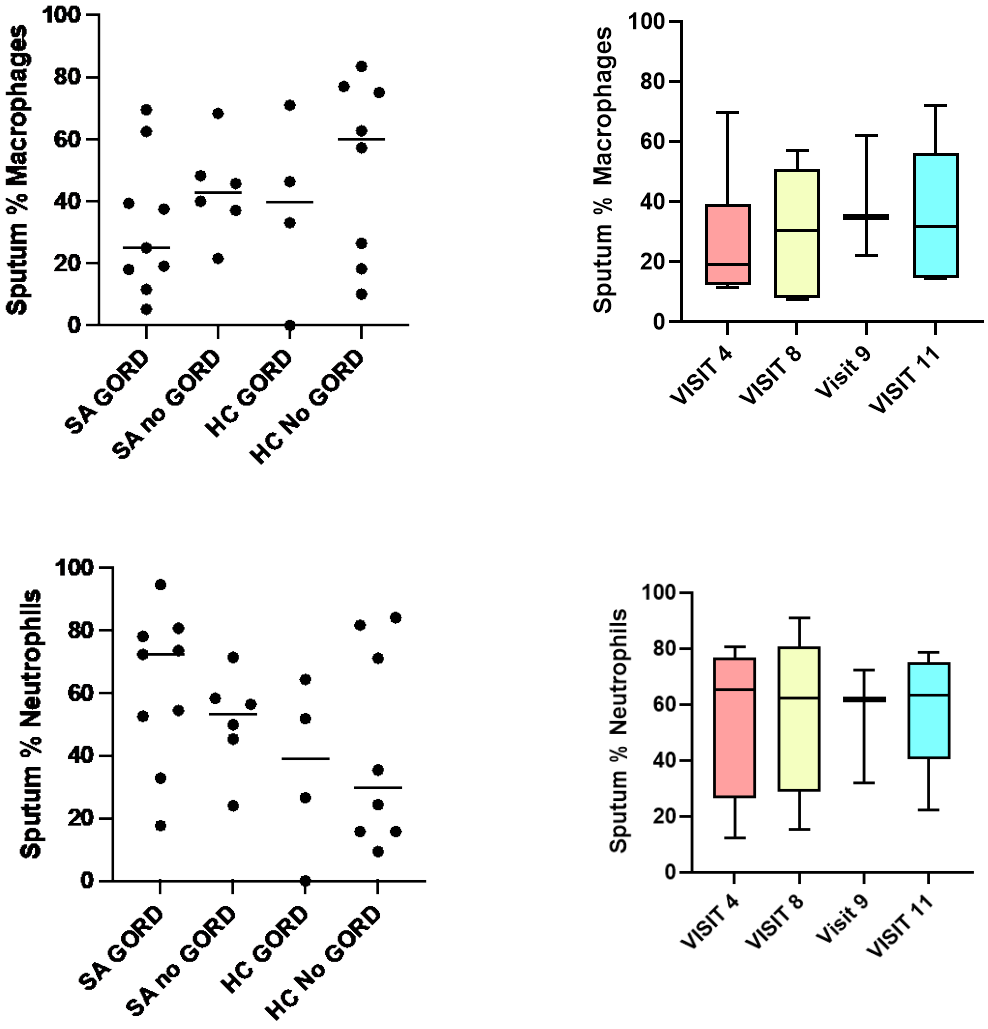

Figure 7-1: Differential cell counts for all groups (left) and SA-GORD with anti-reflux treatments (right)

Sputum differential counts - continued

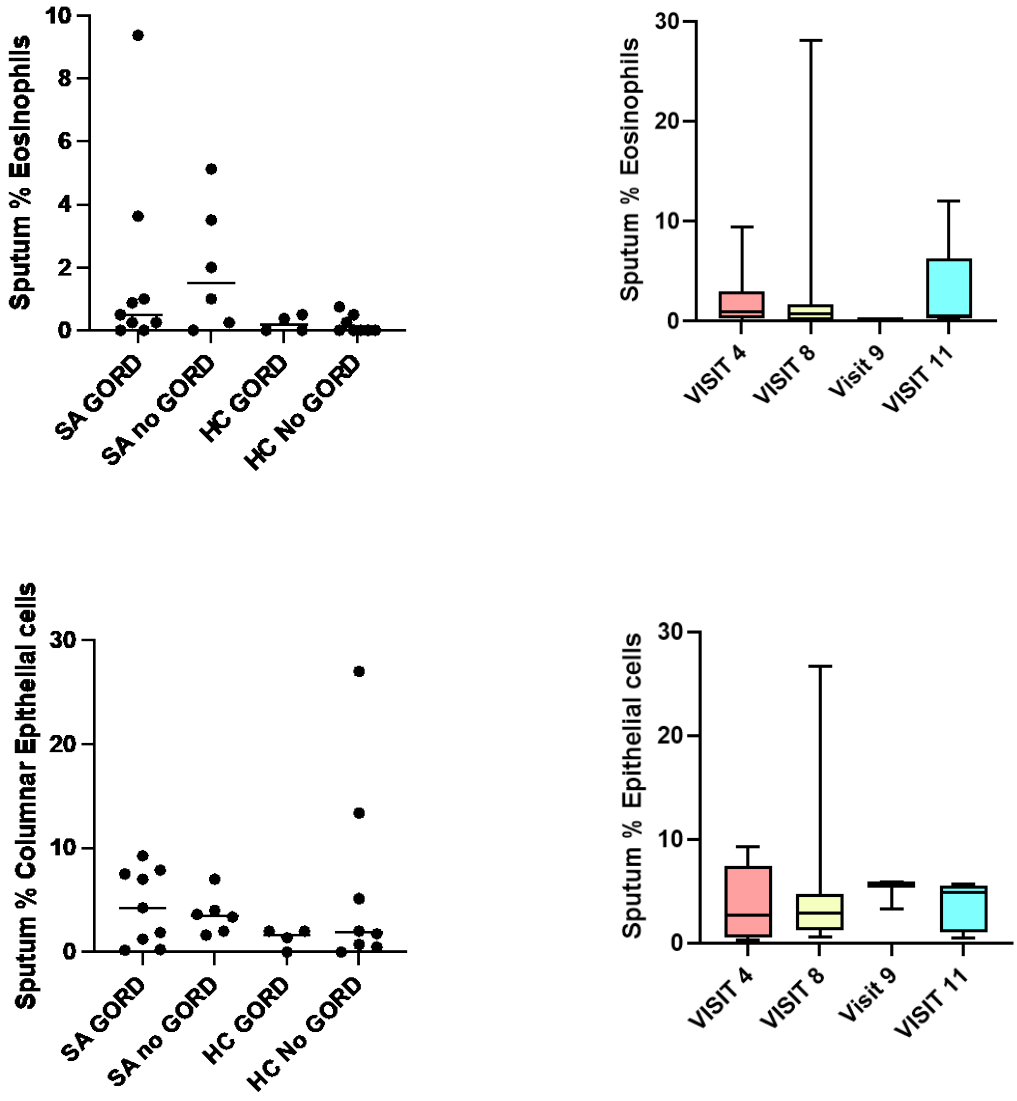

Figure 7-2: Differential cell counts across all the groups and trends in severe asthma GORD group across visits 4 (Post-PPI), Visit 8 and Visit 11

BAL differential cell counts

BAL differential counts did not show any significant changes across the health groups and severe asthmatics with or without GORD. The differential counts showed mean (SD) macrophage (%) 84.8(7.5), 94.4(3) for SA-GORD and SA-no GORD and 79.3(23.3) and 92.3(3) for HC-GORD and HC-no GORD (see Figure 7-3). The neutrophils (%) differential count was 4.1(4.3) and 1.6(0.5) for SA-GORD and SA-no GORD and 7(10.4) and 1.3(0.6) for HC-GORD and HC-no GORD respectively. Similarly epithelial cells and lymphocytes were not significantly different among the groups. Eosinophil count higher in BAL in SA-no GORD compared to SA-GORD (median (IQR) 0.5(0.13-1) and 0(0-0.25) respectively) but did not reach significance (p=0.5).

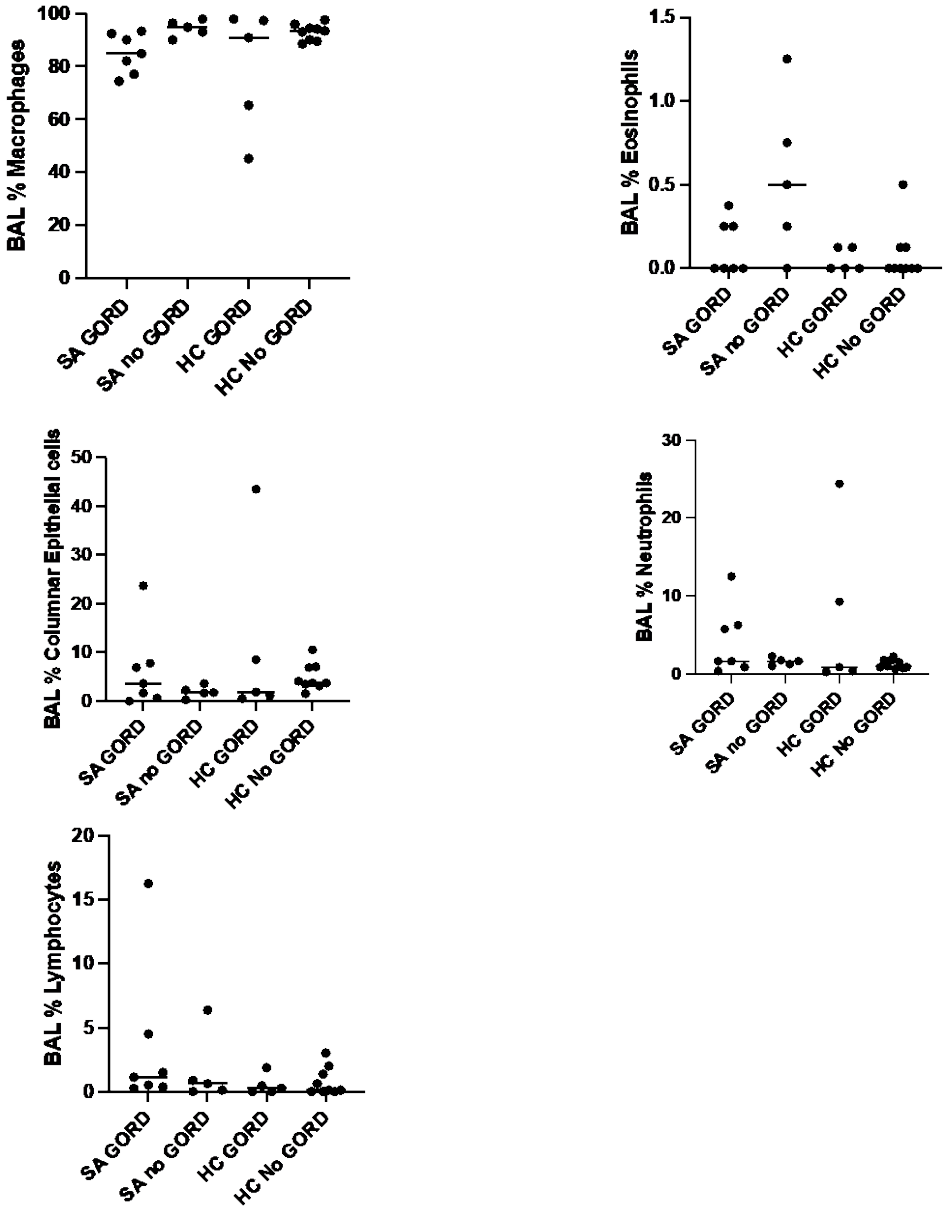

Figure 7-3: Differential cell counts in BAL from all participant groups

BAL cytokine analysis

BAL cytokine analysis did not show any significant differences between SA-GORD and SA-no GORD. Similarly, there were no differences between SA-GORD and HC-GORD. IL12 showed a significant difference between SA-GORD and HC-no GORD [(0.08(0.05-0.1) and 0.12(0.1-0.15) p=0.009]. Please see figures 7-4 and 7-5 for the cytokines assessed.

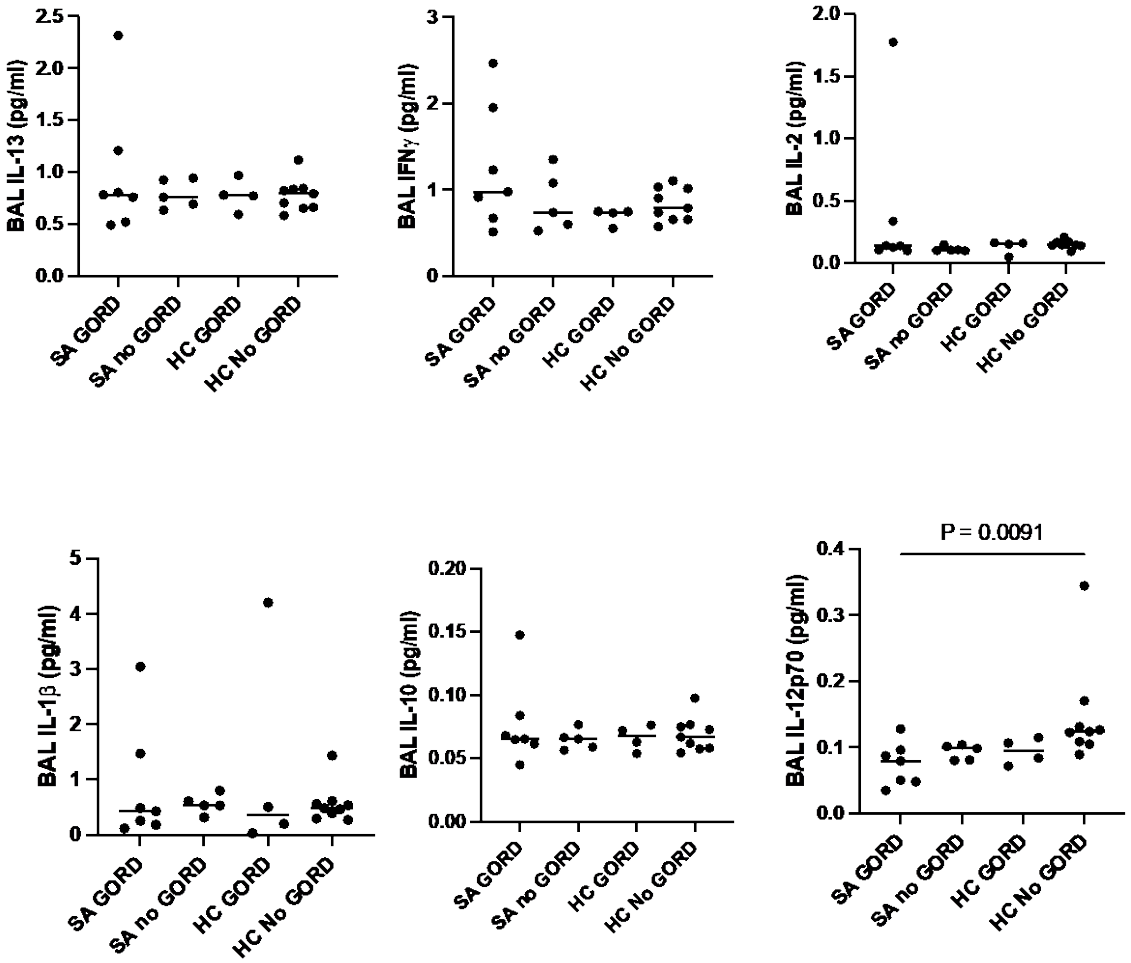

Figure 7-4: BAL cytokines analysed in all groups

BAL cytokine analysis – continued

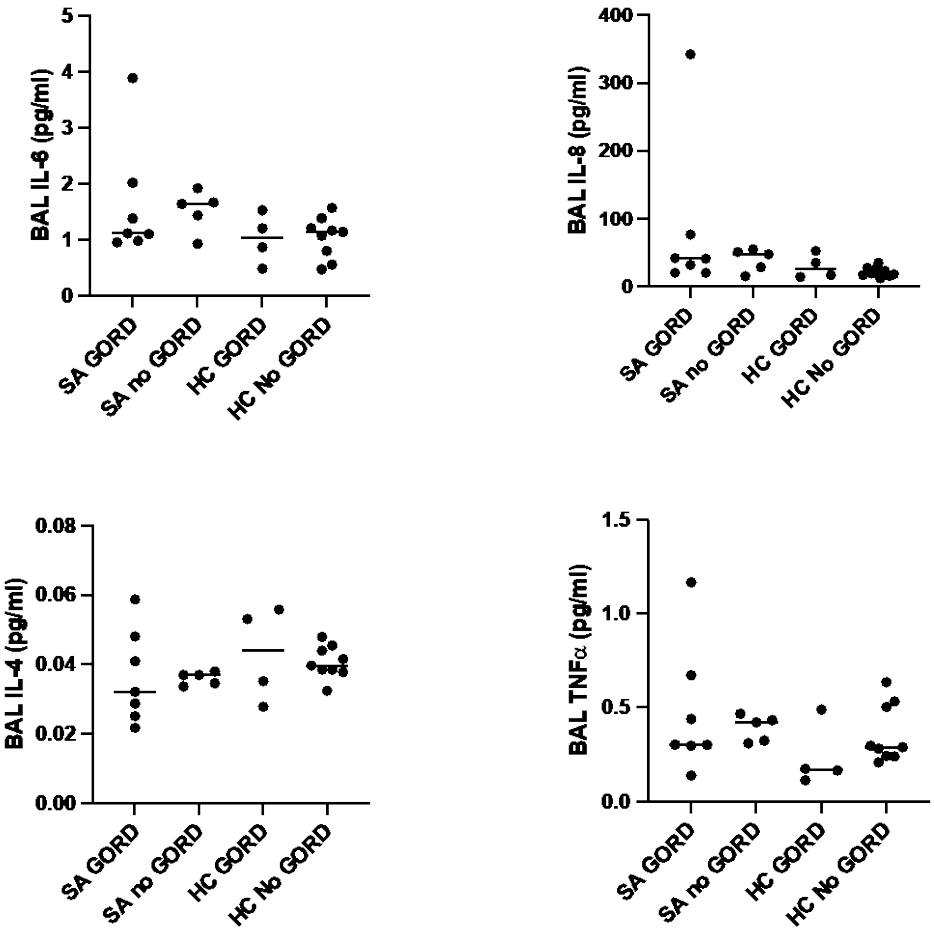

Figure 7-5: BAL cytokine analysis continued

Immune histochemical analysis of bronchial biopsies

Immunohistochemical analysis done on bronchial biopsies also did not show any significant differences between SA-GORD and SA-NO GORD patients, or between SA-GORD and HC-GORD except for the submucosa, where macrophages were observed to be significantly increased in SA-GORD when compared to HC-GORD (see Figures 7-6 and 7-7).

