GASTRO-OESOPHAGEAL REFLUX AND ITS RELATIONSHIP TO CYSTIC FIBROSIS LUNG DISEASE

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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ANCOVA	Analysis of covariance
BAL	Bronchoalveolar lavage
BCC	*Burkholderia cepacia complex*
BMI	Body mass index
CC	Chicago classification
CF	Cystic Fibrosis
CFRD	CF-related diabetes
CD	Crural diaphragm
CDP	Contractile deceleration point
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
DCI	Distal contractile integral
DL	Distal latency
DTT	Dithiothreitol
FEV1	Forced expiratory volume in 1 second
FEF 25-75	Forced expiratory flow 25-75%
FVC	Forced vital capacity
GLI	Global Lung Function Initiative
GOR	Gastro-oesophageal reflux
HbA1C	Haemoglobin A1c
IBS	Irritable bowel syndrome
IBS-SSS	IBS-symptom severity score
IPF	Idiopathic Pulmonary Fibrosis
IOM	Ineffective oesophageal motility
IQR	Interquartile range
HRM	High resolution manometry
LC	Liquid chromatography
LOS	Lower oesophageal sphincter
MII	Multichannel intraluminal impedance
MS	Mass spectrometry

MS-MS	Tandem MS
OGD	Oesophago-gastro duodenoscopy
OGJ	Oesophago-gastric junction
OGJ-MP	OGJ-mean pressure
OGJ-CI	OGJ-contractile integral
PCA	Principal Component Analysis
pH-MII	Combined pH and multichannel intraluminal impedance
PEG	Percutaneous endoscopic gastrostomy
PA	*Pseudomonas aeruginosa*
PROM	Patient reported outcome measure
NG	Nasogastric tube
RESQ-7	Reflux symptom questionnaire, 7-day recall
TLOSR	Transient lower oesophageal sphincter relaxation
UOS	Upper oesophageal sphincter
WB	Western blot

Abstract

Introduction: A number of studies have shown an increased prevalence of gastro-oesophageal reflux in adult and paediatric cystic fibrosis (CF) populations. To date it remains unproven if the increased amounts of reflux shown can affect CF lung disease. The most commonly proposed mechanism by which this may occur is reflux aspiration. The study's aim was to assess if a relationship exists between reflux and measures of lung disease severity.

Methods: A prospective observational study was conducted in stable adult CF patients, measuring reflux with combined oesophageal pH-impedance (n=41). This allowed the following analyses: (I) The reflux measures were described for the entire cohort; (II) The influences of various factors on the amount of reflux were examined, including oesophageal motor function, prescribed medications and co-morbidities; (III) The effect of reflux on respiratory endpoints was assessed; (IV) Mass spectrometry was used to assess the effect of reflux on relative protein abundance within sputum.

Results: Reflux was increased in 54% of this cohort using total reflux events. Reflux characteristics assumed to be high-risk for reflux aspiration (proximal and/or supine events) were raised in 41%. Dysfunction of the oesophago-gastric junction correlated with acid exposure. Methylxanthines but no other medications correlated with the number of reflux episodes. No correlation was shown between reflux measures and any respiratory endpoint tested. No differences in relative protein abundance within sputum were demonstrated between those with the highest and lowest measures of reflux.

Conclusions: Although there was a high prevalence of increased reflux using pH-impedance, no effect of reflux was demonstrated on the tested respiratory endpoints or relative protein abundance within sputum. This may reflect that it is currently not possible to directly measure the amount of reflux aspiration, which is a major limitation.

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Dedication

I would like to dedicate this thesis to my father, Michael Lord, and to my grandparents, Hubert and Joan Lord. I am forever grateful to the opportunities you provided me with.

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The author

Robert Lord grew up in Accrington, a small Lancashire town close to Blackburn. He attended Queen Elizabeth's Grammar School and then studied Medicine at the University of Manchester. He moved slightly further afield to Merseyside to complete Foundation training at Whiston Hospital. This was followed by a slightly longer journey to Australia where he was employed at the John Hunter Hospital, a large regional teaching hospital in Newcastle, New South Wales. He worked initially at a junior grade within the Emergency departments and then middle grade within the Medical department. Returning back to the Northwest he commenced in 2008 Core Medical, followed by Respiratory and General Internal Medicine training. He took time out of program to work as a clinical fellow within the Manchester Adult Cystic Fibrosis Centre. During this time he undertook his PhD.

1 Preface

This study was designed in order to investigate the relationship between gastro-oesophageal reflux and lung disease in adult CF patients. To achieve this, the amount of gastro-oesophageal reflux and measures of lung disease severity were compared. In addition, a number of specific areas were studied: the prevalence of reflux; the potential mechanism(s) that may account for the increased amounts of reflux; and pilot data exploring the role of several proteomic methods to help identify subjects with aspiration of their reflux.

The background covers three major areas. There is an introduction to CF and discussion of the factors that may influence lung disease (Chapter 2). Next the physiology and pathogenesis of reflux in healthy individuals and in those with gastro-oesophageal reflux is discussed (Chapter 3). To finish there is a literature review of the effect of gastro-oesophageal reflux on CF lung disease (Chapter 4).

The results and discussion are divided into specific topics. Chapter 7 focuses upon the reflux prevalence and characteristics within an adult CF population. Chapter 8 seeks to identify factors using high resolution manometry – pressure readings within the oesophagus – that lead to increased reflux. This includes motor function of the oesophagus and impairment of the oesophago-gastric junction. Chapter 9 then examines the effect of co-morbidities and medications on reflux.

Chapter 10 explores the associations between the amount of reflux and measures of severity of lung disease. Chapter 11 then attempts to further refine this analysis, by identifying subjects in whom reflux aspiration is occurring.

Finally, conclusions are made based on the findings from the results of this study (Chapter 12).

2 Cystic fibrosis lung disease

2.1 Epidemiology

Cystic fibrosis is an inherited multi-system disease. Within Caucasian populations, it has a frequency of one in 2500 live births[1]. In 2016 the UK registry data showed there were 10,461 individuals with the disease, of which 247 were new diagnoses [2].

2.2 Pathogenesis of CF

2.2.1 The Cystic Fibrosis Transmembrane Conductance Regulator protein

Cystic fibrosis is caused by a defect in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. The protein is located within the apical membrane of epithelial cells and controls transport of chloride and bicarbonate [3]. It plays a role in controlling the movement of water across the cell membrane, as well interacting with cellular pathways such as those related to inflammation and pH [4, 5].

2.2.2 Genetics

A single gene codes for the CFTR protein [6]. Of this CFTR gene nearly 2000 mutations have been described [7]. However, only around 200 of these have been shown to be associated with disease [8]. These mutations can be divided into six functional classifications: absence of synthesis (class I); processing (class II); disordered regulation (class III); defective conductance (class IV); reduced number of transcripts (class V); and accelerated turnover (class VI) [9]. Class I, II and III mutations are associated with no residual CFTR function, whereas IV, V and VI have some residual function [10].

2.2.3 Complications of CF

Dysfunction of CFTR leads to thick mucus accumulating within the respiratory tract. This sets forth a repeated cycle of inflammation and chronic bacterial infection [11]. This leads to respiratory failure and ultimately death.

CF also has the potential to damage organ systems beyond the respiratory tract. The accumulation of thickened secretions has been documented to affect the pancreas, gastrointestinal tract, liver, reproductive systems and sinuses [1]. These all occur mainly as a consequence of luminal obstruction due to viscous secretions.

2.3 Assessment of severity of CF lung disease

The ability to accurately measure severity and capture progression of CF lung disease is vitally important in both clinical practice and research. The measures of lung disease severity are in fact surrogates for the key clinical outcomes: how long a patient will survive; how they feel; how they are able to function[12]. These are of course largely impractical and intangible to measure, in either the clinical or research setting, hence the need for alternative surrogate outcome measures. These can include physiological, radiological or 'patient-reported' outcome measures. The most commonly used endpoints to reflect severity and progression of CF lung disease are percentage forced expiratory volume in 1 second (FEV1%) and rate of pulmonary exacerbations [13, 14].

2.4 Factors affecting the severity of CF lung disease

2.4.1 Genetic factors

Importantly the genotype – the combination of the two CFTR mutations possessed – has been shown to poorly predict the severity and course of pulmonary disease[15]. *Ergo*, other factors must shape the clinical phenotype. Studies in monozygous and dizygous CF affected twins have shown that non-CFTR related genetic factors can affect severity of lung disease [16]. The genes highlighted include Transforming Growth Factor Beta 1 (TGFB1), Mannose-Binding Lectin 2 (MBL2), Epithelial Transcription Factor (EHF) and Apoptotic Protease Activating Factor 1 Interacting Protein (APIP) [17].

2.4.2 Other factors

Beyond genetics a number of other factors have been highlighted that can influence respiratory function in CF patients. Exposure to tobacco smoke and

air pollution has been shown to have a detrimental effect on lung function [18, 19]. Colonisation of the respiratory tract with a number of respiratory of respiratory pathogens, including *Pseudomonas aeruginosa* and *Burkholderia cepacia complex*, have been shown to be associated with greater lung function decline [20, 21]. In addition, a number of the other acquired complications of CF have been shown to negatively impact on lung disease including diabetes mellitus, exocrine pancreatic insufficiency and nutritional status [21-23]. Importantly gastro-oesophageal reflux has also been implicated in worsening the progression of lung disease[24].

2.5 Treatment for CF lung disease

A number of treatments exist which attempt to slow the progression toward respiratory failure and death. However, at present no 'silver bullet' exists. Instead it is a multifaceted approach with a heavy treatment burden for patients. Sputum is cleared regularly using a combination of physiotherapy techniques and mucolytics, such as nebulised DNase and hypertonic saline [25, 26]. Airway colonisation is controlled by long-term antibiotic therapy, including nebulised agents. Acute infections are treated aggressively, with a strategy that includes prompt antibiotics, increased chest physiotherapy and respiratory support [27, 28].

Other approaches include optimisation of nutritional status with use of high calorie supplements. Diabetes mellitus is identified and appropriately managed with insulin [27]. A recent development is the introduction of CFTR modulators, which have been shown to improve CFTR function in specific genotypes[29]. This improvement in CFTR function has then been shown to lead directly to significant improvement in lung function. It may also indirectly improve lung function, as a result of an improved body mass index [30]. As yet it is unclear if treatment of gastro-oesophageal reflux can improve the outlook for CF patients[24].

2.6 Conclusion

There is a variable progression of lung disease in CF. This goes beyond genotype, and is influenced by a number of factors. A number of treatments exist, with some aimed at negating downstream consequences of CFTR dysfunction that have been identified within the specific patients, such as diabetes or poor nutrition. To this end it is important to identify further factors influencing progression, as these may represent further therapeutic targets. As such gastro-oesophageal reflux is an area of interest for future research.

3 An overview of gastro-oesophageal reflux

3.1 Definitions

3.1.1 Gastro-oesophageal reflux

Gastro-oesophageal reflux is the passage of gastric fluid into the oesophagus. It is a physiological occurrence [31].

3.1.2 Gastro-oesophageal reflux disease

Gastro-oesophageal reflux moves from the physiological to the pathological with the presence of symptoms, injury to the oesophageal mucosa or both. This is referred to as gastro-oesophageal reflux disease[32].

3.2 The physiology of a reflux event

3.2.1 Anatomy

The oesophago- gastric junction (OGJ) is the barrier between the oesophagus and stomach. It is composed of two main parts: the lower oesophageal sphincter (LOS) and the crural diaphragm (see Figure 3.1). The LOS is a segment of thickened smooth muscle at the distal oesophagus. Upon this is circumferential skeletal muscle from the crural diaphragm, which wraps around the LOS creating a high pressure zone [31].

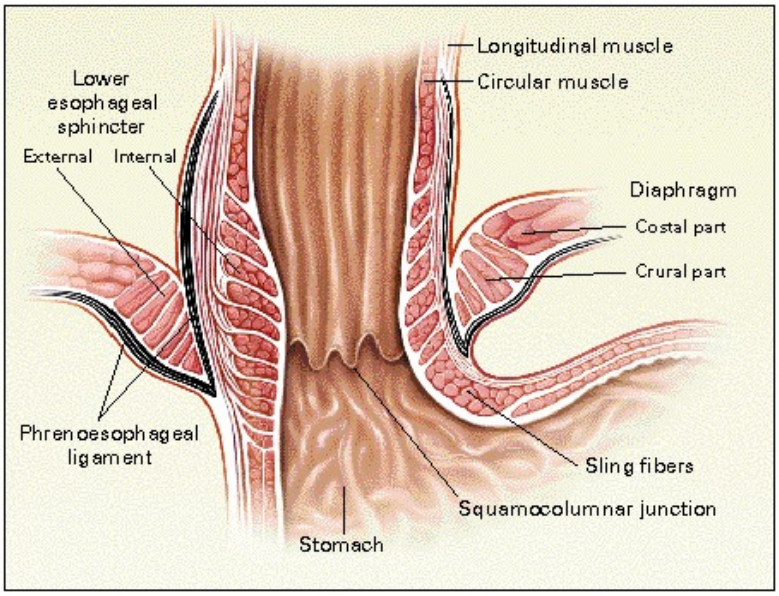


Figure 3.1 The oesophageal gastric junction

Reproduced with permission from Mittal et al, N Engl J Med 1997; 336: 924, Copyright Massachusetts Medical Society [33].

3.2.2 Physiology of the oesophago-gastric junction

The function and role of the OGJ is complex. It has a continuous role whereby it must resist a pressure gradient favoring reflux, which permanently exists between the stomach and the oesophagus – the gastro-oesophageal pressure gradient [34]. To do so the LOS adopts a resting level of contraction, which in healthy individuals is between 15-30mmHg above gastric pressure [35]. The crural diaphragm then augments this pressure when it contracts during inspiration, the point at which the gastro-oesophageal pressure gradient peaks. Thus the OGJ pressure varies throughout the respiratory cycle [36].

The OGJ must also intermittently relax to allow antegrade passage of ingested substances, as well as saliva, toward the stomach. These are known as swallow related relaxations. It also must allow retrograde passage of gas which accumulates during the digestive process known as belching, or more technically as transient lower oesophageal relaxations (TLOSR) [35].

During a swallow-related OGJ relaxation, the trigger is oesophageal peristalsis which leads to inhibitory reflexes causing a short-lived reduction in muscular tone of the crural diaphragm and lower oesophageal sphincter [37]. In contrast the TLOSR occurs as a vagal nerve mediated reflex in response to stimulation by gastric stretch receptors – essentially 'gas-venting' of the stomach [38, 39]. TLOSR last significantly longer than those triggered by swallowing [40].

3.3 Measuring reflux

3.3.1 PH-metry

The first widely used method of quantifying reflux was the traditional pH study, which involved an oesophageal catheter upon which a pH was affixed and remained within the oesophagus for 24 hours. This allows metrics to be produced relative to a pH of >4, essentially measuring the amount of oesophageal acidification. These could then be used individually for a measure of reflux, or commonly as a composite to generate a DeMeester score.

It is now surpassed by a newer modality for measuring reflux known as oesophageal multi-channel intraluminal impedance (MII), or alternatively just as impedance. When pH studies were compared to MII, the former were shown to miss a significant number of reflux events [34, 35]. This is because pH studies can only detect a reflux event when pH falls below 4. It has been shown subsequently, by using combined oesophageal pH and impedance (pH-MII), that there are frequent episodes of reflux that occur with a pH above 4 [36]. Further benefits of pH-MII are that it can differentiate between acid reflux and swallowed acidic food. It also does not require acid suppressive medications, such as proton pump inhibitors (PPI) to be stopped. These are not the case with pH-metry [41, 42].

3.3.2 Multi-channel intraluminal impedance

MII is a validated method for quantification of reflux and has been shown to be superior to pH studies or manometry [34, 35]. The MII catheter, which has regularly spaced electrodes, is placed within the oesophagus. A small voltage electrical current is conducted between adjacent electrodes. As the catheter is non-conducting the luminal components bridge the gap. At baseline it is the oesophageal mucosa bridging, but this can be replaced by a gas or liquid bolus. The ability of each of these has a specific ability to resist the flow of electricity, also known as its impedance. Liquid more readily allows electrical flow, and as such lowers impedance. The opposite occurs for gas. As the electrodes are placed throughout the length of the oesophagus the direction of a bolus can be identified. This allows detection of reflux events, and differentiation from swallowed boluses [37, 38].

3.3.3 Combined pH-impedance

For most research now pH and impedance are combined on one catheter. This also allows the reflux events by MII to be classified according to their pH [37, 38]. These events can be defined as acid with pH <4, weak acid with pH 4-7 or weakly alkaline with pH>7 [6].

3.4 Physiological reflux

Reflux is a physiological occurrence. Using pH-MII the amount of reflux has been studied in 72 healthy individuals [43]. This allows physiological reflux to be quantified and upper limits of normal to be calculated, using the 95th centile (see Table 3.1).

Physiological reflux events occur relatively frequently, with a median number of 44 reflux episodes every 24 hours. Most of these events are acidic. Only about a fifth of events reach the proximal oesophagus (22%) and supine events are uncommon. Proximal events which occur whilst supine are extremely rare with a median of one per 24 hours. The reflux events are short lived, with a median

duration 11 seconds [43]. Almost all reflux that occurs in healthy individuals are thought to relate to TLOSR [44, 45].

Table 3.1 PH-impedance values derived from a healthy cohort

	All reflux	**Acid reflux**	**Weakly acid reflux**	**Weakly alkaline reflux**
Total				
Median (25^{th},75^{th})	44(25,58)	22(10,35)	11(5,18)	3(1,7)
95^{th} centile	75	50	33	15
Upright				
Median (25^{th},75^{th})	40(23,52)	20(10,31)	10(4,15)	3(1,6)
95^{th} centile	64	45	31	14
Supine				
Median (25^{th},75^{th})	3(1,6)	1(0,4)	1(0,2)	0(0,1)
95^{th} centile	14	8	5	3

	Proximal extent		**24-h bolus exposure**	
	Time (min)	**Percent time (%)**	**Time (min)**	**Percent time (%)**
Total				
Median (25^{th},75^{th})	9(4,17)	22(12,39)	11(6,16)	0.8(0.4,1.2)
95^{th} centile	30	64	27	2.0
Upright				
Median (25^{th},75^{th})	8(3,15)	21(10,38)	10(5,14)	1.0(0.6,1.8)
95^{th} centile	27	64	21	2.7
Supine				
Median (25^{th},75^{th})	1(0,2)	11(0,50)	10(0,1)	1.0(0.0,0.3)
95^{th} centile	3	100	5	0.9

The data in the above tables are taken from a study of healthy volunteers by Zerbib et al. [43].

3.5 Oesophageal sequelae of reflux

3.5.1 Oesophageal syndromes

GORD encompasses a number of oesophageal syndromes (see figure 3.2). The initial and main division is made endoscopically between those with and without evidence of mucosal injury - erosive reflux disease as opposed to non-erosive reflux disease. Non-erosive reflux disease is often differentiated from two other syndromes by using pH-impedance and symptom association analysis [31]. Oesophageal hypersensitivity occurs with normal amounts of reflux, but a temporal relationship is noted to reflux events. Oesophageal hypersensitivity is often included within non-erosive disease. However not included are a further group that has typical symptoms but no temporal relationship to reflux events. This is referred to as functional heartburn, a form of functional gut disease[46].

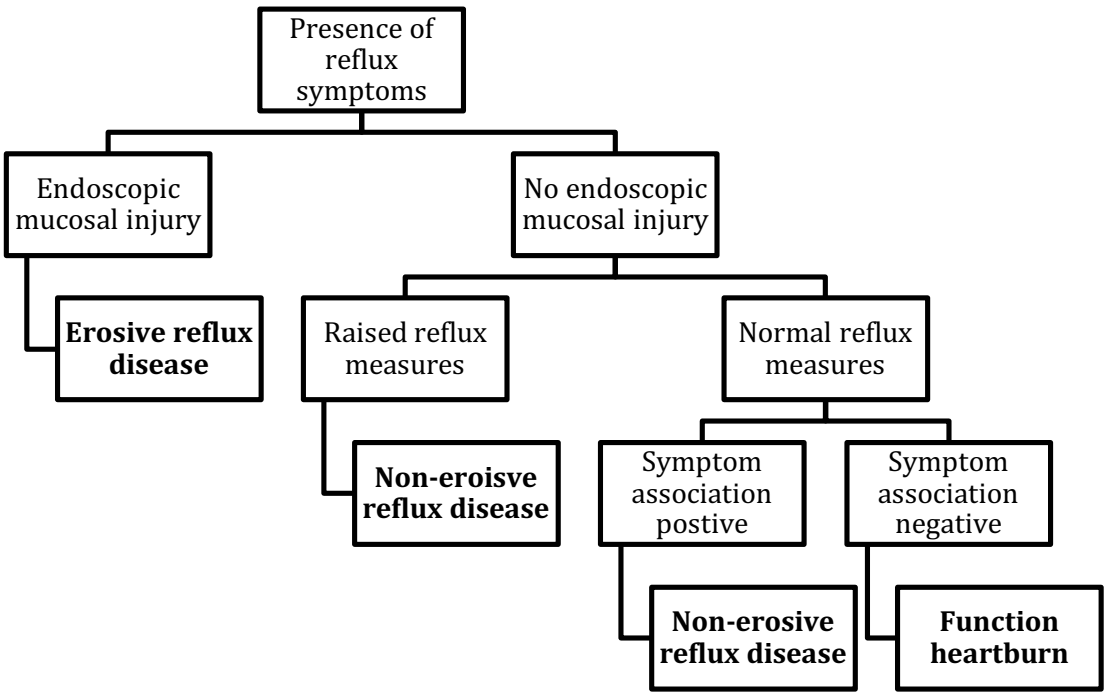


Figure 3.2 Classification of oesophageal reflux syndromes

3.5.2 Symptom generation

Two main oesophageal symptoms are experienced: acid regurgitation and heartburn. Acid regurgitation occurs when gastro-oesophageal refluxate is tasted in the mouth. Heartburn is a painful retrosternal sensation [31]. Development of heartburn is mediated by four major pathophysiological determinants: the amount of gastric refluxate entering the oesophagus; the efficacy of the oesophagus to both clear the liquid and neutralise the acidity following an event; the constituents of the reflux e.g. the acidity or presence of digestive enzymes; and the sensitivity of the mucosa and its threshold to allow triggering of pain [47].

3.6 Mechanism of increased amount and impaired oesophageal clearance of refluxate in GORD

3.6.1 Mechanisms of increased reflux

The mechanism of reflux changes between those with physiological and pathological amounts of reflux. There is an increasing role for alternative mechanisms other than TLOSR [45, 48-50]. Importantly patients often exhibit more than one of these mechanisms [49].

3.6.2 Reflux during lower oesophageal sphincter relaxations

The number of TLOSR associated events remain constant in GORD populations compared to healthy subjects, however the proportion associated with a reflux event rises [32, 38]. One study showed that this percentage increased from 36% in healthy individuals to 65% in those with erosive esophagitis [38].

3.6.3 Gastro-oesophageal pressure gradient

The positive pressures within the stomach and negative pressures within oesophagus lead to the creation of a gastro-oesophageal pressure gradient. Several studies have shown TLOSR associated with reflux have a greater gastro-oesophageal pressure gradient [39-41]. In GORD patients this gradient appears

to be influenced by body mass index (BMI), as a consequence of increased gastric pressures [42, 43]. Their gradients vary throughout the respiratory cycle, with the most negative pressures seen within inspiration. It has been shown that exaggerated ventilatory effort, such as occurs with exercise or in respiratory disease, can result in gastro-oesophageal reflux [44].

3.6.4 Oesophago-gastric junction integrity

A small percentage of the total amount of reflux is made up by events that overcome the OGJ, rather than during TLOSR. These events often occur during periods of increased gastro-oesophageal pressure gradients, such as straining [35, 40]. Scheffer et al. have shown in 31 reflux patients using pH and standard manometry, that 14% (+/- 3) of events were due to low LOS tone but these accounted for 28% (+/- 6%) of the acid exposure. The events due to a low LOS tone lasted much longer than those related to TLOSR (189 seconds (+/-23) versus 41(+/-1), $p<0.001$) [45]. The likely explanation for this is that the volume of reflux increases. Due to their prolonged nature they are of importance in the pathogenesis of oesophageal reflux disease.

Numerous factors have been implicated in reducing OGJ integrity. These include reduced lower oesophageal pressures, reduced extrinsic compression by the crural diaphragm, the intra-abdominal location of the LOS, compliance of the OGJ, integrity of the phrenoesophageal ligament and maintenance of the acute angle of His promoting the 'flap-valve' [26]. However, a major pathological occurrence, which frequently results in a number of the aforementioned factors, is presence of a hiatus hernia [50-53].

A hiatus hernia occurs due to upward movement of the lower oesophageal sphincter and separation from the crural diaphragm. This interferes with high pressure zone normally created when the components of the OGJ are superimposed on one another [46]. As such presence of a hiatus hernia reduces the OGJ pressure [47]. The reduced pressure then allows it to be overcome by

abrupt rises in the gastro-oesophageal pressure gradient, leading to increased amounts of reflux [37, 48, 49]

3.6.5 Impaired oesophageal clearance of reflux

Although the amount of refluxate entering the oesophagus is clearly crucial, the speed with which it is removed is also thought to be important. The first stage is mechanical clearance, whereby the volume is cleared from the oesophagus by secondary peristalsis. The second involves neutralisation of oesophageal acidity by saliva and secretion of bicarbonate into the lumen, and is known as chemical clearance [71, 72]. There is an increased frequency of peristaltic dysfunction in reflux disease, and it has been shown to associate with worsening damage to the oesophageal mucosa [50-52].

3.7 Conclusion

Gastro-oesophageal reflux is a physiological occurrence. It becomes pathological when it is associated with symptoms or results in organ damage, most commonly affecting the oesophagus. At this point it is referred to as Gastro-oesophageal Reflux Disease (GORD). Although increased amounts of reflux are of major importance, other factors are also implicated in the pathogenesis of GORD. A number of mechanisms have been highlighted as being responsible for the increase in reflux in GORD populations. The next chapter will explore if these have been shown to be similar in CF populations.

4 A literature review of effect of gastro-oesophageal reflux on CF lung disease

4.1 The prevalence of gastro-oesophageal reflux in adult CF populations

4.1.1 The influence of method and population characteristics on prevalence

The prevalence of gastro-oesophageal reflux has been reported in adult CF populations as between 62% - 91%, depending on the methodology used and the population studied (see table 4.1). The methods utilised to quantify reflux include measures derived from impedance studies, pH studies or symptoms. The population recruited may also vary depending on severity of the lung disease or frequency of symptoms within the cohort.

Table 4.1 Overview of the main gastro-oesophageal reflux studies in CF adults

Author	Study methods			cohort		Prevalence
	Number of patients	Method	Parameters	FEV1 (mean(IQR))	Recruitment criteria	
Button et al [54]	11	pH only	Not stated	31.3% (7.8)	Pre-transplant assessment	91%
Blondeau et al [55]	23	pH-MII	Combination	2.6L (1.1)	Random from CF outpatient clinic	87%
Ledson et al [56]	10	24-hour pH	DeMeester score	50.1% (25-100)	Reflux symptoms	80%
Pauwels et al [57]	42	pH-MII	Combination	58% (30-78)	Random from CF outpatient clinic	67%
Sabati et al [58]	201	Validated questionnaire	Symptoms	68.9% (NS)	Any patient	63%

4.1.2 Reflux prevalence using impedance

Combined pH-impedance is the current gold-standard for measurement of reflux. From the impedance measures prevalence is often calculated using the total number of reflux events in a 24-hour period. The two studies in adult CF populations have reported a prevalence of 67% (n=42) and 87% (n=23) [55, 57]. However, both calculated prevalence based on a combination of both impedance and pH measures. A major advantage of pH-impedance is that it can be performed on the patient's usual medications. This is particularly useful when exploring the relationship between reflux and lung disease, as performing the study on the patient's usual medications will give a more representative measure of daily reflux. However, the protocol for both reported studies was that the patient had to be off acid suppression [55, 57].

4.1.3 Reflux measures using pH

Prevalence has been reported using pH measures in several adult CF studies. Button et al reported a prevalence of 91% in a cohort with advanced lung disease awaiting transplant assessment, although it was not reported what parameters were exactly used [54]. In another study Ledson et al. reported a prevalence of 80% in a symptomatic population drawn from an outpatient CF clinic, using the DeMeester score [56]. DiMango et al. report a prevalence of 62% in an asymptomatic outpatient cohort not taking antacids, using an endpoint of combination of pH measures [59]. No study has reported prevalence based solely on acid exposure. It must be highlighted that all three studies involved small samples sizes (<11).

4.1.4 Reflux prevalence using symptoms

Prevalence can be assessed using typical symptoms of reflux, using either clinical history or a validated questionnaire. Sabati et al. used a validated questionnaire for reflux symptoms and found a prevalence of 63% [58]. However, assessing prevalence of increased reflux using symptoms has its limitations. Symptoms are not solely generated based on the amount of reflux,

but are affected by other factors including oesophageal sensitivity and clearance of refluxate from the oesophagus[47]. This is supported in CF populations, where previous studies have shown that increased reflux often fails to generate typical symptoms [54, 55, 57, 59].

4.2 Mechanisms of increased gastro-oesophageal reflux in CF

4.2.1 Overview

The increased reflux in cystic fibrosis is most likely multifactorial with a number of possible mechanisms (see Figure 4.2). For some there is supporting evidence, whereas others are almost entirely hypothetical. Importantly no mechanism has been conclusively proven.

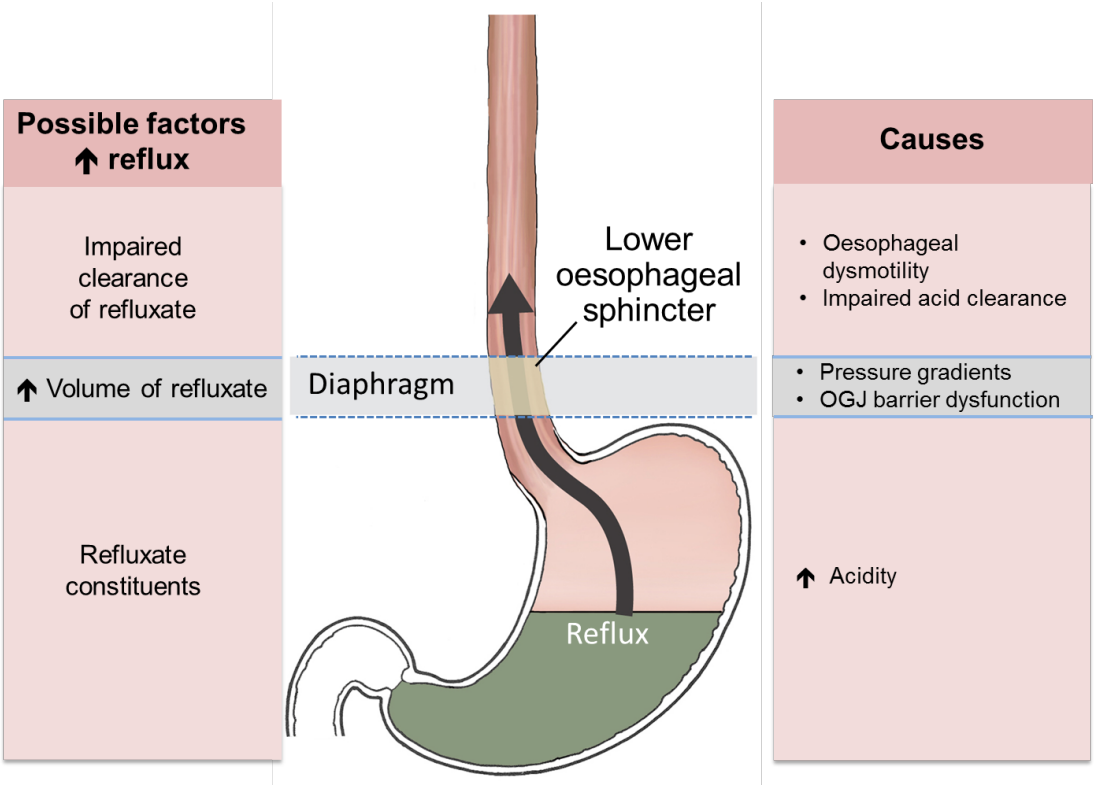


Figure 4.2 Proposed factors leading to the increased gastro-oesophageal reflux seen in CF.

The mechanisms can be divided into factors that increase the amount of reflux entering the oesophagus and those that impaired its clearance. Composition of refluxate is also potentially important.

4.2.2 Factors increasing the amounts of reflux

There is currently no way to quantify volume of reflux. As such total reflux events and total acid exposure are used as surrogates. However how they relate to each other is unknown.

4.2.3 Transient lower oesophageal relaxations

Most reflux events do not overcome the OGJ. They in fact occur during periods when the OGJ relaxes to allow gas venting – known as transient lower oesophageal relaxations (TLOSR). In healthy individuals these are responsible for almost the entirety of physiological reflux (See Chapter 3). In GORD these do not increase in number compared to healthy subjects, but a greater proportion are associated with reflux events [60]. It is thought this occurs as a consequence of a greater gastro-oesophageal pressure gradient [61, 62].

A small study of twelve adult CF patients found similar findings to those previously noted within GORD patients. Using high resolution manometry impedance (HRM-MII) the incidence TLOSR was similar to healthy subjects. However, reflux was associated with 90% of TLOSRs in adult CF patients, compared to only 42% in healthy subjects [63].

4.2.4 Oesophago-gastric junction barrier impairment

Events that overcome the OGJ, rather than occur during a TLOSR, are believed to be of greater volume. This is supported by a previous study that has shown that these events are responsible for a disproportionate rise in acid exposure compared to TLOSR (See Chapter 3). Pauwels et al. have described in twelve CF adults using HRM that OGJ pressures were significantly lower than healthy volunteers in the pre-prandial and post-prandial periods [63]. It has been

described in other studies of CF patients but using short periods of manometry performed prior to a reflux study [56, 64].

Frequently the cause of OGJ dysfunction in non-CF GORD patients is a hiatus hernia [51, 52]. It has been proposed that respiratory disease may lead to a hiatus hernia, through altering the position of the crural diaphragm by a reduction in volume or hyperinflation of the lungs[65]. This is supported by a number of studies that have demonstrated a high prevalence of hiatus hernia in respiratory disease, such as pulmonary fibrosis and chronic obstructive pulmonary disease, often using computed tomography (CT) [66-68]. This could explain some of the increased reflux seen in various respiratory diseases. This has not been studied in depth in CF. However, one small study noted that in two of twelve CF subjects a hiatus hernia was seen on HRM [63]. It must be noted that there are differences between the traditional endoscopic diagnosis, and other methods such as CT and HRM [69, 70].

Medications may also increase reflux. Some commonly used in CF have been linked to reflux. β2 agonists, methylxanthines and anti-cholinergics have been shown to reduce OGJ pressure [71-74]. Methylxanthines have also been shown to increase acid exposure when measured by pH studies [73]. However, the effect in CF patients has not been specifically investigated.

4.2.5 The gastro-oesophageal pressure gradient

The gastro-oesophageal pressure gradient is thought to be an important determinant of the amount of reflux in GORD patients (Chapter 3). It is believed that it may also play a role in the increased amount of reflux seen in a wide range of respiratory diseases. The gastro-oesophageal pressure gradient is created by the difference between positive pressures seen within the stomach and negative pressures within the oesophagus. The oesophageal pressures are reflective of the thoracic pressures, and studies have shown that these are significantly lower in lung disease in particular during inspiration [63, 75]. A

recent study has suggested that restrictive, rather than obstructive lung disease, creates a far greater gastro-oesophageal pressure gradient [76].