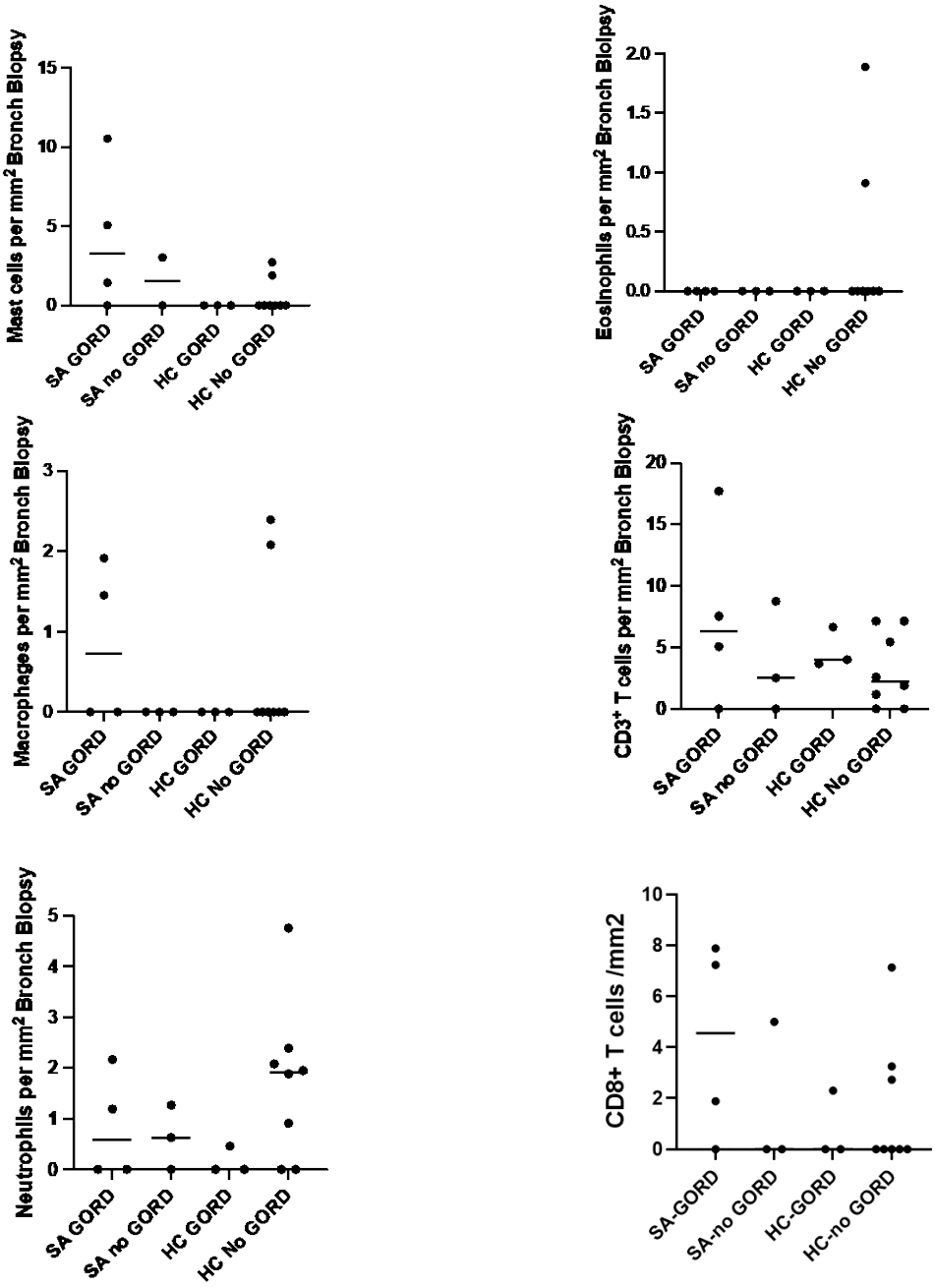

Figure 7-6: Immuno- histochemical analysis of the bronchial biopsy – epithelium in all groups

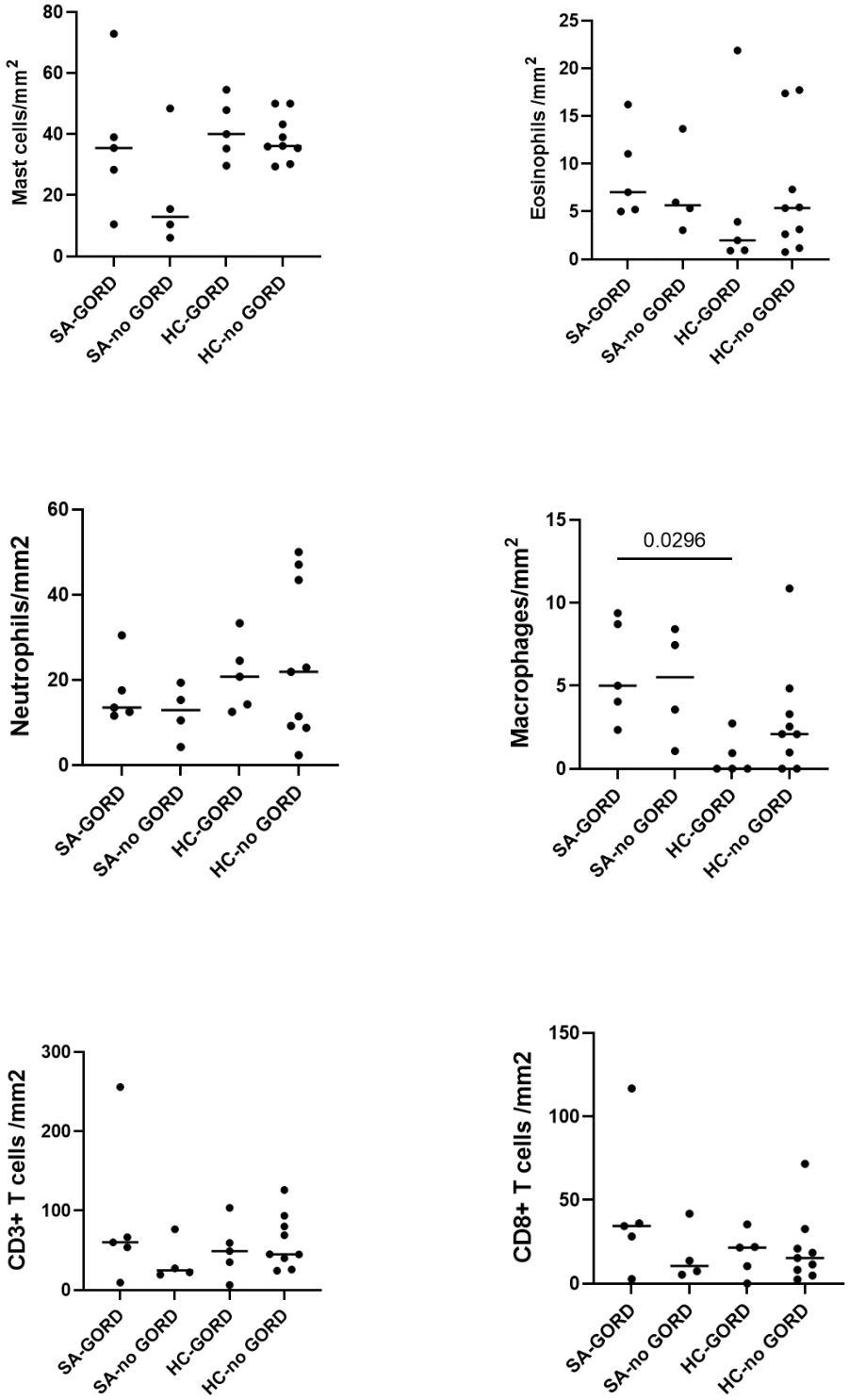

Figure 7-7: Immunohistochemical analysis of bronchial biopsy sub-mucosa in all groups

Immune histochemical analysis of laryngeal biopsies

Statistical analysis did not show any significant differences in laryngeal biopsy cell counts between SA-GORD patients and SA-noGORD patients, as well as between the former group and HC-GORD patients (see Figure 7-8).

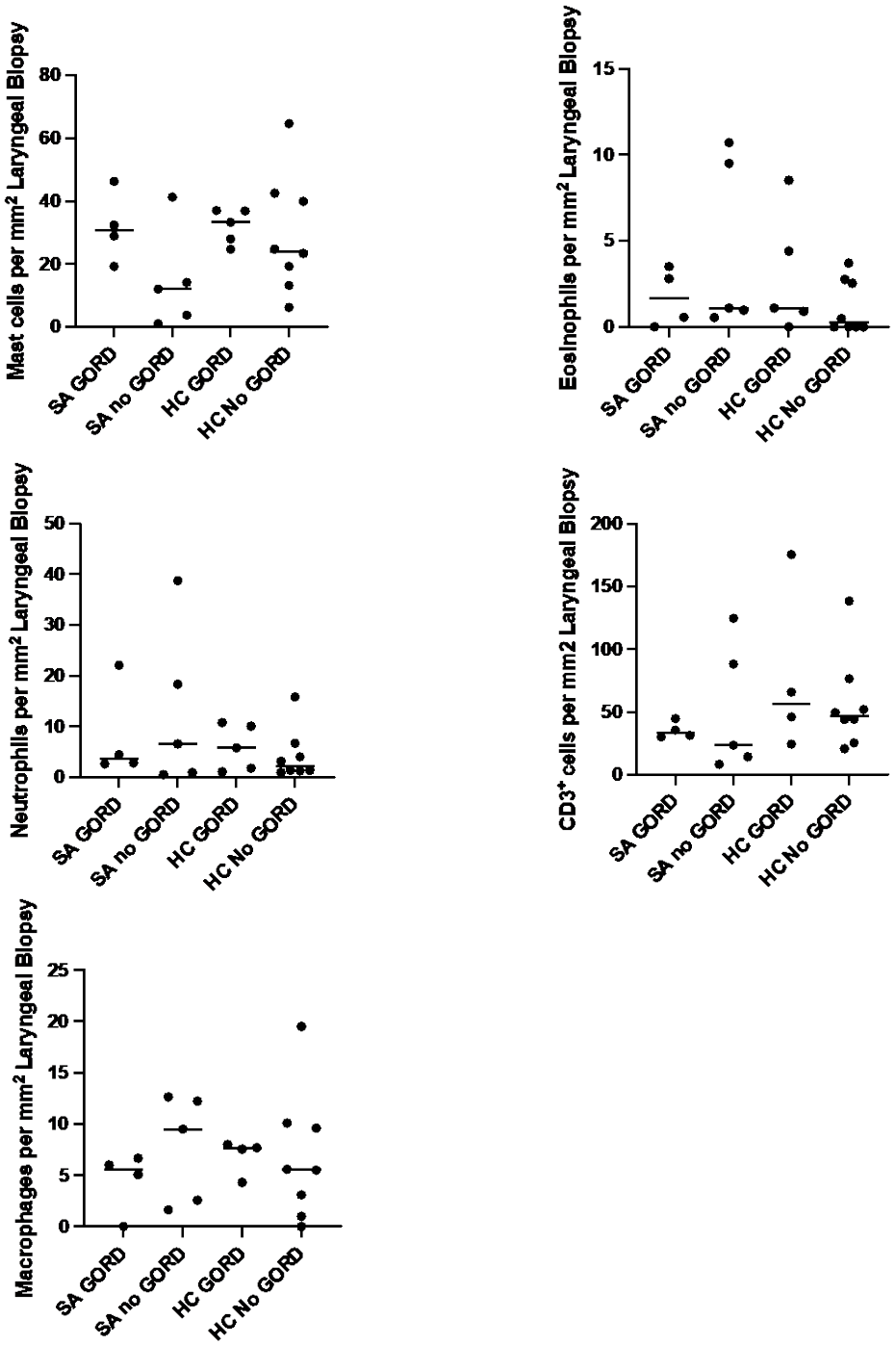

Figure 7-8: Immune histochemical analysis of laryngeal biopsies

Pepsin assessment in throat clearate and saliva

Pepsin was assessed in both saliva and throat clearate. The median level of pepsin in the throat clearate samples was increased in SA-GORD when compared to all the groups (HC-GORD, HC-no GORD and SA-no GORD) it was observed to be statistically significant when compared to HC-no GORD (p=0.003). Comparison of Pepsin in throat clearate of SA-GORD with SA-no GORD also showed a significantly elevated level of pepsin (see Table 7-5 and Figure 7-9). In the analysis of pepsin levels in SA-GORD with anti-reflux treatment, pepsin levels were noted to be lower with anti-reflux treatment but it did not reach significance. No significant differences were observed with salivary pepsin or pepsin assessment in BAL.

Table 7-5: Throat clearate pepsin assessment using Peptest in all groups

	SA-GORD	**SA-no GORD**	**HC-GORD**	**HC-no-GORD**
V4	93(12-189)	0(0-24) (p=0.04)	0(0-85)	0(0-0)
V8	20(0-41.5) (p=0.12)	n/a	n/a	n/a
V9	24(0-25)	n/a	n/a	n/a
V11	0(0-153.5)	n/a	n/a	n/a

Table 7-6: Saliva Pepsin assessment using Peptest in all groups

	SA-GORD	**SA-no GORD**	**HC-GORD**	**HC-no-GORD**
V4	31(0-209.8)	16(0-30) (p=0.51)	0(0-12.5)	0(0-0)
V8	0(0-500) (p=0.9)	n/a	n/a	n/a
V9	0(0-58)	n/a	n/a	n/a
V11	0(0-98.5)	n/a	n/a	n/a

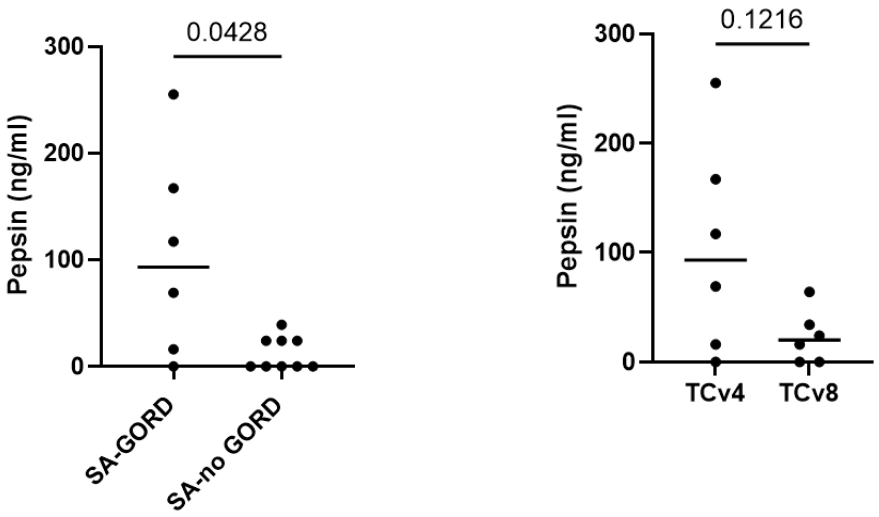

Figure 7-9: Throat clearate pepsin analysis using Peptest™. TC – Throat clearate

Oil Red O stain sputum and BAL

Sputum and BAL were analysed with Oil Red O stain with a view to observing lipid laden macrophages (LLM). There were no significant differences observed in the LLM percentage in SA-GORD and SA-no GORD in sputum or BAL (see Table 7-7 and 7-8 and Figure 7-10).