One small study in CF patients (n=12) a far larger gastro-oesophageal pressure gradient was observed than in healthy volunteers. This was shown to be driven by more negative oesophageal inspiratory pressures. These reflect intrathoracic pressure changes generated by lung disease. However, only a correlation was noted between proximal reflux events and inspiratory pressures [63].

Additional factors that may alter the gastro-oesophageal pressure gradient have been examined, but as yet their impact is inconclusive. Cough has the potential to acutely alter the gastro-oesophageal pressure gradient [77]. However, a temporal relationship between a cough and then a reflux episode – the cough-reflux sequence – was rare in both CF adults and children. In both studies only a very small amount of reflux could potentially be attributed to cough [55, 78]. Chest physiotherapy has also been implicated. Button showed that postural drainage worsens reflux [79]. However, subsequent studies in CF and non-CF lung disease have contradicted this [80, 81].

It has also been proposed that other factors may increase gastric pressure in CF, in particular increased gastric fluid volumes due to impaired gastric emptying and enteral feeding. In certain individuals these may increase reflux. Enteral feeding has been shown to increase reflux symptoms in non-CF patients, but has not been investigated in CF patients [82]. Delayed gastric emptying has been described in seven of twenty enrolled (35%) in a study of adult CF subjects, but no correlation was noted with any measures of reflux [57]. Similar results have been seen in paediatric CF populations [83]. It is also of note that delayed gastric emptying is not a universal finding in all CF studies[84].

4.2.6 Impaired oesophageal clearance

Although the amount of refluxate entering the oesophagus is clearly crucial, the speed with which it is removed is also thought to be important. The first stage is mechanical clearance, whereby the volume is cleared from the oesophagus by

secondary peristalsis. The second involves neutralisation of oesophageal acidity by saliva and secretion of bicarbonate into the lumen, and is known as chemical clearance[85, 86].

Several small studies have noted that peristalsis is impaired in some CF patients using standard and high resolution manometry [56, 63]. No study in CF has examined if abnormal peristalsis affects clearance of reflux from the oesophagus. It has been demonstrated in CF children that chemical clearance is delayed when compared to controls with symptomatic reflux [87]. This is in keeping with studies supporting an overall increase in acidity in the upper gastrointestinal tract presumably as a consequence of impaired CFTR transport of bicarbonate [88].

4.3 The relationship between gastro-oesophageal reflux and CF lung disease

4.3.1 Lung disease causing increased reflux

Increased reflux has been described using both impedance and pH measures in a number of respiratory conditions: interstitial lung disease, chronic cough, cystic fibrosis, chronic obstructive pulmonary disease, bronchiectasis, asthma and lung transplant recipients [55, 57, 89-94]. A logical explanation for this is that lung disease is a cause of increased gastro-oesophageal reflux. As discussed above this maybe a consequence of an altered gastro-oesophageal pressure gradient, an increased prevalence of hiatus hernia or use of medications that lead to increased reflux (see section 4.2). It is then possible that the increased reflux detected may exert a negative effect upon CF lung disease. As such a bi-directional relationship exists.

4.3.2 The effect of gastro-oesophageal reflux on CF lung disease

Data from the European Registry of cystic fibrosis shows that the clinical diagnosis of GORD predicts a 5-10% worse lung function [100]. However, when measured objectively in two studies in adults using pH-MII, no link was shown

between reflux parameters and baseline lung function. However, no comparison was made with longitudinal change in lung function and number of exacerbations, which are important endpoints for assessing stability of CF lung disease [4, 7]. In contrast a study in paediatric patients showed that baseline lung function correlated both with total reflux events ($r = -0.474$, $p = 0.009$) as well as with non-acid events ($r = -0.397$, $p = 0.03$). This might reflect that the relatively less diseased paediatric lungs allow an effect of reflux to be more easily observed.

Two observational studies have examined the effect of fundoplication – surgical reinforcement of the OGJ known to reduce the amount of reflux. One retrospective analysis from 48 patients, both adults and children, showed a post-operative reduction in pulmonary exacerbations requiring antibiotics as well as an initial increase in FEV1 at one year. This was not sustained at two years, but the preceding decline did not recur [17]. In six patients with CF undergoing fundoplication for refractory cough, a reduction in number of exacerbations and a small, but statistically significant, improvement in lung function was demonstrated [18].

There are two potential mechanisms by which reflux is thought to exert an effect on CF lung disease. The most likely is reflux aspiration, where gastric contents extend beyond the oesophagus and are inhaled into the lungs. The alternative is neuronal cross-talk. This is thought to occur as a consequence of shared innervation of the airways and upper gastrointestinal tract, with afferent signals “crossing over” as they travel to the brain. As such stimuli within the oesophagus may trigger an efferent response within the respiratory tract, such as a cough or episode of bronchoconstriction[65].

4.3.3 Reflux aspiration

The characteristics of reflux in CF intuitively favour aspiration. There are significantly more episodes of proximal reflux. In normal subjects 22% of reflux episodes are proximal with a median of 9 per 24 hours [43]. In contrast in adult

CF patients, a median of 22 episodes per 24 hours extended proximally [55]. Almost half of all episodes detected have proximal extent [63]. Logically the higher the reflux extends the greater the likelihood of extra-oesophageal extension.

In CF patients there are also more episodes of reflux in the supine position, something that rarely occurs in physiological reflux [54]. This is important because the majority of the time a person spends supine during a 24-hour period is whilst they are asleep. It has been shown that sleeping depresses protective mechanisms that reduce the risk of aspiration [95]. In addition, reflux can be asymptomatic, so it may go unrecognized, and potentially untreated, for large periods of time [59]. This may further increase the risk of aspiration.

In many studies the levels of pepsin and bile acids are tested within respiratory samples [96-98]. The premise is that pepsin and bile acids are of gastro-intestinal origin and increased amounts within the lungs is consistent with reflux aspiration. However, there are significant concerns about the precision of the assays used and the accuracy of these tests for the diagnosis of reflux aspiration [65, 99-101]. There is variability in the reported values in respiratory samples obtained from various respiratory diseases and healthy controls, and the complex nature of these samples has been shown to interfere with retrieval of pepsin [99]. As such studies reporting with these should be interpreted with caution.

As a consequence of the lack of a well-validated measure there are limitations in the understanding of reflux aspiration. It is unclear how pH-impedance measures of reflux relate to reflux aspiration. The mechanisms responsible for reflux aspiration are also poorly understood. It is thought that the integrity of laryngeal protective mechanisms may influence the amount of reflux aspiration. Clearly these must be overcome to allow passage of refluxate into the airways.

However little, if any, knowledge exists about the actual contribution of these upper airway protective mechanisms [65].

A different study reported on reflux aspiration using an alternative, and novel, approach in 11 CF subjects. Reflux aspiration was assessed using a tracheal pH monitor. Simultaneous oesophageal pH monitoring was also performed. In four subjects there was evidence of tracheal acidification, suggestive of reflux aspiration. All of those with tracheal acidification had raised DeMeester scores [102]. However, it is unknown if the tracheal pH monitor has the potential to compromise upper airway protective mechanisms responsible for preventing aspiration.

4.3.4 Reflux aspiration and lung inflammation

Pepsin has been detected in the BAL of paediatric CF patients at levels above that seen in healthy volunteers. The levels of pepsin showed a moderate correlation with increased levels of IL-8, a measure of neutrophilic lung inflammation ($r=0.48$, $p=0.04$) [96]. Bile acids have been detected in sputum, at levels above that of healthy volunteers and correlate with increased neutrophil elastase, another measure of lung inflammation ($r=0.60$, $p=0.002$). Interestingly bile acids were also associated with worse lung function ($r=-0.53$, $p=<0.01$) and increased antibiotic usage ($r=0.58$, $p=0.009$) [98]. This could suggest that reflux aspiration has the potential to be pro-inflammatory and result in worse respiratory outcomes. Alternatively, those with worse lung function and more exacerbations may have greater amounts of reflux, which in turn may increase the amount of reflux aspiration.

In vitro studies provide further support of the detrimental effect of gastric contents on the respiratory tract. Exposure of gastric fluid has been shown to provoke increased levels of IL-8 in both human and CF primary bronchial epithelial cells [103, 104]. Other in vitro experiments have examined the effect of specific components of gastric fluid, namely bile acids and pepsin, on the

lungs. The exposure of bile acids to human alveolar and bronchial cells resulted in increased levels of pro-inflammatory and pro-fibrotic cellular mediators including Prostaglandin E2 (PGE-2) and Transforming Growth Factor Beta 1 TGF-B1 [105-107]. Exposure of pepsin to bronchial epithelial cells also caused significant inflammation with raised IL-6 and IL-8 levels, which was more marked at lower pH [108]. The effect of acidity on the lungs appears more complex. Exposure of acid has been shown to lead to inflammation in bronchial epithelial cells [109]. However, the gastric fluid in patients taking PPI with a higher pH induces a far greater inflammatory reaction than those off PPI [103, 104].

4.3.5 Reflux aspiration and the effect on lung microbiology

Reflux aspiration may exert an effect on the microbiology of the CF lung. Observational data from a retrospective paediatric cohort have demonstrated that CF patients with a clinical diagnosis of GORD acquire *Pseudomonas aeruginosa* and *Staphylococcus aureus* earlier[110]. Again in a paediatric cohort significantly more total reflux events were seen in subjects with *Pseudomonas aeruginosa* than those without. Furthermore, this difference was also seen for non-acid but not acid reflux events [111]. This suggests that reflux, in particular if the pH has been supressed by PPI, increases the risk of *Pseudomonas aeruginosa* acquisition. This is in keeping with a previous small randomized control study in CF patients which showed a trend toward earlier and more frequent exacerbations in those receiving PPI compared to placebo [59]. Another study in support found that PPI prescription was associated with both increased annual decline in lung function and frequency of exacerbation in paediatric CF patients, during a 5 year longitudinal follow up[112].

Acid suppression has also been shown to alter the microbiology and microbiome of gastric fluid [113, 114]. Of particular note, *Staphylococcus aureus* was found more commonly in the routine culture of gastric fluid of subjects taking PPI [114]. Using 16S rRNA gene sequencing there was a non-significant increase in the relative abundance of both *Pseudomonas aeruginosa* and

Staphylococcus aureus [113]. A study in CF patients has also identified a number of respiratory pathogens, including *Pseudomonas aeruginosa*, in gastric fluid using routine culture. Most of the subjects were on acid suppression medications [115]. Another study in CF has interestingly demonstrated that *Mycobacterium abscessus* can also be cultured in gastric fluid [116]. These findings potentially suggest that gastric fluid, in particular if acid suppressed, may act as a reservoir for respiratory pathogens which can be inoculated into the lungs via reflux aspiration.

Other studies have suggested alternative mechanisms by which reflux may alter lung microbiology. An *in vitro* study demonstrated that when bile acids were added to growth media they led to a significant increase in biofilm formation of cultured CF pathogens including *Burkholderia cepacia complex*, *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*[117]. In another study the sputum microbiome obtained by sequencing the 16s ribosomal ribonucleic acid genes was compared between subjects with and without bile detected in sputum. Bile acids were detected using a mass spectrometry technique. The bacterial diversity was significantly reduced between those with bile acids in sputum, compared to those without. There was also a dominance of respiratory pathogens in three of five samples with bile acids: *Stenotrophomonas maltophilia, Ralstonia Mannolytica* and *Pseudomonas aeruginosa* [105]. As such introduction of bile acids into the CF airway by reflux aspiration may modulate the microbiology.

4.3.6 Neuronal cross-talk

Neuronal cross-talk is believed to effect reflex arcs within the respiratory tract – mainly cough and bronchoconstriction. There is limited research examining if this is an important mechanism in CF patients. Several studies support this mechanism in non-CF patients. When acid is instilled into the distal oesophagus it causes increased cough hypersensitivity and bronchial hyperactivity in chronic cough and asthma patients [118-120].

In patients with chronic cough a temporal relationship between cough and reflux has been demonstrated, importantly with the reflux event confined to the distal oesophagus and in most instances the reflux events preceding the cough [121]. This temporal relationship with reflux preceding cough has also been demonstrated in adult and paediatric CF populations [55, 78]. In addition, in adult CF patients there was a significant correlation between number of coughs and oesophageal acid exposure over 24 hours [55]. These studies use manometry to detect cough rather than acoustic cough recording, which is often considered to be better validated [65]. However, in a previous study manometry has been shown to perform well against acoustic cough recording [122]. No studies have attempted to examine the neuronal effect of reflux on bronchoconstriction in CF populations.

4.4 Alternative mechanisms linking increased reflux with CF lung disease severity

An additional consideration is that a factor may exist that impacts both on the quantity of reflux, as well as the severity of the lung disease. Potential factors may include body mass index (BMI), which when raised is known to influence the amount of reflux in non-CF patients and can also affect ventilation [123]. However, it is important to note in CF patients that nutritional status, and therefore being underweight, has a direct relationship with survival and has correlated closely with lung function [124, 125]. Alternatively, certain interventions that are used more frequently in CF patients with more severe lung disease such as airway clearance techniques or use of enteral feeding, have been previously linked to increased reflux [79, 82].

4.5 Conclusion

Gastro-oesophageal is highly prevalent in CF and the causes are multi-factorial. There is also potentially a complex bidirectional relationship existing whereby lung disease drives increased reflux, and then this increased reflux exerts a negative effect on the lung disease. Further work is required to provide definitive proof supporting this relationship. Current evidence would seem to

suggest that a number of mechanisms may be responsible for both how the lung disease may lead to increased reflux, as well as how reflux might affect lung disease.

5 Study hypotheses, aims and objectives

5.1 Hypothesis

The increased gastro-oesophageal reflux seen in CF patients exerts a negative effect on lung disease.

5.2 Aims

To determine if a relationship exists between gastro-oesophageal reflux and CF lung disease.

5.3 Objectives

The study's objectives are:

1) To evaluate the prevalence of reflux in an adult CF population

2) To evaluate if characteristics of reflux support proposed mechanisms by which reflux may affect lung disease

3) To evaluate if oesophageal gastric junction (OGJ) function and oesophageal motility affect reflux

4) To evaluate factors that could affect the amount of reflux

5) To evaluate the effect of reflux on CF lung disease

6 Methods

6.1 Study overview

Forty-one CF patients completed a cross-sectional, observational, single-centre study. Participants attended for a single research visit as described below in Section 6.4.1. For all subjects this included oesophageal high-resolution manometry (HRM) and 24 hour combined pH impedance. At the research visit a large data set was compiled that allowed a subsequent number of separate analyses to be performed. Twenty healthy volunteers were enrolled to provide induced sputum. The study was conducted between February 2016 and September 2017.

6.2 Participants

6.2.1 CF patients

The study was advertised using posters in the CF outpatient department (see appendix 1) and on social media (Facebook ®). Participants were approached either whilst attending outpatient appointments or during an inpatient stay. Presence of reflux symptoms was not a requirement for the study. Patients undergoing clinical investigation for reflux could be included. Pre-specified eligibility criteria were:

Inclusion criteria

- Patients with a confirmed diagnosis of cystic fibrosis on genetic testing and/or sweat testing with typical phenotypic features
- Aged over 18 years
- Provision of signed, written and dated informed consent, prior to any study specific visits

Exclusion criteria

- Defined clinically unstable by a physician
- Pregnant
- Previous lung transplantation
- Previous fundoplication
- Contraindication to HRM
    - Unexplained swallowing difficulties without preceding oesophago-gastro-duodenoscopy (OGD)
    - Presence of an oesophageal pouch on imaging or OGD
    - Liver cirrhosis without an OGD to exclude varices

If interested, all eligible participants were provided with a patient information sheet (see appendix 2) and at least 48 hours to consider. Further discussion then took place either by telephone or at their next clinic appointment. If agreeable a study visit was arranged.

6.2.2 Healthy volunteers

Healthy volunteers were identified from staff members. If interested a participant information leaflet was provided (see appendix 3). After 48 hours they were contacted for a final decision regarding participating. If agreeable a study visit was booked.

Inclusion criteria:

- Aged over 18 years
- Provision of signed, written and dated informed consent, prior to any study specific visits.

Exclusion criteria:

- No respiratory or gastro-intestinal past medical history

- Any past medical history or medications suspected of altering gastro-oesophageal reflux
- Current smoker
- Spirometry demonstrating percentage forced expiratory volume in 1 second (FEV1%) <80% predicted
- Ratio of forced expiratory volume in 1 second (FEV1) to forced vital capacity (FVC) of < 0.7

6.3 Ethical considerations

Ethical approval was granted by the Greater Manchester West NHS Ethics Committee (reference 15/NW/0655) (see appendix 6). All participants provided written consent before enrollment.

6.4 Study visit

6.4.1 CF patients

The first visit lasted approximately 2 hours and entailed:

- **Height and weight**
- **Assessment of clinical stability (see section 6.5)**
- **Information captured from the medical notes**
    - **List of co-morbidities and medications**
    - **Two years of retrospective data recorded for:**
        - **Number of intravenous (IV) antibiotic episodes**
        - **Duration of IV antibiotic episodes**
        - **FEV1%**
- **Patient reported outcome measures**
    - **Reflux questionnaire (see section 6.6.1)**
    - **Bowel habit questionnaire (see section 6.6.2)**

- **Spirometry (see section 6.7)**
- **Venous blood sample**
    - **HbAIc**
- **Sputum sample stored for proteomic analysis (see section 6.8.1)**
- **Oesophageal high resolution manometry (HRM) (see section 6.9)**
- **24 hour combined pH-impedance (see section 6.10)**

6.4.2 Study visit for healthy volunteers

Healthy controls underwent a single study visit. If they met criteria they underwent the following:

- **Height**
- **Weight**
- **Medical history**
- **Reflux questionnaires (see section 6.6.1)**
- **Spirometry (see section 6.7)**
- **Induced sputum (see section 6.8.2)**

6.5 Assessment of clinical stability

Clinical stability was assessed by a brief clinical examination and review of baseline observations (heart rate, blood pressure, temperature, respiratory rate and oxygen saturations).

6.6 Patient reported outcome measures

6.6.1 Gastro-oesophageal reflux symptoms

The Reflux Symptom Questionnaire, 7-day recall (RESQ-7) is a validated patient reported outcome measure for use in patients with GORD who are partial responders to proton pump inhibitor (PPI) therapy [126]. It is available in two versions: an electronic version to be completed daily; and paper version to be

completed weekly [126, 127]. The paper version was deemed to be the most appropriate for this study (see appendix 7 for copy of questionnaire).

The RESQ-7 was developed by a process of exploratory interviews and followed by refinement of an initial draft by a panel of experts. By principal component analysis these were organized into four domains: regurgitation, heartburn, burping and extra-oesophageal symptoms – which included difficulty swallowing, hoarse voice and cough.

Each symptom is scored (0-5) for severity (absent-severe) and frequency (absent-daily). The average is calculated for each domain. This was then validated in a population with GORD partially responsive to PPI [126]. It has been used subsequently in studies looking at gender difference in partial responders for PPI and in evaluating the effect of lesogaberan, a peripherally selective $GABA_B$ agonist [128, 129].

Permission for its use in this study has been granted permission by RWS life sciences, on behalf of Astra Zeneca.

6.6.2 Bowel symptoms

At the time of study design there was no current validated patient report outcome measure for bowel disease in CF. There is significant overlap in the symptoms experienced by irritable bowel syndrome (IBS) patients, such as diarrhoea, constipation, abdominal pain and distension [130, 131]. There are a number of patient reported outcome measures that have been validated in IBS[132].

To capture these symptoms the IBS symptom severity score (IBS-SSS) was used. This is a simple and quick scoring system using visual analogue scales, to capture abdominal pain, distension, satisfaction with bowel habit and inference with life. It was validated in 141 patients and 40 healthy controls[133].

Permission for its use in this study has been granted by Professor PJ Whorwell, Manchester University Hospitals NHS Foundation Trust (see appendix 8 for copy of questionnaire).

6.7 Spirometry

Spirometry was performed using the Easy on-PC (NDD, Zurich) system. This is a fully-integrated PC-driven spirometer. It comprises a disposable mouthpiece, an ultrasound flow sensor and a PC installed with an integrated software platform, EasyOne Connect (NDD, Zurich). The system has been shown to retain inhalation and exhalation volume accuracy of better than three percent over at least four years [134].

Calibration was performed in the morning before every study visit. Spirometry was performed as per ATS/ERS guidelines [135]. Predicted values were calculated for lung function measures by the software using the 2012 Global Lung Function Initiative reference equations [136]. The results were stored within an electronic database — the EasyWare Pro™ (NDD, Zurich).

6.8 Sputum sampling for storage for proteomic analysis

6.8.1 Sputum sampling for CF patients

Sputum was expectorated spontaneously into sterile universal containers. The samples were transferred on ice to our laboratory within one hour for processing. This involved removing the salivary component from the sample. The mucoid component was then stored in a one ml Nalgene® storage tube and frozen at -30^{o}C.

6.8.2 Sputum induction for healthy volunteer

Sputum induction is a safe and widely used procedure [137]. It allows sampling of lower airway secretions from subjects who do not produce sputum spontaneously [138]. It has been used in various respiratory diseases, including

asthma and chronic obstructive pulmonary disease, as well as in healthy volunteers [139-141].

There are a number of described methods for sputum induction. However, at present, there is no accepted superior method. As such decisions were made about the key methodological factors including strength of the hypertonic saline, nebuliser device and use of adjunct physiotherapy. This was based on available evidence and expertise of the respiratory physiotherapists based within the Manchester Adult CF Centre.

The technique chosen was based on the experience of Langridge et al.. They used chest physiotherapy to help expectorate sputum (63%), followed by nebulised 7% hypertonic saline if unsuccessful (33%). They achieved a sputum sample in 353/364 (97%) of patients with pulmonary aspergillosis [142]. They avoided hypertonic saline where possible based on the risk of bronchospasm in patients with respiratory disease [139, 140].

In our study because the cohort was of healthy subjects 7% hypertonic saline was used in all, with two to three breaks during the period of nebulisation for physiotherapy techniques to assist in sputum clearance. It was felt routine pre-treatment with a bronchodilator was not required, but it would be administered if there was a greater than 10% decline from baseline FEV1.

Previous guidelines have emphasised the importance of the type of nebuliser used for sputum induction and recommended an ultrasonic device [138]. However jet nebulization has been shown to be comparable in terms of aerolised output (Khatri 2001)[143]. Ultrasonic nebulisers are reusable and require cleaning between subjects whereas disposable systems, such as the Pari Sprint™, are single use. As such due to familiarity and perceived hygiene issues with the ultrasonic devices available, the Pari Sprint™ jet nebulisation system was used.

6.9 High resolution manometry

6.9.1 Background of procedure

Oesophageal high resolution manometry (HRM) is a technique that allows continuous pressure recordings throughout the oesophagus, from the upper oesophageal sphincter (UOS) to below the oesophago-gastric junction (OGJ). This is achieved by the manometry catheter having regularly spaced sensors along its entire length [144]. This can be visualised in real-time as a Clouse plot: time is plotted on the x-axis; oesophageal position on the y-axis; with pressure amplitude expressed as colour changes [145] (see figure 6.1).

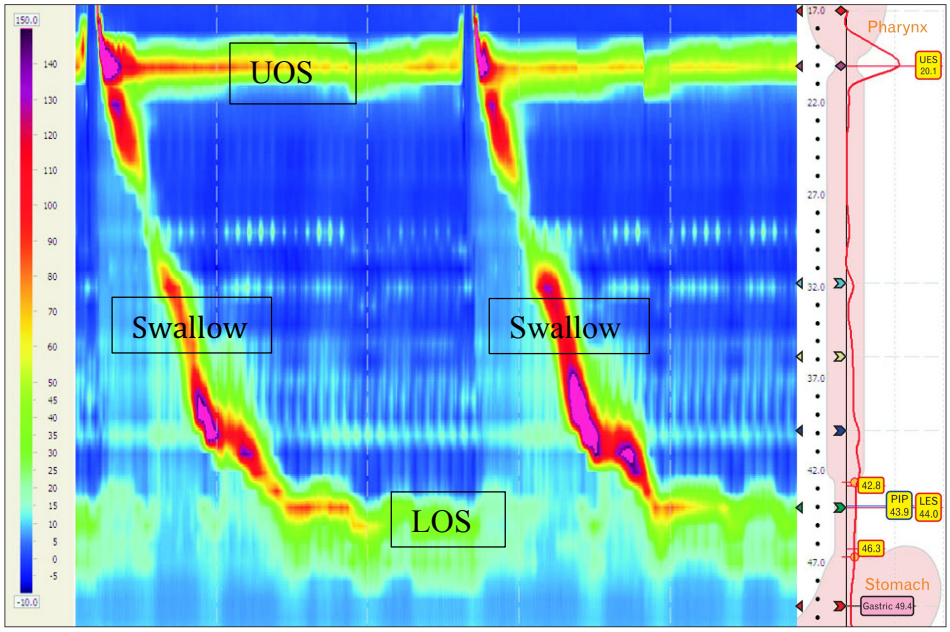


Figure 6.1 The Clouse plot and the appearance of oesophageal swallows
Abbreviations: UOS (upper oesophageal sphincter); LOS (lower oesophageal sphincter). Reprinted and adapted with permission from the J Neurogastroenterol Motil 2016;22:6-13 [144].

The Clouse plot allows identification and localisation of oesophageal anatomy (see figure 6.1). This knowledge is an essential prerequisite for placement of the pH-impedance catheter for reflux monitoring at the required five cm above the OGJ. It is also used as the gold-standard for assessing oesophageal motor function. [146].

6.9.2 Equipment

A water-perfused HRM system was used with a Single-use 24 channel 12F catheter (Mui Scientific). In addition to the catheter a water-pump, data logger and standard PC loaded with the appropriate software were required.

6.9.3 Study procedure

A qualified GI physiologist performed all studies. All visits were performed between 10am and midday. The patients fasted from midnight, but were allowed small amounts of water and could take medications. All patients were offered local anesthesia to the nose and throat. The catheter was lubricated and then inserted intranasally whilst the patient was sitting. The passage of the catheter was achieved by the patient taking continuous sips of water. It was passed a sufficient distance that allowed both the upper and lower oesophageal sphincter to be visualized and the distal sensor to be in the stomach. Slight adjustments were made to position the circumferential sensors over the OGJ. Once in the correct position the catheter was taped in place. The patient was then placed in the semi-recumbent position for the study.

6.9.4 Analysis using high resolution manometry

A recent classification system has been proposed by an international consensus working group specifically for evaluation of motor function in GORD [146]. The first two steps were followed (see **Table 6.1**).

Table 6.1 Classification of oesophageal motor function in GORD

		Possible diagnoses
Step 1	**Evaluation of OGJ**	Intact
		Hypotensive
		Hiatus hernia
		Both hypotensive & hiatus hernia
Step 2	**Evaluate oesophageal body**	Intact
		Fragmented peristalsis
		Ineffective oesophageal motility
		Absent contractility

As proposed by Gyawali et al. [146].

6.9.5 Evaluation of the oesophago-gastric junction

A detailed evaluation of the OGJ can be performed using HRM. It has been used to describe the relationship between the LOS and crural diaphragm and as such categorised into three distinct morphological subtypes (see **Table 6.2**)[36]. These subtypes can be used to accurately predict the presence of a hiatus hernia, when compared to endoscopy and barium swallow [70].

Table 6.2 Classification of oesophago-gastric junction morphology

		Diagnostic criteria – Degree of lower oesophageal sphincter and crural diaphragm separation
Type1	Normal	Non discernible
Type 2	Hiatus Hernia	There is minimal, but discernible separation: >1cm and <2cm
Type 3a		Separation was greater than 2cm with the pressure inversion point proximal to the crural diaphragm
Type3b		Separation was greater than 2 cm with the pressure inversion point proximal to the lower oesophageal sphincter

As proposed by Pandolfino et al. [36].

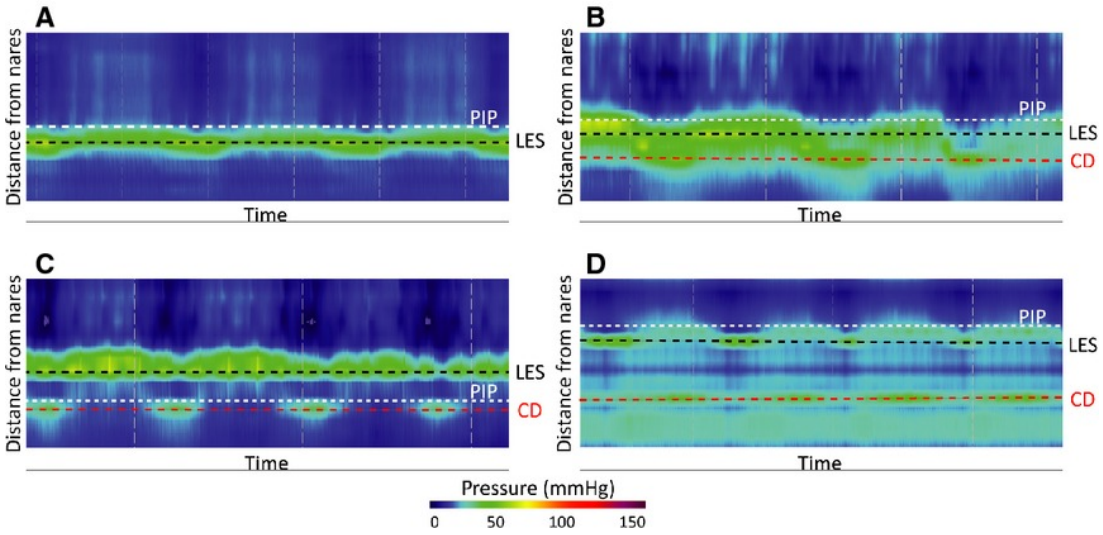


Figure 6.2 High resolution manometry appearances of hiatus hernia. In the absence of a hiatus hernia the lower oesophageal sphincter (LES) and crural diaphragm are indistinguishable from each other (A). As the degree of separation of the LOS and crural diaphragm increases consistent with a hiatus hernia this is visible on the HRM tracing (A-D). *Reprinted and adapted with permission from the J Neurogastroenterol Motil 2015;27:293-299 [70]*

Evaluation of OGJ function can be performed using HRM. However, at present there is no widely accepted metric that can be recommended [146]. Until recently the mean lower oesophageal sphincter pressure (LOSP) was often used. However in reality this metric measures the pressure within the entire OGJ, as such it is referred to as the OGJ mean pressure (OGJ-MP).

The OGJ-MP is calculated over a resting period – a period of tidal breathing where there are no swallows. However, there is significant temporal variation in OGJ pressure throughout the respiratory cycle, as a consequence of cyclical crural diaphragm contracting superimposed on basal LOS pressure. As such OGJ-MP is a composite measure of both these elements.

An alternative metric is the OGJ contractile integral (OGJ-CI), which measures contractile vigour as generated by contraction of the crural diaphragm. It can

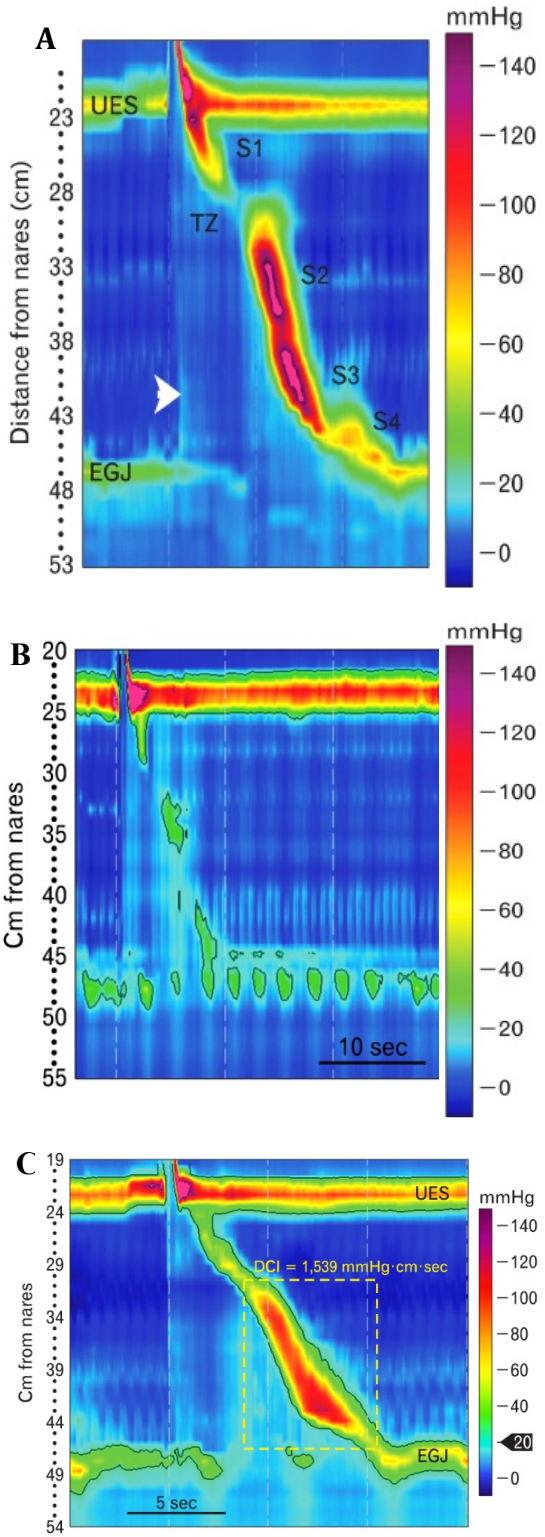
be calculated using most HRM software packages. The mean pressure change is calculated above a defined threshold - set at 2mmHg above gastric pressure - over three respiratory cycles. It creates an aggregate of this pressure over the length of the OGJ, which is then standardised to yield a metric in units of mmHg.cm [147] (see section 8.1).

All subjects who had tolerated HRM had a detailed evaluation of the OGJ using the classification proposed by Gyawali et al.[146]. The OGJ borders were localised as abrupt pressure increases relative to intra-oesophageal and intra-gastric pressure. Previous studies had assessed morphology over a five minute period or a 30 second period when the patient was asked not to swallow [36, 70]. However, many subjects struggled to tolerate the procedure. As such periods of at least 10 seconds without any swallows either before or after the 10 wet swallows were analysed. The morphology was then classified as described above. Automated software was used to calculate OGJ-MP and OGJ-CI.

6.9.6 Evaluation of the oesophageal body

Oesophageal body motility was assessed using a standard method of 10 swallows of five millilitres of water. At least 20 seconds was left between swallows [148]. A qualified gastro-intestinal physiologist performed all procedures and interpretation the manometry trace.

The manometry traces were interpreted using the Chicago classification version 3. This is a classification devised by an international HRM working group of experts [149]. Each swallow was analysed individually for contractile vigour and contractile pattern based mainly on two different metrics: presence of breaks (>5cm) in the 20mmHg isobaric contour and distal contractile integral (DCI) (see Figure 6.2).



(A) This HRM section represent a normal swallow passing between the upper oesophageal sphincter (UES) and oesophageal gastric junction (EGJ). S1 represents striated muscle. Striated muscle changes to smooth muscle at the transition zone (TZ). The smooth muscle portion (S2 and S3) eventually meets the OGJ at S4.

(B) The black line surrounding the swallow is the 20mmHg isobaric pressure line. Fragmented peristalsis is classified by gaps in this isobaric pressure line within the smooth muscle of the oesophagus. Only those greater than 5cm are now considered significant.

(C) The distal contractile integral (DCI) is a measure of contraction vigour. It is calculated by measuring all the contraction associated with the S2 and S3 smooth muscle segments.

Figure 6.3 Overview of high resolution manometry metrics pertaining to the analysis of a 5ml swallow of water. *Reprinted and adapted with permission from the J Neurogastroenterol Motil 2013;19:281-294 [150].*

The DCI informs on contraction vigour [149]. An area around the S2 and S3 parts of the swallow was selected, which corresponded to the proximal and distal parts of the smooth muscle. Within this area the DCI was calculated, by an algorithm within the software, that added all pressures greater than 20mmHg over the entire length and duration of these segments [150].

The presence of peristaltic breaks informed on the contractile pattern, with an additional measure of distal latency (DL) used to assess if the contractions occur prematurely [149]. The DL was measured as the duration of time from the start of swallow-induced UOS opening to the arrival of peristalsis at the contractile deceleration point (CDP). The CDP was defined as the time point at which the peristaltic wave appears to slow[151]. Peristaltic breaks were assessed by application of a 20mmHg isobaric contour line around each swallow. Any breaks between the upper oesophageal sphincter and lower oesophageal sphincter were measured [150]. Only large breaks above 5cm were considered significant (see figure 6.2) [149].

Each swallow was then classified first by contraction vigour. Only if the vigour was normal (DCI 450-8000mmHg.cm.s) then was it classified according to the contractile pattern (see Table 6.3). In the final stage, the characterisations of all 10 swallows were used to assign a final overall classification, which in the context of GORD related to peristalsis: absent, ineffective oesophageal motility (IOM) or normal [149] (see table 6.4).

Table 6.3 Overview of Chicago classification version 3 for the analysis of individual swallows

Contraction vigour	**Criteria**
Failed	DCI <100
Weak	DCI >100, but <450
Ineffective	Failed or weak
Normal	DCI > 450, but <8000mmHg.cm.s
Hypercontractile	>8000
Contractile pattern	**Only applied if normal contraction vigour**
Premature	DL <4.5 seconds
Fragmented	Large break (>5cm) in the 20mmHg isobaric contour with DCI >450
Intact	Not achieving any of the above diagnostic criteria

Table 6.4 Chicago classification version 3 for the analysis of peristalsis

Classification	**Criteria**
Absent	100% failed peristalsis
Ineffective	>50% ineffective swallows
Fragmented	>50% fragmented contractions
Normal	Not fulfilling any of the above criteria

6.10 Combined pH and impedance

6.10.1 Background of procedure

Combined pH-impedance is considered the gold standard for measurement of gastro-oesophageal reflux (see section 3.3).

6.10.2 Equipment

A pH-MII catheter with 6 impedance channels and a single distal pH sensor was attached to omega™ data logger.

6.10.3 Study procedure

Once HRM had located the position of the OGJ PH-MII was performed. The catheter was lubricated and inserted intranasally. The local anaesthesia was reapplied if the effect had worn off. Continuous sips of water were taken to encourage catheter progression. It was attached at the nose with tape when the first sensor was located at 5cm above the upper margin of the OGJ, which was measured previously using the HRM. The catheter was attached to a portable data-logger. The patient then left to return in 24 hours for removal. The data was uploaded for subsequent analysis.

Patients recorded symptoms, meals and periods of being supine both by pressing buttons on the data logger and keeping a paper diary. They were encouraged to maintain both normal activity and diet.

6.10.4 Assessment for reflux characteristics

The data uploaded was analysed by automated software, but then reanalysed by a gastro-physiologist to confirm correct identification of swallows and reflux events, categorised as gas, liquid or mixed. From this the pH-impedance software generates a number of metrics (see section 3.3).

6.11 Planned analyses

Information was collected at the research visit to allow for multiple different analyses (see Figure 6.4).

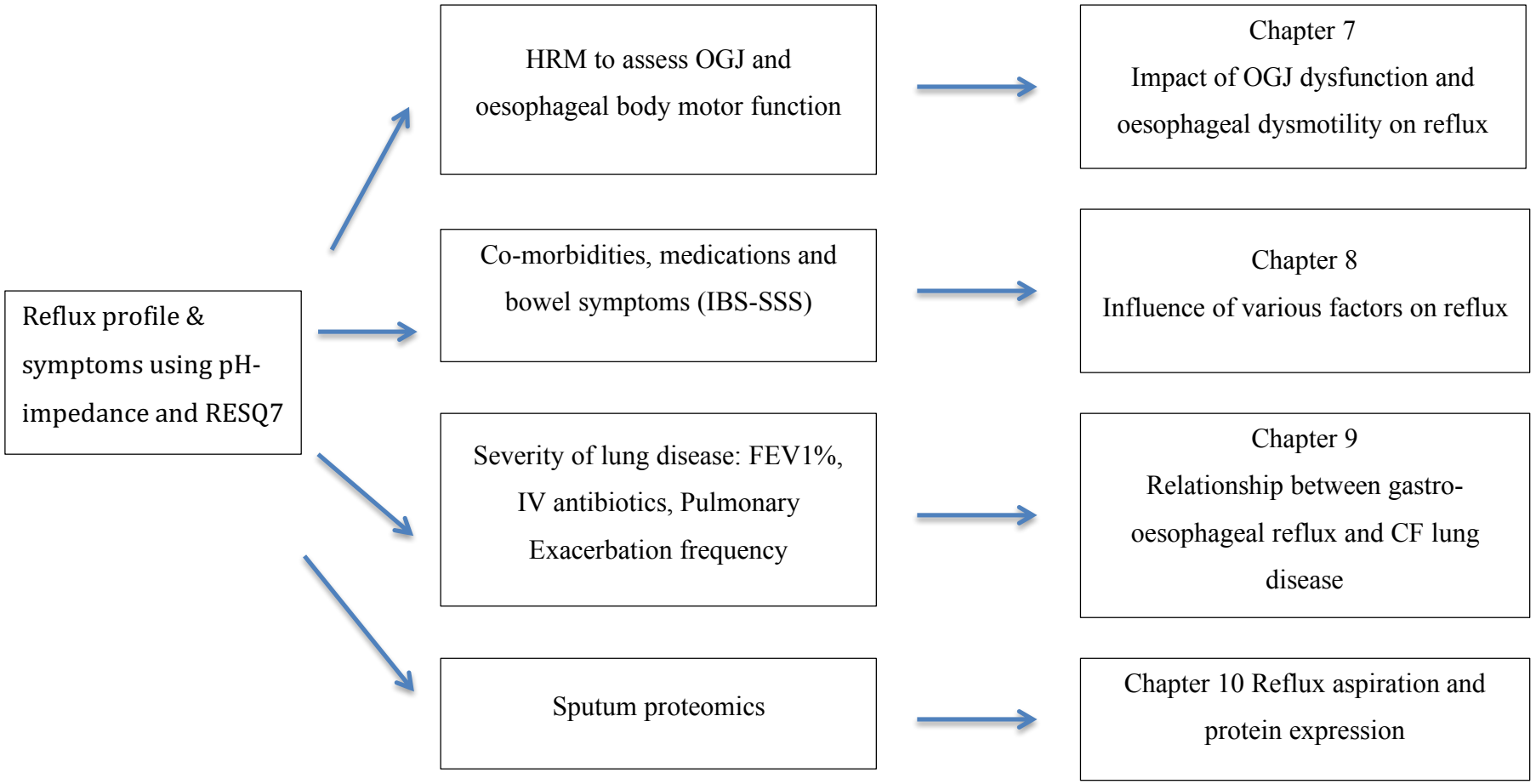


Figure 6.4 Overview of analyses contained within chapter

6.12 Statistical analysis

6.12.1 Power calculation

Due to the exploratory nature of this work it was not possible to perform a power calculation. Therefore, the target was to recruit as many subjects as possible.

6.12.2 General statistics

All data analysis was performed using SPSS ® version 22 (IBM, New York, USA). Data were summarised as a mean (standard deviation) if parametric, or alternatively as median (interquartile range) if non-parametric. All significance testing involved non-parametric data. A significance level of <0.05 was used for analyses.

All analyses involved non-parametric data. Therefore, statistical methods were based on rank order. The Mann-Whitney U was used to test the difference between two cohorts. Bivariate correlations were tested by Spearman's rank.

For multivariate analysis the non-parametric data were transformed using the natural logarithm. Adjustments were then made using an ANCOVA.

6.12.3 General estimating equations

Longitudinal data for the impact of reflux on lung endpoints, such as FEV1%, were analysed using a general estimating equation (GEE). This statistical method takes into account the repeated measurements made for each individual at each time point i.e. baseline, one year and two years preceding. For each time point it treats the variability in recorded values as nuisance and plots a line based on the mean values (see figure). From this plotted line the effect of a covariate, such as total number of reflux episodes, can then be tested. The

model is appropriate for this data set because it is robust when used on non-parametric data and can make allowances for missing data points.

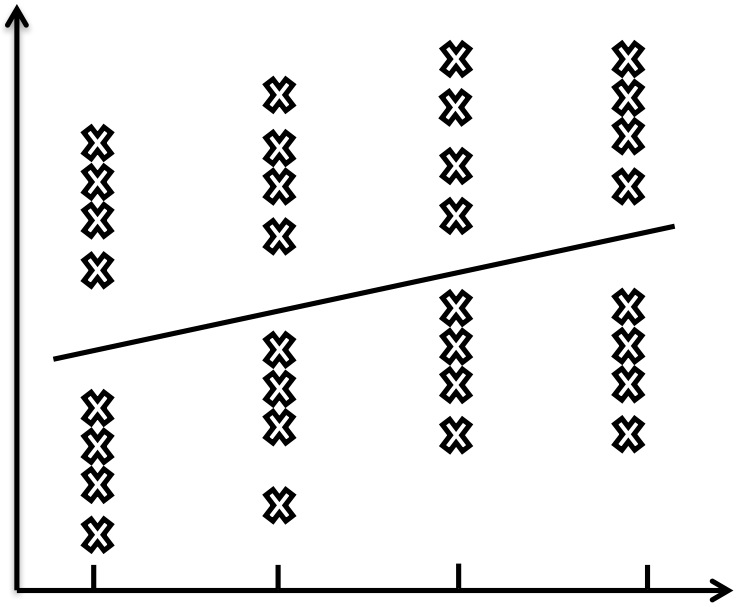


Figure 6.5 The theory of a General Estimating Equation

6.12.4 Statistical proteomic analysis

Progenesis QI® is a software package used for proteomic analysis. It has a statistical analysis package incorporated. This performs an analysis of variance (ANOVA) to compare outputs of protein quantification between selected groups. It also makes adjustments to the p-value to take into account the large number of analyses performed, creating a q-value. This minimises the false discovery rate.

The software within Progenesis QI® also allows principal component analysis. This is a transformation from a number of different variables between 2 groups, into 2 or more combined variables. These are created in such a way as to maximise the difference between the two groups.

6.13 Individual contributions to the work leading to this thesis

This research project was undertaken at the Manchester Adult CF Centre (MACFC). The author of the thesis (RL) was the principle investigator and was supervised by Professor Andrew Jones (AJ) and Professor Jacky Smith (JS).

- The study design and set-up was by RL, under the supervision of AJ and JS.
- Recruitment and all study visits were performed by RL.
- HRM and pH-impedance were performed by the staff at the Neurogastroenterology laboratory, Manchester University NHS Foundation Trust. RL attended Salford Royal NHS Hospital to learn the procedure by observing and assisting with clinical patients. RL assisted with insertion of the intra-oesophageal catheters and the measurements taken.
- James Pearson and Sam Treadway analysed all the HRM and pH-impedance tracings. Both are registered gastrointestinal physiologists. RL observed analysis in a number of research patients to learn the methodology.
- Additional HRM analysis (OGJ morphology classification and OGJ-CI) was performed by RL, John Casey (JC) and Professor Lesley Houghton (LH). JC and LH are qualified gastrointestinal physiologists.
- Statistical supervision was by JS and Philip Foden, medical statistician.
- Proteomic work is a collaborative project with Prof Rob Beynon, Rosie Maher (RM) and Victoria Harman (VH) of the Centre for Proteomic Research (University of Liverpool) and Prof Paul McNamara (Alder Hey Hospital, Liverpool).
- RM and VH performed sample preparation and the proteomic analysis. RL observed and assisted in all aspects of this process by spending a week in the laboratory. This included sample preparation, use of the

mass spectrometry equipment and interpretation of the data from the machine which was analysed by specific proteomic software packages.

7 The prevalence and characteristics of gastro-oesophageal reflux measured by combined pH-impedance in adults with cystic fibrosis

7.1 Introduction

Although there are several studies supporting an increased prevalence of gastro-oesophageal reflux in adult CF patients [54-57, 59], what is lacking is clarity about the exact nature of the relationship between reflux and CF lung disease. It is not known if lung disease leads to increased amounts of reflux through mechanisms such as altered gastro-oesophageal pressure gradients, or if lung disease is negatively affected by increased quantities of reflux. It is also feasible that both could be correct, in which case a complex bi-directional relationship needs to be explored. Alternatively, it must not be discounted that reflux is entirely unrelated to lung disease. Mechanistic studies are limited and it is unproven what factors may lead to increased reflux in CF. As it has not been possible to definitively demonstrate that reflux affects the lung, proposed mechanisms for lung damage remain hypothetical. Two mechanisms are thought to be most likely: introduction of gastric contents into the respiratory tract (reflux aspiration); and reflux stimulating oesophageal afferent nerves which as a consequence of shared vagal innervation stimulate airway efferent nerves that evoke reflexes such as cough or bronchoconstriction (neuronal crosstalk). To progress in this area further exploratory work is required. To begin the reflux characteristics in an adult CF population were described.

Two studies have reported using pH-impedance in adult CF patients [55, 57]. A number of others have studied paediatric CF patients, however these are not comparable as increased reflux is a common entity in non-CF children which often settles by adulthood [78, 80, 83, 111, 152, 153]. Both adult cohorts studied reported an increased prevalence of reflux, however rather than calculating prevalence based on normative values for a single measure of reflux,

e.g. total reflux episodes, they used a combination of several measures [55, 57]. This potentially overestimates the prevalence of reflux. Blondeau et al reported in 21 adult CF patients a median number of 66(51-85) total reflux episodes in 24 hours. Of these 22(16-37) reflux episodes reached the proximal oesophagus [55]. Pauwels et al. reported in 42 CF patients, but did not publish details of the actual impedance figures [57]. Neither study reported specifically on supine reflux [55, 57]. Both studies reported that over half the patients lack typical reflux symptoms despite demonstrating a raised amount of reflux on pH-impedance [55, 57]. As such it is widely believed that patients with increased amounts of reflux with these properties – proximal, supine and/or lacking typical symptoms – could be at increased risk of reflux aspiration, which in turn is believed could negatively affect lung disease. However, the relationship between these measures and standard clinical measures of lung disease severity – baseline and longitudinal change in lung function and number of exacerbations - has either not been tested or produced conflicting results [57, 78, 111, 152]. At present there are no studies in CF that have investigated neuronal crosstalk. There is some evidence in asthma that neuronal crosstalk may cause bronchoconstriction[119]. Most evidence comes from studies in chronic cough [118, 121, 154].

Despite combined pH-impedance producing a number of additional measures of reflux, the utility of most have not been shown to relate to oesophageal disease [155]. Thus they are not widely used in clinical practice. Given the potential differences between the impact of reflux on lung and oesophageal disease, it could be possible that they have a utility, not hitherto noted. This chapter begins the process of exploring in detail the relationship between gastro-oesophageal reflux and CF lung disease, using the reflux profile from oesophageal pH-impedance.

7.2 Hypotheses

1. CF patients have an increased prevalence of gastro-oesophageal reflux compared to previously established normative values.

2. The reflux characteristics favour aspiration.
3. There are identifiable mechanisms to explain the increased amounts of reflux in CF patients.

7.3 Aims

1) To establish the prevalence of gastro-oesophageal reflux amongst CF patients.
2) To establish if gastro-oesophageal reflux characteristics in CF are those that favour aspiration – proximal, supine and asymptomatic.
3) To gain mechanistic insights from the reflux profile

7.4 Methods

7.4.1 Subjects

Patients under the care of a specialist cystic fibrosis service were pre-screened and then approached. All subjects had confirmed cystic fibrosis on genetic testing and with a typical clinical phenotype, were aged over 18 years and deemed clinically stable. Patients were excluded if they were pregnant or had undergone lung transplantation or fundoplication. In addition, they were excluded if they had a contraindication to undertaking of oesophageal pH-impedance (see Chapter 5 Methods for details). The study was conducted in line with ethical approval granted by The Greater Manchester West Research Ethics Committee (15/NW/0655). As such interested patients were provided with a patient information leaflet (appendix 2). If they decided to proceed a consent form was completed (appendix 4).

7.4.2 Study design

A prospective observational study was conducted recruiting patients from the Manchester Adult CF centre. All subjects attended for a single visit, the details of which are described in Chapter 5. The information captured during this visit was used for the analyses described in this chapter. This included background

information captured from the patient's notes, baseline spirometry and gastro-oesophageal reflux symptoms assessed using the RESQ-7 questionnaire. Gastro-oesophageal reflux was measured using impedance-pH monitoring. Data were expressed as median and inter quartile range or mean and standard deviation as appropriate.

7.4.3 Oesophageal impedance-pH monitoring

Gastro-oesophageal reflux was measured using combined pH-impedance as described in detail in Methods section (Chapter 5). Each study and interpretation was performed by a qualified gastrointestinal physiologist. The reflux measures for primary and secondary endpoints were decided *a priori* (Table 7.1). These were compared to normative values that had been previously established using normative data from a healthy European cohort [43]. Specifically, subjects were excluded if they were symptomatic of reflux, had previously undergone thoracic or digestive surgery, had a diagnosis of diabetes mellitus, neurological or gastrointestinal disease, ingested greater than 40 grams per week of alcohol or smoked more than 10 cigarettes per day. No subjects were taking acid suppressive medications or any medications that altered gastrointestinal motility. Just over 50% were men with a mean age of 34.2 (range 18-72) and BMI 22 (range 16-36) [43].

Prevalence was calculated using the total number of reflux events. The numbers of events that reached the proximal oesophagus and/or occurred whilst supine were recorded. These were described individually, but also as a composite endpoint described as 'high-risk reflux' for aspiration.

Table 7.1 Table of pH-impedance derived reflux endpoints

Measure	Outcome	Comments
Total reflux events	Primary	Measure of total number of reflux events detected in the distal oesophagus by using impedance over 24 hours. Changes are required in the two most distal impedance sensors.
Proximal reflux events	Secondary	Measure of total number of reflux events detected in the proximal oesophagus (at sensor located at 15cm above the LOS) using impedance over 24 hours.
Supine reflux events	Secondary	Measure of total number of events detected in the distal oesophagus whilst supine over 24 hours.
Supine-Proximal reflux events	Secondary	Measure of total number of events detected in the proximal oesophagus whilst supine over 24 hours.
Total bolus exposure	Secondary	Measure of the percentage of time the oesophagus is exposed to the refluxate bolus. It relates to mechanical clearance. This can be divided into supine and upright periods.
Median bolus exposure per event	Secondary	Measure of bolus exposure duration for each impedance defined event. Advantage over bolus exposure, is that it is not affected by the total number of reflux events.
Total acid exposure	Secondary	Measure of percentage of time the distal pH sensor is below 4 for the study. It relates to chemical clearance. This can be divided into supine and upright periods.
Longest acid reflux event	Secondary	The longest duration of a reflux events, detected by the distal pH sensor dropping below 4. Not as well described as total acid exposure, but not influenced by number of reflux episodes.
DeMeester score	Secondary	Composite endpoint of six pH derived measures: total acid exposure, supine acid exposure, upright acid exposure, total reflux events, total greater than 5 minutes and longest event.
pH of reflux events	Secondary	Using the impedance and pH sensors all distal events can be classified by pH: acid pH <4, non-acid pH 4-7 or weakly acid pH >7.
Composition of reflux events	Secondary	All events can be classified by physical state (gas, liquid or mixed). Gas events are not included in any impedance measure of reflux.

7.4.4 Statistical analysis

Data were presented as either a mean (standard deviation) or median (interquartile range) as appropriate. The sensitivities and specificities of reflux symptoms captured, using the RESQ-7, were calculated for measures on the oesophageal pH-impedance study.

7.5 Results

7.5.1 Study participants

Forty-one patients were recruited and completed the study (see Figure 7.1). Appropriate subjects were screened (n=134) and in those approached 58/116 (50%) agreed. Of those declining 43/58 (74%) cited the invasive nature or the prospect of catheter insertion as the main reason. Of those who agreed 41/58 (71%) completed the study. Three patients were enrolled who were already undergoing combined pH-impedance for investigation of reflux symptoms.

Baseline demographics are detailed in Table 7.2. They are compared to CF registry data describing Manchester Adult CF Centre patients (n=421) downloaded on the 31/12/2017. Acid suppressive medications were defined as at type of proton pump inhibitors (PPI) and H-2 receptor antagonists (H2 antagonists). The study population was representative of an outpatient CF population for the majority of baseline demographics tested (Table 7.2). The FEV1% was significantly higher in the cohort recruited (p=0.024). Males appeared over represented, but this did not reach significance (p=0.084).

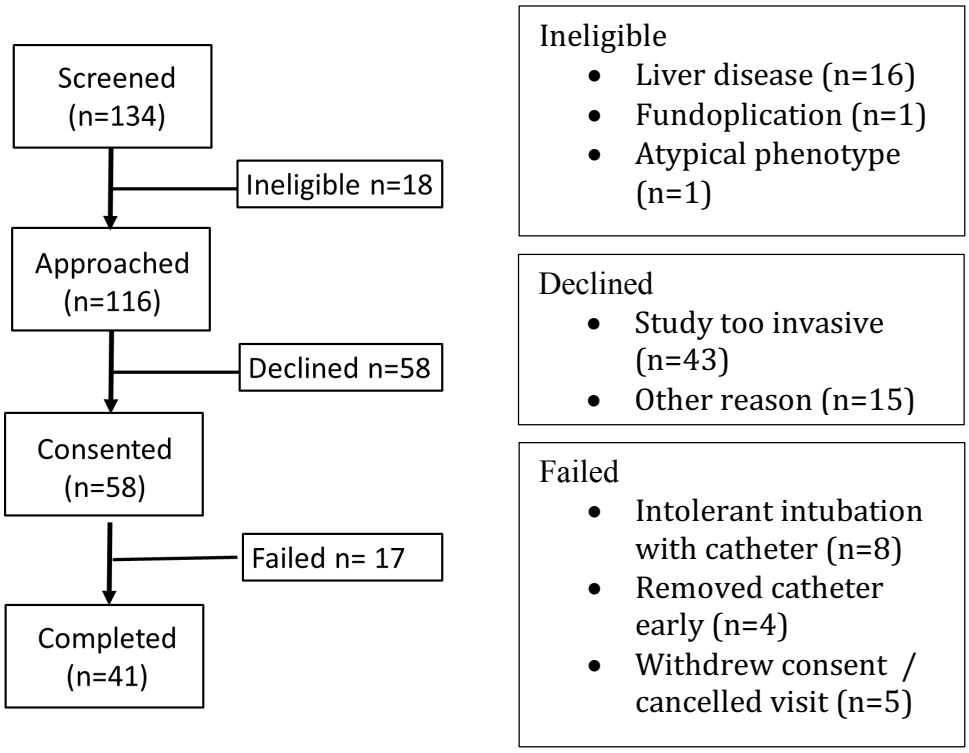


Figure 7.1 Consort diagram for recruitment

Table 7.2 Table of cohort demographics

	Study cohort (n=41)	**MACFC cohort (n=421)**	**p value**
Age (years)	30.9(8.2)	31.3 (10.3)	0.737
Male	70.7%	56.8%	0.084
Baseline FEV1%	51.7(17.5)	58.6 (24.7)	0.024
Baseline BMI	22.4 (3.1)	21.4 (5.8)	0.277
Chronic infection Pa	73.2%	63.7%	0.224
Chronic infection Bcc	12.2%	9.5%	0.295
Prescribed acid suppressive medication	78%	Not recorded	NA

The data are presented as mean (standard deviation) or percentage as appropriate. Pa: *Pseudomonas aeruginosa*; Bcc: *Burkholderia cepacia complex*

7.5.2 Prevalence of total and 'high-risk reflux'

The total number of reflux events was raised in 54% of CF patients when compared to established normative values (see **Table 7.3**). High-risk characteristics for reflux aspiration were considered as an increased number of proximal, supine and supine proximal events. These were seen in 20%, 24% and 37% respectively (see **Table 7.3**). Considered in combination high-risk characteristics were present in 41% of the study population.

Table 7.3 Impedance profile for study participants (n=41)

		Normal Range	Reflux Events	Percentage raised
Total Reflux events/24 hours	**Total**	<75	78.1 (49.2-102.3)	54%
	Upright	<64	65 (41.3-88.5)	56%
	Supine	<14	6.4 (3.2-14.8)	24%
Proximal reflux events/24 hours	**Total**	<30	10.2 (5.1-20.9)	20%
	Upright	<27	9.6 (3.3-19.1)	17%
	Supine	<3	1.2 (0-3.9)	37%

The data are presented as median (IQR) and percentage as appropriate.

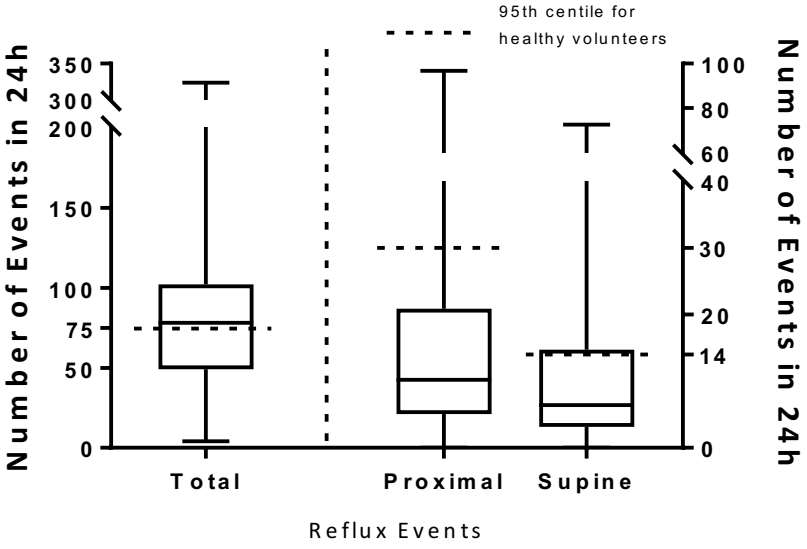


Figure 7.2 Box plot of total and high risk reflux measures showing median/IQR and range.

7.5.3 Incidence of reflux symptoms

All patients completed the RESQ-7, with the domains of heartburn and regurgitation used to represent typical reflux symptoms. Heartburn was present in 70% (30/40) and regurgitation in 67.5% (29/40) despite usual acid suppressive medications. The median severity and frequency of symptoms were rated as mild and occurring one day per week (see Table 7.4). There was no difference in heartburn and regurgitation between those taking or not taking acid suppressive medications.

Table 7.4 Reflux symptoms experienced using the RESQ-7 (n=41)

	Heartburn			Regurgitation		
	Frequency	**Intensity**	**Percentage reporting**	**Frequency**	**Intensity**	**Percentage reporting**
Range	*0-5*	*0-5*		*0-5*	*0-5*	
CF cohort (N=41)	1.1 (1.3)	1.2 (1.2)	70%	1.1 (1.2)	1.1 (1.2)	67.5%

Data are presented as mean (SD) and percentage where appropriate.

Table 7.5 RESQ-7 scoring system

Score	**Frequency in last week**	**Intensity**
0	Not present	Not present
1	1 day	Very mild
2	2 days	Mild
3	3-4 days	Moderate
4	5-6 days	Moderately severe
5	7 days (Daily)	Severe

Typical symptoms, defined as the presence of either heartburn or regurgitation on the RESQ-7, were present in 85% (35/41) of subjects. Using typical symptoms on the RESQ-7 to identify increased total events has a sensitivity of 82%, but specificity of only 11%. This was again similar for raised proximal events (sensitivity 88%, specificity 15%), supine events (sensitivity 92%, specificity 17%), acid exposure (sensitivity 87%, specificity 15%) and acid events (sensitivity 83%, specificity 16%), i.e. reflux symptoms were highly prevalent even in those without raised amounts of reflux.

Table 7.6 Data of typical symptoms captured by the RESQ-7 and increased total reflux (n=41)

	Normal Total Reflux events (<75 episodes)	Increased Total Reflux events (>75 episodes)	Total Total number
Presence of typical reflux symptoms	17	18	35
Absence of typical reflux symptoms	2	4	6
Total number	19	22	41

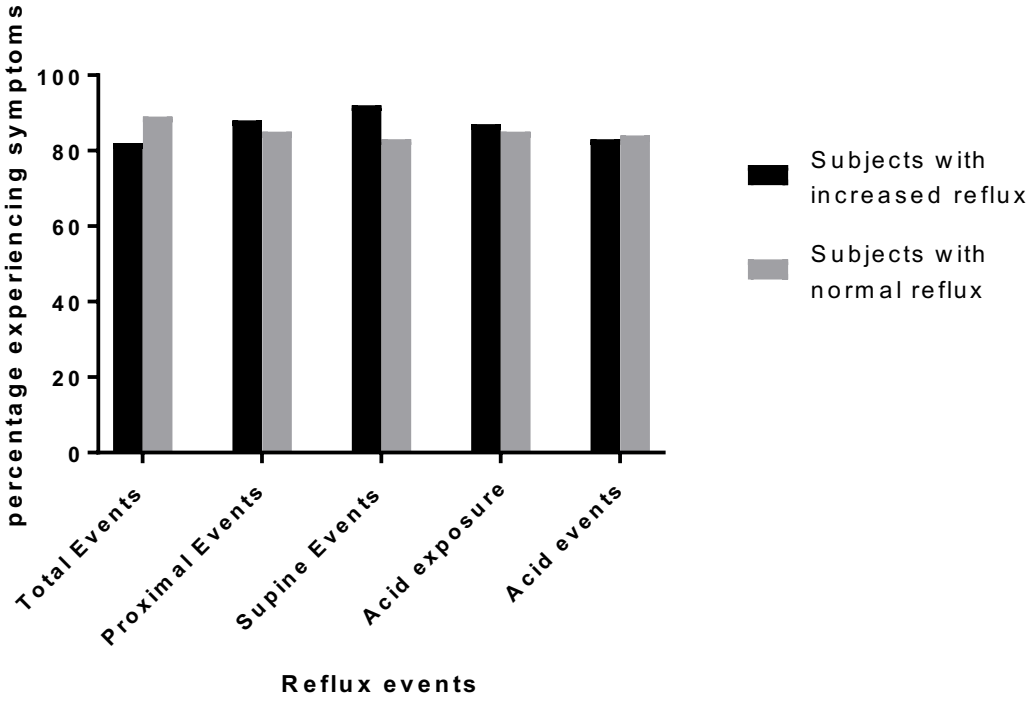


Figure 7.3 The percentage of subjects experiencing typical reflux symptoms on the RESQ-7 in those with and without raised reflux measures

7.5.4 Prevalence of acid and weakly acid events

There were greater numbers of weakly acid reflux events in 24 hours (33.9[11.9-72]) than acid events (21.5[10.1-45.2]). As such a greater percentage of subjects had increased weakly acid events (51.3%) than acid events (15.4%) above the normal range (see Table 7.7). It must be noted that these measures were obtained on acid suppressive medication in 30/39 of the subjects included. Unfortunately, the pH sensors failed in 2 patients. For these no pH derived metrics (total acid exposure and reflux event pH) were available for analysis.

Table 7.7 PH of impedance defined reflux events (n=39)

	Normal range	Events	Percentage raised
Acid	<50 events	21.5 (10.1-45.4)	15.4%
Weakly Acid	<33 events	33.9 (11.9-72.0)	51.3%
Weakly Alkaline	<15 events	0 (0-1.2)	5.1%

Data are presented as median (IQR) and percentage where appropriate.

7.5.5 Composition of reflux events

The raised total reflux episodes were driven by mixed (liquid and gas) events (55.5[32-74.4]) rather than liquid events (18[9.3-35.3]). As such only 10% of subjects had an increase in liquid events, compared to 60% with mixed events above the normal range. The number of gas events (44.8[22.1-86.6]) was increased in 65.9%.

Table 7.8 Table of composition of reflux events (n=41)

	Normal range	Events	Percentage raised
Liquid	<55	18.0 (9.3-35.3)	12.2
Mixed	<42	55.5 (32.0-74.4)	61.0
Gas	<30	44.8 (22.1-86.6)	65.9

Data are presented as median (IQR) and percentage where appropriate.

7.5.6 Chemical and volume clearance

The total bolus exposure was prolonged in 29.3% of subjects (median 1.4%, IQR 0.9-2.3). However, when volume clearance was calculated for each reflux event (median bolus clearance) no subjects had a result above the upper limit derived from healthy data. Acid exposure was raised in 38.4% (median 2.9%, IQR 1.1-10.7). The results for DeMeester score were similar with 41% with a raised value. Forty-six percent of subjects had an acid reflux event lasting above the accepted upper limit of normal. This suggests that within this cohort volume clearance is normal but chemical clearance is prolonged.

There were differences between the upright and supine periods for both clearance measures. During the supine period more subjects had increased acid exposure (60%) but less had increased bolus exposure (17.1%). There was a more marked reduction in the percentage of time spent between upright and supine for bolus exposure (2.3% vs 0.2%) than for acid exposure (2.8% vs 1.9%).

Table 7.9 Acid and bolus exposure of study participants (n=41)

		Normal Range	Reflux Events	Percentage raised
Acid Exposure*	**Total**	<4.2	2.9% (1.1-10.7)	38.4%
	Upright	<6.3	2.8% (1.3-7.3)	25.6%
	Supine	<1.3	1.9% (0-6.1)	60.0%
Longest reflux event*	**Total**	<9.3	7.4 secs (2.9-34.5)	46.0%
DeMeester score*	**Total**	>14.72	11.46 (3.52-33.0)	41.0%
Bolus Exposure	**Total**	<2	1.4% (0.9-2.3)	29.3%
	Upright	<2.7	2.3% (1.2-3.3)	39.0%
	Supine	<0.9	0.2% (0.1-0.7)	17.1%
Median bolus clearance	**Total**	<20	8.2 secs (6.8-9.6)	0%

Data are presented as median (IQR) and percentage as appropriate. *only n=39 in these cohorts.

7.6 Discussion

7.6.1 Prevalence

Within this study of adult CF patients there is an increased prevalence of reflux events with 54% of subjects exhibiting excess numbers of reflux events compared with the 95th centile for healthy controls. This supports the findings of previous combined pH-impedance studies in adult CF patients. However, in contrast we calculate the prevalence of reflux using a single measure — the

total number of reflux events — as opposed to the two previous pH-impedance studies that used a combination of several different measures[55, 57]. This prevalence exceeds that seen in these previous adult CF studies [55, 57]. The total number of reflux events was greater in this cohort (median 78.1, IQR 49.2-102.3) compared to that reported previously (median 66, IQR 51-85)[55].

There are a number of considerations when comparing the reflux measures between studies. There are differences between the study populations, such as method of cohort selection and severity of lung disease. The patients remained on their usual acid suppressive medication regimen. Enrolled patients had a range of severity of lung disease. Methodological differences amongst these studies vary for all of the aforementioned.

There were also reasons within the specific methodology of this study that could have led to an increased prevalence of reflux. Three study subjects were included who were undergoing reflux measurement for clinical investigation of GORD symptoms. There may also have been a selection bias, as patients with troublesome reflux symptoms may be more likely to enrol. The study advert itself may have further contributed to this with its wording.

There are several limitations in using this method for assessing prevalence of reflux aspiration. The reproducibility of 24-hour pH-impedance has been shown to be acceptable for a time period of between four to ten days [43]. Another study in healthy volunteers showed that pH studies were reproducible up to a period of one to four weeks[156]. It is unknown how much variation in reflux can occur over longer time periods, such as months or years, and whether the study population, i.e. GORD versus healthy, makes any significant difference. It is also unknown whether having pH-impedance catheter *in situ* effects the amount of reflux.