Table 7-7: Sputum Oil Red O stain showing LLM % in all 4 groups

	SA-GORD	SA-no GORD	HC-GORD	HC-no-GORD
V4	0(0-8.5)	0(0-12)	6.5(1.5-27.3)	3.5(0.8-18.3)
V8	0(0-4.8)	n/a	n/a	n/a
V11	0.5(0-30.3)	n/a	n/a	n/a

Table 7-8: BAL Oil Red O stain showing LLM % in all 4 groups

	SA-GORD	SA-no GORD	HC-GORD	HC-no-GORD
Oil Red stain %	0(0-0)	0(0-5.5)	0(0-0)	7(1.5-9)

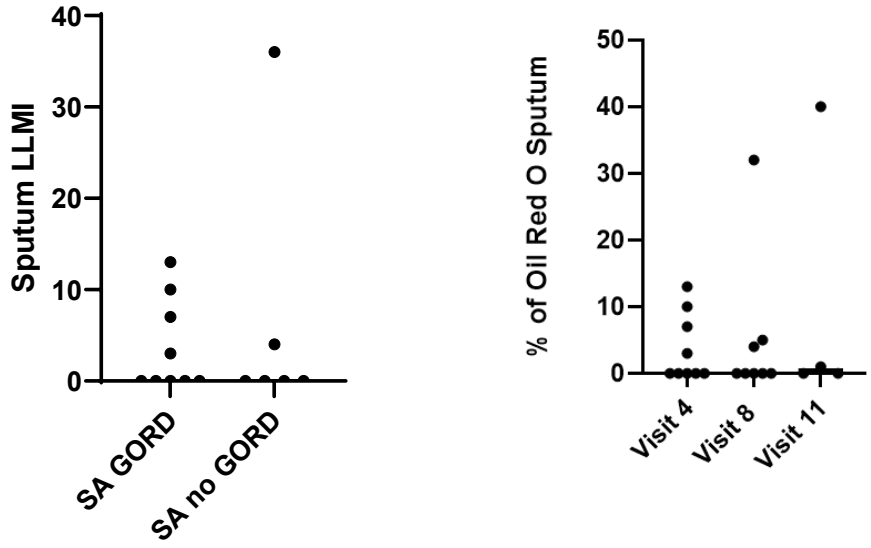

Figure 7-10: Sputum LLMI on Oil Red O stain

7.4 Discussion

This study was the first attempt to comprehensively look for potential biomarkers that would point to possible mechanisms that are altered in patients with severe asthma who also have GORD as an important co-morbidity. Detailed analysis using well validated methods, such as sputum induction and bronchial biopsy, failed to show even slight trends in differences between patients with GORD and those without, suggesting that these biomarkers are not sensitive enough to pick up any subtle changes that may arise out of exposure to reflux. The major finding was the demonstration of higher concentrations of pepsin in the throat clearate in severe asthmatics with GORD, compared to those without GORD [Pepsin (ng/ml) 93(12-189) vs 0(0-24) p=0.04] (see Figure 7-9 and Table 7-5). This suggests a significantly higher prevalence of presence of pepsin in the back of the throat of severe asthmatics with GORD. Comparison with levels in non-asthmatic individuals with GORD (not pre-specified as a statistical test prior to analysis) showed no significant differences between asthmatics and non-asthmatics who share the diagnosis of GORD. Furthermore, comparison with healthy individual without GORD showed even greater difference with severe asthmatics (p=0.003). This suggests that this test is good at picking up reflux which contains pepsin and that it could be useful test in the assessment of reflux in any population. Of note, treatment with PPI and H2-antagonists showed no statistically significant effect on pepsin levels in patients with asthma. Given the significant reduction in DeMeester score and AET in SA-GORD patients this is somewhat surprising. However, the impedance events did not change despite treatment, neither the non-acidic reflux, which would suggest that pepsin does make its way into the throat despite adequate control of acid reflux. Whether and to what extent this has an effect on asthma control is unclear and would require further study.

The cytokine analysis of BAL showed a significant difference in IL12p70 between SA-GORD compared to HC-NO GORD. IL12 family of cytokines are involved in regulation of T-cell responses in infections. In particular, it is required for induction of IFN-gamma production required for induction of Th1 cells. A lower level of IL-12p70 may pre-dispose to increased risk of infections. In our case the level of IL-12p70 was low in both SA-GORD and SA-NO GORD compared to HC-NO GORD (see Figure 7-4). Although, statistical significance was only reached in SA-NO GORD there is a high possibility that this subdued response is a result of increased use of steroids (oral and inhaled) in the severe asthmatics in general. This is suggested by absence of any significance when comparing IL12p70 between SA-GORD and SA-NO GORD.

Immune histochemical analysis of bronchial biopsies to identify inflammatory changes attributable to GORD has been few and far between with unclear outcomes and largely have failed to show a significant pattern of inflammation and/or remodelling that can be confidently attributed to

GORD[177]. I performed laryngeal biopsies for all participants undergoing bronchoscopy. This was a break from tradition where performing laryngeal biopsies as day case procedure is uncommon. While the analysis of laryngeal biopsies did not show a significant difference in inflammatory cell types, more detailed studies of the structure of epithelial cells, including tight junctions along the lines of the last study in this thesis are likely to result in more valuable findings.

I performed Oil Red O stain for lipid laden macrophages on cytospins from sputum and BAL from all groups. The LLM from sputum have been attributed to GORD and possible aspiration in previous studies [120,130]. There were no significant differences noted in the sputum in SA-GORD when compared to other groups in the context of LLM (see Table 7-7 and 7-8 and Figure 7-10). Additionally, I could not find any significant differences in LLM in BAL of the severe asthmatics and healthy cohorts. The lack of any statistical difference in LLMI in severe asthmatics and healthy with and without GORD suggests that this method may not be valuable in the assessment of GORD as previously believed.

Chapter 8 Vulnerability to acid reflux of the airway epithelium in severe asthma

8.1 Introduction

Severe asthma is associated with multiple co-morbidities, including gastro-oesophageal reflux disease (GORD) which is particularly common and is associated with exacerbation frequency and poor quality of life [183]. Until recently, this association was explained by three mechanisms: vagal reflex [184], neuroinflammation [185], and microaspiration directly triggering airway inflammation [186-189]. While studies of reflux in animal models [187-190] and cultures of bronchial epithelial cells [143,191] have shown varying impact of gastro-oesophageal refluxate on mediators of inflammation and airway remodelling, direct in vivo evidence for these mechanisms in patients with asthma has been limited. We recently undertook an in-depth analysis of sputum proteomics in severe asthmatics and identified 11 proteins differentially abundant in patients with GORD, including elevated levels of anti-microbial proteins and reduced levels of proteins involved in systemic inflammatory responses and epithelial integrity [192], providing the first direct evidence that reflux is associated with changes in the microenvironment on the epithelial surface of the airways. Recognising that defective epithelial barrier function, dysregulated repair mechanisms, and modified epithelial immune responses to pathogens and allergens are important features in asthma [193], we further hypothesised that the presence of GORD in severe asthma would significantly influence global epithelial gene expression. Applying unbiased topological data analysis (TDA) of microarray data derived from bronchial brushings, we identified a subset of severe asthmatics with a clinical phenotype defined by obesity, presence of GORD and treatment with proton pump inhibitors (PPI) [194], characterized by upregulated airway remodelling signalling and downregulated mechanisms of immune cell recruitment, possibly linked to both bile acid exposure and PPI treatment [194].

In the current study, we have sought to elucidate further the underlying mechanisms of GORD-associated dysregulation of the airway epithelium in severe asthma using a combination of in vitro and ex vivo approaches. We developed an in vitro model of GORD in which fully differentiated air-liquid interface (ALI) cultures of primary bronchial epithelial cells were exposed to a multiple challenge protocol using pepsin, acid pH and bile acids (MCP-PAB). Consistent with our previous in vivo observations [194], we observed that ex vivo exposure of epithelial cells to refluxate results in significant structural and functional changes. We then extended our studies using bronchial biopsies and bronchial brushings from severe asthmatics with GORD and confirmed the effects on IL-33 and changes in expression of a selection of genes identified from the in vitro study.

8.2 Methods

Study participants and sample collection

Severe asthmatics (Step 4/5 of BTS/SIGN Guidelines) and healthy control participants were recruited prospectively and assessed for GORD by 24-hr pH/impedance studies. The severe asthmatics were further stratified into those with documented GORD but no PPI treatment, those with documented GORD and PPI treatment and those without GORD. There were no statistically significant differences in the clinical characteristics of severe asthmatics although the SGRQ scores indicated worse quality of life in the severe asthmatics with GORD with higher number of exacerbation and a higher ACQ6 score. Comparison of age, BMI, FEV1 and FEV1/FVC ratio and SGRQ scores between the healthy and severe asthma participants did show a significant difference confirming two clinically different groups (see Table 8-1).

Epithelial cells were harvested by bronchoscopic brushings and processed into RNAlater for subsequent RNAseq analysis or used in primary bronchial epithelial cell (PBEC) culture [195]. Bronchial biopsies were also taken and fixed in paraffin for immunohistochemical analyses.

The study was approved by the South-Central Hampshire A, Research Ethics Committee, UK (reference numbers: 13/SC/0182 and 14/WM/1226) and all participants gave their informed consent.

Analysis of the ex vivo effect of a multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB)

Initial dose and time-course studies were conducted with 16HBE cells exposed to MCP-PAB (pepsin and chenodeoxycholic acid at acidic pHs); optimised conditions were subsequently confirmed to be appropriate for fully differentiated air liquid interface (ALI) cultures (please see Appendix B, Figure B1 for full details). MCP-PAB conditions were applied to the apical epithelial surface for 30 minutes before washing twice. After 4h recovery, epithelial permeability was measured using transepithelial electrical resistance (TEER) and FITC dextran 4kDa [195]. Apical supernatants were collected for cytokine measurements. Cells were then lysed with Trizol lysis reagent (Life Technologies, Paisley, UK) and frozen at -80°C until analysis or fixed for immunofluorescence staining or electron microscopy analysis.

Immunostaining and electron microscopy

ALI cultures were fixed with 4% paraformaldehyde and processed for immunofluorescence staining, as previously described [195]. The cultures were also processed for transmission electron microscopy (TEM) and analysed for epithelial integrity.

Bronchial biopsies were processed as previously described [196], embedded into paraffin; sections were stained using goat polyclonal anti-human IL-33 (R&D Systems, Abingdon, UK). Results were expressed as positive nuclei per total number of epithelial cells.

Cytokine analyses

Interleukin (IL)-8 concentrations in conditioned media were measured using an IL-8 DuoSet ELISA (R&D, Abingdon, UK) while IL-6, TNFα and IL-1α were measured using VPlex (MSD, Maryland, USA).

Analysis of gene expression in epithelial brushings and differentiated cells

Total RNA was extracted from epithelial brushings or cultured cell lysates using miRNeasy Mini Kit and RNase-Free DNase Set (Quiagen, Manchester, UK). cDNA libraries were prepared using NEBNext Ultra (non-stranded) mRNA library prep kit with polyA pulldown for mRNA enrichment. Paired-end 150bp sequencing to a depth of 20M reads (epithelial brushings) or 50M reads (differentiated cells) was performed on an Illumina HiSeq2500 by Novogene Inc (Cambridge, UK). FATSQ files were aligned to human genome build GRCh38 using STARv2.6.0, reads were counted with HTSeq and differential gene expression analysis conducted with edgeR.

Details of the methods are in the Appendix B. Data are deposited in GEO.

Statistical analyses

Paired t-tests were applied to transcriptomic data, while clinical and experimental data were analysed by Kruskal-Wallis, Mann-Whitney U or Student t tests depending on data distribution; $p<0.05$ was considered significant. False discovery rate (FDR) correction was applied to the transcriptomic data.

Study contribution

I was the Principal investigator on the protocol that included this study. I recruited, collected clinical data and biological material (biopsies, BAL and brushings) for this study and reviewed the manuscript of the publication with other authors. All the lab work and analyses in the study

including the statistical analyses were performed by Dr Jean-Marie Perotin and Dr Gabrielle Wheway.

Table 8-1: Characteristics of participants

	Healthy controls	Severe asthmatics	p value	SA-no GORD	SA-GORD	SA-GORD+PPI
n	15	18		4	6	8
Age, yrs	41.5 ± 12.9	55.7 ± 8.1	0.0005	52.5 ± 6.2	54.3 ± 9.2	58.25 ± 7.99
Age of asthma onset, yrs		23.7 ± 21.0		24.0 ± 25.7	27.3 ± 23.6	20.75 ± 19.40
Gender (F/M)	11/4	11/7	0.48	3/1	6/0	2/6
Never smoker	11	8	0.28	3	4	7
BMI, kg/m²	25.76 ± 3.68	32.55 ± 6.74	0.001	30.28 ± 5.97	37.0 ± 5.5	30.4 ± 6.9
Obese (BMI>30)	1	10	0.004	2	5	3
Diagnosed GORD	0	14	<0.0001	0	6	8
Atopy, n	4	11	0.08	1	4	6
Sputum						
Eosinophils, % total cells	0.14 [0-0.51]	1.01 [0.27-3.57]	0.10	1.97 [0.26-4.83]	0.51 [0.13-5.23]	1.13 [0.34-2.95]
Neutrophils, % total cells	44.3 [20.1-74.7]	64.1 [37.8-73.1]	0.44	52.9 [31.4-68.3]	65.0[60.3-73.4]	63.4 [11.7-75.3]
FEV1, % predicted	116.6 ± 23.4	89.7 ± 23.6	0.004	82.5 ± 23.0	105.8 ± 24.4	78.3 ± 15.7
FVC, % predicted	121.7 ± 19.6	110.7 ± 20.0	0.14	99.4 ± 12.1	124.8 ± 25.0	104.1 ± 9.4
FEV1/FVC	0.80 ± 0.04	0.66 ± 0.12	0.0001	0.69 ± 0.12	0.72 ± 0.08	59.2 ± 12.2
FeNO		24.2 ± 11.1		33.0 ± 17.0	28.8 ± 11.0	19.8 ± 8.8
Exacerbations (last year)		4.1 ± 3.4		3.3 ± 1.5	3.7 ± 2.0	4.7 ± 4.9
ACQ6		12.8 ± 6.5		6.5 ± 4.8	15.5 ± 6.2	14 ± 5.88
SGRQ						
Activity score	5.16 ± 6.55	58.12 ± 24.52	<0.0001	43.89 ± 12.02	62.10 ± 23.10	72.94 ± 16.66
Impact score	0.32 ± 1.15	36.47 ± 15.82	<0.0001	20.45 ± 10.08	39.10 ± 18.28	41.75 ± 12.94
Symptoms score	8.44 ± 8.93	65.13 ± 22.81	<0.0001	55.51 ± 23.90	65.28 ± 25.56	75.73 ± 13.83
Total score	3.16 ± 2.42	47.79 ± 17.01	<0.0001	30.05 ± 12.85	50.42 ± 17.20	56.84 ± 13.83
Hull Cough questionnaire	1.0 ± 2.0	36.05 ± 21.97	<0.0001	23.75 ± 13.23	32.50 ± 20.54	34.43 ± 13.10
Treatment						
Proton pump inhibitor, n	0	8	0.0036	0	0	8

Results are expressed as numbers, mean ± SD or median [IQ25-75]. No significant difference within the groups of severe asthma patients.

8.3 Results

Multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) causes epithelial damage and alters barrier and secretory function

To analyse the impact of GORD on the airway epithelium of severe asthmatics, ALI cultures derived from bronchial brushings of participants with severe asthma, GORD and PPI treatment (n=8) and healthy controls (n=5) (Table 8-1) were exposed for 30 min to MCP-PAB conditions consisting of 50µg/ml of pepsin and 250µM chenodeoxycholic acid (CDC) at pH 5.

MCP-PAB-induced epithelial damage was characterized by TEM as enlargement of intercellular spaces and beginning of cell detachment. MCP-PAB-exposed ALI cultures also had markedly increased ionic and macromolecular permeability, as shown by decreased TEER (Figure 8-1a) and increased FITC dextran 4kDa passage respectively (Figure 8-1b), with a significantly greater impact of MCP-PAB on permeability of cultures from severe asthmatic donors compared with healthy controls. Analysis of epithelial tight junctions and adherens junctions in ALI cultures from severe asthmatic donors showed a marked disruption of the junctions in MCP-PAB-exposed cultures (Figure E3).

In addition to having a marked impact on epithelial structure, MCP-PAB caused an increase in the secretion of CXCL8, IL1α, and TNFα (Figure 8-2). These results were supported by analysis of epithelial gene expression (Figure E4).

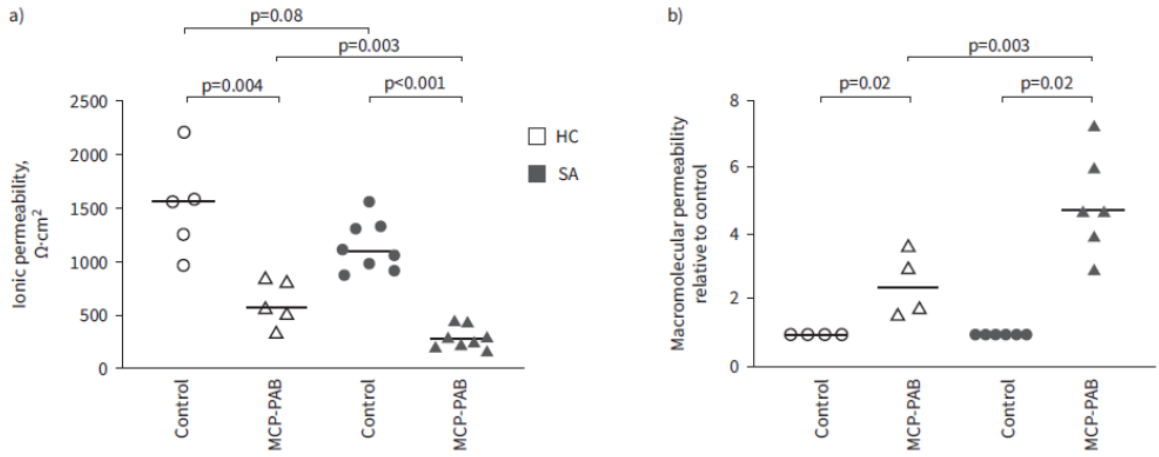

Figure 8-1: Effects of multiple challenge protocol using pepsin, acid pH and bile acid on epithelial permeability. Bronchial epithelial air–liquid interface cultures from healthy controls (HC) (n=5) and severely asthmatic (SA) (n=8) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 h before a) ionic and b) macromolecular permeability were measured.