A *post-hoc* power calculation for the prevalence of reflux, as defined by number of reflux episodes, was subsequently undertaken. To estimate a prevalence of

54% with a precision of 5% (95% confidence intervals between 44%-64%) would require a sample size of 381 subjects [157]. Therefore, the current study with its sample size of 41 subjects allows a sufficient power to demonstrate a prevalence of increased reflux of 54% with a precision of 7.5%, giving a 95% confidence interval 39%-69%.

7.6.2 'High-risk' reflux for reflux aspiration

Within this cohort 19.5% had increased proximal reflux compared to accepted normative values. This is similar to previous studies in CF [55, 63]. A raised number of supine reflux episodes occurred in 24%. Reflux that extends to the proximal oesophagus or occurs whilst lying flat is believed to portend a 'high-risk' for reflux aspiration. Perhaps the highest risk of aspiration may be associated with events that occur whilst supine and extend to the proximal oesophagus. These are a rare physiological occurrence (median 1, IQR 0-2). In this cohort they still occurred infrequently (median 1.2, IQR 0-3.9) but are raised in 36.6% of subjects. In total 41% of CF patients had at least one of these reflux measures raised for 'high-risk' reflux. Thus it would appear that a significant number of CF patients could be at risk of reflux aspiration.

There are limitations to these findings. It is assumed that 'high-risk' reflux is a reasonable surrogate for reflux aspiration. However, since no accurate measure of reflux aspiration currently exists, then this remains unproven. The relationship between 'high-risk' reflux and lung disease severity will be examined and subsequently discussed in Chapter 9.

Unlike in previous studies a high prevalence of increased reflux without symptoms was not demonstrated, despite most patients taking acid suppressive medications [55, 57]. Typical symptoms were identified, using a validated questionnaire, in most patients with increased total (82%), proximal (88%) or supine (92%) amounts of reflux. However typical symptoms were present in large numbers of those without raised reflux, leading to a very poor specificity

(11-17%). Although symptoms are frequently present, they are poorly specific for increased amounts of reflux by any measure. It is unknown whether this relates to visceral hypersensitivity with physiological amounts of reflux generating symptoms, or symptoms occurring independently of reflux. Alternatively, the RESQ-7 questionnaire may be more sensitive for mild symptoms.

There is clearly a need to understand symptom generation better within CF patients. To do so the cohort will be investigated for the association between reflux events and symptoms, and then the probability of reflux events generating reflux symptoms will be calculated using a validated method [158]. In the first instance this will help establish if the symptoms experienced are attributable to reflux episodes.

7.6.3 Prevalence of acid and non-acid reflux events

Within this cohort most reflux events are non-acid, most likely as a consequence of high rates of use of acid suppressive medication. Acid suppression is the main therapy for oesophageal reflux syndromes and is frequently prescribed in CF. Non-acid events have been associated with measures suggestive of poorer lung health, such as lower baseline lung function or earlier acquisition of *Pseudomonas aeruginosa* [111]. Other studies support a detrimental effect of non-acid reflux. CF respiratory pathogens have been cultured from acid supressed gastric juice [115]. A small randomised control trial suggested a trend toward reduced time to first exacerbation in the PPI treated arm [59]. It has also possible that non-acid events may be less irritant to the upper airway and as such more likely to be aspirated. Thus if reflux aspiration is occurring, acid suppression has the potential to compound the issue. This emphasises the importance of assessing both acid and non-acid events when exploring the impact of oesophageal measures on severity of lung disease – as is performed in Chapter 10.

7.6.4 Composition of reflux events

This is the first study to demonstrate that CF patients have large amounts of gas events detected on the oesophageal impedance. Most reflux events were mixed, i.e. containing both liquid and gas, (median 55.5(IQR 32-74.4)) rather than purely liquid (median 18(IQR 9.3-35.3)), meaning that approximately three quarters of all reflux events had a gas component. This is far greater than previous studies in GORD patients in which only approximately half had a gas component [159, 160]. In addition, there are a raised number of gas events in two thirds of CF patients, with a median 44.8 (IQR 22.1-86.6) events per 24 hours compared to normative limits (<30). This again is in excess of previous values seen in GORD patients with a mean of 22.9(SD 6.1) events, albeit in a study of only 12 patients [161]. Given the increased amounts of gas one wonders both the reason for this and if it could be a potential cause of the increased amounts of reflux seen in CF. There are also possible implications of increased gas events on CF lung disease. It has been theorised that gas events may increase the risk of reflux aspiration, possibly as a consequence of aerosolised refluxate [162].

Gas events can be identified on oesophageal impedance as being of gastric or supragastric origin. However identification can be difficult and requires someone experienced in the technique [163]. For our study only gastric gas events were measured, due to the programming of the automated software. The volume of gastric gas has been shown to be increased by air swallowing (aerophagia), when measured by oesophageal impedance [164]. Aerophagia is thought to occur in many respiratory conditions due to work of breathing although this comes from empiricism rather than from previous studies. Retrograde movement of intestinal gas, possibly as a consequence of bacterial overgrowth and malabsorption, may also contribute to gastric gas volume [165]. This is supported by increased reflux symptoms and raised pH-impedance measures seen in IBS, a condition where there is disrupted bowel function [166, 167]. Bowel function is also disrupted in CF patients but the exact pathology is not yet fully understood. However, small bowel bacterial

overgrowth, changes in gut microbiome and widespread gut inflammation have been implicated [168-171]. Thus there are logical explanations why CF patients may have increased gastric gas volumes.

It has been shown that gas events do not lead directly to reflux events: during mixed events the liquid component frequently precedes the gas; and liquid events can occur without gas events [159, 160, 172, 173]. However, it maybe that gastric distension from gas leads to both increased reflux events, and directly causes increased amounts of reflux by stimulating transient lower oesophageal relaxations (TLOSRs). Bredenoord et al. showed that air swallowing increased gastric volume and also the number of subsequent gas events [164]. In a different study they distended the stomach by directly infusing air and this lead to increased gas reflux events as well as increased TLOSRs [161].

It has been shown that that TLOSR allow the passage of gastric contents into the oesophagus independent of movement of gas [172]. Thus should their number increase it may be postulated amount of liquid reflux will rise. However, at present the evidence does not support this. Gastric distension or raised gas events have no reported effect on reflux events [161, 164]. The change in frequency of TLOSR between healthy and those with reflux disease, has been shown to increase in some studies and remain the same in others [174, 175]. These findings may reflect small study size, the methodology used and the cohort selected. It is possible given the heterogeneity of mechanisms that increase reflux, that TLOSR may be raised in a specific group rather than as a gradient across the cohort.

This area requires a more detailed examination. In the first instance in the next chapter the relationship between CF bowel disease, gas events and reflux measures will be explored.

7.6.5 The role of volume and chemical clearance

Oesophageal reflux syndromes occur not just as a consequence of the amount of refluxate entering the oesophagus, but due to a number of additional factors. This includes the ability to clear refluxate from the oesophagus, which requires volume clearance of the liquid by peristalsis and chemical clearance of any associated acidification by bicarbonate-containing saliva [47, 85]. There is no direct evidence to link CF to impaired mechanical clearance, however small studies have shown increased oesophageal dysmotility [56, 63]. Oesophageal motility has been shown to correlate with oesophageal reflux disease. In contrast chemical clearance has been shown to be prolonged in children with CF compared to non-CF children of a similar age [87]. This is in keeping with current knowledge of the CFTR protein, which is known to control bicarbonate movement, and therefore CFTR dysfunction may impair buffering of reflux in the oesophagus.

Within this study the bolus exposure was raised in 29.3%, whereas no subjects had a raised median bolus clearance time. Bolus exposure is the total percentage of time the oesophagus is exposed to refluxate, whereas the median bolus clearance time is the duration of exposure for each reflux event. Bolus exposure will be influenced by both number of reflux events and mechanical clearance of each event. Thus the raised bolus exposure likely reflects the increase in number of reflux events, whereas the median bolus clearance suggests that mechanical clearance is unaffected. This reflects findings by Woodley et al who showed that mechanical clearance was actually better in CF children than non-CF with reflux symptoms [87].

Within our cohort measures of chemical clearance (acid exposure, DeMeester score and longest acid event) are all prolonged. The total overall acid exposure was increased in 38.4% despite acid reflux events being elevated in only 15.4% and a large proportion of patients prescribed acid suppression. In keeping with this the longest period of acid reflux despite acid suppressive medication, defined as the duration for which an events pH falls below 4, was raised above

that seen in healthy volunteers in 46% of subjects. Interestingly increased acid exposure was seen more often during the supine period, with 60% of subjects having levels raised above normative values.

There are limitations in using bolus exposure to represent oesophageal volume exposure. It has not been shown to correlate well with oesophageal reflux disease[155]. To explore this further, additional metrics thought to better represent mechanical and chemical clearance will be used. The post-swallow peristaltic wave index has been shown to predict oesophageal reflux disease severity, unlike any current measure of bolus exposure [176].

Using total acid exposure also has limitations. Although it has been shown to correlate well with oesophageal pathology, it may not be the best metric to inform on mechanisms of CF reflux. Acid exposure is influenced by a number of confounding factors: the number of reflux events that enter the oesophagus; the initial pH of the reflux events which is in turn influenced by acid suppressive medication; the time for mechanical clearance; and the volume of these events which at present is unable to be measured. To control for some of these the chemical clearance can be calculated, which measures the time following mechanical clearance, when impedance baseline normalises, to the when chemical clearance occurs, i.e. pH returning to above 4 [87]. This has already been shown to be increased in a study of paediatric CF patients [87]. Also importantly is there is only one sensor which is located in the distal oesophagus. As such only have information about chemical clearance is available in this region of the oesophagus. To overcome this, a second proximal pH sensor could be used in future studies.

7.6.6 Conclusion

Within this cohort this study has shown that reflux is increased, as defined by an increased total number of reflux episodes in CF patients compared to previously reported normative values. A significant proportion of CF patients also have reflux characteristics that are at a presumed high risk for reflux

aspiration. The consequence of this will need exploring in subsequent chapters. In addition, several mechanistic areas have been highlighted for further investigation: the high prevalence of symptoms may involve visceral hypersensitivity; chemical clearance appears to be prolonged despite acid suppressive medications and may lead to prolonged oesophageal acidification; and the increased number of gas events could represent an important and as yet undescribed mechanism for increased reflux in CF patients.

8 Exploring the mechanisms of increased reflux in CF patients using high resolution manometry

8.1 Introduction

There are a number of proposed mechanisms in CF that may lead to the increased amounts of reflux (see Figure 8.1). Further work is needed to explore this further. A recent consensus guideline has concluded that high resolution manometry (HRM) is capable of performing a detailed evaluation of the function of the OGJ and oesophageal body in patients with GORD [146]. Dysfunction of the OGJ can lead to increased amounts of reflux entering the oesophagus, whereas impairment of motor function can result in impaired clearance [31, 177]. In small studies of CF patients, dysfunction of the OGJ and oesophageal body has been noted to be common [56, 63, 84].

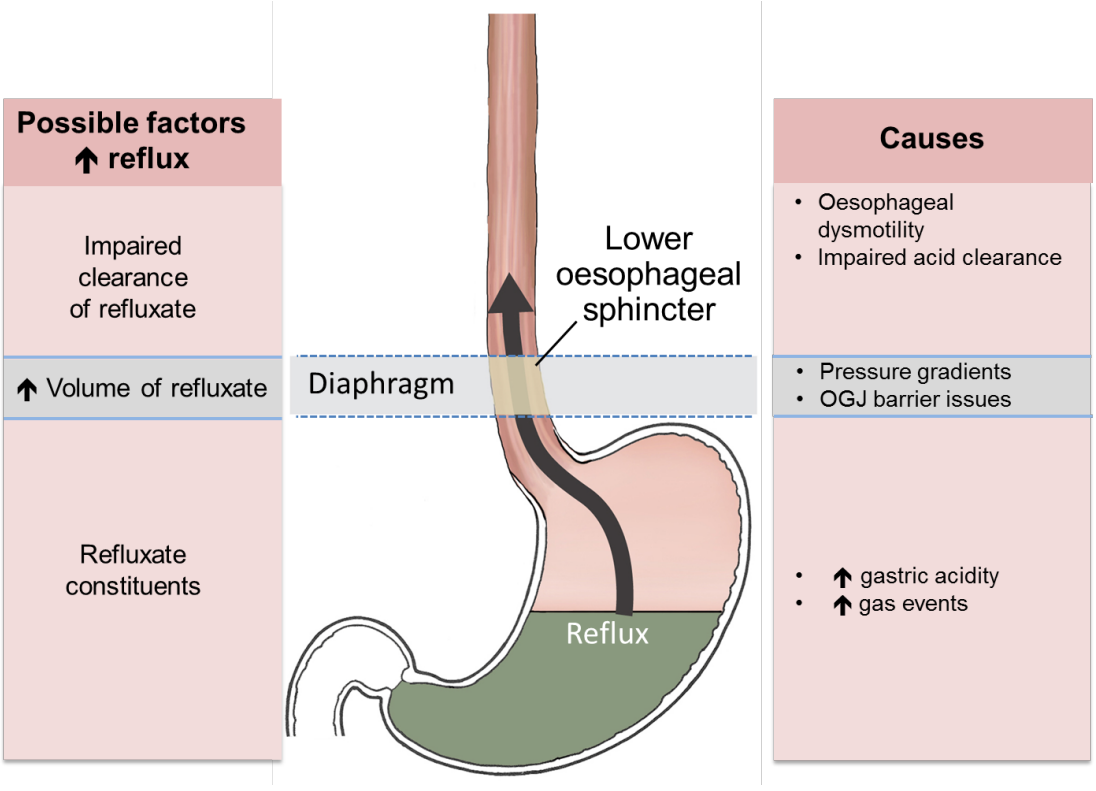


Figure 8.1 The potential factors leading to increased reflux in CF

Transient lower oesophageal relaxation (TLOSR) is responsible for the majority of physiological reflux events in healthy individuals [45, 48, 159]. In GORD, TLOSR remains the predominant mechanisms but others, in particular those that occur by reflux overcoming a hypotensive OGJ, become more frequent [48, 49, 159]. Reflux events related to OGJ hypotension have been shown to result in a greater acid exposure compared to those events occurring during TLOSR. In one study examining the post-prandial period they were responsible for only 14% of reflux episodes, but caused 28% of acid exposure [178]. This may reflect that an increased volume of reflux occurs during these events. As such, although OGJ hypotension may not be the most frequent mechanism it is still of importance in the pathogenesis of GORD.

There is no consensus on the best method for measuring OGJ pressure [146]. An often quoted measure is the mean OGJ pressure (OGJ-MP), also referred to inaccurately as the lower oesophageal sphincter pressure (LOSP) (see figure 8.2). LOSP is an inaccurate term since it measures the combined effect of both elements of the OGJ, the LOS and the crural diaphragm. This was the measure used in the previous studies of CF patients [56, 63, 84]. However, studies evaluating its ability to assess OGJ barrier function are lacking [146]. A promising new metric is the OGJ contractile integral (OGJ-CI) (see figure 8.2). This measures the contractile vigour of the OGJ, reflecting the crural diaphragm's contribution to OGJ function. It has been shown to accurately discriminate between healthy controls and reflux disease, as defined by endoscopy and pH-impedance studies [179]. In GORD patients it negatively correlates with increased reflux events and total acid exposure [180].

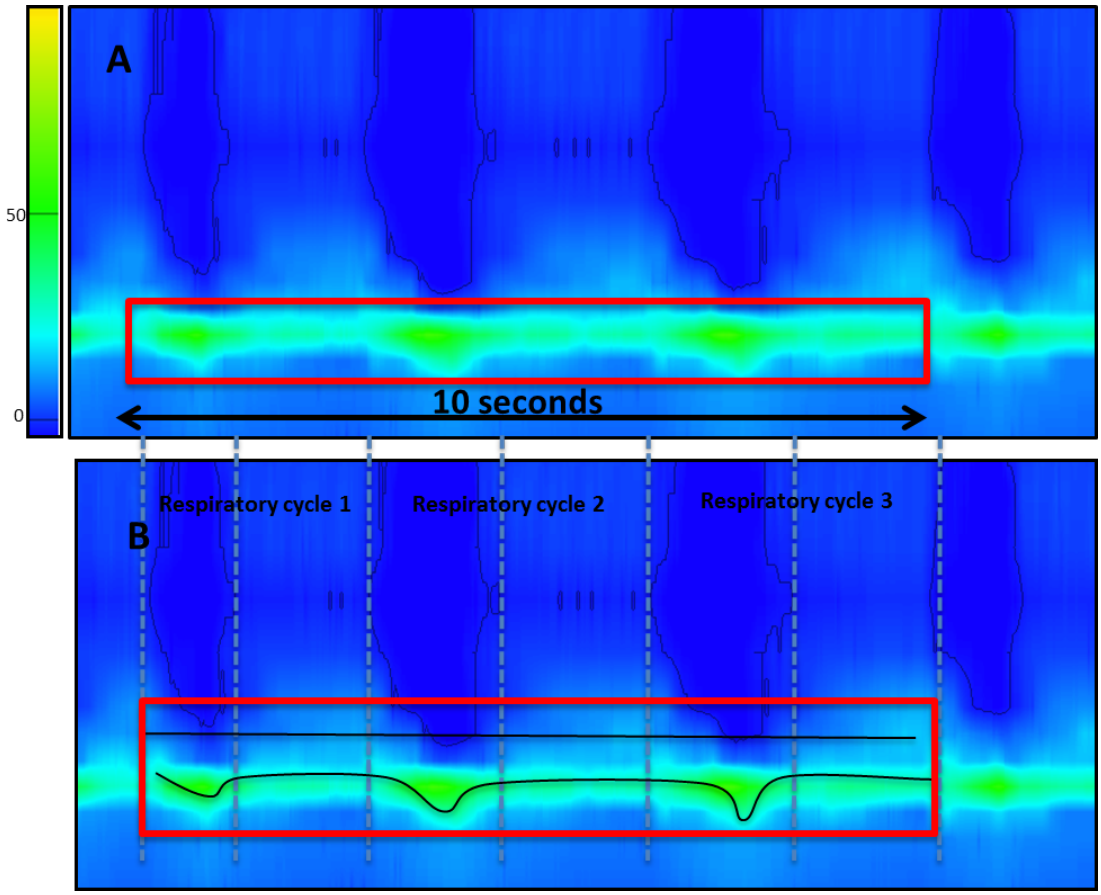


Figure 8.2 Calculation of the OGJ-MP (A) and OGJ-CI (B) using HRM. The red box denotes the area of the HRM tracing used for the analysis of OGJ-MP (ten seconds) and OGJ-CI (three respiratory cycles).

Frequently the cause of OGJ hypotension is a hiatus hernia [181]. When a hiatus hernia exists there is separation of the LOS and crural diaphragm [53]. This causes a loss of their synergistic configuration with a resultant reduction in the peak OGJ pressure generated[51]. HRM is able to identify separation of pressure peaks generated by the crural diaphragm and LOS, and as such has been shown to be highly sensitive and specific for identification of a hiatus hernia [36, 70].

A previous small study in an adult CF cohort (n=12) reported HRM evidence of hiatus hernia in two patients which equates to a prevalence of 17% [63]. Using computed tomography (CT) one study (n=79) in idiopathic pulmonary fibrosis (IPF) has shown a prevalence of hiatus hernia of approximately 50% [66]. In other chronic respiratory disease CT has been used to report slightly lower

hiatus hernia prevalence including asthma (17%), chronic obstructive pulmonary disease (13%) and bronchiectasis (36%) [67, 182]. It has been proposed that changes in lung volumes can alter the position of the crural diaphragm relative to the LOS causing their separation, thus explaining the increased prevalence of hiatus hernia seen in various respiratory diseases [65]. This could lead to increased reflux.

Oesophageal peristalsis is responsible for volume clearance, i.e. removal of the gastric fluid from the oesophagus, following a reflux event [183]. When there is peristaltic impairment it has been demonstrated that volume clearance is impaired [184, 185]. Impaired peristalsis has been shown to be highly prevalent in GORD patients [186, 187] and in some studies associates with increased acid exposure [188-190]. HRM can be used to assess and classify oesophageal motility, using the Chicago Classification (CC), based on the peristaltic patterns during ten swallows of five millilitres boluses of water [149]. From this, three motor phenotypes have been identified: impaired oesophageal motility (IOM); absent peristaltic activity; and fragmented peristalsis where breaks in the swallows occur as they propagate down the oesophagus [146]. However, it remains unclear of the importance of these HRM diagnoses in the context of reflux disease, and as such IOM and fragmented peristalsis are classified as minor abnormalities in the most recent CC version.

An increased prevalence of oesophageal dysmotility has been described in CF as well as other respiratory conditions, such as idiopathic pulmonary fibrosis (IPF), chronic cough and lung transplant [56, 63, 191-194]. As in non-CF reflux disease it is unclear if dysmotility leads to prolonged exposure of the oesophagus to refluxate. In support one study showed that absent peristalsis – the most severe classification of dysmotility – in IPF patients was associated with a raised DeMeester score [191].

Our intention in this chapter is to explore using HRM the prevalence of OGJ oesophageal body dysfunction in adults with CF, and correlate this with oesophageal reflux as measured by pH-impedance.

8.2 Hypothesis

Abnormalities of the OGJ and oesophageal body motor function are contributing factors to the increased reflux seen in cystic fibrosis.

8.3 Aims

1) To describe the prevalence of oesophago-gastric junction (OGJ) abnormalities.
2) To describe the prevalence of motor abnormalities of the oesophageal body.
3) To explore if OGJ and motor abnormalities of the oesophageal body affect amount of reflux.

8.4 Methods

8.4.1 Subjects

Patients were identified from a cohort of 41 CF patients participating in a prospective observational study to investigate the prevalence of reflux in CF patients (Chapter 6). This entire cohort required high-resolution manometry (HRM) to allow accurate placement of the pH-impedance catheter. In those tolerating the HRM procedure further assessment was performed and the results form the basis of this chapter. The study was conducted in line with ethical approval granted by The Greater Manchester West Research Ethics Committee (15/NW/0655).

8.4.2 Study design

Each patient had a detailed analysis of the OGJ and oesophageal body motor function using HRM. This was performed as described in the Methods (Chapter 5). This analysis was based upon recent consensus guidelines for investigation of oesophageal motor disease in GORD [146]. The findings were then compared

with oesophageal reflux measures for number of events and clearance, which were obtained from their previous pH-impedance investigation detailed in Chapter 6.

8.4.3 Assessment of oesophago-gastric junction function

The OGJ tone was assessed by two different measures: the traditional measures of mean resting tone; and the novel metric of the OGJ-contractile integral (OGJ-CI). These were both calculated using the Sandhill© software package for HRM analysis.

Hiatus hernia was assessed by classifying the OGJ morphology [36]. Type 1 morphology was taken as normal. Types 2 and 3 represent a hiatus hernia. Classification was independently performed by a researcher (myself) and a qualified gastrointestinal physiologist, Mr John Casey. In the case of lack of consensus, a professor of gastrointestinal physiology, Professor Lesley Houghton, decided the class.

Within the Methods is a more detailed description of the assessment of OGJ function (Chapter 6).

8.4.4 Assessment of oesophageal body motor function

The series of 10x 5ml swallows were analysed and the integrated relaxation pressure (IRP), distal contractile integral (DCI) and distal latency (DL) were calculated by the software package. From these the motility was classified as per the Chicago Classification version 3[149]. The DCI was recorded separately for analysis. Further detail is provided within the Methods section (Chapter 5).

8.4.5 Statistical analysis

All continuous measures are non-parametric thus presented as median (inter-quartile range). Statistical significance was assumed at the conventional level of $p<0.05$. Statistical analysis involved a Spearman's rank analysis when testing

the bivariate correlation between the continuous variables (OGJ-CI, OGJ-MP and DCI) and measures of reflux. The values were natural logarithm transformed to allow linear regression by ANCOVA to calculate the R^2 and adjust for covariates.

A Mann-Whitney U test was used to compare the difference in reflux variables between two groups: Chicago classification (normal versus abnormal); OGJ dysfunction based on OGJ-MP based on a normal threshold of 10mmHg [146]; and OGJ-CI based on the lower limit of normal of 38.5mmHg.cm [195].

All analysis was performed using SPSS ® version 22.0 (IBM, New York, USA).

8.5 Results

8.5.1 Study participants

Baseline characteristics are shown in Table 8.1. Of the original cohort (n=41) two were unable to tolerate the HRM catheter for a sufficient period of time to allow analysis. There was no significant difference between the subjects able and unable to tolerate HRM. The thirty-nine patients included in the analysis had 24-hour pH-impedance recordings and a manometry trace suitable for analysis.

Table 8.1 Characteristics of the study participants.

	Study cohort
N	39
Age (years)	31.1(8.1)
Male	74.4%
Baseline FEV1%	51.9 (17.7)
Baseline BMI	22.3 (3.2)
Chronic infection Pa	68.3%
Chronic infection Bcc	15.4%
Acid suppressive medication	77%

The data are presented as mean (standard deviation) or total number (percentage of total study population) as appropriate. Pa: *Pseudomonas aeruginosa*; Bcc: *Burkholderia cepacia complex*.

8.5.2 Oesophago-gastric junction evaluation

Table 8.2 shows the results of the OGJ evaluation. The prevalence of OGJ hypotension when measured by mean resting pressure was found in 6/39 (15%). However, when measured by OGJ-CI, 27/39 (69%) subjects were hypotensive. All subjects with low mean OGJ-MP had a low OGJ-CI.

Hiatus hernia, as defined by OGJ morphology type 2 or 3, was present in 4/39 (10%) subjects. All subjects with hiatus hernia had hypotension on OGJ-CI, but only 1/4 (25%) using OGJ-MP. The OGJ hypotension was not due to a hiatus hernia in 5/6 (83%) cases when measured by mean pressure, whereas in OGJ-CI hiatus hernia could play a role in 23/27 (85%) cases.

Table 8.2 Results of the evaluation of the oesophago-gastric junction

	Cohort (n=39)
OGJ tone	
Mean resting	16 (11-28) mmHg
Hypotensive (<10mmHg)	6 (15%)
OGJ-CI	30.0 (22.0-42.0) mmHg.com
Hypotensive (<38.5mmHg.cm)	27 (69%)
Pandolfino classification of OGJ morphology	
Type 1	35 (90%)
Type 2	4 (10%)
Type 3a	0 (0%)
Type 3b	0 (0%)
Hiatus Hernia present	4 (10%)

The data are presented as median (interquartile range) or total number (percentage) as appropriate.

8.5.3 Oesophageal body motor evaluation.

Table 8.3 shows the results of the evaluation of oesophageal body motor function. Abnormal motility was found in 66% patients: 54% IOM, 10% absent contractility and 3% oesophageal spasm. None had fragmented peristalsis. Of those with OGJ hypotension, using either OGJ-MP or OGJ-CI, 16/26 (62%) had an associated abnormality of oesophageal motor function.

Table 8.3 Results of oesophageal body evaluation

	Cohort (n=39)
Oesophageal body contractility	
Distal contractile integral	438 (274-687) mmHg.s.com
Chicago v3.0 classification for oesophageal body motor abnormalities[b]	
Normal	13 (33%)
Impaired oesophageal motility	21 (54%)
Fragmented	0 (0%)
Absent contractility	4 (10%)
Oesophageal spasm	1 (3%)
Combination of OGJ hypotension and oesophageal body dysmotility[b]	
Both normal	6 (15%)
Normal OGJ, abnormal oesophageal body motor function	17 (44%)
Abnormal OGJ, normal oesophageal motor function	7 (18%)
Both abnormal	9 (23%)

The data are presented as mean (standard deviation) or percentage as appropriate.

8.5.4 Effect of oesophago-gastric junction hypotension on measures of reflux

Correlations between OGJ-MP and reflux events using pH-impedance

Correlations are shown in Figure 8.3. There was a significant relationship between OGJ-MP and total acid exposure (r_s =-0.371, p=0.024). There were weak non-significant correlations between OGJ-MP and total reflux (r_s=-0.154, p=0.349), proximal reflux (r_s =-0.119, p=0.471) and bolus exposure (r_s =-0.086, p=0.602).

Total acid exposure was natural logarithm transformed to a parametric distribution to calculate the amount of variation in acid exposure attributable to OGJ-MP. Correlation between the natural logarithm of OGJ-MP and log total acid exposure was assessed by linear regression using ANCOVA. The natural logarithm of OGP-MP explained 13% of the variance (adjusted r^2=0.125) of the natural logarithm of total acid exposure (p=0.018).

Due to an expected relationship, a multivariate analysis was performed for the natural logarithm of OGJ-MP, the natural logarithm of total reflux events and use of acid suppressive medications. The natural logarithm of OGJ-MP was best determinant of natural logarithm of total acid exposure and remained statistically significant (p=0.028). Within the model there was a non-significant trend between natural logarithm of total acid exposure and natural logarithm of total reflux events (p=0.055) and use of acid suppression (p=0.103). In combination the three variables accounted for 23% of variability (adjusted r^2=0.227) in natural logarithm of total acid exposure.

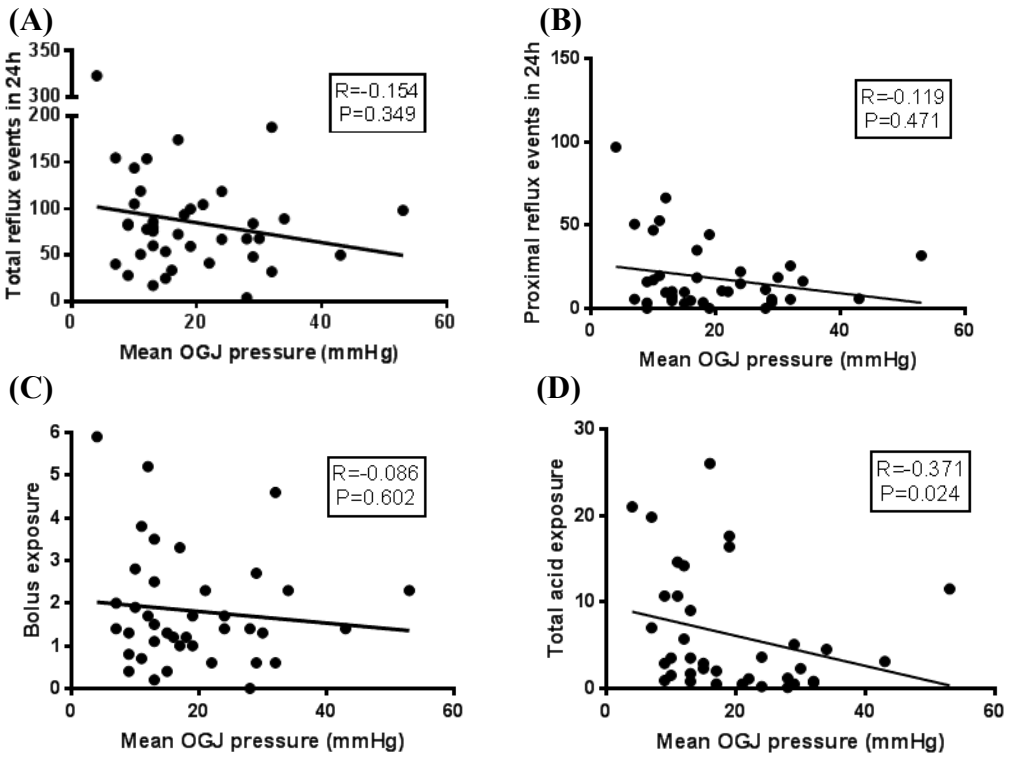


Figure 8.3 Correlation between mean OGJ pressure (OGJ-MP) and pH-impedance measures
(A) Total reflux events, (B) Proximal reflux events, (C) Bolus exposure and (D) acid exposure

Correlations between mean OGJ-CI and reflux events using pH-impedance.

OGJ-CI showed a trend toward a significant correlation with acid exposure (r_s =-0.274, p=0.101). There were weak non-significant correlations with all other measures: total reflux (r_s =-0.153, p=0.352), proximal reflux (r_s =0.023, p=0.888) and bolus exposure (r_s =-0.040, p=0.810).

As with OGJ-MP, total acid exposure was natural logarithm transformed. Correlation between the natural logarithm of OGJ-CI and natural logarithm of total acid exposure was assessed by linear regression using ANCOVA. The natural logarithm of OGJ-CI explained 5.2% (R^2=0.052) of the variance of the natural logarithm of total acid exposure (p=0.093).

A multivariate analysis was performed for the natural logarithm of OGJ-CI, the natural logarithm of total reflux events and use of acid suppressive medications. In this model the most important determinant of the natural logarithm of acid exposure was the natural logarithm of total reflux events (p=0.039). Within the model there was a trend toward correlation between the natural logarithm of OGJ-CI (p=0.126) and use of acid suppression (p=0.137). In combination the three variables accounted for 17% of variability (adjusted r^2=0.166) in the natural logarithm of total acid exposure.

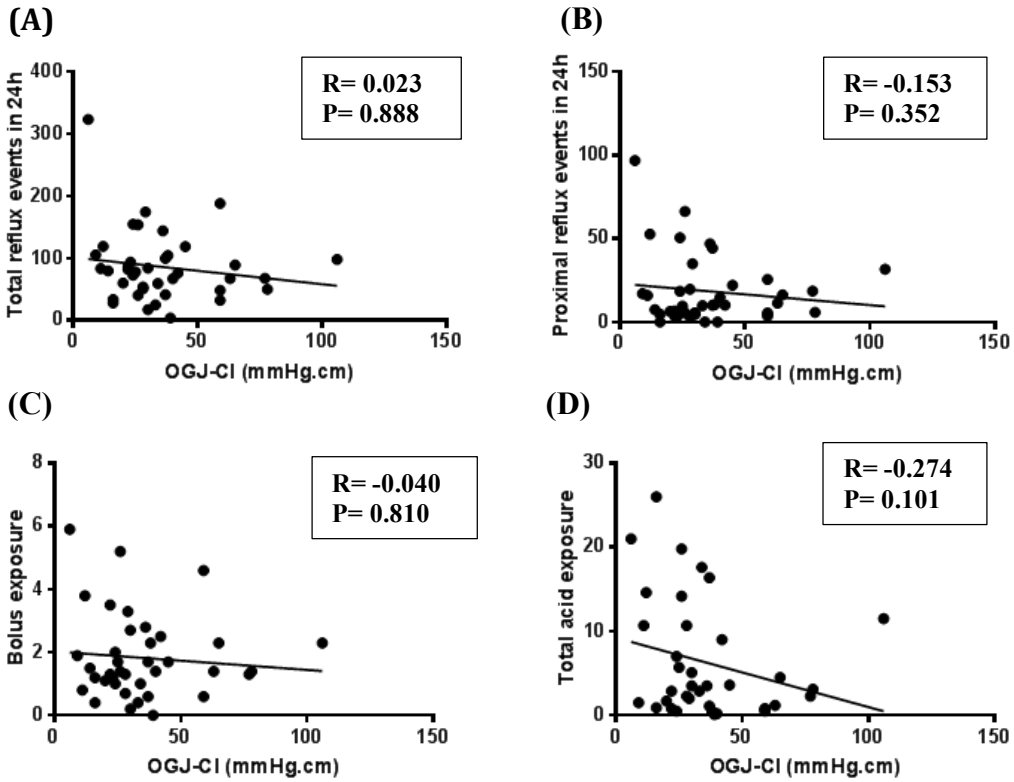


Figure 8.4 Correlation between mean OGJ pressure and pH-impedance measures

(A) Total reflux events, (B) Proximal reflux events, (C) Bolus exposure and (D) acid exposure

Differences in pH-impedance measures between those with and without OJG hypotension using mean OGJ pressure.

The pH-impedance measures were compared between subjects with a mean OGJ pressure above or below 10mmHg. This is based on the lower limit of normal as derived from the 5th centile of a healthy population [195]. The results are shown in Table 8.4. Only acid exposure appeared different between the groups (8.9% vs 2.9%), but not statistically significant. A power calculation was used to detect a difference of this magnitude, with the same proportion in both arms (1:5), significance <0.05 and a power of 0.8. To achieve double the number of patients would be required to complete the study – 13 with hypotension and 65 with normotension. No other variables suggested a difference between the groups.

Table 8.4 Differences in HRM and reflux measures between those with a low and normal OGJ-MP

	Low OGJ-MP	Normal OGJ-MP	p value
N=	6	33	
HRM findings			
OGJ-CI	9.75 (6.0-19.0)	18 (13.0-28.5)	0.003
Mean OGJ pressure	8.0 (6.25-9.0)	34 (24.5-52.0)	0.000
Hiatus Hernia (n=4)	1/4	3/4	
DCI	623 (223-803)	435 (266-686)	0.805
Reflux events			
Total Events	83.0 (40.4-154.9)	76.1 (50.5-102.3)	0.690
Proximal Events	10.7 (3.3-50.5)	10.2 (5.5-20.9)	0.924
Reflux exposure			
Bolus Exposure	1.35 (0.8-2.0)	1.4 (1.0-2.4)	0.835
Acid Exposure	8.9 (2.4-20.1)	2.9 (0.8-9.0)	0.125

Differences in pH-impedance measures between those with and without OJG hypotension using OGJ-CI.

To investigate if those with OGJ hypotension had worse reflux, pH-impedance measures were compared between subjects with an OGJ-CI above or below 38.5mmHg.cm. This is based on the lower limit of normal as derived from the 5th centile of a healthy population[195]. The results are shown in Table 8.5. Again, only acid exposure appeared different between the groups (6.4 vs 2.9), but was not significantly different. A power calculation was used to detect a

difference of this magnitude, with the same proportion in both arms (1:5), significance <0.05 and a power of 0.8. To achieve this more than three times the number of patients would be needed to complete the study– 78 with hypotension and 51 with normotension. No other variables were significantly different between the groups.

Table 8.5 Differences in HRM and reflux measures between those with a low and normal OGJ-MP

	Low OGJ-CI	**Normal OGJ-CI**	**p value**
N=	27	12	
HRM findings			
OGJ-CI	25 (16-30)	59 (43-74)	0.000
Hiatus Hernia (n=4)	4	0	
DCI	435 (246-682)	512 (319-1175)	0.425
Reflux events			
Total Events	82.3 (51-105.4)	67.7 (48.8-96.0)	0.480
Proximal Events	9.8 (4.7-34.9)	13.1 (5.5-21.2)	0.940
Reflux exposure			
Bolus Exposure	1.4 (1.0-2.7)	1.4 (0.8-2.3)	0.940
Acid Exposure	3.5 (1.6-14.4)	1.8 (0.6-4.3)	0.053

8.5.5 Reflux and motility

Correlations between DCI and pH-impedance measures

The DCI did not correlate significantly with total reflux (r=-0.025, p=0.879), proximal reflux (r=-0.072, p=0.655), bolus exposure (r=-0.136, p=0.395) and acid exposure (r=-0.086, p=0.602) Table 8.6.

Differences in pH-impedance measures between those with and without oesophageal dysmotility as per the Chicago Classification

The pH-impedance measures were compared between subjects who had either normal (n=13) and had a classification of IOM or absent contractility (n=28). The results are shown in Table 8.6. There was no significant difference for any of the tested pH-impedance measures.

Table 8.6 Differences in HRM and reflux measures between those with normal and abnormal (absent peristalsis or IOM) oesophageal motility

	Normal	**Abnormal**	**p value**
N=	13	25	
HRM findings			
DCI	1152 (672-1595.5)	315 (171.5-440.5)	.00
OGJ-CI	26 (22.5-49.0)	33 (21-43.5)	.74
Reflux events			
Total Reflux Events	84.2 (44.4-126.1)	72.7 (50.5-102.7)	0.63
Supine events	9.0 (4.2-15.7)	5.5 (3.1-14.0)	0.41
Reflux exposure			
Bolus Exposure	1.4 (0.7-3.1)	1.4 (1.0-2.1)	0.79
Supine bolus exposure	0.2 (0.1-1.5)	0.3 (0.1-0.8)	0.76
Acid Exposure*	6.1 (1.2-13.5)	2.9 (1.1-8.2)	0.38

*Note 2 no pH metrics

8.6 Discussion

The exact pathogenesis for the increased reflux seen in CF is not fully understood. This is the first study in CF patients to use HRM to describe the prevalence and impact on reflux of OGJ and oesophageal body dysfunction. The main findings of the study are: (i) OGJ dysfunction as measured by OGJ-MP and OGJ-CI is highly prevalent; (ii) OGJ dysfunction appears to influence acid exposure, as evidenced by a significant correlation with OGJ-MP and a trend with OGJ-CI; (iii) oesophageal dysmotility is highly prevalent; (iv) occurrence of oesophageal dysmotility showed no correlation or trend toward correlation with any tested measure of reflux.

8.6.1 Oesophago-gastric junction function

The prevalence of OGJ dysfunction was found to be much higher using OGJ-CI (59%) as opposed to OGJ-MP (15%). This difference presumably reflects that each measure quantifies a slightly different aspect of OGJ function. The OGJ-MP is a composite of both basal LOS tone and crural diaphragm augmentation, whereas OGJ-CI assesses OGJ contractility mainly due to crural diaphragm contraction. A useful additional metric of OGJ function would be basal LOS tone. This would allow better delineation of how each aspect is affected in CF. From our data using OGJ-MP and OGJ-CI it would appear that crural diaphragm contraction could be the aspect most affected.

Contrary to studies in other respiratory disease the prevalence of hiatus hernia was low, being identified in only 4/39 (10%) subjects. Therefore, a significant proportion of those with OGJ hypocontractility did not have a hiatus hernia 22/26 (85%). The separation of the crural diaphragm and LOS that occur in hiatus hernia, results in reduced OGJ augmentation[51]. Thus within this cohort, it would appear that a problem exists with diaphragmatic contraction, not explained by misalignment with the LOS. Several explanations are possible. The diaphragm is composed of skeletal muscle, and this type of muscle has been reported to be directly affected by CFTR dysfunction [196]. In addition, it has been shown that CF patients have decreased diaphragmatic strength but no

difference in muscle mass, when compared to healthy volunteers [197]. As such impairment of diaphragmatic skeletal muscle impairment may be a major contribution to OGJ dysfunction. Factors identified that are related to CFTR dysfunction, could be amenable to CFTR modulation by medications such as lumacaftor and ivacaftor.

Another factor affecting diaphragmatic function could relate to changes in lung volumes. It has been demonstrated that increased lung volumes impair diaphragmatic contractility[198]. However, the pattern of CF lung disease varies between patients with some demonstrating air trapping with increase lung volumes, and others having a reduction in lung volumes [199].

Both measures of OGJ function suggested a relationship to acid exposure, but not with other measures of reflux. This is in keeping with previous studies that suggested that OGJ hypotension although increasing reflux events lead to a disproportionate rise in total acid exposure [178]. The natural logarithm of OGJ-MP reached statistical significance, with explaining 12% of the variation in the natural logarithm of acid exposure. Whereas for the natural logarithm of OGJ-CI there was only a non-significant trend for the natural logarithm of acid exposure, explaining only 5%. As such although OGJ-CI abnormalities maybe more prevalent in the CF population, they may not have the same amount of influence as OGJ-MP.

A mentioned early a useful additional metric would be measuring basal LOS pressure. This would allow a more comprehensive evaluation of each aspect of OGJ function. A previous study has described a simple method for calculating basal LOS pressure by measuring the mid-expiratory OGJ pressure. However in this study of non-CF patients basal LOS pressure was not found to be a significant predictor of GORD [36]. This could be different in CF. The relationship between basal LOS tone and contractility of the diaphragm requires further exploration, and could help explain the increased reflux seen.

8.6.2 Oesophageal body motor evaluation

The high prevalence of dysmotility in this CF cohort, is in keeping with that seen in other respiratory diseases. 21/39 (54%) had a minor disorder of peristalsis, whereas only 4/39 (10%) had absent contractility. Over 23% had an abnormality of the OGJ and oesophageal body, whereas only 15% had neither. However, dysmotility had no obvious effect on worsening reflux, using measures taken both over the entire period and only the supine period.

The reason for the increased dysmotility seen both in CF and other respiratory conditions remains unclear. Even if CFTR dysfunction resulted in abnormalities of smooth and skeletal muscle, it would not explain the high prevalence seen in other respiratory diseases. It seems improbable that in all respiratory diseases this is a unique primary phenomenon, whereas a shared acquired mechanism related to respiratory impairment would seem more likely.

A novel hypothesis linking all respiratory disease with dysmotility could involve neuronal cross-talk. In a similar way to the hypothesis which suggests stimulation of the cough reflex by oesophageal afferents, airway nerves maybe stimulated by respiratory disease which cross-talk with the reflex arc involved in control of oesophageal peristalsis – a consequence of both afferents being transmitted through the vagal nerve to the nucleus solitarius [65, 200]. This propensity for 'cross-talk' is supported by numerous studies that have shown that oesophageal acidification influences cough, airway hyper responsiveness and bronchoconstriction within the airways [119, 120, 201, 202].

The lack of impact on reflux could be because of the potential for mild peristaltic abnormalities – in this case defined as IOM – to cause little or no effect on any measure of reflux exposure. There were insufficient subjects with absent peristalsis (4/39) to perform analyses or draw meaningful conclusions. The only study in respiratory disease to demonstrate an effect of peristalsis on reflux exposure did so between those with and without severe disruption. In

patients with ILD, those with absent peristalsis (n=28) had a significantly higher DeMeester score (p=0.024) than those with normal peristalsis (n=32).

There are a number of limitations with this method of assessing oesophageal body function and attempting to relate to amount of reflux. Although it would seem clear that IOM does not lead to a significant change in reflux exposure, the numbers recruited are insufficient to generate a cohort of CF patients with absent motility to be analysed. However, to generate a sample size of 10, providing the prevalence of absent peristalsis within this cohort reflects the larger population (4/39), a 2.5-fold increase in overall recruitment would be required – i.e. rather than n=56 agreeing to participate, n=140 would be needed. This would be a significant challenge given the invasive nature of the procedure involved in the study.

A further limitation is that CC swallowing assessment using HRM provides a measure of oesophageal contractility, but no measures of bolus clearance. This can be overcome by addition of impedance to the HRM catheter[203]. This technology was not available at our hospital site. However, it is possible to extract useful measures of oesophageal reflux clearance from the 24-hour pH-impedance study completed by all subjects. The post-reflux swallow induced peristaltic wave index calculates the percentage of reflux events that are followed by a swallow [204]. This metric presumably reflects peristaltic clearing of the reflux along with chemical neuralisation by saliva. This could be calculated for the entire cohort (n=39) and correlated with acid exposure, as well as compared to a healthy or GORD control group.

Another pH-impedance derived metric, the mean nocturnal baseline impedance, can be used to assess for evidence of oesophageal mucosal damage [204, 205]. Baseline impedance has been shown to correlate with acid exposure and histopathological changes [206]. This could be compared to the CC swallowing assessment, rather than acid exposure. The benefit is that mucosal damage

suggests a more longstanding exposure to reflux, and overcomes the limitations of using a single 24-hour study.

8.6.3 Conclusion

This study has identified a potential relationship between OGJ function and acid exposure. There is a high prevalence of OGJ dysfunction despite low prevalence of hiatus hernia. This does not support that altered lung volumes are disrupting OGJ function. Instead CFTR dysfunction may be directly affecting the skeletal muscle of the diaphragm causing impaired contractility. As such CFTR modulation could be used to treat this aspect of reflux pathogenesis.

Although a high prevalence of oesophageal body abnormalities were demonstrated, there was no obvious effect on reflux measures. This could represent the wrong measure or that it does not play an important role in the pathogenesis of reflux in CF. The finding of a high prevalence of oesophageal motility abnormalities, consistent with other respiratory disease, raises the possibility of a shared mechanisms, perhaps that of neuronal cross-talk. Further exploration of mechanisms and impact of impaired oesophageal motility in CF as well as all respiratory disease is required.

9 The effect of co-morbidities and medications on gastro-oesophageal reflux in cystic fibrosis

9.1 Introduction

Within this cohort of CF patients recruited reflux is increased when compared to previous established normative values (Chapter 7). There is also an increased prevalence of OGJ and oesophageal body dysfunction, with our data suggesting that OGJ hypotension leads to increased acid exposure (Chapter 9). A number of factors have been implicated in the pathogenesis of reflux in CF populations. It is possible that some of the comorbidities or medications administered may play a role. Bowel disease and CF-related diabetes (CFRD) as potential co-morbidities that could affect the amount of reflux.

Bowel symptoms, such as bloating and disturbed habit, are frequently experienced by CF patients [130]. In our experience the patients frequently reporting these symptoms often complain of gastro-oesophageal reflux. Currently it has not been shown how bowel disease could lead to increased reflux. Chapter 7 demonstrated that CF patients have raised amounts of gaseous reflux events on pH-impedance, which is theorised to be of intestinal origin. The increased intestinal gas has the potential to increase reflux by causing gastric distension that in turn alters the gastro-oesophageal pressure gradient, or by increasing the frequency of TLOSR, which is the mechanism to 'vent' the gastric gas.

Within CF populations CFRD is also common, with a prevalence of 40-50% of adults [207]. In non CF populations diabetes mellitus has been linked to increased reflux symptoms and acid exposure on pH studies [208, 209]. This is thought to reflect the adverse effect of neuropathy and hyperglycaemia on OGJ pressures, oesophageal motility and gastric emptying [210].

Another potential causal factor leading to increased reflux is the medication taken by CF patients. Studies have shown that bronchodilators, specifically inhaled β2- agonists and oral methylxanthines, can reduce OGJ pressure [71-73]. The effect of inhaled anti-cholinergics is less well established, but oral preparations have been shown to reduce OGJ pressures [74]. However only methylxanthines have been shown to actually increase acid exposure when measured by pH studies [73]. It has been postulated that mucolytic medications and inhaled antibiotics may lead to increased amounts of reflux, possibly as a consequence of increased coughing. The pressures generated during cough are thought to be sufficient to overcome the OGJ. However it is yet to be proven in respiratory conditions that coughing is an important mechanism for reflux generation[65].

By identifying the mechanisms responsible for the increased amounts of reflux seen in CF patients the range of current therapeutic interventions may be able to be expanded. At present these are limited to acid suppression or surgical intervention. This chapter focusses on establishing if two co-morbidities of interest, bowel disease and CFRD, and/or any medications, are associated with reflux in CF patients.

9.2 Hypothesis and aims

9.2.1 Hypothesis

Co-morbidities and medications are contributing factors to the increased reflux seen in CF.

9.2.2 Aim

1) To investigate if bowel disease is related to reflux.
2) To investigate in CFRD is related to reflux.
3) To investigate if medications increase reflux.

9.3 Methods

9.3.1 Subjects

All subjects were included from the main observational study described in Chapter 6. As such all subjects had a confirmed diagnosis of cystic fibrosis on genetic testing and a typical clinical phenotype, were aged over 18 years and deemed clinically stable. Patients were excluded if they were pregnant or had undergone lung transplantation or fundoplication. In addition, they were excluded if they had a contraindication for oesophageal pH-impedance studies. The study was conducted in line with ethical approval granted by The Greater Manchester West Research Ethics Committee (15/NW/0655).

9.3.2 Study design

All subjects had completed 24-hour pH-impedance and RESQ-7 reflux symptom scores recorded at the research visit (Chapter 7). Results from the high resolution manometry (HRM) measures were also included (Chapter 8). Additional data collection occurred at the research visit, as described in Chapter 6, which was used for this analysis. Background information was collected from the case notes regarding diagnosis of CFRD and the list of prescribed medications. The diagnosis of CFRD is based on the clinical diagnosis found within each patient's medical notes. The original diagnosis would have been made by a consultant endocrinologist in line with our unit's policy, which utilises a combination of glucose tolerance test, HbA1C and continuous glucose monitoring in borderline cases. To obtain a measure of bowel symptoms the IBS-SSS questionnaire was completed. The HbA1C was measured from a venous blood sample.

9.3.3 The adapted irritable bowel syndrome – symptom severity score

At the time of study design no validated questionnaire existed for the capture of CF related bowel symptoms. The IBS-SSS is a validated scoring system used to capture severity of gut symptoms in irritable bowel syndrome (IBS) [133].

These symptoms are very similar to those described in CF. As such the IBS-SSS was adapted and used to capture bowel symptoms in subjects. They were instructed to ignore all reference to IBS within the questionnaire and instead complete all questions based on their bowel symptoms. As such the questionnaire was referred to as the adapted IBS-SSS (aIBS-SSS).

The total score for all four questions and the individual domain scores were used for analysis. The presence of bowel symptoms were taken as a score >75, which was the upper limit of scores from healthy volunteers and then subsequently validated against the IBS cohort [133].

9.3.4 Medications

All prescribed medications were recorded from the clinical notes. The prevalence was calculated for medications that have the potential to affect reflux. They were included for analysis if they met two further criteria: they must be sufficiently prescribed amongst the cohort to allow comparisons between those on and off treatment; it must allow a binary answer i.e. prescribed or not prescribed.

9.3.5 Statistical analysis

All variables were non-parametric so the appropriate non-parametric statistical tests were used. The effect of binary variables on reflux measures was assessed using a Mann-Whitney U test. The effect of continuous variables was assessed by correlative analysis using the Spearman's rank co-efficient. Where appropriate for acid exposure an adjustment was made for the effect of total reflux events using an ANCOVA. All analysis was performed using SPSS® version 22 (IBM, New York, USA).

9.4 Results

9.4.1 Subjects

All patients enrolled in the study described in Chapter 7 were included in this analysis. Thus the demographics are the same, as shown in Table 9.1. The prevalence of the co-morbidities and prescribed medications identified as potentially increasing reflux as shown in Table 9.2. The aIBS-SSS was used to detect bowel symptoms (defined as total score >75). Thirty-nine patients completed the aIBS-SSS. The questionnaire was added to the protocol shortly after commencement of the study. As such two patients completed the study prior to its introduction. Bowel symptoms were demonstrated in 20/39 (51%).

Table 9.1 Characteristics of the study population

	Study cohort (n=41)
Age (years)	30.9(8.2)
Male	70.7%
Baseline FEV1%	51.7(17.5)
Baseline BMI	22.4 (3.1)
Chronic infection Pa	73.2%
Chronic infection Bcc	12.2%
Prescribed acid suppressive medication	78%

The data are presented as mean (standard deviation) or total number (percentage of study population) as appropriate. Pa: *Pseudomonas aeruginosa*; Bcc: *Burkholderia cepacia complex*

Table 9.2 Prevalence of factors of interest

	Study cohort (n=41)
Comorbidities	
CF related diabetes	22 (54%)
Medications	
Methylxanthines	15 (37%)
Aminophylline	8 (20%)
Theophylline	7 (17%)
Inhaled anti-muscarinic	16 (39%)
Inhaled beta-2 agonists	38 (93%)
Oral corticosteroids	6 (15%)
Hypertonic saline	13 (32%)
Dornase alpha (DNase)	26 (63%)
Aerosolised antibiotics	37 (90%)

The data are presented as total number (percentage of study population). *Two patients did not complete the aIBS-SSS.

9.4.2 The effect of bowel symptoms on reflux

There was no significant correlation between total or bloating domain scores and total reflux events or total acid exposure (see Table 9.3 and Table 9.4). There was however a trend seen between total aIBS-SSS and gas events (R_s=0.245, CI -0.103–0.550, p=0.113), but not for the bloating domain (R_s=0.134, CI-0.193–0.444, p=0.417).

Interestingly severity of reflux symptoms was strongly associated with bowel symptoms. Overall severity of bowel symptoms on aIBS-SSS (total score) showed a highly significant correlation with heartburn severity on the RESQ-7 (R_s=.678, 95% CI .430–.840, p=.000). Similar correlations were observed between both total and bloating scores from the aIBS-SSS and the other RESQ-7 scores for heartburn and regurgitation (Table 9.3 and Table 9.4).

Table 9.3 Correlations between aIBS-SSS total score and measures of pH-impedance measures and reflux symptoms

	R_s	95% CI	p value
Total Reflux Events	-0.014	-0.303–0.297	0.935
Total acid exposure	-.073	-0.413–0.280	0.667
Total gas events	0.245	-0.103–0.550	0.113
Heartburn frequency (RESQ-7)	0.649	0.380–0.825	0.000
Heartburn severity (RESQ-7)	0.678	0.430–0.840	0.000
Regurgitation frequency (RESQ-7)	0.627	0.361–0.802	0.000
Regurgitation severity (RESQ-7)	0.638	0.374–0.808	0.000

Table 9.4 Correlations between aIBS-SSS bloating domain and measures of pH-impedance measures and reflux symptoms

	R_s	95% CI	p value
Total Reflux Events	-0.073	-0.413–0.280	0.667
Total acid exposure	-0.142	-0.466–0.188	0.402
Total gas events	0.134	-0.193–0.444	0.417
Heartburn frequency (RESQ-7)	0.495	0.185–0.721	0.001
Heartburn severity (RESQ-7)	0.538	0.261–0.735	0.000
Regurgitation frequency (RESQ-7)	0.482	0.179–0.705	0.002
Regurgitation severity (RESQ-7)	0.486	0.139–0.734	0.002

9.4.3 The effect of CF related diabetes on reflux

Contrary to expectation in patients with CFRD there was actually a significant reduction in total acid exposure compared to non-CFRD subjects (median 2.1% (IQR 0.8-3.8) versus 9.0% (IQR 1.9-15.5), p=0.015). This difference was not noted for total reflux events (median 73 episodes (IQR 40-108) versus 80 (IQR 51-100), p=0.657). There were no significant differences between CFRD and non-CFRD for reflux symptoms (see table 9.5). Importantly more patients were on acid suppressive medication with CFRD (95.5%) compared to those without CFRD (57.9%). Presence of CFRD did not influence OGJ pressures, using either OGJ-mean pressure (OGJ-MP) or OGJ-contractile integral (OGJ-CI), or oesophageal motility measured using the distal contractile integral (DCI) (see table 9.5).

To explore further the relationship between CFRD and acid exposure a multivariate analysis was performed using natural logarithms of total acid exposure, natural logarithms of total reflux events and diagnosis of diabetes. Adjusting for the total number of reflux episodes, CFRD remained statistically significant (p=0.017). When further adjusted for the use of acid suppression the difference was no longer significant (p=0.355).

There was no significant correlation between glycemic control and total reflux events, total acid exposure or reflux symptoms (see Table 9.6).
However, there was a non-significant trend between HbA1C and heartburn frequency (R_s=-0.280, CI -0.554–0.027, p=0.088) and heartburn severity (R_s=-0.299, CI -0.564–0.001, p=0.068). The HbA1C did not influence OGJ pressures, using either OGJ-MP or OGJ-CI, or oesophageal motility measured using the DCI (see Table 9.6).

Table 9.5 Differences between subjects with and without CFRD for selected pH-impedance, high resolution manometry measures and reflux symptoms

	CFRD	Non CFRD	p value
Total acid exposure	2.1 (0.8–3.8)	9 (1.9–15.5)	0.015
Total reflux events	73.0 (43.3–115.3)	78.8 (51.0–99.9)	0.657
OGJ-MP	18.5 (12–28)	13 (11.0–19.0)	0.496
OGJ-CI	36.5 (24.5–55.5)	28 (14–37)	0.149
DCI	413 (268.5–1036.5)	443 (274–682)	0.875
Heartburn frequency	0.9 (0–2.1)	0.6 (0–1.8)	0.979
Heartburn severity	1.1 (0–2.2)	0.8 (0–2.2)	0.780
Regurgitation frequency	1.0 (0–1.9)	0.5 (0.3–2.8)	0.534
Regurgitation severity	0.9 (0–2.2)	0.5 (0.3–2.5)	0.671

Table 9.6 The relationship between HbAIc, selected pH-impedance measures, high resolution manometry measures and reflux symptoms

	Rs	95% CI	p value
Total reflux events	-0.158	-0.445–0.142	0.342
Total acid exposure	-0.001	-0.313–0.340	0.993
Heartburn frequency (RESQ-7)	-0.280	-0.554–0.027	0.088
Heartburn severity (RESQ-7)	-0.299	-0.564–0.001	0.068
Regurgitation frequency (RESQ-7)	-0.174	-0.464–0.145	0.297
Regurgitation severity (RESQ-7)	-0.190	-0.504–0.158	0.252
Distal contractile integral	-0.247	-0.584–0.104	0.146
OGJ-mean pressure	-0.212	-0.494–0.131	0.216
OGJ-contractile integral	-0.147	-0.445–0.200	0.392

9.4.4 Medications influencing risk factors

Subjects were prescribed a number of medications with the potential to increase reflux (see Table 9.2). The only medication that appeared to be associated with an increased number of total reflux events was methylxanthines (median 97.3 episodes (interquartile range 72.7-154.9) versus 67.5 (38.8-90.4), p=0.015), but no difference was noted for acid exposure.

Although the measures of total reflux are higher for oral corticosteroids and nebulised hypertonic saline, there is overlap in values especially within the

lower quartile. No significant difference was noted for any medication other than methylxanthines and total reflux events or acid exposure (see Table 9.7 and Table 9.8). There was no significant difference noted for any medications and OGJ pressures or oesophageal motility (see Table 9.9).

Unfortunately, it was not possible to perform analyses on patients prescribed aerosolised antibiotics, beta 2 agonists or inhaled anti-cholinergics due to the heterogeneity of the groups. Different aerosolised antibiotic are used and some, such as tobramycin and colistin, can be a dry powder or nebulised wet preparation. There were only four patients not prescribed nebulised antibiotics meaning there were insufficient numbers for a control cohort. Aerosolised anti-muscarinic and beta 2 agonists again come in different preparations (dry powder and nebulised) and had different durations of action (short and long-acting preparations). They are also taken as both regular and on an as required basis.

Table 9.7 The effect of medications on total reflux events

	Positive	**Control**	**p value**
Methylxanthines	97.3 (72.7-154.9)	67.5 (38.8-90.4)	0.015
Oral corticosteroids	106(53-222)	78(48-98)	0.319
Hypertonic saline	82(46-137)	70(49-99)	0.413
DNase	63.4(34-95)	83(71-105)	0.116

Table 9.8 The effect of medications on total acid exposure

	Study group	**Control**	**p value**
Methylxanthines	2.6 (1.4-8.1)	3.5 (1.0-10.7)	0.874
Oral corticosteroids	2.1(1.1-7.9)	3.1 (1.0-10.7)	0.718
Hypertonic saline	2.9 (1.2-9.5)	3.1 (0.8-10.7)	0.940
DNase	2.9 (1.1-5.1)	3.6 (1.0-13.1)	0.605

Table 9.9 The effect of medication on high resolution manometry measures

		Study group	Control	p value
Methylxanthines	**OGJ-MP**	15 (10.5–28)	17 (11–28)	0.826
	OGJ-CI	26 (22–52)	33.5 (22.3–40.5)	0.627
	DCI	438 (324–729.5)	439 (228.8–687.8)	0.429
Oral corticosteroids	**OGJ-MP**	28 (7–31)	15.5 (12–24)	0.729
	OGJ-CI	59 (21–70)	28.5 (22–39)	0.223
	DCI	391 (339–1249.5)	440.5 (259–687)	0.505
Hypertonic saline	**OGJ-MP**	12.5 (11–20.75)	18 (13–28)	0.313
	OGJ-CI	27 (17.5–36)	34 (23–45)	0.327
	DCI	342 (47–507.5)	568 (304–1152)	0.070
DNase	**OGJ-MP**	17.5 (11.25–29)	13 (11–21)	0.283
	OGJ-CI	31.5 (24.5–54.25)	25 (14–42)	0.246
	DCI	548.5 (306.75–1036.5)	351 (177–618)	0.091

9.5 Discussion

9.5.1 Overview of findings

The pathogenesis of the increased amounts of gastro-oesophageal reflux seen in CF populations remains unclear. This is also true for other respiratory diseases, including pulmonary fibrosis, asthma and chronic obstructive pulmonary disease. It has been suggested the associated co-morbidities and numerous medications taken by CF patients may be a contributing factor. This study attempted to ascertain if presence of bowel symptoms or CFRD, as well as taking any specific medications, are associated with increased amounts of reflux. The main findings are: (i) bowel symptoms strongly correlate with reflux symptoms, but appear not related to the amount of reflux; (ii) CFRD did not

appear to correlate with increased amounts or symptoms of reflux; (iii) there was a trend toward less heartburn associated with a higher HbA1C; and (iv) use of methylxanthines is associated with increased total reflux events, but not reflux exposure.

9.5.2 Bowel symptoms and reflux

In clinical practice, patients that frequently report bowel symptoms also report symptoms attributed to reflux. In support of this within this cohort symptoms scores for overall bowel symptoms and bloating correlate with reflux symptoms on the RESQ-7. To our knowledge this has not previously been reported in CF. Interestingly there was no correlation, nor suggestive trend, between bowel symptoms and amount of reflux measured. This highlights a crucial point about the complexity of symptom generation in reflux disease, in particular heartburn. Although amount of reflux and oesophageal acid exposure are key components, mucosal and visceral nervous sensitivity play an important modulating role in symptom generation [47]. This is highlighted in patients symptomatic of reflux where it is possible to identify large numbers with oesophageal hypersensitivity (temporal association with normal amounts of reflux) and functional heartburn (no temporal association between heartburn and reflux)[46, 211]. Put another way, it is possible to complain of reflux symptoms that are attributable to either physiological amounts of reflux or not temporally linked to reflux at all.

Similar findings have been shown previously in IBS patients. IBS is a heterogeneous entity with likely pathophysiology that includes visceral hypersensitivity, gut inflammation and bacterial overgrowth[212]. It has been shown that symptoms of reflux frequently occur in patients with IBS [213]. However, when using pH-impedance with symptom association it has been shown that in IBS these are most often due to functional heartburn and oesophageal hypersensitivity. Our findings suggest that oesophageal visceral hypersensitivity is an important cause of typical reflux symptoms in CF patients, meaning that reflux, and possibly bowel symptoms, are perceived with a severity disproportionate to the degree of stimuli. This is also in keeping with

our findings in the previous chapter showing that reflux symptoms have poor specificity for identifying CF patients with increased reflux. To further explore the relationship between symptoms and reflux, the temporal relationship could be assessed between symptoms recorded during the pH-impedance study. This will allow it to be determined which patients have functional heartburn, or oesophageal hypersensitivity.

To our knowledge no study has reported increased visceral hypersensitivity in CF patients. In IBS it is thought that visceral hypersensitivity can occur as a consequence of psychological stress and gut inflammation [214, 215]. It was shown that anxiety was more common in patients with functional heartburn and oesophageal hypersensitivity, than GORD subtypes related to increased amounts of reflux [167]. Anxiety is a common problem in CF patients [216]. Several studies have also demonstrated increased gut inflammation in CF [169, 170]. These may explain a potential increase in prevalence of visceral hypersensitivity in CF. Neither anxiety or gut inflammation were measured in our cohort, but present interesting areas for future work. If CF patients are proven to have visceral hypersensitivity, this has several implications: treatments targeting this mechanism may be useful in appropriately identified individuals; symptoms are not an appropriate surrogate for quantifying the amount of reflux seen in CF patients; and could visceral hypersensitivity could share mechanisms with neuronal-crosstalk.

A trend was noted, suggesting a link between the overall aIBS-SSS score and total gas events. Intestinal gas composition has shown to be altered using breath testing in a number of gastro-intestinal conditions, such as IBS and small bowel overgrowth, as well as in cystic fibrosis [217-219]. Although patients with these conditions frequently complain of increased belching, no previous study to our knowledge has objectively measured belching using impedance measured gas events. Although intestinal gas composition maybe altered in these conditions, it is unclear if overall gas production is actually increased.

Our data showed no significant relationship between bloating and gas events. This is in keeping with current knowledge. The bloating sensation is believed to occur as an imbalance between gut tone and volume of contents. There is also a likely role for individual perception, with varying degrees of sensitivity to the 'bloating' stimuli [220]. Although it would appear there is an increased intestinal gas within this CF cohort based on pH-impedance, the high prevalence of bloating reported may be generated by additional mechanisms. This could be a consequence of heightened sensory perception, due to visceral hypersensitivity.

Our findings support a need for further work looking into the role of altered gas production within the small bowel and its relationship to reflux, including examining the possibility of a global increased sensitivity of the gut.

9.5.3 CF related diabetes and reflux

No significant increase was detected in the amount of reflux associated with the presence of diabetes. In fact, the opposite was observed with a statistically significant reduction in acid exposure in those with CFRD. Although one could theorise of potential reasons, this was most likely a chance finding. There was no difference between presence of CFRD and total reflux episodes, nor correlation noted between HbA1C and either measure of reflux. What is more when the result was adjusted for use of acid suppression it was no longer significant.

Interestingly there was a trend toward a negative correlation between HbAIc and RESQ7 captured symptoms of heartburn — suggesting that with poorer glycaemic control patients experienced less heartburn. There was however no significant difference between in scores between those with and without CFRD. Microvascular complications are noted in CFRD patients with a frequency similar to that seen in Type 1 diabetics [221]. It has been shown that in non-CF patients with microvascular diabetic complications, oesophageal innervation can be effected and interferes with generation of typical reflux symptoms [222].

This could lead to an even more complicated mechanism of reflux symptom generation, with both visceral hypersensitivity as well as denervation occurring due to diabetes.

The prevalence of diabetes in this cohort (54%) was high compared to that reported in the CF registry (33%) [2]. This may reflect the more advanced disease seen within the research cohort. Although the mean age of the cohort was similar to the overall population at the Manchester Adult CF Centre, the FEV1 percent predicted was noted to be significantly lower within the research cohort (mean (standard deviation) FEV1 percent predicted 51.7(17.5) % versus 58.6(24.7) %.

9.5.4 Medications and reflux

Methylxanthines was the only medication where prescription suggested an increase in reflux. The total reflux events were significantly raised, but not so for total acid exposure. In fact, the median acid exposure was actually higher in those not receiving this medication (3.5% vs 2.6%). One explanation is this finding may be due to the differences in acid suppression prescription. There was a slight increase in those subjects prescribed of any form acid suppression who were on methylxanthines (13/15, 86.7%) compared to those who were not (19/26, 73%). However, there were much higher prescriptions of dual acid suppression — prescription of PPI and H2 antagonist — for those on methylxanthines (7/15, 47%) compared to those who were not (5/26, 19%). This could have occurred as a consequence of those subjects on methylxanthines being increasingly symptomatic and requiring more aggressive acid suppression.

Alternatively, this could represent a type 1 statistical error. This is particularly important given the performance of multiple analyses in this cohort. Should a Bonferroni adjustment have been undertaken this would no longer have been significant, with the level required to reach significance being <0.013. However

our results are in keeping with a previous study that demonstrated that methylxanthines were associated with increased reflux, albeit acid exposure in a cohort not on acid suppression therapy [73]. These finding will need confirming in a repeat study, before any firm conclusions can be drawn.

9.5.5 Study limitations

There are several limitations of this study. Firstly, there are clearly multiple interacting effects on the amount of reflux. Some of these will increase and others decrease the amount. The sample size (n=41) may not be sufficient to appreciate competing effects of different medications and co-morbidities.