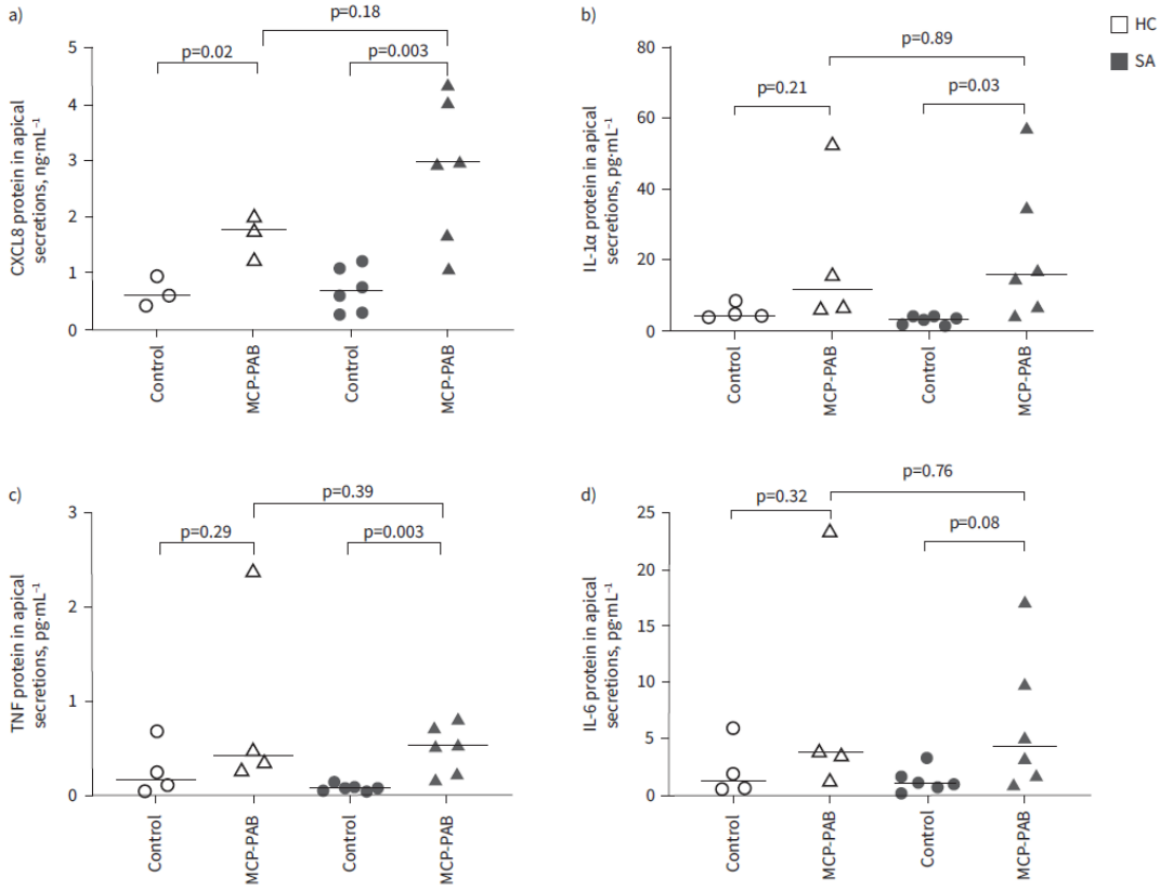

Figure 8-2: Stimulation of epithelial cytokine release by multiple challenge protocol using pepsin, acid pH and bile acid. Bronchial epithelial air–liquid interface cultures from healthy controls (HC) (n=4) and severe asthmatic (SA) (n=6) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 h before a) CXCL8, b) interleukin (IL)-1α, c) tumour necrosis factor (TNF)-α and d) IL-6 protein release was measured in apical secretions.

Artificial refluxate upregulates unfolded protein responses, damage-responses and epithelial remodelling mechanisms.

To further analyse the mechanisms involved in MCP-PAB-induced epithelial dysregulation in ALI cultures, we analysed mRNA transcriptomes obtained by RNAseq.

Comparison of gene expression in unstimulated ALI cultures showed 147 genes upregulated and 266 downregulated in cultures from severe asthmatics when compared with healthy control (Figure 8-3, Appendix B, Table B-1). Application of MCP-PAB resulted in a profound effect on gene expression, especially in ALI cultures from severe asthmatic donors which had a significantly higher number of differentially expressed genes (DEGs) (n=599) compared to cultures from healthy donors (n=87 DEGs). Amongst the most prominent modulated genes were IL1RL1 (Interleukin 1 Receptor Like 1, the receptor for IL33), CHAC1 (cation transport regulator-like protein 1), involved in oxidative balance and unfolded protein response (UPR), and SERPINB9, a serine protease inhibitor.

Gene ontology analysis (AmiGO) of all MCP-PAB-induced DEGs identified a number of differentially controlled biological processes ($p<0.05$). Taking a cut-off of 2-fold increase in gene expression, we found 57 processes upregulated in cultures from severe asthmatic donors and 25 in those from healthy donors. In order to identify the processes with the greatest impact, we undertook a further selection of gene expression with a cut-off of 5-fold; this showed 16 processes upregulated in cultures from severe asthmatic donors and 11 in cultures from healthy controls (Table 8-2).

The most significant enrichment due to MCP-PAB exposure was in the PERK-mediated UPR; this was significant in cultures from both asthmatics and healthy donors but was three times greater in healthy participants. Cultures from healthy individuals exposed to MCP-PAB were also enriched in other stress response processes (Table 8-2). In contrast, MCP-PAB-exposed epithelial cultures derived from severe asthmatic donors were enriched in EGFR signalling, cell migration and vasculature development, suggesting upregulation of tissue repair and remodelling responses.

Having established MCP-PAB-induced epithelial damage, we next explored the impact of MCP-PAB on damage-signalling. We analysed the damage-associated cytokine IL-33 and found that AR caused increased nuclear IL-33 staining cultures from severe asthmatic donors (Figure 8-4). We confirmed IL-33 expression in ALI cultures using qPCR (HC, n=5; SA, n=6), and showed that MCP-PAB was associated with a 67% increase in IL-33 expression in SA and a -11% change in HC ($p=0.01$ for between group comparison).

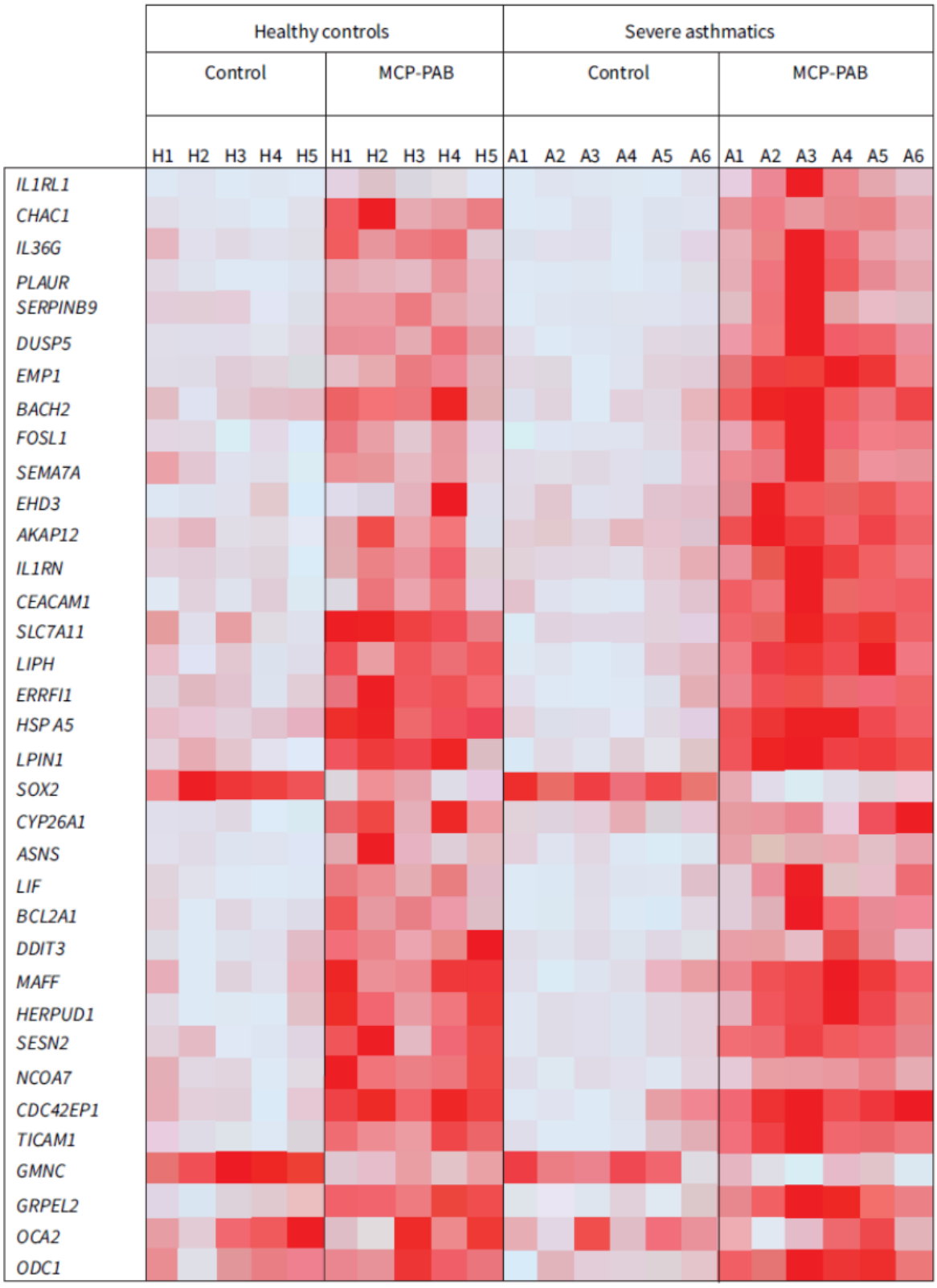

Figure 8-3: Changes in epithelial gene expression caused by multiple challenge protocol using pepsin, acid pH and bile acid in vitro. Bronchial epithelial air–liquid interface (ALI) cultures from healthy controls (n=5) and severely asthmatic (n=6) donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 h before harvesting for RNA sequencing. Heatmap of the top dysregulated epithelial genes from low (blue) to high (red) levels of expression.

Table 8-2: Top upregulated biological processes in multiple challenge protocol exposed cultures when compared with control cultures (details provided if fold enrichment >5, p-value<0.05)

	HC		SA	
GO biological process	**fold enrichment**	**P value**	**fold enrichment**	**P value**
PERK-mediated unfolded protein response (GO:0036499)	> 100	0.0000	31.53	0.0020
cellular response to glucose starvation (GO:0042149)	45.1	0.0243		
cellular response to biotic stimulus (GO:0071216)	13.21	0.0099		
negative regulation of intracellular signal transduction (GO:1902532)	7.7	0.0208	3.27	0.0024
regulation of response to stress (GO:0080134)	6.25	0.0000		
cellular response to lipopolysaccharide (GO:0071222)			4.55	0.0476
epidermal growth factor receptor signalling pathway (GO:0007173)			11.58	0.0024
positive regulation of cytokine biosynthetic process (GO:0042108)			8.22	0.0313
regulation of epidermal growth factor receptor signalling pathway (GO:0042058)			7.42	0.0210
regulation of cell junction assembly (GO:1901888)			6.78	0.0439
positive regulation of epithelial cell migration (GO:0010634)			6.22	0.0015
positive regulation of vasculature development (GO:1904018)			5.37	0.0013
regulation of protein kinase B signalling (GO:0051896)			4.48	0.0122
positive regulation of cellular catabolic process (GO:0031331)			3.39	0.0362

HC: healthy controls; SA: severe asthmatics

Comparison of in vitro findings with in vivo epithelial changes in severe asthma with GORD

To determine the relevance of our in vitro findings with epithelial changes in severe asthma with GORD, we first performed IHC for IL-33 using bronchial biopsies from severe asthmatic (n=9) or healthy control subjects without GORD (n=4); the severe asthmatics were sub-grouped as follows: (i) SA with no GORD (n=5), and (ii) SA with documented GORD but who had abstained from their regular PPI treatment for 2 weeks to avoid potential bias of systemic or local (through micro-inhalation) impact of PPI on epithelial gene expression (n=4). As shown in Figure 8-5, there was a significantly higher number of IL-33 positive nuclei in SA-no GORD compared to healthy controls with a further significant increase in SA-GORD.

Finally, we analysed mRNA transcriptomes of bronchial brushings from SA-GORD no PPI (n=6), SA-no GORD (n=4) and healthy control subjects (n=12). RNAseq analysis identified that of the top 37 genes whose expression was modified in ALI cultures in response to MCP-PAB, 15 were similarly modified ex vivo in patients with GORD (Table 8-3). Of note, the expression of CHAC1, the top upregulated gene involved in the UPR process which was identified as the main mechanism induced by MCP-PAB in ALI cultures (Appendix B Table B-1), was also increased in bronchial brushings obtained from SA-GORD when compared to SA-no GORD, confirming a similar impact of refluxate on epithelial responses to endoplasmic reticulum stress in vivo.

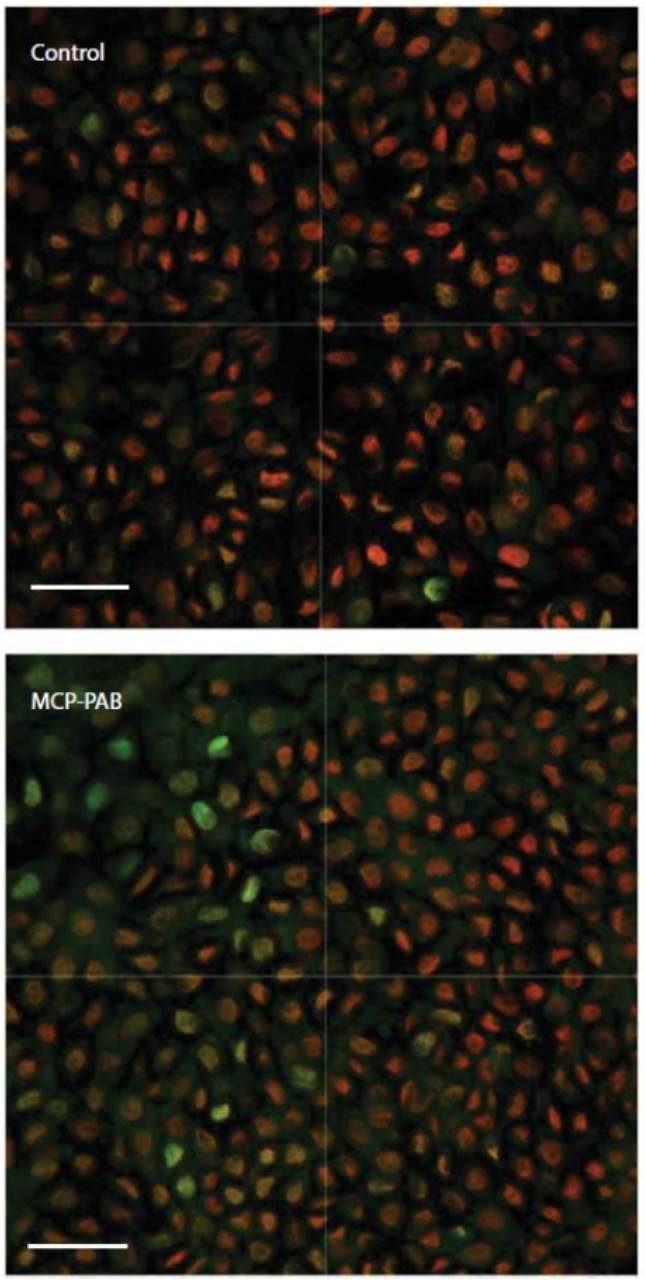

Figure 8-4: Regulation of epithelial expression of interleukin (IL)-33 by multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB). Bronchial epithelial air–liquid interface (ALI) cultures from severely asthmatic donors were untreated (control) or exposed to MCP-PAB for 30 mins, washed and allowed to recover for 4 h before fixing and immunofluorescence staining. Images show IL-33 nuclear staining (green) and 4',6-diamidino-2-phenylindole (DAPI) (red). Images are representative of experiments using ALI cultures from six donors. Scale bars=25 μm.