The pH-impedance measures were performed to reflect the patients usual reflux profile, as such they continued on their regular acid suppression medications. There were a number of different regimens made up of different agents and doses, which in turn leads to a variable amount of acid suppression. Had assessing the impact of various factors on reflux been the main aim of the study rather than a sub-analysis, acid suppression would have been withheld or the dosing standardised for each subject.

A further issue was the use of the aIBS-SSS as a measure of CF bowel symptoms. Although symptoms are similar to IBS, this questionnaire has not been validated in a CF population. However, at the time of conception no validated CF questionnaire existed. In an attempt to overcome this issue a study within the Manchester CF Centre is validating both the original aIBS-SSS and an amended CF-bespoke version — known as the CF bowel score (CF-BS). Furthermore the aIBS-SSS was used to capture symptoms as a surrogate measure for the extent bowel disease, whereas findings from this study suggest that in the upper gastro-intestinal tract visceral hypersensitivity may be occurring. As such the symptoms reported in the lower gastrointestinal tract may also be effected by disordered sensory reflexes. This may explain our failing to detect correlation between reflux and bowel symptoms. A future approach may be a more objective measure of bowel disease, such as breath testing for small bowel

bacterial overgrowth, stool microbiology for dysbiosis, intestinal transit time assessments or general measures of bowel mucosal inflammation.

Diagnosis of CFRD is dichotomous and based upon a threshold set by current guidelines. However glycaemic control is a spectrum with a number of patients displaying disordered glucose handling but failing to reach the cut off for diabetes. When a patient is diagnosed with CFRD there will be a number of interventions, such as dietary modification or prescription of insulin, which will potentially improve glucose handling. An improvement could then result in that patient with a CFRD diagnosis, having better glycaemic control than one on the cusp of the diagnostic threshold.

The HbA1C overcomes some of these limitations by providing a measure of glycaemic control. However, a single result neither provides a measure of control on the day of the reflux study nor does it provide historic information beyond several months prior to the study. Thus it does not assess the impact of fluctuations in blood glucose on the pH-impedance or HRM measures. It also is not able to identify the patients with longstanding hyperglycaemia that could have microvascular damage of the upper gastrointestinal with the potential to lead to impairment of OGJ function and gastric emptying.

Finally, the medication data analysed were for prescription rather than administration. This may not reflect accurately the amount of these medications taken by the patients. It has been shown previously that compliance in CF to medications is often below 50% [223].

9.5.6 Conclusion

Analyses within this chapter have provided novel insights into the influences on reflux disease in CF, specifically looking at symptom generation. Our data suggests that visceral hypersensitivity maybe a significant factor in the frequency of reflux symptoms reported. This could have therapeutic implications. Impaired glycemic control may reduce the occurrence of reflux

symptoms possibly due to microvascular damage to the oesophageal innervation.

Methylxanthines could increase the amount of reflux in CF patients, but needs further exploration. None of the other medications or comorbidities examined showed a relationship with increased amounts of reflux.

10 Gastro-oesophageal reflux in cystic fibrosis: characteristics and relationship to lung disease

10.1 Introduction

Although a relationship has been proposed between gastro-oesophageal reflux and CF lung disease, the evidence in support is still accumulating. This relationship maybe one in which increased reflux is driven by lung disease [63]; that the lung disease is worsened by reflux [96, 98]; or both occur — a bi-directional relationship. In turn there are two proposed mechanisms by which reflux may exert an effect on lung disease: the reflux extends proximally and is 'breathed in' to the lungs (reflux aspiration) or reflux stimulates afferent nerves within the oesophagus with efferent effects in the lungs (neuronal cross-talk) [65]. This is discussed more comprehensively in chapter 4.

Currently there is a lack of robust evidence supporting the presence of a relationship between reflux and CF lung disease. The majority of studies in adults and children with CF have failed to show a significant relationship between measures of reflux derived from pH-impedance and CF lung disease [57, 78, 152]. The only exception is a single study in a paediatric cohort that showed total number of reflux episodes (R=-0.474, p=0.009) and number of non-acid events (R=-0.397, p=0.03) correlated with baseline FEV1 percent predicted [111]. Importantly all studies that examined lung function did so using baseline FEV1 percent predicted, none looked at longitudinal change [57, 78, 152]. Of note only one study examined intravenous antibiotic requirements [78]. As such, a thorough analysis of all standard measures of respiratory health in CF compared with reflux measurements using pH-impedance is still outstanding.

The crux is that current evidence does not provide conclusive proof of the presence of a relationship between reflux and CF lung disease. This study

attempts to define the relationship between reflux, as measured by pH-impedance, and severity of CF lung disease.

10.2Hypothesis

In CF patients increased amounts of reflux are related to more severe lung disease.

10.3Aim

The aim is to assess the relationship between reflux and CF lung disease.

10.4Methods

10.4.1 Subjects

All subjects from the main observational study, described in chapter 6, were considered for this analysis. As such all were confirmed cystic fibrosis on genetic testing and a typical clinical phenotype, aged over 18 years, clinically stable and able to undergo oesophageal pH-impedance studies. Patients had been excluded if they were pregnant or had undergone lung transplantation or fundoplication. In addition, patients were excluded from the analysis if during the period of follow-up (two years retrospectively) they had been commenced on CFTR modulation therapy or had been pregnant. The study was conducted in line with ethical approval granted by The Greater Manchester West Research Ethics Committee (15/NW/0655).

10.4.2 Study design

All subjects had 24 hour-impedance measurements available from the main study research visit. Additional data were collected at this visit, as described in chapter 5, for use in this analysis. Baseline lung function was recorded at the visit. Retrospective measures of CF lung disease severity were collected from the clinical notes for the two years preceding enrolment.

10.4.3 Baseline FEV1

Baseline spirometry was measured using the Easy on-PC (NDD, Zurich) system. The predicted values were calculated for forced expiratory volume in one second (FEV1), forced vital capacity (FVC) and forced expiratory flow at 25-75 (FEF 25-75%) using the Global Lung Function Initiative reference equations (see Methods section).

10.4.4 Longitudinal FEV1 percent predicted change

Lung function is routinely collected at every clinical interaction. Only measures collected at a routine outpatient appointment whilst clinically stable were included for analysis. Clinically stable was defined as not currently receiving, nor planned to receive, antibiotics and no acute respiratory issues highlighted.

To calculate longitudinal change, the baseline percent predicted FEV1 was defined as the maximum value occurring within three months before or after the date of the study visit. This method was chosen, as the lung function measures achieved at the study visit were often lower than those seen from adjacent clinic appointments. Presumably this reflects a combination of dehydration and disruption of the usual treatment regimen, as the subject was starved overnight and attended early in the morning. The same method was used to calculate FEV1% at one and two years prior to the study visit.

10.4.5 Intravenous antibiotic requirements

Intravenous antibiotic requirements were used as a surrogate for pulmonary exacerbations. The number of courses and actual days of IV antibiotics were recorded from the clinical notes. This was calculated for one and two calendar years prior to the study visit.

10.4.6 Statistical analysis

All continuous measures are non-parametric thus presented as median (inter-quartile range). Statistical significance was assumed at the conventional level of $p<0.05$. Statistical analysis involved a Spearman's rank analysis when testing

the bivariate correlation between baseline percent predicted FEV1 and reflux variables.

General estimating equation (GEE) models were used with each reflux variable in turn as the independent variable changing with time. The respiratory variables were used as the outcome variables. Duration of IV antibiotic courses were the only non-parametric outcome variable so were natural logarithm transformed.

All analysis was performed using SPSS ® version 22.0 (IBM, New York, USA).

10.5 Results

10.5.1 Subjects

A total of 39 patients were included from the original cohort of 41 (Chapter 6). Two patients were excluded due to being commenced on CFTR modulation therapy during the follow-up period. Data for another were limited to only one year immediately before the study visit, as she was pregnant before this. Characteristics are shown in Table 10.1.

	Study cohort (n=41)
Age (years)	30.9(8.2)
Male	70.7%
Baseline FEV1	2.0(1.0)
Baseline FEV percent predicted	51.7(17.5)
Baseline FVC	3.3(1.2)
Baseline FVC percent predicted	70.2(18.8)
Baseline FEF 25-75	1.2(1.1)
Baseline FEF 25-75 percent predicted	27.1(21.5)
Baseline BMI	22.4 (3.1)
Chronic infection Pa	73.2%
Chronic infection Bcc	12.2%
Prescribed acid suppressive medication	78%

Table 10.1 Characteristics of study population

The data are presented as mean (standard deviation) or total number (percentage of study population) as appropriate. Pa: *Pseudomonas aeruginosa*; Bcc: *Burkholderia cepacia complex.*

10.5.2 Raw data for longitudinal lung function

Longitudinal results were calculated for the three time intervals and the raw data are plotted for each tested lung function parameters (see figure 10.1 below). For some subjects no lung function was available for inclusion at certain time intervals. At baseline results were available for 40 of 41 subjects; at 1 year prior 38 of 41 subjects; and at 2 years prior 29 of 41 subjects. The missing data points occurred for some subjects because they did not have stable lung function available within the specified time frame. For the remaining subjects with missing data points, they were all enrolled at the start of the trial and retrospective data was not available for the entire two year time interval. This

was because the Easy on-PC (NDD, Zurich) system for monitoring and recording lung function was introduced into routine clinical toward the start of the two year retrospective data collection period.

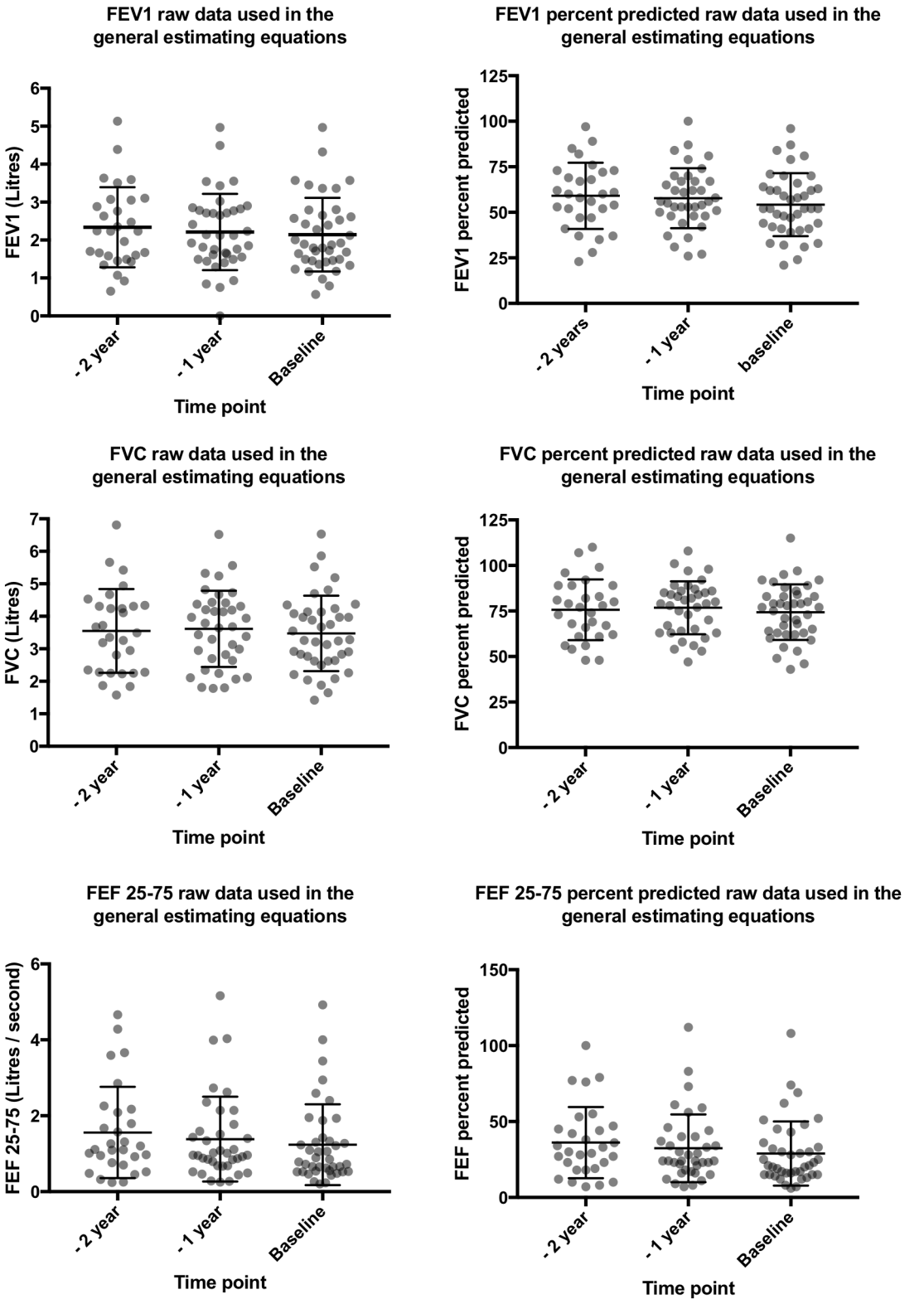


Figure 10.1 Scatter plots showing raw lung function data used for the general estimating equations. The horizontal lines show the mean values and the error bars the standard deviation.

10.5.3 Effect on lung function

Using a Spearman Rank analysis no significant correlation was demonstrated between lung function (FEV1 absolute, FEV1 percent predicted, FVC absolute, FVC percent predicted, FEF 25-75% absolute, FEF 25-75% percent predicted) and any tested reflux parameter. Results are shown in tables 10.2 - 10.7. For FEV1 percent predicted only the correlations are plotted (see Figure 10.2**Error! Reference source not found.**). GEE showed only one significant correlation ($p<0.05$) between longitudinal lung function (FEV1 absolute, FEV1 percent predicted, FVC absolute, FVC percent predicted, FEF 25-75% absolute, FEF 25-75% percent predicted) change and any of the reflux measures. Proximal supine events correlated with a change in absolute FEF 25-75% (beta co-efficient 0.031, p value 0.04). However, given the large number of analyses performed and that this association was not present between FEF 25-75% percent predicted and proximal supine events then this likely represents a false discovery i.e. a type 1 error. If the p value for significance ($p<0.05$) was adjusted by Bonferroni (adjusted to $p<0.001$) the relationship would be no longer be, or trending toward, significance.

	R_s	95% CI	p value
Total reflux events	.157	-.220 – .464	.325
Proximal reflux events	.082	-.305 – .419	.611
Supine reflux events	.045	-.297 – .417	.780
Proximal supine reflux events	.118	-.213 – .437	.462
Total acid reflux events	.008	-.003 – .175	.961
Total non-acid reflux events	.237	-.074 – .537	.146
Acid exposure	.081	-.240 – .401	.624
Bolus exposure	.133	-.233 – .457	.408

Table 10.2 Correlations between FEV1 absolute and pH-impedance measures of reflux.

	R_s	95% CI	p value
Total reflux events	.127	-.237 – .451	.430
Proximal reflux events	-.013	-.398 – .323	.938
Supine reflux events	-.051	-.363 – .310	.752
Proximal supine reflux events	-.004	-.330 – .314	.981
Total acid reflux events	.037	-.318 – .374	.823
Total non-acid reflux events	.213	-.129 – .520	.192
Acid exposure	.043	-.301 – .367	.795
Bolus exposure	.040	-.314 – 361	.805

Table 10.3 Correlations between FEV1 percent predicted and pH-impedance measures of reflux.

	R_s	95% CI	p value
Total reflux events	.144	-.227 – .484	.371
Proximal reflux events	.137	-.221 – .458	.393
Supine reflux events	.037	-.307 – .399	.821
Proximal supine reflux events	.150	-.171 – .439	.349
Total acid reflux events	.052	-.250 – .388	.752
Total non-acid reflux events	.162	-.163 – .472	.323
Acid exposure	.174	-.130 – .481	.290
Bolus exposure	.187	-.193 – .515	.241

Table 10.4 Correlations between FVC absolute and pH-impedance measures of reflux.

	R_s	95% CI	p value
Total reflux events	.107	-.232 – .430	.505
Proximal reflux events	-.005	-.375 – .318	.976
Supine reflux events	-.118	-.433 – .242	.464
Proximal supine reflux events	-.055	-.376 – .259	.735
Total acid reflux events	.026	-.303 – .338	.874
Total non-acid reflux events	.178	-.205 – .492	.278
Acid exposure	.096	-.226 – .408	.568
Bolus exposure	.144	-.199 – .444	.368

Table 10.5 Correlations between FVC percent predicted and pH-impedance measures of reflux.

	R_s	95% CI	p value
Total reflux events	.202	-.148 – .494	.204
Proximal reflux events	.101	-.264 – .420	.528
Supine reflux events	.048	-.251 – .388	.766
Proximal supine reflux events	.108	-.228 – .413	.501
Total acid reflux events	.006	-.308 – .345	.973
Total non-acid reflux events	.310	-.044 – .580	.055
Acid exposure	.006	-.312 – .352	.972
Bolus exposure	.116	-.226 – .429	.471

Table 10.6 Correlations between FEF 25-75% absolute and pH-impedance measures of reflux.

	R_s	95% CI	p value
Total reflux events	.160	-.182 – .457	.317
Proximal reflux events	.035	-.343 – .374	.829
Supine reflux events	-.012	-.327 – .341	.942
Proximal supine reflux events	.043	-.286 – .364	.790
Total acid reflux events	-.028	-.349 – .302	.868
Total non-acid reflux events	.305	-.029 – .580	.059
Acid exposure	-.064	-.381 – .291	.697
Bolus exposure	.054	-.284 – .369	.739

Table 10.7 Correlations between FEF 25-75% percent predicted and pH-impedance measures of reflux.

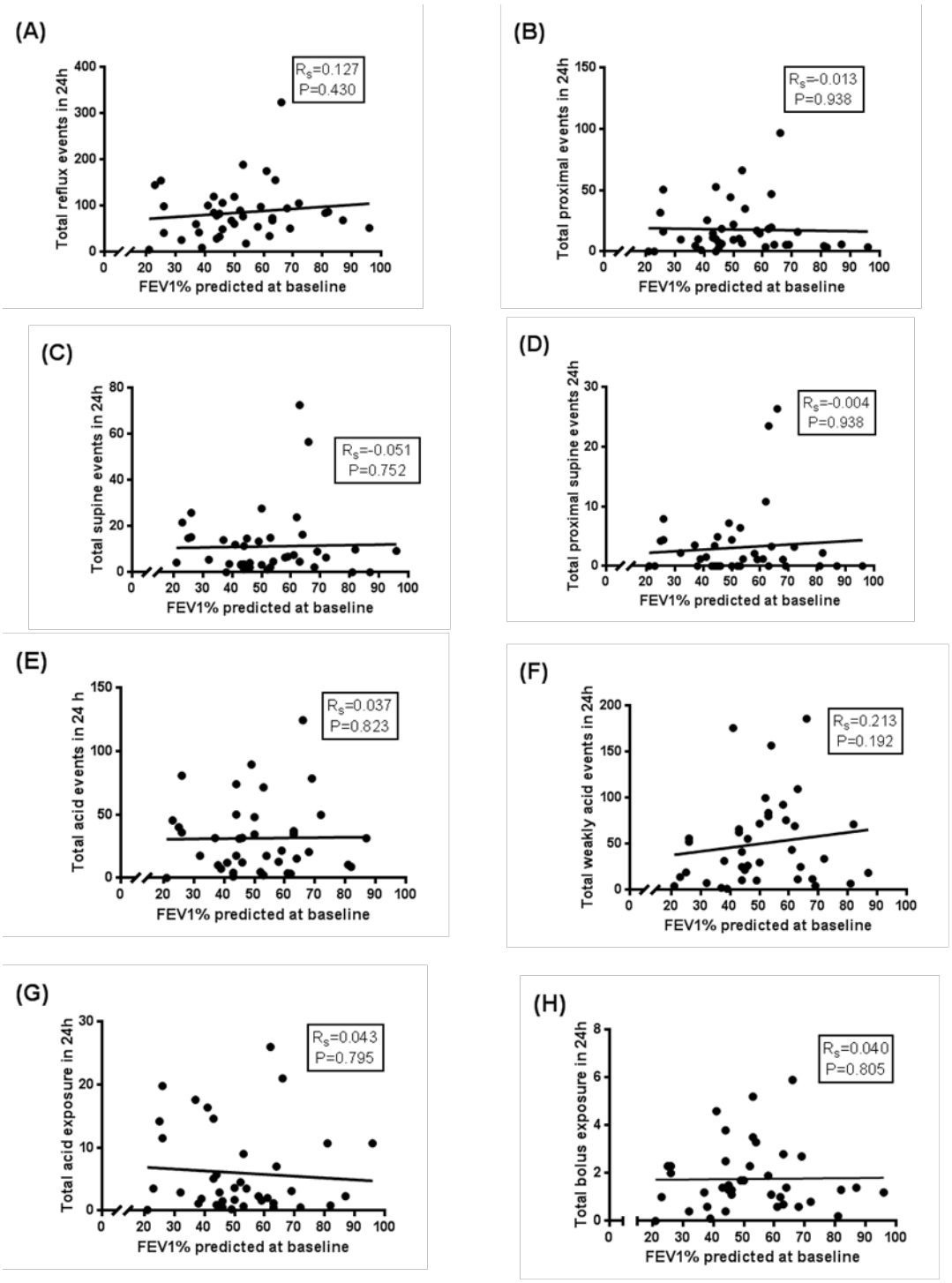


Figure 10.2 Correlation between reflux measures derived from pH-impedance and correlation with FEV1 percent predicted

	Beta co-efficient	95% confidence interval	P value
Total reflux events	0.002	-.003–.006	.419
Proximal reflux events	0.001	-.013–.016	.847
Supine reflux events	0.007	-.008–.023	.355
Proximal supine reflux events	0.030	-.004–.064	.084
Total acid reflux events	0.003	-.006–.012	.526
Total non-acid reflux events	0.002	-.004–.007	.540
Acid exposure	0.016	-.020–.052	.379
Bolus exposure	0.039	-.158–.236	.699

Table 10.8 GEE results examining the effect of reflux variables on longitudinal absolute FEV1.

	Beta co-efficient	95% confidence interval	P value
Total reflux events	-.003	-.085–.079	.942
Proximal reflux events	-0.094	-.350–.163	.474
Supine reflux events	-.004	-.232–.224	.974
Proximal supine reflux events	.163	-.349–.676	.532
Total acid reflux events	-0.05	-.166–.155	.949
Total non-acid reflux events	0.005	-0.086–.095	.923
Acid exposure	0.010	-.646–.666	.976
Bolus exposure	-1.075	-4.681-2.530	.559

Table 10.9 GEE results examining the effect of reflux variables on longitudinal change in FEV1 percent predicted.

	Beta co-efficient	95% confidence interval	P value
Total reflux events	0.002	-.005–.010	.587
Proximal reflux events	.005	-.015–.024	.626
Supine reflux events	.008	-.018–.033	.550
Proximal supine reflux events	.039	-.020–.097	.196
Total acid reflux events	.005	-.008–.017	.481
Total non-acid reflux events	.002	-.004–.007	.540
Acid exposure	.030	-.017–.076	.216
Bolus exposure	.098	-.199–.395	.518

Table 10.10 GEE results examining the effect of reflux variables on longitudinal change in absolute FVC.

	Beta co-efficient	95% confidence interval	P value
Total reflux events	-.010	-.098–.078	.818
Proximal reflux events	-.066	-.304–.172	.589
Supine reflux events	-.087	-.337–.163	.493
Proximal supine reflux events	-.006	-.631–.619	.986
Total acid reflux events	-.011	-.172–.151	.896
Total non-acid reflux events	-.013	-.116–.090	.804
Acid exposure	.092	-.478–.662	.752
Bolus exposure	-.277	-3.874–3.320	.880

Table 10.11 GEE results examining the effect of reflux variables on longitudinal change in FVC percent predicted.

	Beta co-efficient	95% confidence interval	P value
Total reflux events	.002	-.002–.005	.321
Proximal reflux events	-.002	-.015–.012	.823
Supine reflux events	.0101	-.003–.023	.126
Proximal supine reflux events	0.031	.002–.061	.040
Total acid reflux events	.003	-.004–.011	.372
Total non-acid reflux events	.001	-.003–.006	.565
Acid exposure	.005	-.027–.037	.747
Bolus exposure	-.022	-.198–.154	.807

Table 10.12 GEE results examining the effect of reflux variables on longitudinal change in absolute FEF 25-75%.

	Beta co-efficient	95% confidence interval	P value
Total reflux events	.017	-.044–.078	.582
Proximal reflux events	-.094	-.349–.162	.472
Supine reflux events	.133	-.141–.408	.342
Proximal supine reflux events	.395	-.271–1.061	.245
Total acid reflux events	.023	-.115–.162	.740
Total non-acid reflux events	0.020	-.064–.103	.644
Acid exposure	-.048	-.682–.586	.883
Bolus exposure	-1.220	-4.524–2.084	.469

Table 10.13 GEE results examining the effect of reflux variables on longitudinal change in FEF 25-75% predicted.

10.5.4 Effect on intravenous antibiotic requirements

GEE showed no significant correlation between IV courses or days duration for up to two years prior to baseline for any reflux measure. However, there was a potential trend toward significance between total duration of IV courses and both total non-acid events (p=.084), and bolus exposure (p=0.099) (Table 10.14 and Table 10.15).

	Beta co-efficient	95% confidence interval	P value
Total reflux events	0.002	-0.003–0.008	0.418
Proximal reflux events	0.008	-0.010–0.025	0.384
Supine reflux events	0.003	-0.012–0.019	0.678
Proximal supine reflux events	0.000	-0.039–0.038	0.992
Total acid reflux events	0.003	-0.009–0.014	0.666
Total non-acid reflux events	0.003	-0.003–0.010	0.314
Acid exposure	-0.023	-0.069–0.023	0.324
Bolus exposure	0.187	-0.078–0.453	0.167

Table 10.14 GEE results examining effect of various reflux variables on intravenous antibiotic requirements (total courses) over 2 years.

	Regression co-efficient	95% confidence interval	P value
Total reflux events	0.001	0.000–0.003	0.185
Proximal reflux events	0.003	-0.001–0.007	0.154
Supine reflux events	0.001	-0.002–0.005	0.489
Proximal supine reflux events	0.002	-0.007–0.010	0.669
Total acid reflux events	0.001	-0.002–0.004	0.561
Total non-acid reflux events	0.001	0.000–0.003	0.084
Acid exposure	-0.007	-0.019–0.006	0.281
Bolus exposure	0.054	-0.010–0.118	0.099

Table 10.15 GEE results examining the effect of various reflux measures on antibiotic requirement (total duration in days) over two years.

IV antibiotic duration was natural logarithm transformed.

10.6Discussion

In this study it was attempted to demonstrate if reflux, as measured by oesophageal pH-impedance, related to conventional measures of lung disease severity. The main finding of the study was that none of the measures of reflux derived from pH-impedance significantly correlated with: (i) any tested measure of baseline lung function; (ii) longitudinal change in any tested measure of lung function (iii) IV antibiotic requirements. There was however a trend toward significance for non-acid events and bolus exposure with total duration of IV antibiotics.

Within our study the findings fail to support a significant relationship between reflux measures and severity of CF lung disease. This is in keeping with findings from a number of previous studies [57, 78, 152]. However, unlike these previous studies we provide a more comprehensive comparison, by including baseline and longitudinal change in lung function, as well as IV antibiotic requirements.

Importantly our results contrast with those of Palm et al. [111]. They reported a significant relationship between FEV1 and both total reflux events ($R=-0.474$, $p=0.009$) and non-acid reflux events ($R=-0.397$, $p=0.03$), as well as more reflux episodes in those who were positive for Pa in sputum ($p=0.001$). There are number of important differences between the two studies: in age group (adult versus paediatric); and in subject selection (prospective and random, versus retrospective with symptoms of reflux or declining respiratory health). In keeping with the findings of Palm et al. a non-significant trend was noted between non-acid events and total duration of IV antibiotics ($p=0.84$). However due to the number of analyses ($n>20$) at a significance of 0.05 at least one variable would be expected to reach statistical significance.

It is quite possible that the potential effect of reflux differs between children and adults. Palm et al. proposed that their results may be a consequence of inoculation of the lung with gastric fluid containing Pa and leading to infection

[224]. The prevalence of Pa colonisation in CF increases with age [225]. As such Pa inoculation may have a greater impact if occurring in those patients with intermittent isolation i.e. children. The cohort selection may also be important. By including presumably more subjects that have an increased likelihood of having reflux associated effects on the respiratory tract — achieved by having a cohort that was investigated for reflux symptoms or declining respiratory health — this may have also helped unmasked a relationship. In our study most patients are already colonised with Pa and had established lung disease. This may explain the difference between this study and that of Palm et al..

There are a number of limitations with this study. By far the most important is that it is unknown if oesophageal reflux measures derived from pH-impedance are an appropriate endpoint for this study. Although they provide an accurate measure of oesophageal reflux[226], it is unknown how these may relate to reflux aspiration or neuronal cross-talk. They maybe a poor surrogate for one or both of these endpoints. For reflux aspiration to occur, a number of protective mechanisms would have to be overcome in the upper airway [65]. Knowledge is limited about these. It is presumed that reflux events that extend to the proximal oesophagus and/or occur whilst supine would lead to an increased risk. This will not be established until an accurate measure of reflux aspiration is developed.

The alternative mechanism, neuronal cross-talk, may prove even more complex. If there are similarities to oesophageal symptom generation, then it is likely that interplay will exist between various factors, beyond that of oesophageal exposure[47]. In Chapter 9 the results suggested an important role for neuronal sensitivity in generation of oesophageal symptoms. Presumably a measure of oesophageal acid or bolus exposure would be most appropriate, but it is not possible to be sure. It is our future intention to analyse cough recording data made in (n=11) subjects who had simultaneous acoustic cough and reflux recordings, and assess temporal association.

There were also limitations in the method by which endpoints reflecting CF lung disease severity were calculated. Collection of data from the notes, whether it is retrospective or prospective, relies heavily on the quality and accuracy of the information recorded. It is not as accurate as prospective follow-up from specific research visits. However, it offers significant advantages with respect to reducing time burden on recruits and reducing the work-load for researchers. In this study prospective data collection was not an option due to the time period available — limited to three years from inception to completion. The patients were also often prepared to enroll based upon the need for only a single research visit.

A further limitation is by measuring reflux at a single time point. Although reflux parameters have been shown to not significantly alter when measured at points two weeks apart the effect of longer time intervals is unclear [43]. Reflux measures may be highly variable when measured over a time frame of months. As such, a subsequent change could have occurred in the amount of reflux over the two years from recording of clinical data to the study visit. To explore further it is our intention to collect prospective data on lung function for two years following the study visit.

Finally, the study was also limited by the sample size, which reflected the difficulty in both recruitment and then study completion for subjects. This reflected that pH-impedance is an invasive test which can be difficult to tolerate, coupled with the relatively small pool of appropriate recruits within our centre. Respiratory health is affected by a number of factors in CF and it is possible that insufficient numbers were analysed to unmask a relationship.

For future work an accurate measure of reflux aspiration is required. At present, although a number of studies have reported using various methods it is unclear how accurately these are at quantifying reflux aspiration. Specifically, pepsin and bile acids have been measured in respiratory samples in CF [55, 78, 96-98, 227]. However, there are concerns about how robustly these methods

have been validated (see Chapter 11 for details) and as such the results they produce should be viewed with caution [65, 99-101].

In the first instance an accurate test to confirm the occurrence of reflux aspiration is required, which may be a biomarker. With this knowledge the effect of reflux aspiration on CF lung disease could be studied directly, rather than using pH-impedance derived measures as a surrogate. If this measure was a sputum biomarker it would remove the need for an invasive test and allow increased recruitment of subjects to studies. Having identified those patients with reflux aspiration it may be possible to establish the relationship to pH-impedance, as well as informing about the potential role of protective mechanisms within the upper airways.

The next chapter provides preliminary data of our attempt to identify patients with reflux aspiration, as part of a collaborative project with the Centre for Proteomic Research at the University of Liverpool. By using a combination of mass spectrometry for global and individual protein identification, as well designing immunological assays designed to identify biomarkers for reflux aspiration.

In summary the findings of this chapter rather than prove a lack of relationship between reflux and adult CF lung disease, highlight the need for more knowledge and better investigatory tools. In the first instance a measure of reflux aspiration is needed.

11 Using the sputum proteome to establish if reflux aspiration occurs in CF patients

11.1 Introduction

Our results, as reported in Chapter 9, failed to show a relationship between CF lung disease and gastro-oesophageal reflux. However, there is the potential for a relationship to be masked as a consequence of using measures of oesophageal reflux, rather than a measure of reflux aspiration. It is at present unknown how the amount of reflux in the oesophagus relates to any of the proposed mechanisms by which an effect is exerted on the lungs. The most frequently surmised mechanism is reflux aspiration. For this to occur, the refluxate must overcome numerous upper airway protective mechanisms [65]. These potentially prevent refluxate to enter the lungs, but the efficacy may vary between patients. For example, in one patient copious amounts of reflux are repelled by highly effective upper airway barriers, whereas in another a single reflux event is able to pass unimpeded. To explore this further a direct and accurate measure of reflux aspiration is required.

A number of methods have been described to measure reflux aspiration. The original involved calculation of the lipid laden macrophages index (LLMI) in bronchoalveolar lavage fluid (BALF)[228]. However this has been shown to lack specificity and is raised in many respiratory conditions in the apparent absence of reflux aspiration[229]. An alternative is using scintigraphy, which has been most frequently used in paediatric populations [230]. A radiolabeled tracer is ingested and the lungs are scanned. However, it has poor sensitivity and is not readily available [230, 231]. By far the most utilized methods have been measuring bile acids and pepsin within respiratory samples.

Bile acids have been measured and shown to be elevated in several CF [55, 78, 98] and multiple lung transplant studies [232-235] using enzymatic assays. Unfortunately, these assays were designed to measure serum bile acid levels

and have not been validated for use in respiratory samples. Parikh et al compared these enzymatic assays with mass spectrometry and showed them to be poorly sensitive especially at lower levels [100]. It is also important to note that bile acids are duodenal in origin rather than gastric, requiring an additional pathophysiological step for them to reflux into the oesophagus. Furthermore, they are present in serum and introduction of blood into the airway would allow their detection. Macroscopic haemoptysis is well documented in CF, however the occurrence of microscopic haemoptysis due to smaller arterial bleeds or dysfunction of the alveolar-capillary membrane is unknown but likely to occur [236, 237].

Pepsin has been measured in the BAL of patients with CF [96], asthma [238], chronic cough [154], idiopathic pulmonary fibrosis [239] and lung transplant recipients [232, 240-245]. It also been measured in the sputum of chronic obstructive pulmonary disease [93], bronchiectasis [93] and chronic cough patients [246]. These results have been used to draw conclusions about the increased prevalence of reflux aspiration in the various respiratory diseases, as well as more specifically about increased inflammation in CF lungs [96] and a greater likelihood of allograft rejection in lung transplant patients [242]. However, there are methodological failings in the validation process performed in all of these assays, and perhaps most telling not one has been published in a peer-reviewed biochemical journal.

Cross-reactivity is the first major concern for pepsin detection in respiratory samples. There are two types of pepsin found in humans (pepsin A and pepsin C) as well as precursor forms for both. Pepsin A, and its precursor pepsinogen, are believed to only originate from the stomach and are ultimately the aim of detection for any biomarker assay of reflux aspiration [247]. Although pepsin C (gastricin) and pepsinogen C are produced within the stomach, they are also believed to be produced within the alveoli of the respiratory tract [248-250]. In addition using spectrometry 459 proteins have been identified in CF BAL, with the potential cross-reactivity with a pepsin antibody of each unknown[251].

Although attempts are made in some studies using pepsin assays to assess for cross-reactivity [232, 242, 243, 252, 253], none do so using a respiratory sample. A proteomic group based at the Centre for Proteomic Research at Liverpool University have demonstrated that a pepsin antibody can cross-react with several different proteins found within CF and healthy volunteer sputum (see Figure 11.1).

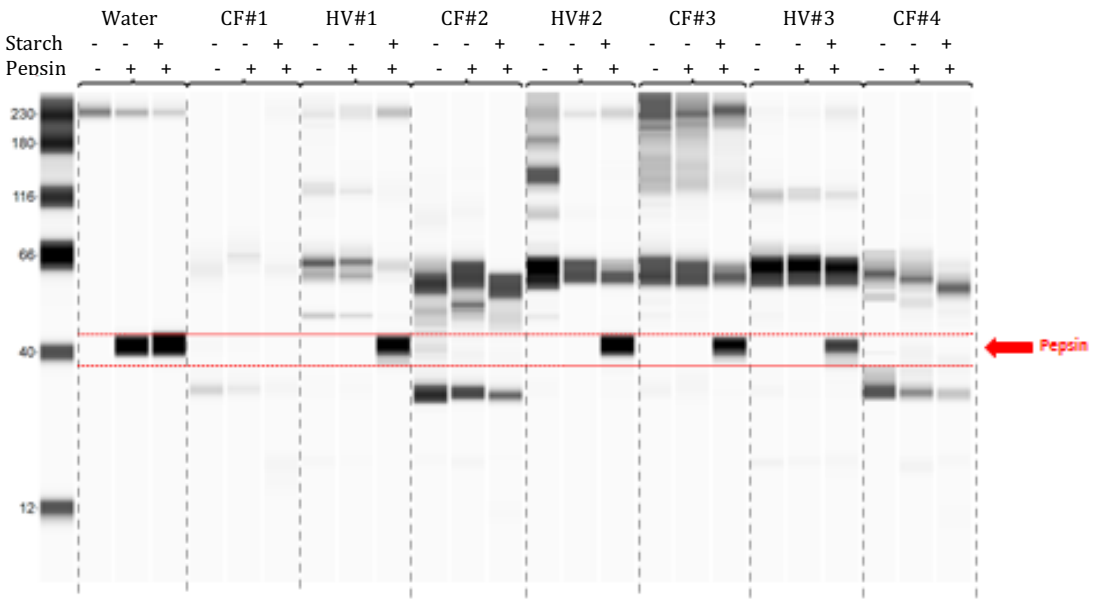


Figure 11.1 Western blot (WB) of CF and healthy volunteer sputum spiked with pepsin

A WB was performed on seven subjects (four CF and three healthy) with pepsin spiked in at 10fmol (80ng/ml). In addition, starch added to remove alpha amylase, previously noted to cross-react with the antibody. Cross-reactivity with other proteins is noted and significant variability in detection of the pepsin spike. CF: cystic fibrosis; HV: healthy volunteer. Unpublished data courtesy of Maher et al., Centre for Proteomic research, University of Liverpool.

Spiking experiments are also lacking in any of the aforementioned studies in sputum or BAL — for this pepsin is spiked into samples at a known concentration and retrieval is recorded. It has been shown that retrieval of pepsin when spiked into BAL causes significant assay interference (see Figure

11.2) [99]. This is supported by similar findings with spiking into saliva with good recovery at 1000ng/ml spikes (89-95%) but with large variability at 100ng/ml (50-93%), 10ng/ml (27-82%) and throughout lower concentrations [254].

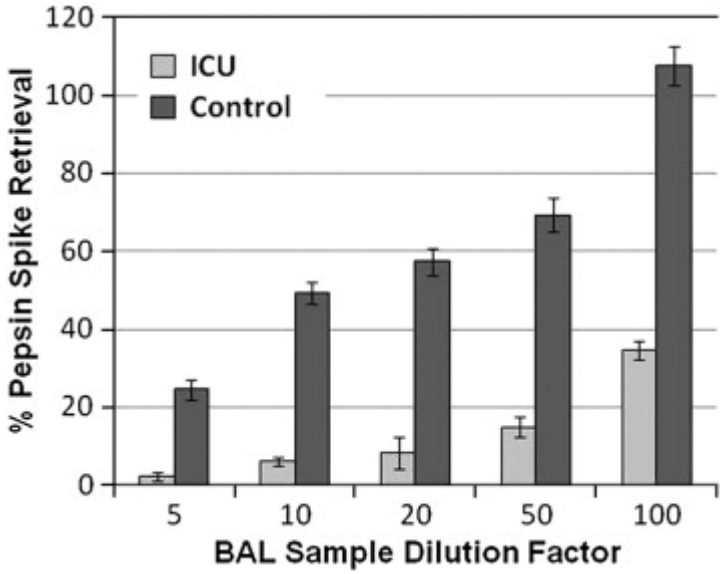


Figure 11.2 Pepsin spike retrieval from broncho-alveolar lavage (BAL) fluid of ITU paediatric patients

Reprinted and adapted with permission from Journal of Paediatric Surgery 2012;47:291-298 [99].

A further problem shared by both bile and pepsin relates to the overlap seen between assay results generated in healthy volunteers and the disease group of interest [154, 242, 253, 255]. Aside from accuracy of assay, this highlights the need for established normative data, as it is possible that small amounts of reflux aspiration could be physiological [99]. Finally, the 'snap-shot' value generated will need to be related to the potentially longitudinal process of reflux aspiration [101]. This will only be achieved with a reliable assay that can be performed at repeated intervals. This will help establish the behaviour of pepsin levels within the airways, in terms of both clearance of the aspirate and the effect of repeated episodes.

Our reservations about the current methods of measuring reflux aspiration are in line with experts in this area [99-101]. As such a collaborative project was undertaken with Professors Rob Beynon and Paul McNamara at the Centre for Proteomic Research, University of Liverpool. The aim is to explore if a better understanding of reflux aspiration can be garnered using proteomic approaches. In the first instance mass spectrometry (MS) will be used to identify global protein presence in sputum — the sputum proteome. It will be investigated if this is altered in those with higher amounts of reflux. The preliminary data from this study is presented in this chapter.

The respiratory tract proteome has previously been reported in a study analysing the BAL of CF patients, using a form of proteomics known as shotgun MS [251, 256]. This method involves tryptic digestion of proteins, separation using liquid chromatography and then tandem mass spectrometry [257]. 459 proteins were identified, of which 144 were differentially expressed compared to healthy controls [251]. The sputum proteome has previously been described in smokers and chronic obstructive pulmonary disease patients [258, 259]. To our knowledge it has never been used in CF to identify patients with reflux aspiration.

11.2Hypothesis

Reflux aspiration can be identified by examining the sputum proteome of CF patients.

11.3Aim

1) To establish if the sputum proteome is altered in those with more gastro-oesophageal reflux.
2) To establish if any proteins found in CF sputum are of gastric origin.

11.4Methods

11.4.1 Subjects

The sputum samples were collected from subjects whilst attending the research visit described in chapter 5. Samples from 20 subjects were selected to give a variety of measures captured on the pH-impedance (as described in Chapter 6). Heathy volunteers provided a further 20 samples for comparison. Gastric fluid was obtained from a single healthy volunteer. The study was conducted in line with ethical approval granted by 15/NW/0655.

11.4.2 Study design

Shotgun MS was used to provide global protein expression on the samples from the CF patients and healthy volunteers. The protein expression in the CF patients was compared to their pH-impedance results. The expression of proteins was examined for co-expression with gastric proteins.

11.4.3 Sputum sampling and processing

The samples were prepared and stored initially on site in freezers at -30 ^{o}C (as described in sections 6.8.1and 6.8.2). The samples were transferred frozen to The Centre for Proteomic Research, University of Liverpool. Sample preparation is described in detail in appendix 9.

In brief the samples underwent an initial step that involved addition of dithiothreitol (DTT), followed by filtration and centrifugation. This began the process of unfolding the protein and removing cellular debris. Further protein unfolding is achieved by addition of Rapigest® and then the protein was finally digested using trypsin to provide peptides for the MS analysis.

11.4.4 Gastric fluid

Gastric fluid was obtained by inserting a nasogastric catheter into the stomach and aspirating. The subject was fasted for 12 hours.

11.4.5 Mass spectrometry

The samples were analysed using a process of liquid chromatography (LC) and tandem mass spectrometry (MS-MS) using a Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (ThermoFisher Scientific, USA) (see figure 11.3). In summary the mixture of peptides, created by the trypsin digest, were separated using LC. The peptides were then ionized using an electronspray, which works by applying a high voltage to the liquid sample. Initial separation by mass-to-charge ratio (m/z) is performed by the quadrupole mass filter (MS1). The ions then pass into a C-trap, where they move to and from the HCD cell which leads to further fragmentation (MS2). These then pass into the Orbitrap Mass analyser[260]. The species of a particular m/z ratio is then used to identify peptides using MASCOT®, and then the protein of origin identified using Progenesis® QI, a commercial small molecule discovery analysis software package. This was performed by Miss R Maher under the supervision of Professor Beynon.

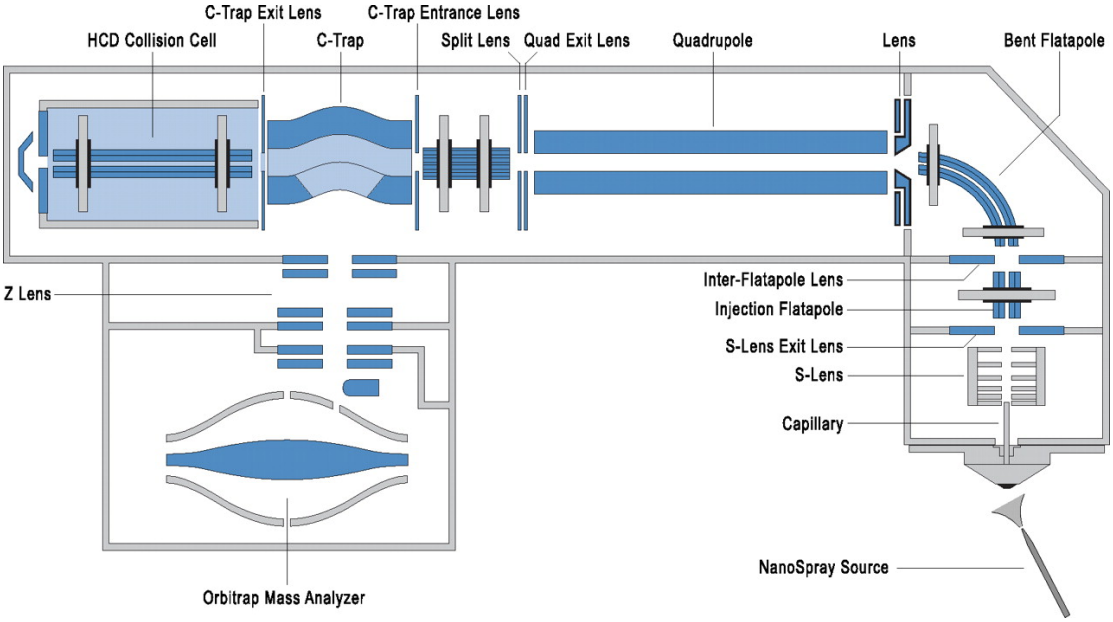


Figure 11.3 The Q Exactive mass spectrometer

The nanospray source is a form of electronspray ionization. Separation of peptides is achieved by the quadrupole, C-Trap and HCD Collision cell. They then pass into the Orbitrap Mass analyser.

This image was originally published in Molecular & Cellular Proteomics. Michalski et al. Mol Cell Proteomics.2011;10 © the American Society for Biochemistry and Molecular Biology or *© the Author(s) [260].*

11.4.6 Statistical analyses

Progenesis QI®, using an inbuilt statistical package, identified proteins with differing detection using analysis of variance (ANOVA) to compare between selected groups: healthy versus CF; and highest reflux values versus lowest. Adjustments are made to the p-value to take into account the large number of analyses performed, creating a q-value. This minimises the false discovery rate.

PCA plots. This was performed using Progenesis QI®. Only proteins with a Q-value <0.05, power >0.8 and at least two unique peptides were included.

Volcano plots. These were produced on R software GG plots 2. Only proteins with a Q-value <0.05 and at least two unique peptides were included.

11.5 Results

11.5.1 Subjects

The samples from 20 patients were included in this analysis. Baseline demographics are shown in table X. The pH-MII reflux measures are shown in table 11.2. The demographics of the healthy are shown in table 11.3.

Table 11.1 Demographics of the study population

	Study cohort (n=20)
Age (years)	31.2 (7.6)
Male	17 (85%)
Baseline FEV1%	50.1 (17.6)
Baseline BMI	22.0 (2.7)
Chronic infection Pa	13 (65%)
Chronic infection Bcc	3 (15%)
Prescribed acid suppressive medication	16 (80%)

The data are presented as mean (standard deviation) or total number (percentage of study population) as appropriate. Pa: *Pseudomonas aeruginosa*; Bcc: *Burkholderia cepacia complex*

Table 11.2 PH-impedance results of interest for the study population

	Study cohort (n=20)
Total reflux events	93.8 (65.6-125.4)
Proximal reflux events	19.1 (10.1-32.5)
Supine reflux events	11.7 (3.2-15.6)

The data are presented as median (IQR).

Table 11.3 Demographics of healthy control cohort

	Control cohort (n=20)
Age (years)	34.7 (10.6)
Male	4
Baseline FEV1%	101.1 (9.9)
Baseline BMI	24.0 (4.3)

The data are presented as mean (standard deviation) or total number (percentage of study population)

11.5.2 Protein abundance within sputum

628 proteins were identified within the sputum samples. Of these 384 proteins could be confidently identified by the presence of at least two unique peptides. 276 showed statistically significant ($q<0.05$) differences in protein abundance between CF and healthy volunteer sputum samples, with 101 increased and 175 decreased in the CF samples relative to those of healthy volunteers (see Figure 11.4). Using PCA analysis there were obvious differences in the clustering of protein expression between CF compared with healthy volunteer samples (see Figure 11.5).

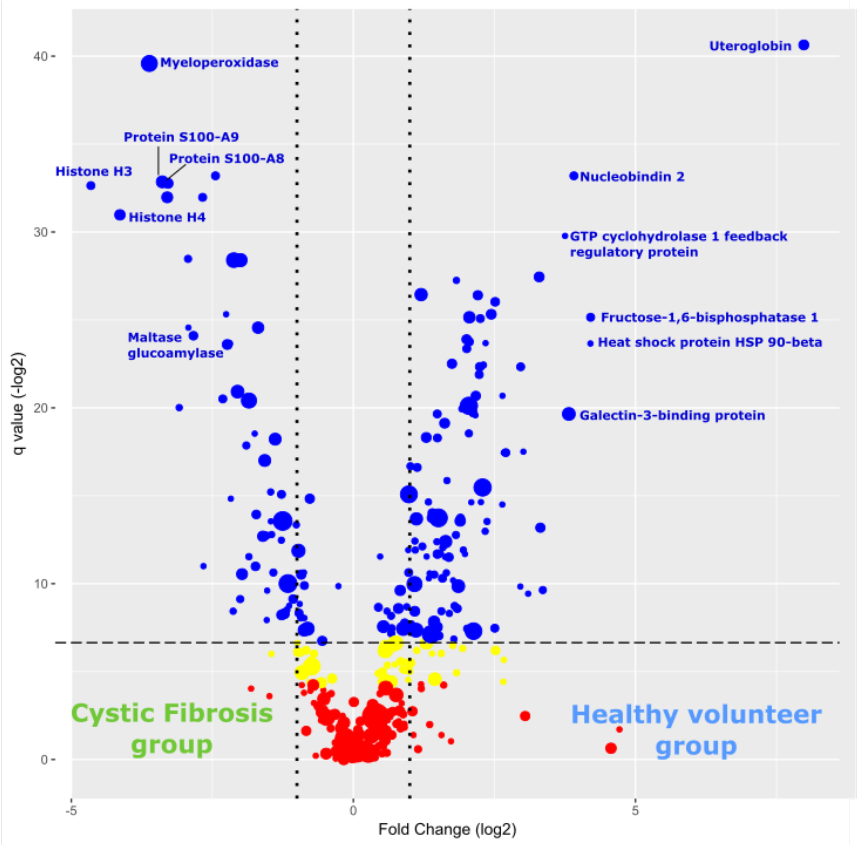


Figure 11.4 Volcano plot demonstrating the difference in proteins identified using shotgun MS between CF and healthy volunteer sputum. The X axis specifies the log2 fold change and the Y axis the ANOVA -log 2 adjusted p values (q values). The filtering criteria are reflected by the vertical dotted (log2 fold change +/- 2) and horizontal dashed lines (-log2 q value < 0.01). The colour of the dots represents the level of significance of the q values: blue (<0.01), yellow (0.01-0.05) and red (>0.05).

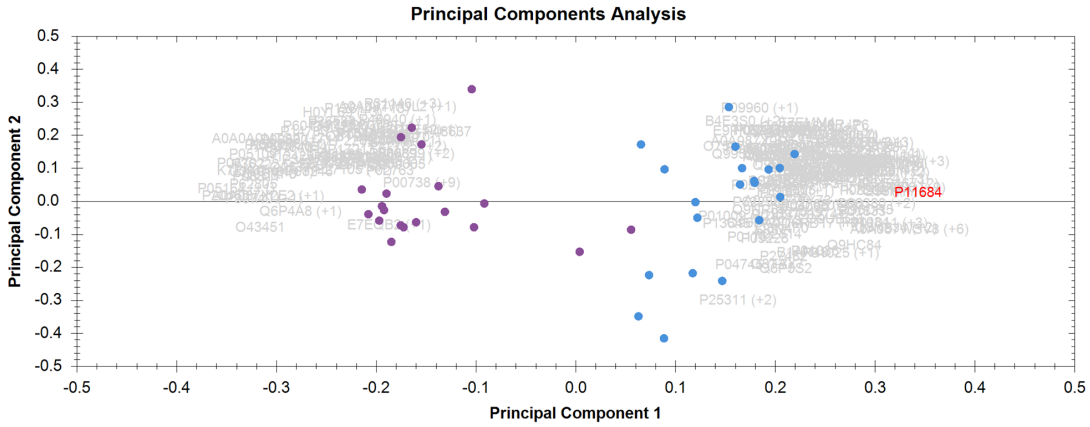
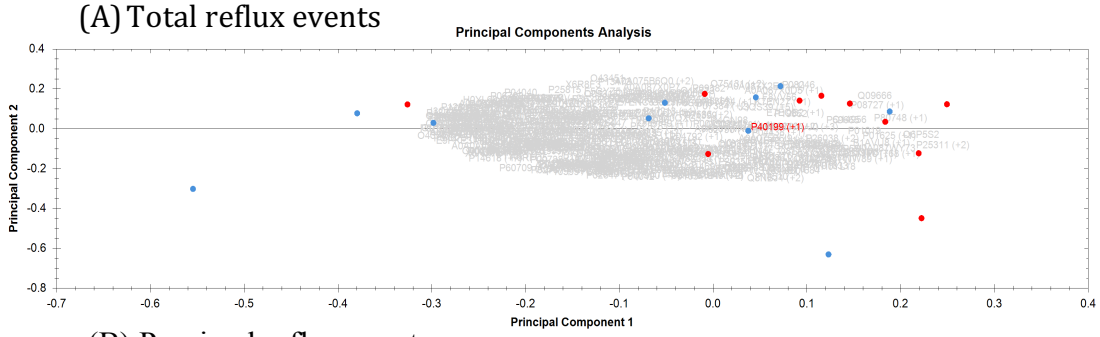


Figure 11.5 Principal component analysis for CF versus healthy volunteer sputum samples. Each point represents an individual sample, the purple dots CF and blue dots healthy volunteers. Only proteins are included that were differentially expressed with at least two unique proteins, q value (<0.05) and a power (>0.8). The grey figures in the background represent the loading plot for the individual proteins.

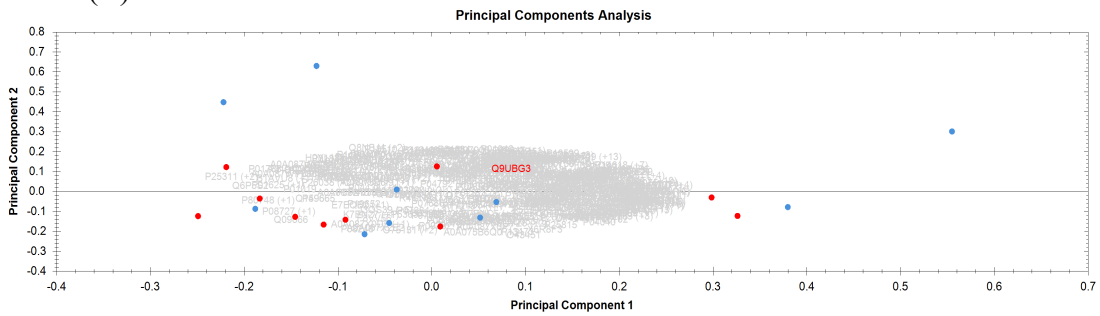
11.5.3 Difference in protein expression in sputum between patients with the highest and lowest amounts of reflux

The CF subjects (n=20) were divided into two cohorts, those with the highest reflux measures (top 50%) compared to those with least (bottom 50%). There was no significant difference for individual protein expression (q<0.05). Using PCA there was no obvious separation between the cohorts (see Figure 11.6).

(A) Total reflux events



(B) Proximal reflux events



(C) Supine reflux events

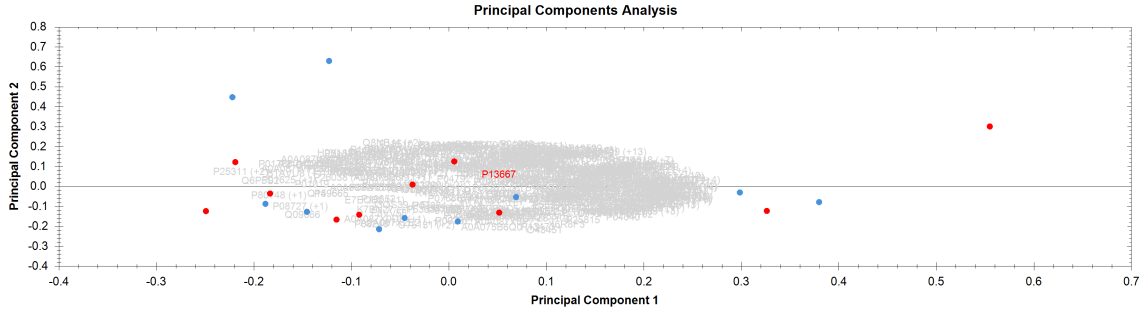


Figure 11.6 Principal component analysis for highest 10 reflux values versus lowest 10 reflux values in CF subjects

Total reflux events; (B) Proximal reflux events; (C) Supine reflux events.

Each point represents an individual sample, the red dots the highest and blue dots lowest amounts of reflux. Only proteins are included that were differentially expressed with at least two unique proteins, q value (<0.05) and a power (>0.8). The grey figures in the background represent the loading plot.

11.5.4 Comparison of the CF sputum and healthy gastric proteome

There were a total of 23 co-expressed proteins identified in both sputum and gastric samples. Of these 11 had at least two unique peptides identified. These 11 proteins were compared against previous work that has detailed the human tissue proteome [261] — www.proteinatlas.org/human proteome. From this it was noted if the proteins were found to be expressed in salivary gland, lung and gastric tissue (see figure 11.7, figure 11.8 and table 11.4).

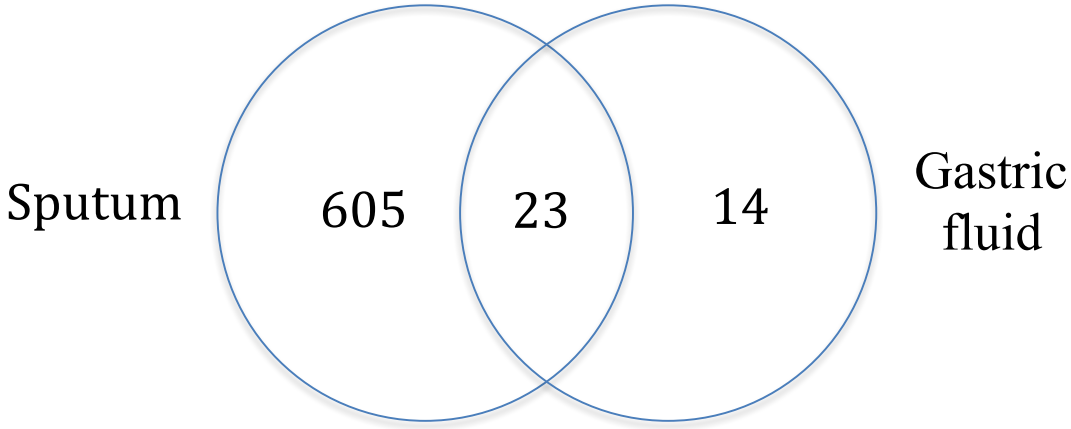


Figure 11.7 Venn diagram showing the proteins identified in sputum from CF and healthy volunteers (n=40) and gastric fluid from a healthy volunteers (n=1).

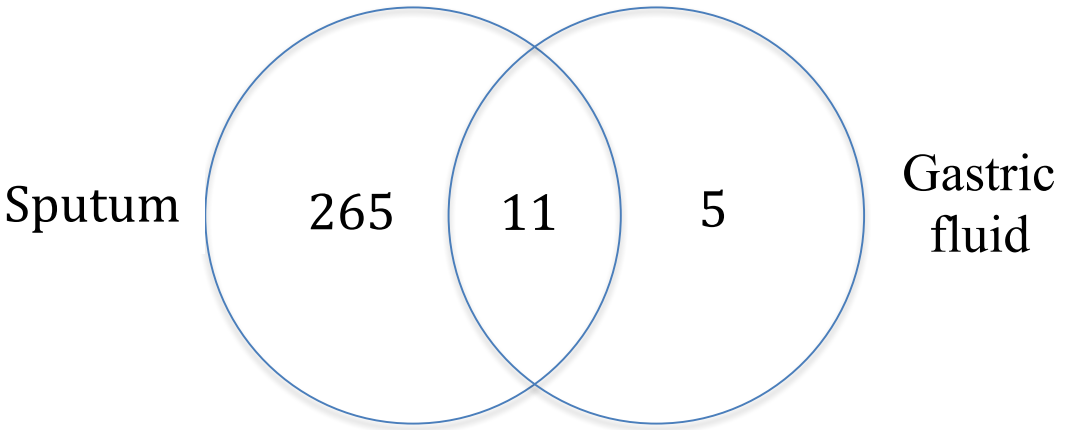


Figure 11.8 Venn diagram showing expression of proteins with at least 2 unique peptides in sputum from CF and healthy volunteers (n=40) and gastric fluid (n=1) from a healthy volunteer.

	Upregulated in CF or healthy sputum	Expression demonstrated in tissue			Other tissues of note
		Lung	Stomach	Salivary gland	
Keratin 1	Healthy				Skin, oral mucosa, oesophagus
Keratin 10	Healthy				Skin
Lysozyme C	Healthy	✓	✓	✓	
Keratin 9	Healthy				Skin
Keratin 5	Healthy	✓	✓		
Zymogen granule protein 16 homolog	Healthy			✓	
UPF0762 protein	Healthy		✓	✓	
Submaxillary gland androgen-regulated protein 3B	Healthy			✓	
Keratin 13	Healthy				Oral mucosa and oesophagus
Ig alpha-1 chain C	Healthy				No data available
Basic salivary proline-rich protein 2	Healthy				RNA found in salivary gland

Table 11.4 Proteins found in both CF sputum and healthy gastric fluid.

This table shows proteins and their previous detection within lung, gastric and oral tissue based on a previous study categorising the human tissue proteome [261]

11.6Discussion

This chapter summarises the progress to date of our experiences using global protein expression captured by MS to inform about reflux aspiration. The key findings are that: (i) a large number of proteins were noted to be differentially expressed between the sputum of CF and healthy subjects; (ii) no difference was noted in the sputum proteome between those with the highest versus lowest amounts of reflux; (iii) a number of proteins were co-expressed but none appeared to be of solely gastric origin.

The MS analysis identified a large number of proteins present in both healthy and CF sputum. There were more proteins (n=384) that could be confidently recognised, i.e. had two or more peptides identified, than had been previously reported in studies of smokers and a number of respiratory diseases [258, 262-264]. A large number of proteins (n= 276) were identified that were differentially expressed in CF compared to healthy samples. It would appear that this method used for tandem mass spectrometry using the Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass spectrometer (ThermoFisher Scientific, US) offers an effective method for global protein analysis in sputum. It is noted that there are more females in the healthy cohort compared to the CF cohort but it is felt that will not significantly alter the results.

If reflux aspiration was occurring it might be expected that protein expression may change due to the effect on introduction of refluxate into the respiratory tract, i.e. leading to an increase in inflammatory proteins. However, there was no significant difference in protein expression observed between subjects having the most reflux (i.e. top 10 on pH-impedance) versus the least reflux. There also was no clustering noted between the reflux groups on the PCA plots. It is possible that this is a consequence, as suggested in Chapter 9, that the relationship between pH-impedance and reflux aspiration is complicated by the upper airway protective mechanisms. The role of these protective mechanisms

is to limit movement of gastric fluid out of the oesophagus and oral contents into the lungs. These could modify the passage of reflux to varying degrees between subjects. If this is true then our method of cohort selection could mask an effect, by assuming pH-impedance is a reliable surrogate for reflux aspiration.

No proteins were identified within the sputum proteome that appeared to be of gastric origin. Although a number of proteins were co-expressed in sputum and gastric samples it appeared that they were either normally present in both samples or were introduced by salivary contamination. However, there are a number of limitations with this approach. There is only a single gastric fluid sample from a healthy volunteer. The intention would be to increase this number. Although there were more sputum samples, 20 CF samples and 20 healthy volunteers, this is still a small sample size. In the future the aim is to increase the sample size in both cohorts. It would also be helpful to have some comparative data from BALF, which should be less likely to be contaminated with saliva during sampling.

Further insights could be gained by performing shotgun MS on saliva samples. These have been collected at the research visit and stored for each patient enrolled. As such, examining co-expression of proteins between the three compartments (lung, stomach and oral cavity) may provide additional information. It may show that gastric proteins can be detected within the oral cavity, due to reduced distance to travel for refluxate.

It must also be noted there are some limitations of shotgun mass spectrometry. Not all proteins present within the sample can be identified. It also preferentially identifies peptides present at higher abundances whilst missing those at lower abundance[257]. Given that presumably small amounts of gastric fluid will be entering the respiratory tract, it is likely that gastric proteins will be low abundance. This unfortunately is a limitation that cannot be easily overcome.

Differences in the method of collection of sputum samples between those from CF and healthy volunteers must also be acknowledged. Unlike CF subjects who provided samples spontaneously, the healthy volunteers performed sputum induction with nebulised hypertonic saline. As such this may have led to differences within the proteome between the two groups. Currently the effect of hypertonic saline on the sputum proteome is unknown. Concerns have been highlighted that nebulised hypertonic saline may lead to the release of inflammatory mediators [265]. However, this effect was not noted in a study examining the difference between isotonic and hypertonic saline on induced sputum from asthma and COPD patients [265, 266].

Another area to be explored within this project is utilising proteomics to identify gastric proteins within sputum and confirming reflux aspiration. This will involve quantitate mass spectrometry techniques and western blot. To begin with pepsin will be the focus. However initial work has shown antibody cross-reactivity and pepsin spiking retrieval to be highly variable (unpublished work). It is also the intention to proceed with other gastric proteins as biomarkers, and the global protein expression knowledge of gastric fluid, sputum and saliva will be of great utility. This work represents a significant challenge as at present no accepted gold-standard exists.

In summary no evidence for reflux aspiration has been demonstrated in our initial findings. However, this is early in this collaborative project and there are several strategies to be explored. It is imperative that a reliable test for reflux aspiration be developed. Until this point there are limits about what can be concluded based on traditional measures of reflux within the oesophagus, such as pH-impedance.

12 Discussion

The aim of this thesis was to explore the relationship between gastro-oesophageal reflux and cystic fibrosis (CF) lung disease. This is a relationship that is frequently proposed, but in reality lacks a robust body of evidence. It is believed by many that reflux exerts a negative effect on lung function, most likely as a consequence of reflux aspiration. However, the relationship is most likely far more complex: evidence is emerging that supports both lung disease leading to increased reflux (see Chapter 4). What is more there would appear to be multiple potential factors that may drive this bi-directionally, as well as factors that that are able to influence both lung disease and amount of reflux.

12.1.1 Causes of increased reflux: the role of lung disease

In this cohort reflux was highly prevalent with the total number of reflux episodes above normative values in 54% of subjects. Raised amounts of reflux have been previously described in a number of studies in CF across a spectrum of severity of lung disease [54-57, 59], as well in a number of other respiratory conditions including interstitial lung disease [90, 91], chronic obstructive pulmonary disease[267], bronchiectasis[268], asthma [269, 270] and lung transplant [240]. Given the frequency on these findings, logically the most likely explanation is that a common factor or factors related to the lung disease are responsible for at least some of the increased reflux.

Several mechanisms have been proposed to explain how lung disease may lead to increased reflux: more negative inspiratory pressures generated by respiratory disease could lead to an altered gastro-oesophageal pressure gradient in favour of reflux [63, 271]; changes in lung volumes could lead to an altered position of the diaphragm and hiatus hernia [65-68, 182, 272]; and medications given for respiratory diseases may interfere with tone of the lower oesophageal sphincter [71-74].

In chapter 8 OGJ morphology and function using high resolution manometry (HRM) were examined. The prevalence of hiatus hernia was low (n=4), despite

69% (n=27) having oesophago-gastric junction (OGJ) dysfunction, either measured using OGJ-MP or OGJ-CI. This suggests that OGJ dysfunction is occurring due to mechanisms other than hiatus hernia. It was hypothesised that the reason for this could be a consequence of CFTR-related myopathy, particularly affecting diaphragmatic function. However, our results suggest that OGJ dysfunction leads to increased reflux, with OGJ-MP showing a significant association with acid exposure, whereas the OGJ-CI showed a non-significant trend. The low prevalence of hiatus hernia is in contract to other studies in different respiratory diseases. It is possible that the mechanism, or mechanisms, for increased reflux may differ between respiratory diseases.

In chapter 8 oesophageal motility was also examined. By the Chicago classification (CC) 66% of subjects (n=26) had abnormal oesophageal motility on HRM. Abnormal motility is thought to impair clearance of refluxate from the oesophagus, and as such may worsen reflux. A number of studies have identified an increased prevalence of oesophageal motility issues in respiratory disease [56, 63, 191-194]. Abnormal motility is sub classified using the CC as either minor or major motility abnormality. Complete absence of peristalsis on an assessment of 10ml swallows of water is classified as a major abnormality [149]. The effect of minor motor abnormalities on amounts of reflux is unclear. Only in one study in lung disease, which was of idiopathic pulmonary fibrosis (IPF) patients with complete absence of peristalsis, was able to demonstrate an association with raised oesophageal acid exposure [191]. In this cohort no difference was shown between those with and without abnormalities of oesophageal motility. Unfortunately, there was insufficient numbers with absent peristalsis (n=4) to examine this cohort separately. It would be interesting to examine the effect of severe motility abnormalities separately. It was hypothesised that lung disease may lead to oesophageal motor abnormalities, as a consequence of shared vagal afferents. This is a further potential method by which lung disease has the potential to worsen reflux.

In chapter 9 the effect of a number of medications on reflux measures and OGJ function was examined. The medications were methylxanthines, oral corticosteroids, hypertonic saline and DNase. No medications were shown to effect OGJ function. Theophylline was associated with an increased number of reflux events, but not with increased acid exposure. A possible explanation is that the increased prevalence of acid suppressive medications being prescribed to those taking theophylline, potentially as a consequence of the increased symptoms, has reduced the acid exposure.