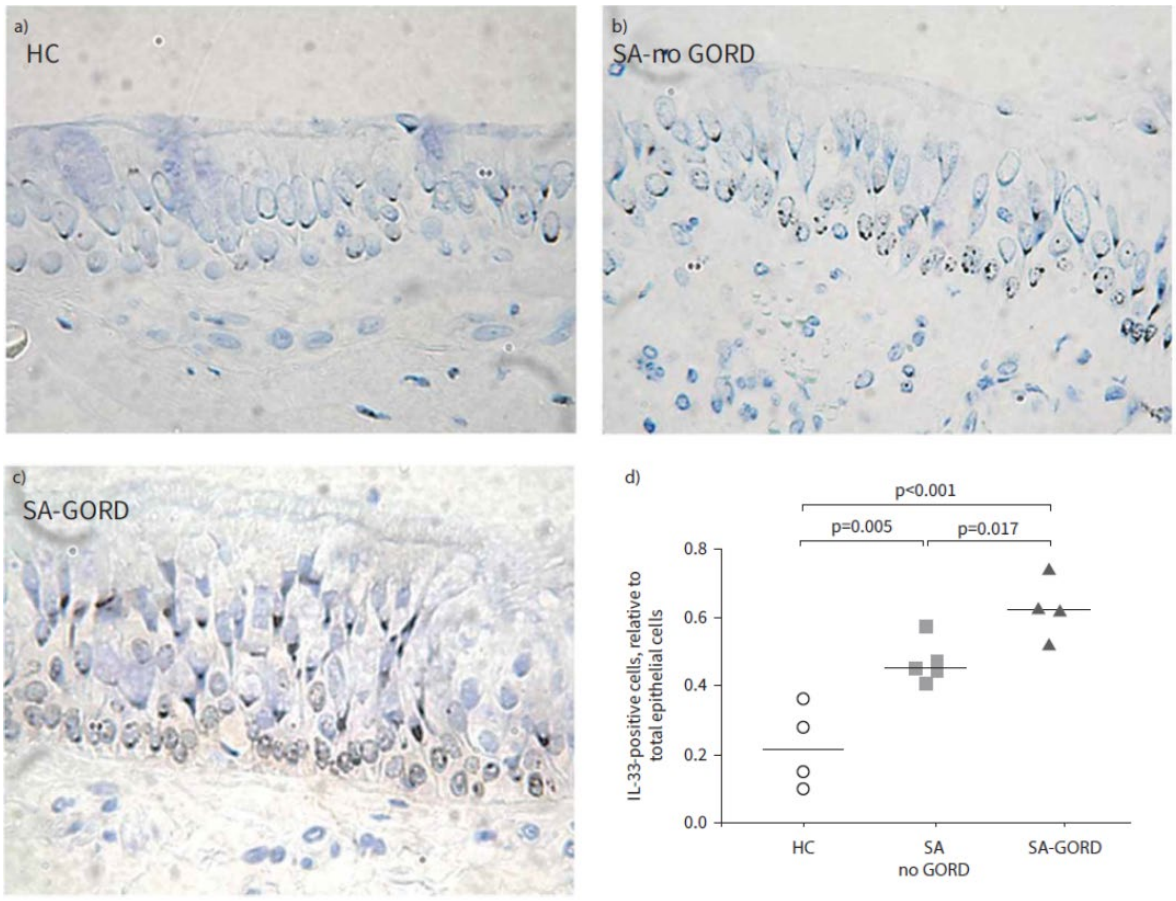

Figure 8-5: Epithelial interleukin (IL)-33 expression is increased severe asthma with gastro-oesophageal reflux disease (GORD). Typical patterns of immunohistochemical staining for IL-33 in bronchial biopsies from a) healthy control participants without GORD (HC); b) severe asthmatics with no documented GORD (SA-no GORD; n=5); and c) severe asthmatics with documented GORD who had abstained from their regular proton pump inhibitor (PPI) treatment for 2 weeks (SA-GORD; n=4). d) Quantitation of positive nuclei expressed as percentage of total epithelial cells.

Table 8-3: Differential epithelial expression of 37 genes in air–liquid interface (ALI) cultures and expression of these genes in bronchial brushings

	ALI cultures				Bronchial brushings					
	SA MCP vs HC CTL		SA MCP vs SA CTL		SA-GORD vs Health		SA-GORD vs SA-no GORD		SA-no GORD vs Health	
	fold change	p value	fold change	p value	fold change	p value	fold change	p value	fold change	p value
IL1RL1	14.22	0.0234	11.84	0.014						
CYP26A1	10.31	0.0102	2.26	0.0226	-0.38	0.2119	-0.59	0.015	0.4881	0.2138
CHAC1	6.38	0.0000	8.53	0.0000	0.57	0.0014	0.4	0.078	0.1204	0.3519
PLAUR	5.06	0.0036	5.71	0.0013	0.78	0.1908	0.22	0.7903	0.4588	0.3107
BCL2A1	4.48	0.0174	7.26	0.006	1.19	0.2114	0.42	0.7148	0.5371	0.3406
LIF	4.39	0.0345	3.49	0.0271	0.03	0.8773	0.28	0.4522	-0.1967	0.2768
ASNS	4.37	0.0000	3.77	0.0000						
EHD3	4.16	0.0001	2.5	0.0001	0.43	0.0314	-0.11	0.6064	0.6105	0.0122
DUSP5	4.13	0.001	4.72	0.0003	0.04	0.8965	0.05	0.9213	-0.0097	0.9756
FOSL1	3.89	0.0011	3.6	0.0005						
AKAP12	3.59	0.0000	2.43	0.0000	0.62	0.0429	0.5	0.2501	0.0834	0.7275
EMP1	3.46	0.0001	3.91	0.0000	0.57	0.0126	-0.32	0.272	1.314	0.008
IL36G	3.33	0.0196	5.99	0.0052						
IL1RN	2.83	0.0003	2.15	0.0002	1.54	0.048	0.04	0.9582	1.4514	0.073
BACH2	2.61	0.0001	3.84	0.0000	0.79	0.0242	0.45	0.3752	0.2311	0.3876
CEACAM1	2.55	0.0001	2.14	0.0000	1.05	0.0031	0.77	0.1259	0.1556	0.3661
SEMA7A	2.51	0.0068	3.36	0.0011	0.86	0.0559	0.67	0.2766	0.1151	0.7938
SERPINB9	2.28	0.0586	5.33	0.0151	0.47	0.1701	0.1	0.827	0.3359	0.4284
MAFF	2.08	0.0002	2.18	0.0001	0.1	0.7534	0.38	0.4754	-0.2083	0.4484
LIPH	1.88	0.0001	2.12	0.0000	-0.22	0.046	0.17	0.5231	-0.3343	0.0044
TICAM1	1.81	0.0001	1.7	0.0001	0.7	0.0171	0.36	0.3158	0.2518	0.2377
HERPUD1	1.72	0.0009	1.65	0.0003	0.17	0.0537	-0.12	0.3178	0.3201	0.007
DDIT3	1.65	0.0096	1.92	0.0023	0.35	0.037	-0.16	0.335	0.6075	0.0004
CDC42EP1	1.52	0.0000	1.49	0.0001	1.63	0.029	1.91	0.1866	-0.0962	0.7368
SESN2	1.42	0.0000	1	0.0000	0.21	0.1479	-0.11	0.517	0.3598	0.0141
SLC7A11	1.41	0.0002	2.13	0.0000	0.25	0.4279	1.25	0.0364	-0.4458	0.2045
LPIN1	1.38	0.0000	1.61	0.0000	-0.01	0.895	0.19	0.2361	-0.1672	0.0609
ERRFI1	1.32	0.0000	1.9	0.0000	-0.03	0.7968	0.12	0.5169	-0.1333	0.1547
GRPEL2	1.15	0.0009	1.42	0.0002	-0.13	0.3447	0.06	0.7801	-0.185	0.2209
HSPA5	1.12	0.0000	1.62	0.0000	-0.05	0.7581	0.1	0.6946	-0.1369	0.3707
NCOA7	0.59	0.0073	0.85	0.0001	-0.35	0.0361	0.34	0.1864	-0.5129	0.0139
ODC1	0.51	0.0119	1.22	0.0001	-0.25	0.0021	-0.2	0.003	-0.0647	0.4584
OCA2	-0.28	0.2787	-0.05	0.8474	0.84	0.0171	-0.37	0.3526	1.9081	0.0177
CYP1B1	-0.32	0.3627	-0.33	0.4407						
CYP1A1	-0.55	0.0343	-0.51	0.1031						
SOX2	-0.7	0.0001	-0.67	0.0000	-0.2	0.038	-0.05	0.7193	-0.1535	0.047
GMNC	-0.81	0.0000	-0.72	0.0047	-0.58	0.0002	-0.14	0.6958	-0.5164	0.0075

Fold changes are coloured from blue (downregulation) to red (upregulation): colour intensity reflects magnitude. SA: severe asthma; MCP: multiple challenge protocol; HC: healthy control; CTL: control; GORD: gastro-oesophageal reflux disease. #: severe asthmatics with documented GORD who had abstained from their regular proton pump inhibitor treatment for 2 weeks; SA-no GORD: severe asthmatics with no documented GORD.

8.4 Discussion

Using a combination of in vitro and ex vivo approaches we have obtained compelling evidence in support of reflux having a significant impact on bronchial epithelial structure and function, with a profound effect on the epithelium of severe asthma patients. Application of MCP-PAB conditions to epithelial ALI cultures caused marked acute structural damage, including disruption of adherens and tight junctions, increased permeability and induction of stress responses, as shown by enrichment of the UPR genes and modulation of the alarmin IL-33 and its receptor IL1RL1. These in vitro findings were supported by observations in bronchial biopsies and by global gene expression analysis of epithelial brushings from severe asthmatics without or with GORD and healthy controls.

GORD is a chronic disorder caused by abnormal reflux of acid, pepsin and bile acids, defined as time of acid exposure >6% during 24-hr monitoring of oesophageal pH [197]. Combined with impedance measurement, pH monitoring allows the detection of acidic (pH<4), weakly acidic (pH 4-7) and non-acid reflux (pH>7), the latter occasionally persisting despite treatment with PPI [198]. Whether and to what extent gastric juice contents penetrate the lungs in subjects with GORD has been uncertain [199], although our own studies have provided evidence that clinical GORD is associated with changes in several biomarkers [192,194]. When deciding on the composition of the ingredients in the MCP-PAB for in vitro testing, we took into account physiological concentrations in gastric secretions of acid [200], pepsin [201] and total bile acids [202] and previous reports of effects of pepsin [191] and bile acids [144] on epithelial cells. Based on dose-ranging experiments, we chose 50µg/ml of pepsin and 250µM chenodeoxycholic acid (CDC) at pH 5 for 30 min because this resulted in measurable damage without causing extreme cytotoxicity. Thus, we observed enlargement of intercellular spaces, disruption of intercellular junctions and increased permeability, effects similar to observations in vivo in oesophageal and laryngeal epithelium exposed to chronic refluxate [203,204]. This, coupled with previous studies showing that ALI cultures derived from severe asthmatic donors exhibit phenotypic features similar to those found in vivo [195,205], led us to conclude that exposure of ALI cultures to MCP-PAB conditions is a reliable model to analyse the effect of reflux on the bronchial epithelium in severe asthma.

Our study revealed a marked reflux-induced increase in the nuclear expression of the alarmin IL-33, as well as upregulation of IL1RL1, the gene encoding the IL-33 receptor. IL-33 is a member of the IL-1 cytokine family localized in the nucleus of airway epithelial cells and its release can be triggered by damage caused, for example, by allergens or viruses [206,207]. It is a known asthma susceptibility gene [208,209] and plays a crucial role in type-2 innate immunity through activation of group 2 innate lymphoid cells (ILC-2) to trigger production of IL-4, IL-5, IL-13 (30). Our findings of IL-33 upregulation in bronchial biopsies from severe asthma with GORD are in concordance with the observed

upregulation of IL-33 nuclear expression in the oesophageal mucosa in patients with reflux oesophagitis [210] and symptoms of heartburn [211].

Refluxate-induced damage also included a response to oxidative stress through PERK-mediated UPR [212]. A recent study, using an oesophageal squamous epithelial cell line, identified bile acid-mediated activation of the PERK-mediated UPR [213]. Our study provides the first evidence of refluxate-triggered UPR activation in the airway epithelium. PERK is a type I endoplasmic reticulum (ER) transmembrane protein activated by misfolded proteins inside the ER. Its stimulation induces transcription of UPR related genes, leading to autophagy, apoptosis and redox homeostasis [214]. UPR is considered a master regulator in inflammatory diseases and its role in asthma development has been suggested [214]. UPR can be activated by various asthma triggers, including allergens, cigarette smoke and viruses, and regulates oxidative stress in asthma [214]. UPR regulates NKκB activity and NFκB-mediated inflammation and can induce apoptosis in case of prolonged activation of ER stress. The relatively limited enrichment in the PERK-mediated UPR process that we observed in exposed cells from severe asthmatics may reflect an ineffective response to multiple types of damage and so explains the vulnerability of the bronchial epithelium in severe asthma to a range of environmental challenges.

We observed MCP-PAB-induced epithelial changes in cultures from both normal and severe asthma donors. However, MCP-PAB had a more pronounced impact on the structural and functional properties of the asthmatic epithelium, including a greater increase in epithelial permeability and a higher number of MCP-PAB-associated DEGs. While our ex vivo transcriptome analysis of bronchial brushings did not completely match our in vitro results from ALI cultures, this may be because the in vitro model represents a single acute stress event caused by exposure to MCP-PAB conditions which do not fully reflect the complexity of gastric juice, whereas the clinical condition of GORD is characterized by repeated exposures to various components of gastric juice. In addition, severe asthma patients with GORD treated with PPI may present a dysregulated aerodigestive microbiome, with a potential role in severe asthma [215,216]. Nonetheless, we were able to identify 15 dysregulated genes in brushings from severe asthmatics with GORD among the 37 top dysregulated genes in MCP-PAB-exposed ALI cultures, with the extent of dysregulation being higher in cultures from severe asthmatics than in those from healthy controls. Amongst the DEGs were genes involved in oxidative stress responses (CHAC1, BACH2), cell adhesion (CEACAM1), cytoskeleton organization (CDC42EP1) and cilia formation (GMNC), pointing to impact on epithelial structure regulation. Exposure of severe asthma cultures to refluxate also caused enrichment in EGFR- and cell migration-related processes that were not changed in cultures from healthy individuals. Our results suggest that refluxate might contribute to defective epithelial barrier function and EGFR-mediated remodelling, key features of asthma [195,217].

In summary, our study has identified a direct impact of refluxate on the airway epithelial structure, barrier permeability and modulation of gene expression, including UPR, responses to oxidative stress and wound healing processes. This suggests a possible role for GORD in increasing exposure of the subepithelial airway mucosa to allergens and infectious pathogens, resulting in increased risk of inflammation and exacerbations, as well as a possible role in airway remodelling, a key feature of severe asthma. These results suggest the need for research into alternative therapeutic management of GORD in severe asthma.

Chapter 9 General discussion

In summary, the main finding in this study was that of pepsin being able to distinguish between presence and absence of GORD which makes this test of potential value as a test to be done when physiological assessment of reflux is not possible. This study did not show significant evidence for a pathobiological change which could explain the role of GORD in severe asthma. While it could be speculated that this was because of limited number of participants, no statistical trends were observed. It is likely that more detailed analysis of the structure of the epithelium would be needed to assess more subtle changes that could be implicated as a cause of cough. Consistent with this speculation, studies of the bronchial epithelium in people with GORD (see study 5) points to evidence of molecular changes that shed light on processes whereby GORD may influence the clinical presentation of severe asthma. Severe asthma remains a concern in clinical practice despite the advent of biologics therapies that mainly support the management of asthma by either modulation of atopy response or the modulation of IL5 pathways. GORD is a morbidity that is observed in 27-45 % of the western population. However, in asthmatics, the prevalence of GORD is higher, estimated between 32-84% according to various studies even in the absence of typical symptoms associated with GORD [99,126,218,219]. In addition to GORD, severe asthmatics suffer from other co-morbidities, such as obesity and psychological issues and these add to the burden of the diseases.

Even in patients where GORD as a co-morbid factor is recognised and treated with acid suppression with PPI and H2 antagonists, the control of symptoms can be elusive. Table 1-2, in the introduction to this thesis, lists some of the major studies that have attempted to manage GORD in asthmatics mainly by using acid-suppression, but also prokinetics e.g. Domperidone and medicines that are known to increase the tone of the LOS i.e. baclofen. The results of these therapies remain ambiguous, so GORD in severe asthma remains as an important risk factor for recurrent asthma exacerbations[12,30].

The importance of weakly acid and non-acid reflux has been raised in the context of manifestations of GORD that persist after PPI treatment; however, identifying and managing such patients remain a challenge as there is very little evidence to inform on causative factors and clinical and patho-biological changes in association with weakly and non-acid GORD. There is evidence from studies in patients with lung transplant and CF patients for the importance of management of acid reflux and weakly acid duodeno-gastric reflux with bile acids to prevent airway inflammation and bronchiolitis obliterans [24,26,27,98,142], which is why patients often have a surgical intervention (fundoplication) following lung transplantation.

Although many studies in asthma have been done with the aim to assess the role of GORD in the pathophysiology of asthma, these studies have been limited in respect of clinical data collected, which is why I undertook a detailed analysis of the relationships between GORD and clinical questionnaires of asthma severity as well as those for cough. There has also been a paucity of simultaneous studies of the underlying pathobiology of the lower airways and, for that matter, the upper airways, which was a major reason for my undertaking the study reported in Chapter 7. In particular, a sequential trial of standard care medicines and responses to this, both clinically and biologically, have been lacking. Moreover, recognition and treatment of weakly acid and non-acid reflux in the same group of patients in sequence has not been done. The mechanism of injury and immune and clinical responses remain unclear.

In this context, I set out to gather further information about the role of GORD in severe asthmatics with the aim to assess the role of GORD and its effects on airway inflammation and symptoms of severe asthma. To understand the mechanisms, I decided to analyse GORD objectively and in detail with 24-hour pH and impedance in all participants in the study including the healthy cohort, with the aim to collect clinical and biological samples at baseline from participants without GORD and untreated participants with GORD. The severe asthmatics with GORD then had standard treatment for acid suppression following which clinical data and sputum samples were collected and measurements of GORD were repeated, guided by the clinical response between visits. I postulated that if patients had a decrease of > 1.5 in ACQ6 score, it would reflect a strong response to treatment with GORD in which case that participant would be able to stop the study.

As a way of getting an overview of GORD in severe asthma I first analysed the raw data from the U-BIOPRED study in which I had taken part as one of the clinical research fellows from Southampton which was one of the key recruitment sites that contributed to the project with a substantial number of study participants. I observed that patients with GORD had a higher BMI, were more likely to be female, and more likely to use oral steroids, suggesting poor control of asthma. Although the ACQ and AQLQ scores did not show any significant difference between non-smoking severe asthmatics with or without GORD, in severe asthmatic smokers with GORD the asthma severity scores ACQ and AQLQ scores were significantly worse. The rhinosinusitis SNOT-20 score were worse in both smoking and non-smoking severe asthmatics with GORD when compared to those without GORD, suggesting a role for GORD that is independent of smoking in sino-nasal disease. The effect of GORD could be observed on clinical assessments to a greater extent in patients who had on-going symptoms of GORD than those with just a past history of GORD.

I then undertook an analysis of the sputum proteomic dataset in the U-BIOPRED study. I was able to identify 3 proteins as good predictors of diagnosis of active symptomatic GORD (ACTIVE-GORD). I observed an increased level of Lipocalin-1 and decreased level of immunoglobulin lambda variable

1-47 and plasma protease C1 inhibitor. At the same time 3 further proteins, immunoglobulin lambda variable 1-47, alpha-1-antichymotrypsin and the heat shock cognate 71 kDa protein were noted as predictors of having a past diagnosis of GORD. Lipocalin, with its primary role in epithelial defence can inhibit bacterial growth and scavenge potentially harmful lipophilic substances but also is implicated in non-allergenic inflammation as well as Th2 response[220,221]. Further work is required to clarify how lipocalin interacts with the inflammatory processes in GORD.

To further elucidate and confirm my findings from the analysis of GORD patients in the U-BIOPRED study I extended the clinical and pathobiological study of GORD in severe asthma in part 2 of my study. I used sputum induction, bronchial and laryngeal biopsies and cytokine analysis to directly assess the changes as a result of untreated GORD in severe asthmatics in comparison with severe asthmatics without GORD and healthy controls. As an improvement on the U-BIOPRED study, I classified the groups not just on clinical grounds but also based on detailed 24-hour pH/impedance test which allowed an insight into the role of overall impedance events, including acid and weakly/non-acid events, as well as proximal reflux and its subtypes based on upright/supine reflux and weakly or non-acidic nature. I saw no significant differences between asthmatics with and those without GORD in either sputum or BAL differential cell counts. Immunohistochemical analysis of bronchial and laryngeal biopsies also did not show any significant differences. I also analysed Oil Red O stain to check the LLMI to see any differentiation between GORD and no GORD, but this also showed no significant results, suggesting that there is no role for assessment of lipid-laden macrophages in this context. The observation lead me to the conclusion that these biomarkers lack the sensitivity to show any changes that may have occurred as a result of GORD. Studies using murine models or airway epithelial cultures and their response to refluxate have suggested that GORD reduces immune response [187,188] but I found no evidence to support this. Again, I highlight that the methods that I used may not be able to detect subtle changes in local immunity that may be relevant for clinical outcomes such as virus induced exacerbations.

The presence of GORD per se does not mean that elements of the refluxate end up in the bronchial tree where they can directly cause damage to the epithelium and induce an inflammatory response that adds to the chronic inflammation that is characteristic of asthma. A recent study by Marshall et al [222] in asthmatics of varying severity identified that more than half of asthma patients had detectable pepsin in their saliva, suggesting that this was evidence of reflux. Unfortunately, that study did not simultaneously assess reflux and, possibly because patient numbers were small, they could not find a correlation between pepsin levels and asthma severity. I sought to build on that study by measuring pepsin in sputum and BAL in all the participants in parallel with physiological assessment of GORD and detailed measurement of asthma severity. The salivary samples from the back of the throat (throat clearate) of severe asthmatics with GORD had a higher concentration of pepsin compared to sampled from severe asthmatics without GORD. The difference between

severe asthmatics with GORD and healthy participants with GORD was even greater suggesting that the test is reliable at picking up GORD. However, when I repeated the pepsin measurements in throat clearate in the severe asthmatics with GORD after treatment with anti-reflux treatment at any stage, I could not find a statistically significant reduction. The median levels reduced from 93 ng/ml to 20 ng/ml but there was a significant overlap in range which meant that overall, there was no significance. Of note, anti-reflux treatment reduced the quantity of acid reflux but did not reduce either the total impedance (acid, weakly acid and non-acid) events or the number of weakly acid reflux events. Thus, although it is tempting to speculate that the pepsin level reduction may have been significant had I had more participants, it is possible that, because the total reflux events did not change, pepsin levels may not change with anti-GOR treatment that is effective at reducing the acidity within the refluxate. Previous studies have suggested that pepsin can remain stable and active as long as pH remains lower than 6.5 but its effect is maximum at pH of 2 [223]. A further study by the same group has shown that there is uptake of pepsin by receptor-mediated endocytosis which can continue to have proteolytic activity despite no ongoing reflux and would be activated after a further episode of reflux with a lower pH. Pepsin can become activated following intracellular uptake when it is within the Golgi bodies to a lower pH [224]. Treatment of reflux by PPI has been shown to reduce pepsin levels in the throat clearate but there is residual pepsin detected in as many as a third of patients[225,226]. Together with my findings from this study, the evidence of persistent pepsin in the upper airways despite aggressive treatment of reflux raises the need for surgical intervention [227].

Respiratory questionnaires assessing disease severity and quality of life impact are an integral part of the clinical management of asthma. In study 3 of my thesis, I have described in detail the results of asthma and respiratory disease questionnaires in the context of management of GORD. Although the questionnaires are very good at describing differences of symptoms and severity of asthma, they seem to be of limited value in predicting quality of life responses to medical treatment of GORD. The ACQ was the best predictor of this response and showed a clear and significant difference between severe asthmatics with GORD and those without GORD; mean (SD) 2.6(1.1) for SA-GORD and 1.7(1.1) for SA-no GORD (p=0.028); this effect was seen to persist after 2 months treatment with PPI and H2 antagonists in severe asthmatics with GORD with a clinically and statistically relevant improvement in ACQ6 score to a mean (SD) 1.9(1) p=0.017. Although the ACQ score did show some further improvement with the addition of baclofen, it did not reach MID or statistical significance. The AQLQ score is very widely used to measure quality of life measures but in my study only the symptoms domain of AQLQ showed a significant difference between severe asthmatics with and those without GORD. While showed a trend toward its reduction with anti-reflux treatment with PPI and H2 antagonists at visit 8, with a small additional benefit with baclofen

at visit 11, these improvements did not lead to a statistically significant improvement. Same trends were observed for SGRQ. A recent Cochrane review has shown that overall response to AQLQ shows a mixed effect to treatment of GORD to control asthma in adults[228] so, overall, I can conclude that effective treatment of GORD has a small impact on quality of life as measured by both AQLQ and SGRQ.

One of my further objectives was to explore the predictive value of reflux disease questionnaire. To my surprise, the RDQ, a well validated questionnaire, was not able to significantly discriminate symptoms of reflux between severe asthmatics with and without GORD although, as expected, it did show a significant difference between severe asthmatics with GORD and healthy participants without GORD. Similarly, there was no change in RDG as a consequence of treatment of GORD in this study. I conclude, therefore, that there is not enough utility for the RDQ in the management of GORD in asthmatics. The study would suggest that in cases of severe or difficult asthma, 24-hour pH and impedance is required as it will give relevant information to support a management plan.

The Hull cough hypersensitivity questionnaire is recommended by its developers as a useful tool in the management of cough hypersensitivity related to GORD [229]. My study extends the clinical utility of the HCHQ to severe asthma. In this study the HCHQ showed significant differences when comparing severe asthmatics with and without GORD and, additionally, it was able to show a significant improvement in cough scores after PPI and H2 antagonist treatment. The HCHQ was not significantly different in healthy participants with GORD compared to those without GORD because in this group cough was not a clinical problem. Assessment of cough using the LCQ, which contains a series of holistic questions, suggested that severe asthmatics have a worse cough-related quality of life but only when compared to healthy individuals (with and without GORD), and this effect is likely impacted largely by the severe asthma component because the LCQ was unable to differentiate on the basis of GORD between severe asthmatics with and without GORD. The changes in scores within the severe asthmatics with GORD did not reach the proposed MID [167] or statistical significance on anti-reflux treatment.

The higher HCHQ scores severe asthmatics with GORD, compared to those without, was not fully confirmed by formal quantification of cough events, with only a trend towards a difference between GORD and no GORD severe asthmatics (p=0.089). The LCM showed that 50% of the participants in severe asthmatics with GORD had a reduction in cough by more than 50% after treatment with PPI and H2 antagonists. This is well above the MID of 30% that is used in study trials of drugs for chronic cough e.g gefapixant and similar to the MID of 54% reduction proposed for acute cough [173]. There was a significant difference in cough rate and cough count between severe asthmatics with GORD when compared to the healthy participants with GORD, and given that this was associated with higher DeMeester scores and AET in the severe asthmatics, would suggest that severe asthmatics

with GORD have worse intensity of cough that is well above the normal rate as defined by Birring et al. [122]. However, the fact that treatment of GORD in the severe asthmatics did not result in a significant resolution of cough, suggests a residual component of the asthma cough that is unrelated to GORD and is due to other factors that are intrinsic to asthma itself.

In summary, severe asthmatics have a higher prevalence of GORD and suffer from worse asthma control and worse quality of life. This is associated with the finding of proteomic biomarkers the role of which requires further elucidation. Pepsin measured in throat clearates, a sample that is easy to collect, is a good predictor of GORD in asthma but does not respond to treatment, and the relevance of this requires further elucidation. Treatment of GORD can have a positive impact on asthma control and symptoms as well as quality of life, but the disease burden related to asthma continues to have a significant role in the quality-of-life measures. Treatment of GORD in severe asthmatics does control the acid reflux but has little or no impact on the weakly acidic and non-acid reflux or the proximal extent of the refluxate as detected by 24-hour pH and impedance tests. This suggests a role of weakly acid and non-acid reflux as well as proximal reflux, and whether this requires further treatment, including surgery remains to be elucidated.

Finally, my study enabled another project to be done, (study 6, chapter, 8) which was led by a colleague (Jeanne-Marie Perotin Collard). The observations I had made in the analysis fo the U-BIOPRED data, provided a rationale and justification for a European respiratory Society Fellowship which funded Dr Perotin Collard. The study began with the development of an in vitro model of GORD using differentiated bronchial epithelial cells (BECs) from normal or severe asthmatic donors exposed to a combination of pepsin, low pH and bile acids using a multiple challenge protocol (MCP). The clinical data and the samples that I had collected (bronchial brushings, biopsies and BAL samples) that I provided were used in this study. RNA-sequencing of bronchial brushings from controls and severe asthmatics without or with GORD was performed and analysed by another colleagues (Gabrielle Wheway). Exposure of BECs to the refluxate (as part of the MCP) caused structural disruption, increased permeability, IL-33 expression, inflammatory mediator release and changes in gene expression that pointed to involvement of several biological processes. Of note, and in keeping with my observations in the in vivo clinical studies that pointed to altered impact of GORD in asthma, the cultures from severe asthmatics were significantly more affected than those from healthy donors. Particularly interesting was the finding in bronchial biopsies was the increased IL-33 expression in severe asthmatics with GORD. RNA-sequencing of bronchial brushings from this group identified 15 of the top 37 dysregulated genes found in MCP treated BECs, including genes involved in oxidative stress responses. These results suggest the need for research into alternative therapeutic management of GORD in severe asthma.

9.1 Study limitations

This was a very ambitious study to build on the data collected from the U-BIOPRED cohort. For a lot of parameters in this study the number of patients or the power of the study limited the conclusions that could be drawn from the analyses. The factors that contributed to this included the overall length of the study. The total duration of treatment with anti-reflux medication spanned 5 months with additional time consumed in screening and a washout period of 2 weeks. This meant that the patient information sheet had to be detailed but very long and that meant there was a risk that patients may not fully engage with the study. I tried to off-set this issue by getting the patient-public involvement representatives to recommend any changes in the patient information sheet or in the study protocol.

Although the collection of data was very comprehensive, it was also time consuming. This was a cohort of patients that was already prone to recurrent exacerbations of asthma and/or infections not just due to GORD but also due to the severity of asthma. A significant number of participants were unable to tolerate the pH/ impedance procedure which is a known limitation factor not just in terms of research but also the difficulty in this patient group in real-time outpatient clinical practice. Since I wanted to exclude the element of bias introduced by subjective diagnosis of GORD this was not a modifiable issue.

Perhaps the most important limiting factor in this study was that this study was directly competing with the large trials of biologic therapy as the study commenced at a time when a new biologic, mepolizumab, directed at interleukin 5, had arrived in the UK. Longitudinal safety trials were recruiting patients, A further competitor was a large study, the anti-IgE biologic, omalizumab. In most cases the study participants had been referred from the severe asthma clinics and it became more and more difficult to recruit patients for standard care of GORD when patients from severe asthma clinics were largely recruiting to trials of biologics. As per GCP regulations the consent for taking part in any study lies with the patient with full details and informed consent.

On the other hand, the study has provided enough information that if such a study were to be redone, I would consider a shorter study which would be focussed on focused knowledge gaps. The trial of multiple treatment options can be very complicated, and I would prefer to limit the number of intervention steps to 3 at maximum. This would mean that patient flow can be quicker. Addition of more recruitment sites for participants where the study investigations could be done independently without any major dependence on the primary centre would provide a larger cohort and it decrease the risk of competing against multiple studies as different sites have different study priorities.

9.2 Future research

My study of the U-BIOPRED data suggested a proteomic signature of GORD with proteins which have a role in the innate immune processes which are still unclear. I would like to look further into the proteomic analysis of clearly classified patient groups as in part 2 of my study based on objective evidence of GORD. Multiple studies have shown alteration of the immune system in the airways as a possible consequence of GORD, but this needs to be confirmed and assessed in cohorts where reflux has been clearly defined whether acidic, weakly acidic or non-acidic.

I would also like to study the role of pepsin and bile acids further in asthmatics where GORD is a significant co-morbidity. Pepsin and bile acids are associated with acid and weakly acid reflux and additionally both have a role in causing upper airway inflammation. I would like to assess the pepsin and bile acids level in sputum and saliva of severe asthmatics with and without GORD, with and without treatment. The sputum samples can then be analysed to see inflammatory cytokine patterns which may give insight into acid and weakly acid/non-acid reflux and airway inflammation.

To extend the work for which I collaborated with colleagues who specialise transcriptomics, I would like to further this work in severe asthmatics as well as healthy participants by collecting laryngeal and bronchial biopsies and analysing how these changes differ between health and asthma.

Appendix A - Supplement to Chapter 3

The following Tables and Figure are the supplementary material for chapter 3.

Table A-1. Mann-Whitney U analysis of severe asthmatics (Cohort A and B) – All GORD *vs* NO GORD. Proteins with p≤0.1 were selected for ULR.

Mann-Whitney U test of proteins between All GORD and NO GORD					
Protein name	**Uniprot ID**	**All GORD Protein Abundance (IU)**	**NO GORD Protein Abundance (IU)**	**Z**	**p value**
Immunoglobulin lambda variable 1-47	P01700	26673 (15028-37586)	38693 (19555-48680)	-3.105	0.002
Heat shock cognate 71 kDa protein	P11142	17474 (12671-31336)	23376 (16362-38133)	-2.317	0.021
Ig lambda-2 chain C regions	P0CG05	452603 (255358-534754)	397731 (238317-481500)	-2.068	0.039
Lactotransferrin (Lactoferrin)	P02788	328357 (259707-405139)	385396 (293012-436902)	-2.049	0.040
Serotransferrin (Transferrin)	P02787	189065 (153260-273889)	178905 (131771-227614)	-1.991	0.047
Alpha-1-antichymotrypsin	P01011	123534 (92929-146781)	130569 (102641-165315)	-1.870	0.062
Immunoglobulin heavy variable 3-13	P01766	57006 (38913-89004)	45428 (34467-77853)	-1.855	0.064
Ig gamma-1 chain C region	P01857	277390 (183825-341790)	238961 (181477-308449)	-1.763	0.078
Pulmonary surfactant-associated protein A2	Q8IWL1	82788 (48836-148667)	69890 (39243-116216)	-1.639	0.101
Alpha-1-acid glycoprotein 1	P02763	32817 (18534-45993)	39145 (28541-46295)	-1.635	0.102

Table A-2. ULR analysis of proteins for All GORD *vs* NO GORD. Proteins with p≤0.05 were selected for MLR

Univariate logistic regression analysis of proteins between All GORD and NO GORD					
Proteins identified	**Uniprot ID**	**p value**	**Odds Ratio**	**95% CI for Odds ratio**	
				Lower value	**Upper value**
Immunoglobulin lambda variable 1-47	P01700	0.004	0.999971923	0.999952969	0.999990877
Alpha-1-antichymotrypsin	P01011	0.029	0.999991593	0.999984069	0.999999117
Heat shock cognate 71 kDa protein	P11142	0.048	0.99997596	0.999952131	0.999999789
Ig lambda-2 chain C regions	P0CG05	0.068	1.000001939	0.999999855	1.000004023
Serotransferrin	P02787	0.072	1.000003671	0.99999967	1.000007673
Pulmonary surfactant-associated protein A2	Q8IWL1	0.075	1.000004237	0.999999574	1.000008899
Lactotransferrin	P02788	0.078	0.999997137	0.999993948	1.000000325
Immunoglobulin heavy variable 3-13	P01766	0.115	1.000007162	0.999998251	1.000016072
Ig gamma-1 chain C region	P01857	0.172	1.000002224	0.999999029	1.000005419
Alpha-1-acid glycoprotein 1	P02763	0.459	0.999994979	0.999981694	1.000008264

Table A-3. ULR analysis of proteins for Active GORD *vs* NO GORD. Proteins with p≤0.05 were selected for MLR

Univariate logistic regression analysis of proteins between Active GORD and NO GORD					
Protein name	**Uniprot ID**	**p value**	**Odds Ratio**	**95% CI for Odds ratio**	
				Lower value	**Upper value**
Immunoglobulin lambda variable 1-47	P01700	0.008	0.999969752	0.999947482	0.999992023
Alpha-1-antichymotrypsin	P01011	0.009	0.999988138	0.999979281	0.999996995
Plasma protease C1 inhibitor	P05155	0.028	0.999973111	0.999949159	0.999997064
Lipocalin-1	P31025	0.046	1.000009386	1.000000171	1.000018601
Ig lambda-2 chain C regions	P0CG05	0.053	1.00000233	0.999999969	1.000004691
Keratin, type I cytoskeletal 10	P13645	0.097	0.999992128	0.999982823	1.000001432
Serotransferrin	P02787	0.124	1.000003501	0.999999042	1.00000796
Lactotransferrin	P02788	0.124	0.999997102	0.999993408	1.000000796
Keratin, type II cytoskeletal 6B	P04259	0.177	0.999987011	0.999968167	1.000005855
Heat shock cognate 71 kDa protein	P11142	0.181	0.999981678	0.999954847	1.000008509
Alpha-1-acid glycoprotein 1	P02763	0.26	0.999991029	0.999975415	1.000006643

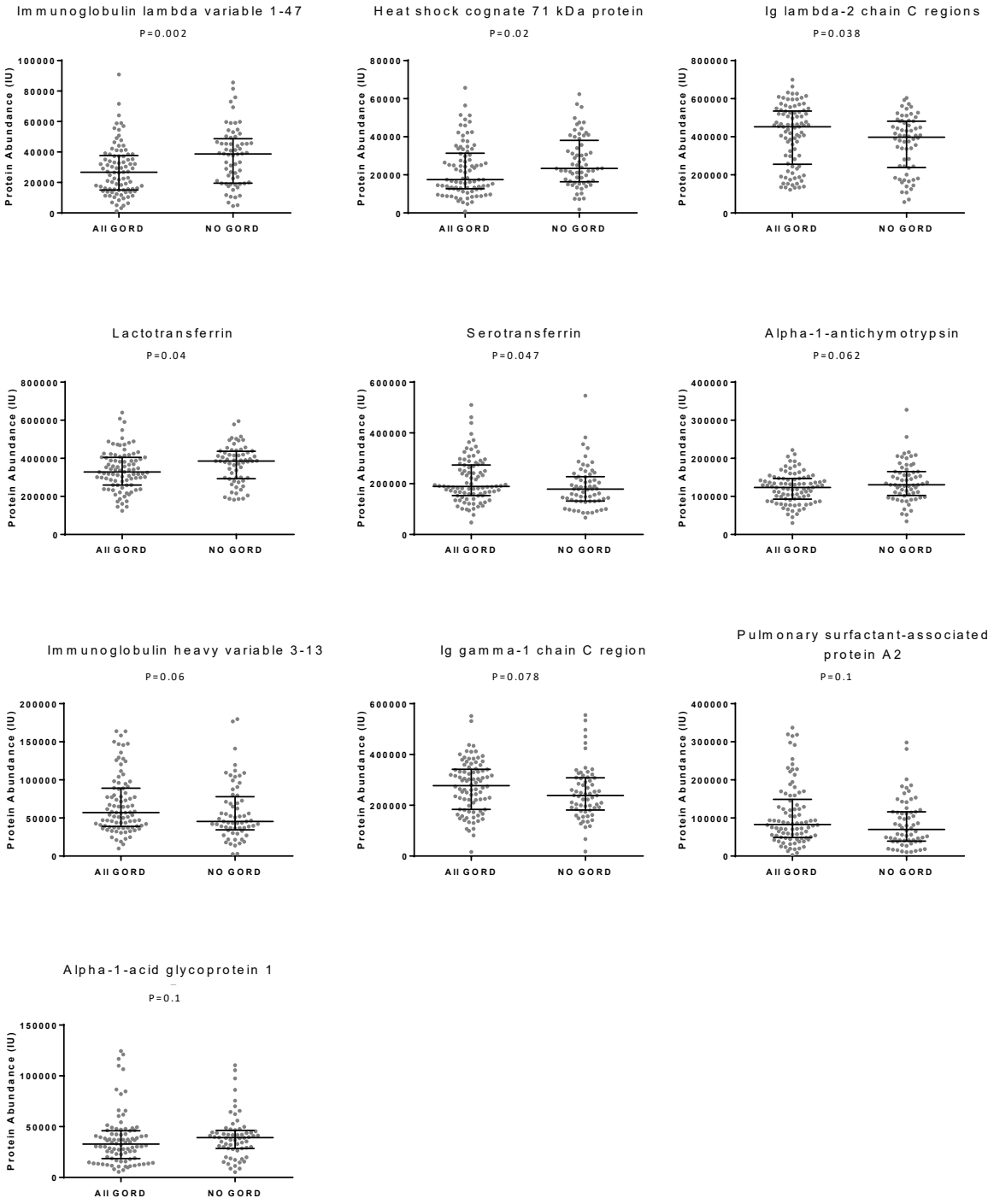

Figure A-1. Proteins predictive of a diagnosis of GORD in severe asthma (All GORD) on Mann-Whitney U up to p≤0.1. *Immunoglobulin lambda variable 1-47* and *Heat shock cognate 71 kDa protein* were found to be the best predictors of a diagnosis of GORD after multiple logistic regression with backward selection.

Appendix B - Supplement to Chapter 8

The following text, tables and figures are the supplementary material to chapter 8.

METHODS

Study participants and sample collection

Healthy participants and severe asthmatics (Step 4/5 of BTS/SIGN Guidelines), aged ≥18, non-smoker or ex-smoker for ≥12 months and <10 pack/years history of smoking, were recruited prospectively. All participants were assessed for GORD by 24-hr pH/impedance studies. The asthmatics were further stratified into those with a documented diagnosis of GORD but not treated with PPI, severe asthmatics with documented GORD and taking PPI treatment and severe asthmatics without a diagnosis of GORD.

Demographic and clinical data as well as results of questionnaires (Asthma Control Questionnaire, Saint Georges Respiratory Questionnaire, Leicester Cough Questionnaire) were recorded on enrolment. Atopy was assessed by skin prick tests (*Dermatophagoides pteronyssinus*, mixed grass pollens, mixed tree pollens, cat dander). Pulmonary function tests, including FEV_1 reversibility to salbutamol, and routine haematology and liver and renal function blood tests were performed. Induced sputum was collected using European Respiratory Society (ERS) recommendations[108]. Fiberoptic bronchoscopy was performed in accordance with the British Thoracic Society guidelines and standard operating procedures of the NIHR Wellcome Trust Clinical Research Facility and NIHR Southampton Respiratory Biomedical Research Unit at the University Hospital Southampton NHS Foundation Trust.

Epithelial cells were harvested by bronchoscopic brushings and samples were either processed into RNAlater for subsequent RNAseq analysis or used in primary bronchial epithelial cells (PBEC) culture. Bronchial biopsies were also taken and embedded into a glycomethacrylate resin for immunohistochemical analyses.

Development of the refluxate mix for use in epithelial culture

Prior to cultures with epithelial cells obtained from patients and controls, dose-ranging and time-course studies were conducted with 16HBE cell line cells using varying compositions of multiple challenge protocol (MCP) containing pepsin (50 to 150 µg/ml; Sigma-Aldrich, St Louis, MO, USA), hydrochloric acid (pH 1.5 to 5) and chenodeoxycholic acid (CDC) (50 µM to 5 mM; Sigma-Aldrich, St Louis, MO, USA). Cells were maintained in minimum essential medium (MEM) with Glutamax and

F12 and supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin (Life technologies, Paisley, UK) on PureCol collagen I (Advanced BioMatrix, San Diego, CA, USA) coated culture flasks. Experiments were carried out using collagen-coated Transwell® permeable supports (diameter 6.5 mm, polyester membrane with 3 μm pores, Corning Life Sciences, Amsterdam, The Netherlands). Cells were seeded at a density of $0.7x10^5$ cells in 200 μL of growth medium; the basolateral compartment contained 500 μL of the same medium. Medium exchange was carried out every 2–3 days. In culture, the 16HBE cells formed a polarized epithelial sheet within seven days, as monitored by measuring the transepithelial resistance (TER). Cells with a TER >700 Ω/cm^2 on day 7 were used for experiments.

Culture media containing varying concentrations of hydrochloric acid, pepsin and/or chenodeoxycholic acid were applied to the apical pole of cells for 5 to 30 minutes. Apical supernatants were collected and centrifuged (10.000 rpm 10 min at 4°C) for assessment of cytotoxicity. The epithelial cells were then washed twice and fresh control culture media was replaced. After 4hr of recovery, micro and macro-molecular permeability were measured by TER and FITC dextran 4kDa respectively. The cells were then lysed using Trizol lysis reagent (Life Technologies, Paisley, UK) and frozen at -80°C until analysed.

Exposure of 16HBE cells to hydrochloric acid was associated with dose-dependent damage of the epithelium (Figure B-1). Comparison between timepoints showed no significant differences in signals (data not shown). Pepsin did not have any additional impact on cell viability at different concentrations (50 to 150μg/ml) or pH (1.5, 2.5 and 5) after 30 minutes of exposure. Chenodeoxycholic acid exposure induced dose-dependent cytotoxicity, with no additional impact of CDC 500 μM at pH5. We, therefore, defined pH5 + pepsin 50μg/ml + CDC 500μM as the working multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) at this stage of culture optimization and chose 30 minutes as the time of exposure. This MCP-PAB model induced a mean 3.5% cytotoxicity in 16HBE cells and a mean 39% decrease in ionic epithelial permeability.

Primary epithelial cultures

Having optimised the culture and AR exposure conditions for 16HBE cells, we went on to optimise the same for primary bronchial epithelial cells (PBEC). PBEC were obtained from healthy and asthmatic donors by fiberoptic bronchoscopy. After adding RPMI, 10% FBS and 2% penicillin/streptomycin, cells were centrifuged at 1200rpm for 5min at room temperature. The cell pellets were re-suspended in Airway Epithelial Cell Growth Medium (Promocell, Heidelberg, Germany) and seeded into PureCol collagen I coated culture flasks. After achieving confluence, the cells were transferred into Transwell® permeable supports and cultured at air-liquid interface (ALI).

After 21 days, the epithelial cells formed a differentiated pseudostratified epithelium. Using trans-epithelial resistance (TER) as a measure of differentiation, cultures with a TER>1000 were deemed fully differentiated and were, therefore, selected for AR experiments. The ALI cultures were starved overnight prior to start of the exposure. In these conditions CDC 500µM was found to be cytotoxic; therefore, its concentration was reduced to 250µM.

The final AR was therefore defined as pH5 + pepsin 50µg/ml + CDC 250µM and this was applied for 30 min.

Analysis of the effect of multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) on epithelial cultures

Assessment of cytotoxicity

Lactate dehydrogenase (LDH) release was measured using a CytoTox 96 Nonradioactive Cytotoxicity Assay (Promega, Southampton, UK), according to the manufacturer's instructions. All samples were assayed in duplicate. Intracellular LDH in control cultures was determined by lysing cells with 1% Triton X-100 in Airway Epithelial Cell Growth Medium. LDH release in each well was calculated as a percentage of total LDH, and values for control wells were subtracted from challenged wells to give the percentage of total LDH released as a result of challenge.

Measurement of epithelial permeability

Transepithelial electrical resistance (TER) was measured using an EVOM voltohmmeter (World Precision Instruments, Hitchin, UK). Fluorescein isothiocyanate (FITC)-dextran 4 kDa (Sigma-Aldrich, Poole, United Kingdom) was applied to cells apically and incubated for 4 hours at 37°C. Basolateral dextran passage was analyzed with a Fluoroskan Ascent FL2.5 reader (Thermo Fisher, London, United Kingdom).

Immunostaining and electron microscopy

Immunostaining

ALI cultures were analysed using immunofluorescence. ALI cultures were fixed with 4% paraformaldehyde and then permeabilized with 0.1% triton X 100 for 15 min at room temperature and blocked with BSA (1% BSA, 0.1% Tween 20 in PBS) for 1 hr. Cells were stained with mouse monoclonal anti-ZO-1 (1/100; BD biosciences, San Jose, CA, USA), mouse monoclonal anti-E-cadherin (1/250; Cell Signaling, London, UK) or goat polyclonal anti-human IL-33 (1/20; R and D

Systems, Abingdon, UK) diluted in 1% BSA, 0.1% Tween 20 in PBS overnight at 4°C. Cultures were then washed with 0.1% Tween 20 in PBS 4 times and incubated for 2 hrs with donkey anti-goat Alexa fluor 488 labelled secondary antibody (Life Technologies, Carlsbad, CA, USA) or goat anti-mouse Alexa fluor 647 labelled secondary antibody (Biolegend, San Diego, CA, USA). Cell nuclei were counterstained with DAPI. Images were acquired using a Leica SP8 laser-scanning confocal microscope (Leica Microsystems, UK).

Bronchial biopsies were analysed using immunohistochemistry. Bronchial biopsies were processed as previously described [196] and embedded into paraffin; 4-µm sections were cut from all suitable biopsies and stained by immunohistochemistry using goat polyclonal anti-human IL-33 (R&S Systems, Abingdon, UK). Epithelial cells were counted and results expressed as positive cells per total epithelial cells.

Electron Microscopy

PBEC in transwells were fixed in 3% glutaraldehyde, 4% formaldehyde, 0.1M PIPES buffer pH7.2 for 30 min at RT, then rinsed with 0.1M PIPES buffer pH7.2 and subsequently post-fixative 1% Osmium tetroxide 0.1M PIPES buffer pH7.2. Transwells were stained enbloc in 2% Uranyl Acetate then dehydrated through a graded series of ethanols into acetonitrile and into embedded in Spurr resin. Ultrathin sections (90nm) were cut and stained with Reynolds lead citrate. Sections were analysed using a Hitachi HT7700 transmission electron microscope.

Cytokine analysis

Interleukin 8 (IL-8) concentrations in culture media from the apical and basolateral chambers were measured using an IL-8 DuoSet ELISA (R&D, Abingdon, UK) in accordance with manufacturer's instructions. Each sample was evaluated using 2 technical replicates and the mean value was used for subsequent statistical analysis. IL-6, TNFα, IL1α were measured using VPlex (MSD, Maryland, USA) according to manufacturer's recommendations.

Analysis of gene expression in epithelial brushings and differentiated cells

RNA extraction

Total RNA was extracted from primary bronchial epithelial samples using the Qiagen RNeasy kit. RNA quality and concentration were measured using an RNA Nano chip on the Agilent Bioanalyser 2100. Samples with total RNA concentration $\geqslant$50ng/µl, RIN $\geqslant$6.8 and OD 260/280 were taken for cDNA library preparation and sequencing.

cDNA library preparation and sequencing

cDNA libraries were prepared using NEBNext Ultra (non-stranded) mRNA library prep kit with polyA pulldown for mRNA enrichment (Novogene Inc). Library quality was assessed using a broad range DNA chip on the Agilent Bioanalyser 2100. Library concentration was assessed using Qubit and q-PCR. Libraries were pooled. Paired-end 150bp sequencing to a depth of 20M reads (epithelial brushings) or 50M reads (differentiated cells) was performed on an Illumina HiSeq2500 by Novogene Inc (Cambridge, UK).

Data processing

Quality control

Raw FASTQ reads were subjected to adapter trimming and conservative quality filtering (reads containing N > 10%, reads where >50% of read has Qscore<= 5) by Novogene Inc. Quality of sequence was assessed using fastqc, aggregated using multiqc.

Alignment

Paired FASTQ files were aligned to human genome build 38 using gencode v29 gene annotations and STAR v2.6.0 splice aware aligner [230], using ENCODE recommend options (3.2.2 in the STAR manual (https://github.com/alexdobin/STAR/blob/master/doc/STARmanual.pdf). Samples aligned at a rate of 88.04 - 93.78% uniquely mapped reads.

Read counting

SAM files were sorted by name using SAMtools [231]. Reads were counted using HTSeq [232] and gencode v29 annotations following guidelines in the HTSeq documentation (https://htseq.readthedocs.io/en/release_0.11.1/overview.html#documentation-overview)

Differential gene expression analysis

Count data from HTseq was subjected to quantile filtration to remove genes with more than 25% of samples with a read count of 0. Count data was normalised by library size to calculate counts per million (CPM). CPM data was normalised by distribution using the trimmed Mean of the M-values (TMM) approach using edgeR [233,234].

Gene ontology was performed by AmiGO [235-237] using the top 300 upregulated differentially expressed genes (DEGs) in the group of severe asthmatic with GORD compared to the group of severe asthmatics with no GORD.

qRT-PCR

RNA was reverse transcribed to cDNA using a Precision Reverse Transcription kit (PrimerDesign, Southampton, UK) according to the manufacturer's instructions. Expression of CXCL8 (Applied Biosystems, Paisley, UK) and IL-33 (Primer Design, Chandler's Ford, UK) was determined using probe-based qPCR, whereas expression of the housekeeping genes ubiquitin C and glyceraldehyde 3-phosphate dehydrogenase was determined using a probe-based duplex primer mix (PrimerDesign). Fold change in gene expression relative to time-matched controls was determined using the ΔΔCt method.

Table B-1. Top DEGs fold changes relative to control in healthy subjects (HC) and severe asthmatics (SA) ALI cultures exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) from negative (blue) to increasingly positive (increasing intensity of red).

	HC	SA	Gene name
IL1RL1		11,8	Interleukin 1 Receptor Like 1
CHAC1	8,9	8,5	ChaC Glutathione Specific Gamma-Glutamylcyclotransferase 1
IL36G	2,9	6	Interleukin 36 Gamma
PLAUR	2,5	5,7	Plasminogen Activator, Urokinase Receptor
SERPINB9		5,3	Serpin Family B Member 9
DUSP5	2,6	4,7	Dual Specificity Phosphatase 5
EMP1	1,3	3,9	Epithelial Membrane Protein 1
BACH2		3,8	BTB Domain And CNC Homolog 2
FOSL1		3,6	FOS Like 1, AP-1 Transcription Factor Subunit
SEMA7A		3,4	Semaphorin 7A (John Milton Hagen Blood Group)
EHD3		2,5	EH Domain Containing 3
AKAP12		2,4	A-Kinase Anchoring Protein 12
IL1RN		2,2	Interleukin 1 Receptor Antagonist
CEACAM1		2,1	CEA Cell Adhesion Molecule 1
SLC7A11		2,1	Solute Carrier Family 7 Member 11
LIPH	1,3	2,1	Lipase H
ERRFI1	1,6	1,9	ERBB Receptor Feedback Inhibitor 1
HSPA5	1	1,6	Heat Shock Protein Family A (Hsp70) Member 5
LPIN1		1,6	Lipin 1
SOX2		-0,7	SRY-Box Transcription Factor 2
CYP26A1	11,7		Cytochrome P450 Family 26 Subfamily A Member 1
ASNS	6,6		Asparagine Synthetase (Glutamine-Hydrolyzing)
LIF	3,9		LIF Interleukin 6 Family Cytokine
BCL2A1	3,8		BCL2 Related Protein A1
DDIT3	2,7		DNA Damage Inducible Transcript 3
MAFF	2		MAF BZIP Transcription Factor F
HERPUD1	1,6		Homocysteine Inducible ER Protein With Ubiquitin Like Domain 1
SESN2	1,5		Sestrin 2
NCOA7	1,5		Nuclear Receptor Coactivator 7
CDC42EP1	1,4		CDC42 Effector Protein 1
TICAM1	1,4		Toll Like Receptor Adaptor Molecule 1
GMNC	-0,7		Geminin Coiled-Coil Domain Containing
GRPEL2			GrpE Like 2, Mitochondrial
OCA2			OCA2 Melanosomal Transmembrane Protein
ODC1			Ornithine Decarboxylase 1

HC : healthy controls ; SA : severe asthmatics

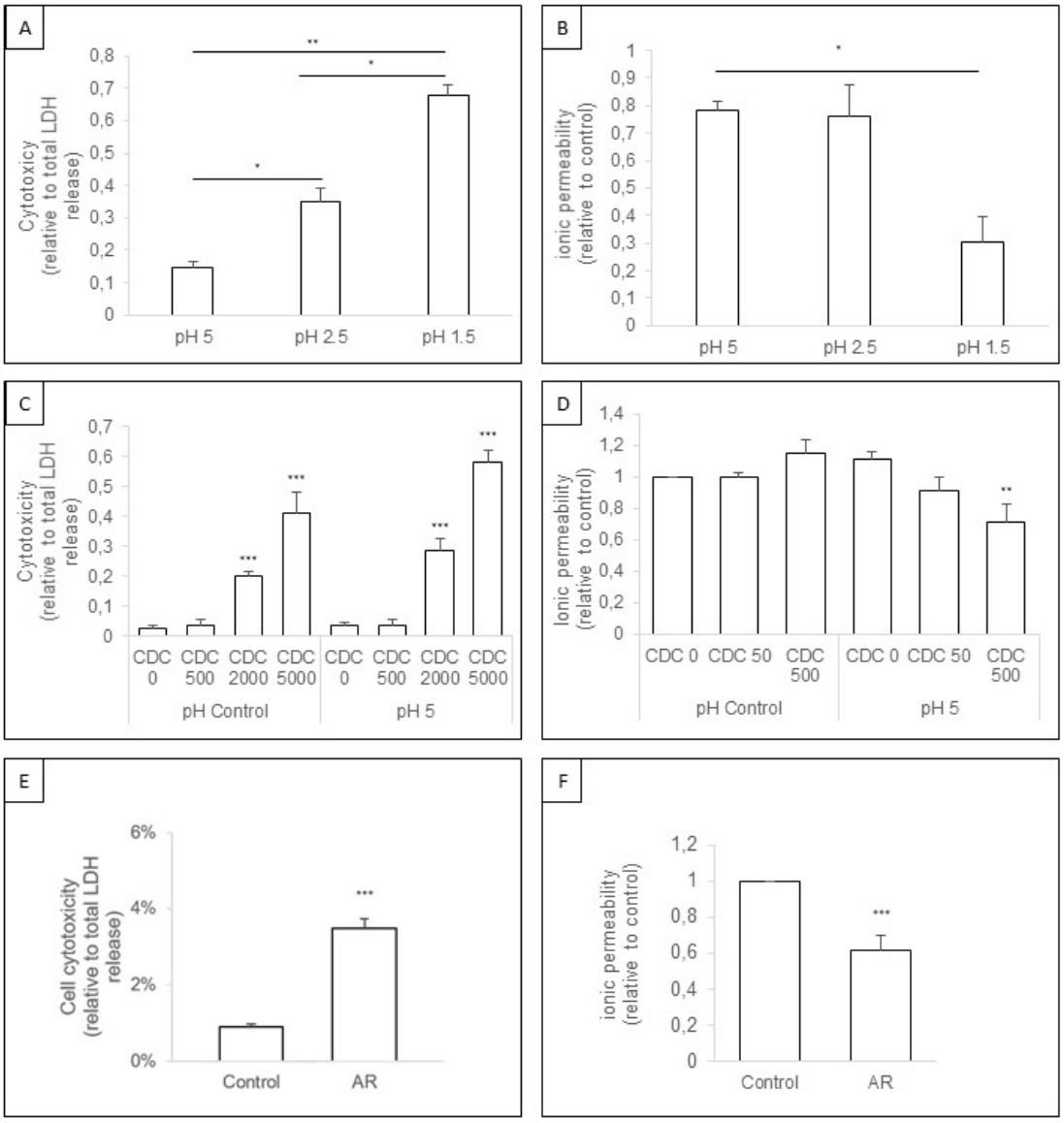

Figure B-1. Development of the refluxate mix. Exposure of 16HBE cell cultures to hydrochloric acid (A, B), chenodeoxycholic acid (C, D) and/or artificial refluxate (E, F) induced cytotoxicity (A, C, E) and increase in epithelial ionic permeability (B, D, F). CDC: Chenodeoxycholic Acid (µM); MCP-PAB: multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) * $p<0.05$; ** $p<0.005$; ***$p<0.0005$

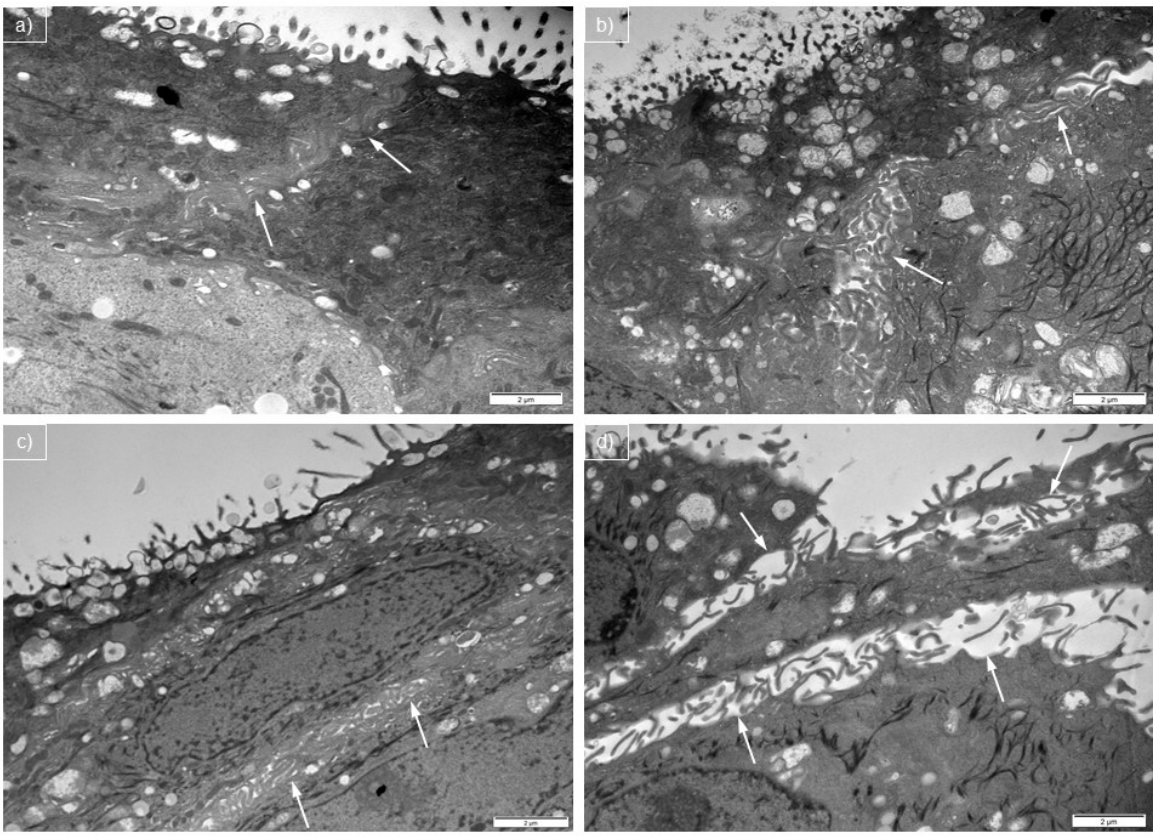

Figure B-2. Enlargement of intercellular spaces by artificial refluxate. Bronchial epithelial ALI cultures from severe asthmatic donors were untreated (A, C) or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) (B, D) for 30 mins, washed and allowed to recover for 4 hours before fixing. TEM photographs of intercellular spaces (white arrows) are representative of experiments using 4 donors.

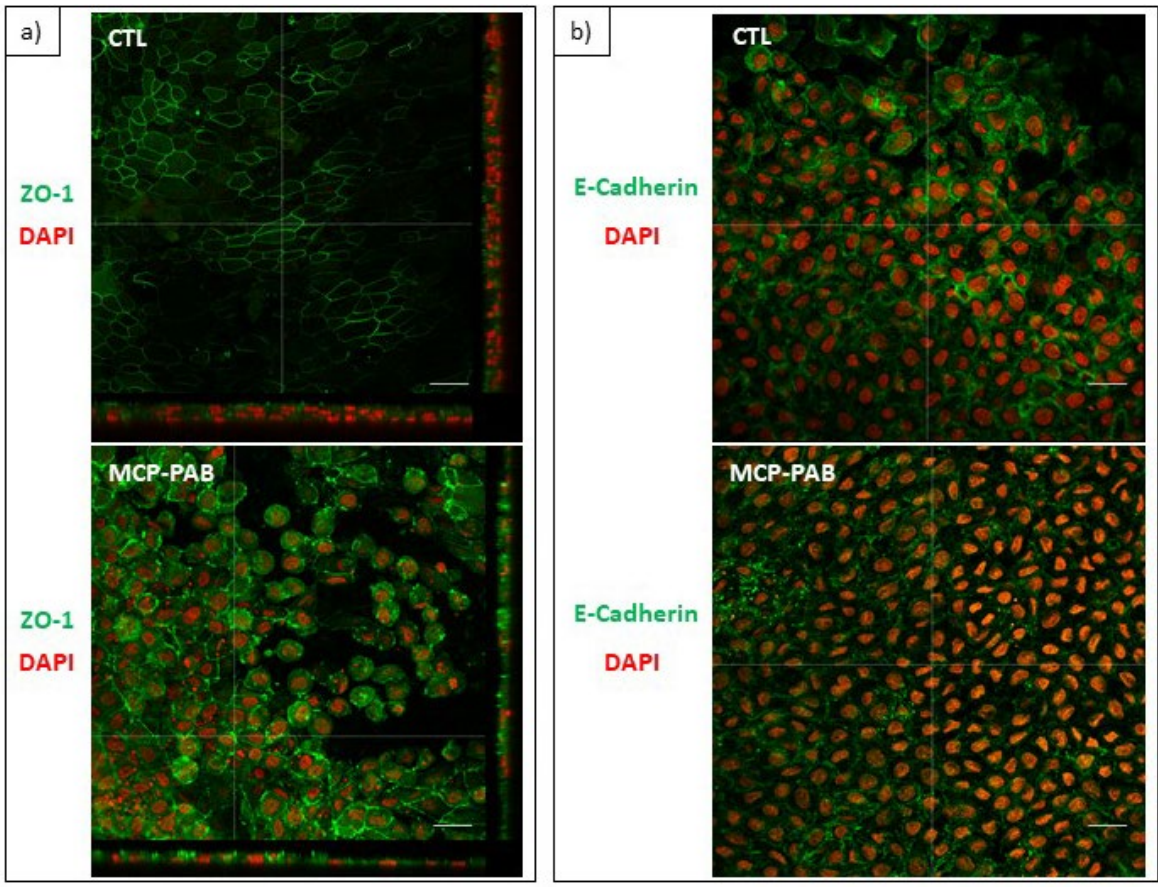

Figure B-3. Disruption of Epithelial Tight junctions by Artificial Refluxate. Bronchial epithelial ALI cultures from severe asthmatic donors were untreated or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 hours before fixing and immunofluorescence staining. Panel A shows ZO-1 (green) and 4',6-diamidino-2-phenylindole (DAPI) (red) and Panel B shows E-cadherin (green) and 4',6-diamidino-2-phenylindole (DAPI) (red). Images are representative of experiments using 6 donors. Scale bar = 25µm.

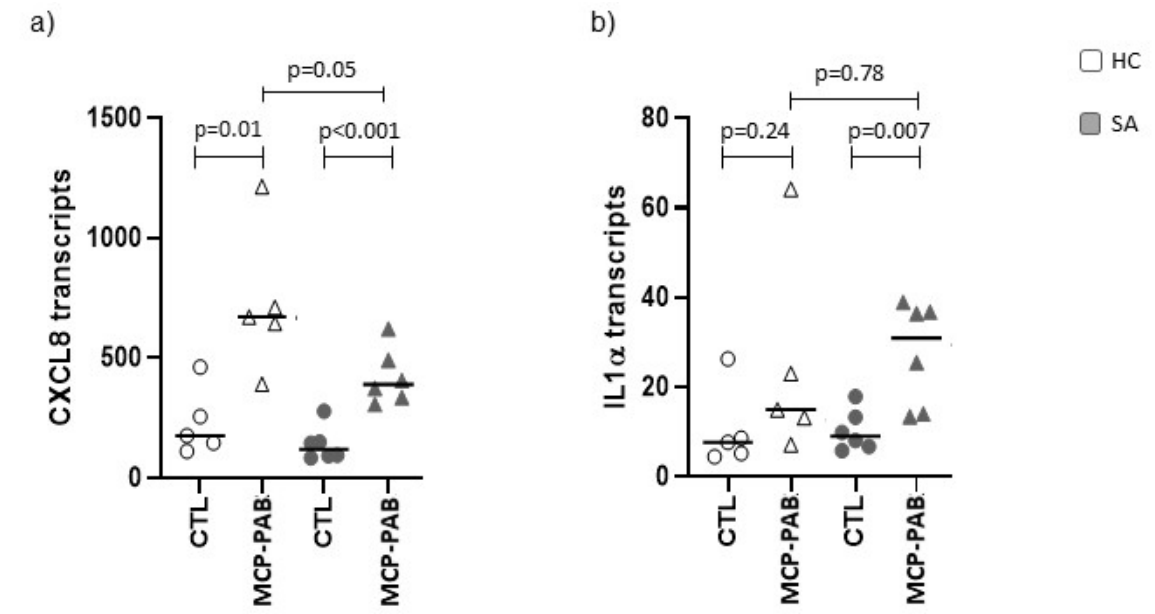

Figure B-4. Stimulation of Epithelial Cytokine expression by artificial refluxate. Bronchial epithelial ALI cultures from healthy controls (HC) (N=5) and severe asthmatic (SA) (N=6) donors were untreated (CTL) or exposed to multiple challenge protocol using pepsin, acid pH and bile acid (MCP-PAB) for 30 mins, washed and allowed to recover for 4 hours before RNA extraction for CXCL8 (A) and IL-1α (B) gene expression analysis. * $p<0.05$ vs control. ** $p<0.005$ vs control.

Appendix C - Abstracts and publications

Perotin JM, Wheway G, Tariq K, Davies DE, Djukanovic R. **Vulnerability to acid reflux of the airway epithelium in severe asthma**. Eur Respir J. 2022 Jan 7:2101634. doi: 10.1183/13993003.01634-2021

Tariq K, Schofield JPR, … Dimitrov B, Djukanović R; U-BIOPRED Study Group. **Sputum proteomic signature of gastro-oesophageal reflux in patients with severe asthma**. Respir Med. 2019 Apr;150:66-73. doi: 10.1016/j.rmed.2019.02.008.

K. Tariq, B.L. Nicholas, R. Lutter, …. PJ Sterk, KF Chung, PH Howarth, R Djukanovic, and the U-BIOPRED Study Group - **Prevalence of gastro-oesophageal reflux (GORD) and associations with clinical phenotypic markers in adult severe asthmatics in the U-BIOPRED cohort**, ATS 2015.

K. Tariq, C. Barber, K. Gove, H. Rupani, T. Brown, R. Kurukulaaratchy, A. Chauhan, R. Djukanovic, P.H. Howarth - **Role of gastro-oesophageal reflux in severe asthma: Experience from the Wessex severe asthma cohort**, ERS 2015.

Tariq K, Brandsma J, Burg D, Nicholas BL, Lutter R, Corfield J, Chung KF, Howarth PH, Ana Sousa, Postle AD, Sterk PJ, Dimitrov BD, Djukanovic R, and the U-BIOPRED Study Group - **Lipid biomarkers predictive of gastro-oesophageal reflux in adult asthma**, ERS 2016.

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