12.1.2 Causes of increased reflux: Cystic Fibrosis Transmembrane Conductance Regulator protein (CFTR) dysfunction related

It is likely that additional factors not related to the underlying respiratory disease are important, which in CF populations most likely relates to CFTR dysfunction. A number of these other factors which may have an additional contributing role were highlighted across various chapters of the thesis. As discussed above, the OGJ dysfunction, which correlates with increased acid exposure, may relate to CFTR dysfunction within the diaphragm.

In Chapter 7 increased acid exposure despite widespread prescription of acid suppressive medications often involving dual agents was noted. This suggests that underlying issues are present with either pharmacological acid suppression and/or neutralisation of acidic refluxate within the oesophagus. This is in line with previous studies that have shown acid clearance following reflux events is abnormal [87] and others demonstrating that gut luminal pH is lower in CF patients [88]. Both these mechanisms if important could be explained by defective bicarbonate transfer associated with CFTR impairment.

There may also be a role for CF bowel disease. Bowel symptoms, measured using the aIBS-SSS, are extremely common in CF and occur in approximately half of the subjects (Chapter 9). The presence of symptoms did not however

correlate with reflux measures. However gut symptoms often are modulated by the sensitivity of the nervous innervation, also known as visceral hypersensitivity. The large number of gas events, which are likely of intestinal origin, support gut disease increasing reflux. For a gas events to occur there must be transient relaxation of the lower oesophageal sphincter (TLSOR). In non-CF patients most physiological and pathological reflux episodes have been shown to occur during TLOSR. Arguably this may relate at least in part to the lung disease and frequent antibiotic usage for treating pulmonary exacerbations, which may lead to small bowel dysfunction through a change in the microbiome.

It was also demonstrated that visceral hypersensitivity is common. The symptoms of gut disease correlated strongly with those of reflux, despite no relationship with pH-impedance results (Chapter 9). This has therapeutic implications for gut and reflux disease, as therapies directed at visceral hypersensitivity maybe useful. It also suggests that the relationship between reflux and symptoms needs closer examination. This is in line with another finding that found typical reflux symptoms although frequently occurring, are very poorly specific for raised pH-impedance results (Chapter 7).

12.1.3 Impact of reflux on lung disease

Reflux was quantified by detecting presence of refluxate within the oesophagus using combined pH-impedance. This is the gold standard for reflux monitoring, but how the measures generated relate to reflux aspiration or neuronal cross-talk is not known. It is thought that reflux episodes with proximal extent should increase risk of reflux aspiration. If an episode of proximal reflux occurs whilst supine or without generating typical reflux symptoms this may further increase risk. Within this cohort 20% had increased proximal reflux, 24% increased supine and 37% proximal supine episodes (Chapter 7). Although reflux symptoms occurred in most subjects, they were frequently mild and poorly discriminating for any measured reflux parameter being increased. This may

exacerbate the risk of reflux aspiration by subjects not being able to modify their behaviour appropriately, for example continuing to eat large meals prior to periods of being supine. Our data suggest that what is thought to represent 'high-risk' reflux characteristics for aspiration is common within this cohort of CF patients.

Previous studies in chronic cough patients have reported a significant relationship between the timings of total, acid or non-acid reflux events and episodes of cough measured using a validated cough monitor. This is thought to occur as a consequence of neuronal cross-talk [121, 154]. Within our cohort there was a global increase in almost all reflux measures. However, no study has examined the relationship between cough and reflux in CF using a validated cough monitor. Insufficient is known to speculate if a specific measure derived from pH-impedance or even symptoms could predict risk of neuronal cross-talk.

There were no significant correlations between any of the reflux measures and any respiratory endpoints tested, including baseline and longitudinal reflux measures as well as number of courses and duration of intravenous antibiotic therapy. A non-significant trend was noted between total non-acid reflux episodes and total duration of antibiotic therapy. As multiple analyses have been performed (n>20), it would be expected that at least one was close or approaching statistical significance by chance alone. However is should be acknowledged that a number of studies have highlighted potential risks of non-acid reflux within CF patients: respiratory pathogens have been grown in the gastric fluid of acid supressed patients [115]; use of PPI was associated with a trend toward earlier exacerbation [59]; and an observational study in paediatric patients found a significant association between non-acid reflux events and baseline lung function [111].

The sputum proteome was examined in order to establish if oesophageal reflux measures resulted in differences in relative abundance of proteins within the

sputum, using shotgun MS. This showed no difference between those that had the most and least reflux measures (total, proximal and supine reflux episodes).

12.1.4 Limitations of the study

The study was limited by the size of the cohort (n=41). Patients frequently declined to enrol in the study due to the invasive nature of the procedure and of those that agreed a number could not tolerate the test. This was coupled with the limited population of CF patients available from which to recruitment at this single centre.

Reflux was measured using pH-impedance only at a single time point. It is unknown how much variability occurs over periods beyond four weeks [43]. For some of the analyses undertaken it was assumed that they remained predictive of reflux for periods of up to two years. This may well be inaccurate. However, it would not be possible to perform serial reflux monitoring with pH-impedance due to its invasive nature. It is unclear if pH-impedance monitoring affects the amount of reflux, either as a consequence of the oesophageal catheter or a change in diet. There are likely a number of factors that could affect the amount reflux, such as use of enteral feeding or other medications, the role of which is not yet clear and as such cannot be controlled for. It is also possible that some of the factors that influence the amount of reflux may also have a relationship to lung disease severity. This would further complicate the nature of the relationship and make delineating it more difficult. Unfortunately, these have yet to be established and as such also could not be controlled for.

A further limitation of pH-impedance is that although it measures number of reflux events, it does not quantify the amount of reflux. The volume of each reflux event is likely to vary and this maybe important. Acid exposure is thought to be reflective of volume of reflux, however there is heterogeneity between subjects in the amount of the prescribed acid suppression therapies. The analyses also used prescribed medication rather than actual administration.

The study attempted to demonstrate if lung disease was responsible for increased amounts of reflux. The presence of hiatus hernia was assessed using HRM. Although, this is a validated technique all of the previous studies made the diagnosis using radiology [65-68, 182, 273]. Gastro-oesophageal pressure gradients were not examined, as at present no universally accepted method for measurement exists. The influences of commonly prescribed medications on reflux was examined, but this was a secondary analysis and there were differences between the subject groups tested including prescription of acid-suppressive medications. The impact of medications was assessed by their prescription, so the actual usage of any of these medications was unknown. We were unable to evaluate the effect of beta-agonist and anti-cholingeric medications. As such we have yet to firmly establish if lung disease is driving reflux.

Reflux was measured using oesophageal combined pH-impedance. This presents a number of problems. Firstly, it is unknown how oesophageal measures may relate to either proposed mechanism by which reflux is thought to negatively affect CF lung disease. For reflux aspiration it is not known how combined pH-impedance measures relate to the amount of contents entering the respiratory tract. There are a number of protective barriers at the proximal oesophagus and within the upper airways that are able to prevent aspiration. For knowledge in this area to progress it must be possible to accurately measure reflux aspiration. Currently no test is adequately validated (see Chapter 11).

The importance of neuronal cross-talk in CF populations is unknown. In chronic cough, a temporal relationship between reflux and cough episodes has been shown using pH-impedance and a validated cough monitor. Other studies have shown that instillation of acid into the oesophagus causes both cough and bronchospasm. No study has looked into this in CF. At present the effect is unquantifiable within this cohort.

There are also limitations with our respiratory endpoints. This included the use of retrospective data for lung function and IV antibiotic requirements. There are many ways to measure lung disease, radiologically and physiologically, and not all were undertaken and used in the analyses.

Protein abundance was only performed in a limited number of patients, with 20 CF and 20 healthy subjects. There are also limitations, as detailed in Chapter 11, in using mass spectrometry (MS) to identify proteins. These include failure to identify all proteins, in particular those at a low abundance. This may cause gastric proteins that have been inoculated into the airways to be missed.

12.1.5 Strengths of study

Despite being a small cohort, the numbers recruited were higher those achieved in most other CF studies using invasive measures of reflux. The largest previously study reported on 42 CF subjects [57]. Although having limitations with respect to establishing a relationship between reflux and CF lung disease, pH-impedance is the gold standard for reflux measurement. Due to its invasive nature and the number of patients eligible it is unlikely that a similarly sized cohort will be able to be recruited in the future.

It was openly declared that this work is exploratory and as such have used multiple endpoints and run multiple analyses. The conclusions made reflect this, and the findings will need repeating to ensure they are correct. However, the purpose of this project is to inform and direct future work, rather than definitively solve whether reflux impacts upon lung disease.

A number of novel elements have been incorporated within the project: HRM was used to assess OGJ function; Chicago classification was used to assess oesophageal motility; bowel symptoms were assessed using the aIBS-SSS; and mass spectrometry was used to measure relative protein abundance in sputum. For each novel aspect experts within the individual area were consulted to ensure the study design was appropriate.

Where required suitably qualified individuals assisted to ensure the methodologies were of the highest standard and results were accurate. Gastroenterologist and physiologists were involved in the design, measurement and interpretation of pH-impedance and HRM. Qualified biochemists performed the mass spectrometry testing and assisted with interpretation of the data.

12.1.6 Areas for future research

No relationship was demonstrated between pH-impedance values and measures of reflux aspiration. This most likely reflects the complexity of the relationship, which maybe bi-directional nature and have multitude mechanisms involved. Future work must examine each of these relationships, possibly in isolation, using methodologies that are appropriate.

Future work is needed to prove if lung disease causes increased amounts of reflux. Currently it is not possible to firmly conclude if lung disease leads to increased reflux. The HRM measurements assessing for presence of hiatus hernia should be performed in other respiratory conditions, such as interstitial lung disease. Ideally the presence of hiatus hernia should be compared to pH-impedance to determine the influence upon the amount of reflux. This will allow firmer conclusions about the important of lung volumes in leading to hiatus hernia.

Gastro-oesophageal pressure gradients can be assessed using HRM. This will be performed in the future within this cohort of CF patients. As yet there is no agreed method for measuring the gastro-oesophageal pressure gradient, so this must be addressed as part of this project. This will allow comparison between other CF cohorts, as well as other groups including other respiratory disease, those with non-respiratory GORD and healthy volunteers.

In our study only theophylline appeared to influence amount of reflux. Further studies could be extended to other commonly prescribed medications for CF

lung disease, including inhaled bronchodilators. These could be specifically designed to assess the impact on OGJ function and on the amount of reflux. Although appealing, recruitment would likely be difficult.

Further understanding is required about the relationship between the presence of reflux within the oesophagus as measured by pH-impedance, and the proposed mechanisms by which reflux can affect lung disease. In the first instance development of an accurate measure of reflux aspiration is required. A global proteomic approach failed to identify any gastric proteins that are present within CF sputum. However, this method is poor at detecting low abundance proteins, which presumably is the level at which gastric proteins would be found. With our collaborators it is the intention to develop and validate a biomarker assay to identify those with reflux aspiration. This would allow the impact of reflux aspiration to be explored on the respiratory endpoints, as well as gain a greater understanding of how oesophageal reflux measures relate to reflux aspiration.

A better understanding of the importance of neuronal cross-talk is required. In the future the relationship between reflux and cough in a CF cohort will be examined. Cough recording have been made using an acoustic sound recorder in a percentage of the enrolled subjects (n =11). The temporal relationship between cough and reflux events will be explored.

By understanding the various mechanisms potentially linking CF lung disease and reflux, it may be possible in the future to demonstrate a relationship. In order to achieve this, a future study would most likely involve a larger cohort and require a multivariate model. This will allow the complexity of the relationship to be captured.

Further analysis of this cohort will be undertaken with the respiratory endpoints collected prospectively. Given concerns about potential changes in the reflux amounts beyond four weeks, time to first exacerbation will be

examined as well change in lung function and IV antibiotics requirement. Also microbiological endpoints will be investigated with microbiome analysis of sputum collected at the research visit.

Finally, two potential interventions are highlighted that require further evaluation. It has been shown that CFTR modulation therapy can raise small bowel pH [88], and reduce the symptoms of reflux [274]. As more patients begin to be started on these medications, there is an opportunity to assess the effect of CFTR dysfunction and its effect on gastro-oesophageal reflux. Another potential intervention is around enteral feeding, via percutaneous endoscopic gastrostomy (PEG) and nasogastric (NG) tube. In our clinical practice patients frequently describe symptoms consistent with aspiration of their enteral feed. A future study could aim to confirm this observation, assess the impact of difference feeding regimens, and compare NG and PEG.

12.1.7 Conclusion

This study has shown that CF patients with stable lung disease have high rates of gastro-oesophageal reflux, as defined by raised total reflux events above normative values. Almost half have increased reflux measures which possess characteristics that are presumed to increase the risk of reflux aspiration. However, in contrast no reflux measures correlated with any tested measures of CF lung disease severity.

This highlights the potential complexity of the relationship between reflux and CF lung disease, and why using pH-impedance alone is potentially inappropriate. To proceed there must be a close examination of all proposed elements of this relationship: reflux aspiration, neuronal cross-talk and lung disease worsening reflux. A key aspect to this will be development of an accurate measure of reflux aspiration.

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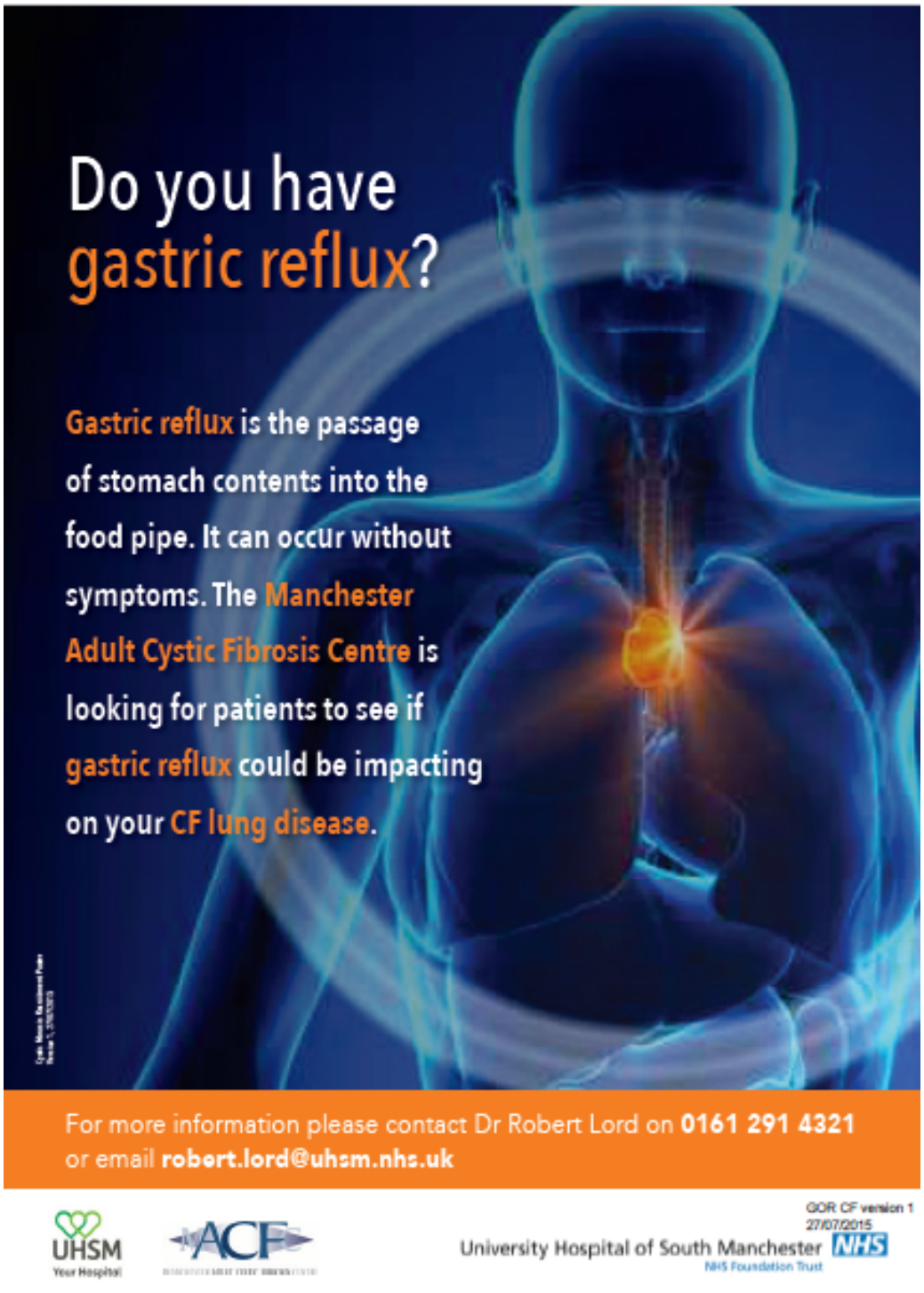
Appendix 1 Recruitment Poster



Appendix 2 Participant information sheet (cystic fibrosis patients)

Gastro-oesophageal reflux (GOR) in cystic fibrosis and its effect on lung function.

Patient Information Sheet

Version 1.2: 24/02/16

Wythenshawe Hospital
Southmoor Road
Wythenshawe
Manchester
M23 9LT

0161 998 7070

Contents	Page

How to contact us

If you have any questions about this study please talk to **Dr Robert Lord:**

Manchester Adult Cystic Fibrosis Centre
Wythenshawe Hospital
Southmoor Road
Manchester
M23 9LT

Tel: 0161 291 4321
Email: robertlord@nhs.net

You are invited to take part in a research study

- This study is designed to look at if gastric reflux has an impact on the lung function of CF patients. It is being carried out by a team of trained doctors, nurses and researchers under the supervision of Doctor Andrew Jones and Professor Jacky Smith at the Adult Cystic Fibrosis Centre, University Hospital South Manchester.
- Before you decide if you would like to take part, it is important for you to understand why this research is being done and what taking part would involve for you.
- Please take your time to read the information carefully.
- Your participation is entirely voluntary so you do not have to take part if you do not want to.
- Please ask a member of the research team if there is anything that is not clear, or if you would like more information before considering taking part.
- Thank you for reading this information. We hope this research will be of interest to you.

Important things that you need to know

- This study is looking at gastric reflux. This is stomach contents travelling up into the oesophagus (also known as food pipe or gullet).
- For most patients taking part would involve 2 visits.
- Taking part involves a physical examination, breathing tests, blood samples and sputum samples similar to performed at usual clinic appointments. In addition oesophageal tests, throat swabs and questionnaires would be completed.
- You can opt out of the study at any time without giving a reason.
- We will provide you with both your own results and the findings of the study at your usual clinic appointments.

What is gastric reflux?

Gastric reflux is a common problem in cystic fibrosis. Stomach acid travels the wrong way back up the gullet. It is commonly associated with heartburn and tasting acid in the mouth. However sometimes it can have no symptoms at all. We think that some of the acid reflux maybe entering the airways and lungs. This could potentially make people's chests worse.

What is the purpose of the research?

We will be testing people to see if acid reflux is really worsening people's lung disease. This will hopefully lead onto further research to offer another treatment to improve lung disease and settle symptoms such as heartburn.

What 'method' is being used?

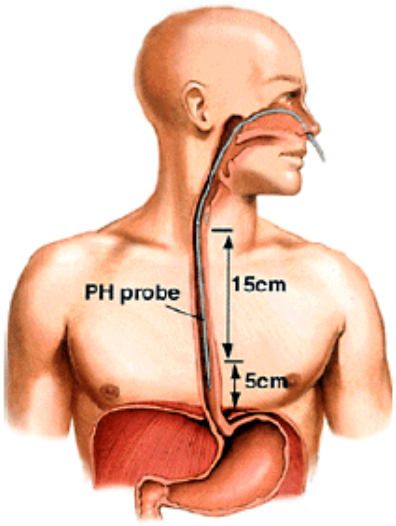
We will be collecting lots of information about patients to help us answer our question. The important part will be looking at what is going on in the gullet. We will be using very small tubes that sit in the gullet and take measurements. They are a little uncomfortable but most patients can manage to have them inserted. They go in through a nostril. Everyone who is involved in the study has had these tests done on themselves to make sure they are okay for any people who agree to the study.

- **Oesophageal manometry**

This is the name of the first part of the gullet tests. The tube stays in place for about 30 minutes. It measures the function and pressures in the gullet. This probe is removed to allow the second part of the test.

- **24 hour impedance / pH monitoring**

The second part of the test involves putting a smaller tube into the gullet and this time it stays in place for 24 hours. This measures the acid levels and how far stomach contents travel up the gullet.



This picture demonstrates how the probe will be placed the gullet to take the acid reading.

Why have I been asked to take part?

To help improve our understanding of cystic fibrosis and deliver better treatments we often ask people to help us.

We are asking all people with cystic fibrosis to be involved. We need people who have no symptoms as well as those people with symptoms. We also need people with lots of different degrees of lung disease.

You have been asked because your doctor believes you are eligible to take part if you wanted.

What happens if I agree to take part?

If you agree to take part we will check whether you can be involved during one of your usual clinic appointments. This is called 'screening '. We would then give you a couple of days to consider and make contact usually via a phone call to book you in for the two extra appointments.

Study visits

First visit

When you attend for your first visit initially we will ask you questions about your health and confirm your details are correct in your medical notes. A number of tests will then be performed;

- A doctor will perform a brief examination and check your vital signs. These are your oxygen saturations, blood pressure and pulse.
- You will be asked to complete several brief questionnaires
- A breathing test, exactly the same as you perform in clinic, will be performed
- Oesophageal manometry & 24 hour impedance / pH monitoring (gullet tests) will be performed
- Sputum samples
- Blood sample
- Throat swab

When you leave you will be asked to keep a diary of symptoms over the next 24 hours. This will be quite a brief process.

Second visit

When you attend the next day we will remove the equipment and take further sputum samples.

Third & Fourth visit

These visits will not be routinely required. If you are receiving overnight feeding or you have commenced on Orkambi (new CF drug) you will be asked to attend. It is optional whether you agree to the additional visits.

Follow up visits

You will require no additional follow up visits. However your clinical notes will be reviewed for 2 years. Both your results and those of the study will be fed back to you at your usual clinic appointments.

Do I have to take part?

No taking part is entirely optional

What happens if I change my mind?

It is okay to agree to take part and then change your mind. You do not need to provide a reason and it will not affect your clinical care in the future. You can also decide if we have already completed some of the study whether you want the data gathered to be withdrawn. However for us to withdraw data the request must be within 2 weeks from collection.

What happens if something goes wrong?

You will be provided with the 24 hour contact details of the doctor running the study. They will be available to advise / see you if required especially if something goes wrong with the oesophageal probe. This study is very safe and we would not expect anything to occur. However the hospital is insured to cover any problems that might arise as a result of the study.

If you have a concern about any aspect of the study, please speak to the research team in the first instance. If you remain unhappy and wish to complain formally, you can do so through the NHS complaints procedure. Details can be obtained from the **Patient Experience Team** on 0161 291 5600.

The hospital is insured to carry out NHS-sponsored research. If something did go wrong and you were harmed during the research due to someone's negligence then you may have grounds for legal action or compensation against the hospital.

Do I need to change my medications?

No you should continue to take the same medications

Additional information about the study

Will I receive a payment for taking part?

You will not receive payment but we will be able to compensate you for reasonable parking/travel expenses.

Will taking part affect my health insurance?

If you have private medical insurance, please check with your insurance company to see if your policy will be affected by taking part in this clinical study.

Will my details be kept confidential?

We follow strict ethical and legal guidance regarding patient confidentiality. Any information we have about you will be handled in confidence. All data recorded will be coded and your name will remain anonymous.

When taking part in research it is occasionally necessary for your details to be made available to authorised research staff who are bound by the same duty of confidentiality. This may include people who conduct quality assurance and quality control checks to confirm that the research was done correctly. These people may include research ethics committees, inspectors, monitors and auditors.

Who has reviewed this study?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee. The Research Ethics Committee is made up of experts, non-experts and members of the general public. Together they review research applications to ensure your safety, rights, wellbeing and dignity are protected at all times. They are happy for us to proceed with this study.

What will happen to the study results?

The results will be stored and used to answer research questions. The information will have no personal information so it will be anonymous.

Who is organising and funding the research?

The Manchester Adult Cystic Fibrosis Centre (MACFC) is organising the research. We have enlisted help from experts in the field of gastro-oesophageal reflux to ensure that our study is as good as it possibly can be. The study will be funded by the MACFC, although if some of the tests prove to be very expensive we will apply for funding from some charities.

What happens to any additional samples that are left over?

The study of cystic fibrosis is a rapidly developing area of research and new methods of analysis are being developed all the time. We will ask that you donate the samples as a gift so that the samples can be stored for future analyses relating to this research programme and for possible use in future (ethically approved) research studies. These samples and any other information held will be anonymised. This will enable us to make the most of any new developments in knowledge and techniques.

Contacts for further information

For more information about this study, you can speak to a doctor supervising the study on 0161 291 4321 (between 9am and 5pm) who will be happy to answer any questions or concerns you might have.

Thank you for reading this information sheet.

Appendix 3 Participant information sheet (healthy controls)

UHSM
MANCHESTER ADULT
CYSTIC FIBROSIS CENTRE

University Hospital of South Manchester
NHS Foundation Trust

Gastro-oesophageal reflux (GOR) in cystic fibrosis and its effect on lung function.

Healthy control Information Sheet

Version 1.1: 25th September 2015

Wythenshawe Hospital
Southmoor Road
Wythenshawe
Manchester
M23 9LT

0161 998 7070

Contents

How to contact us

If you have any questions about this study please talk to Dr Robert Lord:

Manchester Adult Cystic Fibrosis Centre
Wythenshawe Hospital
Southmoor Road
Manchester
M23 9LT

Tel: 0161 291 4321
Email: robertlord@nhs.net

You are invited to take part in a research study

- This study is designed to investigate if gastric reflux has an impact on the lung function of CF patients. It is being carried out by a team of trained doctors, nurses and researchers under the supervision of Doctor Andrew Jones and Professor Jacky Smith at the Adult Cystic Fibrosis Centre, University Hospital South Manchester.
- Before you decide if you would like to take part, it is important for you to understand why this research is being done and what taking part would involve for you.
- Please take your time to read the information carefully.
- Your participation is entirely voluntary so you do not have to take part if you do not want to.
- Please ask a member of the research team if there is anything that is not clear, or if you would like more information before considering taking part.
- Thank you for reading this information. We hope this research will be of interest to you.

Important things that you need to know

- This study is looking at gastric reflux. This is where stomach contents travel up into the oesophagus (also known as food pipe or gullet).
- The group we are investigating are patients with cystic fibrosis
- In order to accurately investigate this group we need to compare some of their results to healthy controls
- Being a healthy control involves a brief medical history, examination and breathing tests to ensure you are suitable for the study. You would then be asked to provide 'induced' sputum samples and a throat swab as well as complete 2 questionnaires.
- You can opt out of the study at any time without giving a reason.

UHSM
Your Hospital

What is gastric reflux?

Gastric reflux is a common problem in cystic fibrosis. Stomach acid travels the wrong way back up the gullet. It is commonly associated with heartburn and tasting acid in the mouth. However sometimes it can have no symptoms at all. We think that some of the acid reflux maybe entering the airways and lungs. This could make people's chests worse.

What is the purpose of the research?

We will be testing people to see if acid reflux is really worsening people's lung disease. This will hopefully lead onto further research to offer another treatment to improve lung disease and settle symptoms such as heartburn.

Why have I been asked to take part?

To help improve our understanding of cystic fibrosis and deliver better treatments we often ask people to help us.

We are asking all people with cystic fibrosis to be involved. We need people who have no symptoms as well as those people with symptoms. We also need people with lots of different degrees of lung disease.

You have been asked because your doctor believes you are eligible to take part if you wanted.

What happens if I agree to take part?

If you agree to take part we will check whether you can be involved during one of your usual clinic appointments. This is called 'screening '. We would then give you a couple of days to consider and make contact usually via a phone call to book you in for the two extra appointments.

Study visit

When you attend we will ask you questions about your health and confirm your details are correct in your medical notes. A number of tests will then be performed;

- A doctor will perform a brief examination and check your vital signs. These are your oxygen saturations, blood pressure and pulse.
- A breathing test will be performed

This information will allow us to ensure that you can take part without any risks. We will then ask you to complete 2 questionnaires and provide us with an induced sputum sample. This involved receiving a nebuliser (vaporised substance) that will make you cough and provide a sample. We will also take a swab from the back of the throat.

Do I have to take part?

No, taking part is entirely optional.

What happens if I change my mind?

It is okay to agree to take part and then change your mind. You do not need to provide a reason and it will not affect your clinical care in the future. You can also decide if we have already completed some of the study whether you want the data gathered to be withdrawn. However for us to withdraw data the request must be made within 2 weeks from collection.

What are the benefits?

Cystic fibrosis is an inherited disease which reduces life expectancy mainly by progressive lung disease. You will

be helping us advance our understanding and this knowledge may lead to further treatments.

What are the risks?

There is a risk with the induced sputum procedures that it may cause chest tightness. This is uncommon. However to minimise the risk we will not include anyone with either a diagnosis of asthma or any abnormality of lung function tests.

What happens if something goes wrong?

The entire process will take place in the hospital. You will be provided with the contact details for the doctor running the study.

If you have a concern about any aspect of the study, please speak to the research team in the first instance. If you remain unhappy and wish to complain formally, you can do so through the NHS complaints procedure. Details can be obtained from the **Patient Experience Team** on 0161 291 5600.

The hospital is insured to carry out NHS-sponsored research. If something did go wrong and you were harmed during the research due to someone's negligence then you may have grounds for legal action or compensation against the hospital.

Do I need to change my medications?

No you should continue to take the same medications

Additional information about the study

Will I receive a payment for taking part?

You will not receive payment but we will be able to compensate you for reasonable parking/travel expenses.

Will taking part affect my health insurance?

If you have private medical insurance, please check with your insurance company to see if your policy will be affected by taking part in this clinical study.

Will my details be kept confidential?

We follow strict ethical and legal guidance regarding patient confidentiality. Any information we have about you will be handled in confidence. All data recorded will be coded and your name will remain anonymous.

When taking part in research it is occasionally necessary for your details to be made available to authorised research staff who are bound by the same duty of confidentiality. This may include people who conduct quality assurance and quality control checks to confirm that the research was done correctly. These people may include research ethics committees, inspectors, monitors and auditors.

Who has reviewed this study?

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee. The Research Ethics Committee is made up of experts, non-experts and members of the general public. Together they review research applications to ensure your safety, rights, wellbeing and dignity are protected at all times. They are happy for us to proceed with this study.

What will happen to the study results?

The results will be stored and used to answer research questions. The information will have no personal information so it will be anonymous.

Who is organising and funding the research?

The Manchester Adult Cystic Fibrosis Centre (MACFC) is organising the research. We have enlisted help from experts in the field of gastro-oesophageal reflux to ensure that our study is as good as it possibly can be. The study will be funded by the MACFC, although if some of the tests prove to be very expensive we will apply for funding from some charities.

What happens to any additional samples that are left over?

The study of cystic fibrosis is a rapidly developing area of research and new methods of analysis are being developed all the time. We will ask that you donate the samples as a gift so that the samples can be stored for future analyses relating to this research programme and for possible use in future (ethically approved) research studies. These samples will be anonymised. This will enable us to make the most of any new developments in knowledge and techniques.

Contacts for further information

For more information about this study, you can speak to a doctor supervising the study on 0161 291 4321 (between 9am and 5pm) who will be happy to answer any questions or concerns you might have.

Thank you for reading this information sheet.

Appendix 4 Patient consent form

University Hospital of South Manchester
NHS Foundation Trust

Centre Number:
Study Number:
Participant Identification Number for this trial:

CONSENT FORM FOR CF PARTICIPANTS (version1)

Title of Project: Gastro-oesophageal reflux (GOR) in Cystic Fibrosis
Name of Researcher: Dr Robert Lord / Other .....

Please initial box

1. I confirm that I have read the information sheet dated ..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from The Manchester Adult Cystic Fibrosis Centre, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers. ☐
5. I agree to my General Practitioner being informed of my participation in the study. ☐
6. I consent to the storage of data and samples for up to 15 years and for these to be used in future research. ☐
7. I agree to take part in the above study. ☐

Name of Participant……..

Date…… Signature……

Name of Person taking consent……..

Date…… Signature……

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix 5 Healthy volunteer consent form

University Hospital of South Manchester
NHS Foundation Trust

Centre Number:
Study Number:
Participant Identification Number for this trial:

CONSENT FORM FOR HEALTHY VOLUNTEERS (version 1)

Title of Project: Gastro-oesophageal reflux (GOR) in Cystic Fibrosis
Name of Researcher: Dr Robert Lord / Other .....

Please initial box

1. I confirm that I have read the information sheet dated ..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers. ☐
4. I consent to the storage of data and samples for up to 15 years and for these to be used in future research. ☐
5. I agree to take part in the above study. ☐

Name of Participant……..

Date…… Signature……

Name of Person taking consent……

Date…… Signature……

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.

Appendix 6 Ethics approval

NHS
Health Research Authority
National Research Ethics Service

North West - Greater Manchester West Research Ethics Committee
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

13 October 2015

Dr Andrew Jones
University Hospital South Manchester
Southmoor Road
Manchester
M23 9LT

Dear Dr Jones

Study title:	**Gastro-oesophageal reflux in patients with cystic fibrosis and it's effect on lung function**
REC reference:	**15/NW/0655**
IRAS project ID:	**177609**

Thank you for your letter of 25 September 2015, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Anna Bannister, nrescommittee.northwest-gmwest@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

A Research Ethics Committee established by the Health Research Authority

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [Trial Poster]	version1	27 July 2015
Covering letter on headed paper [LIST OF CHANGES AS REQUESTED FROM REC MEETING]		
GP/consultant information sheets or letters [GP letter Participants]	version1	27 July 2015
Other [Consent Healthy Volunteers]	version 1	27 July 2015
Other [Website & Facebook text]	1	28 July 2015
Other [Healthy Volunteer PIS]	1.1	25 September 2015

Other [RESQ-7 questionnaire]	1	25 September 2015
Other [Email Clarification 1]		13 October 2015
Other [Email Clarification 2]		13 October 2015
Participant consent form [CF patient consent Form]	1.1	25 September 2015
Participant information sheet (PIS) CF	version 1	30 June 2015
Participant information sheet (PIS) GOR	1.1	24 September 2015
REC Application Form [REC_Form_09102015]		09 October 2015
Research protocol or project proposal	1.1	22 September 2015
Response to Request for Further Information [Response to Provisional]		25 September 2015
Summary CV for Chief Investigator (CI) [Dr Andrew Jones CV]		10 July 2015
Summary CV for student [Dr Robert Lord CV]		08 July 2015
Summary CV for supervisor (student research) [CV Prof Smith]		20 February 2015
Validated questionnaire [RESQ-7 - UK-English - 7 day recall - paper]		06 March 2009
Validated questionnaire [Leicester Cough questionnaire]		27 November 2002

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

15/NW/0655	Please quote this number on all correspondence

With the Committee's best wishes for the success of this project.

Yours sincerely

pp

On behalf of Dr Lorraine Lighton (Chair)
Chair

Email: nrescommittee.northwest-gmwest@nhs.net

Enclosures: After ethical review – guidance for researchers

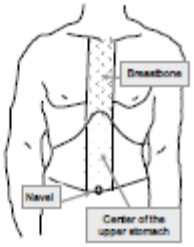
Copy to: *Miss Faye O'Keeffe, UHSM*

Appendix 7 RESQ-7

GOR in CF Version 1 25/09/15

RESQ-7

Please answer the following questions to help us better understand the symptoms you have been experiencing over the past 7 days because of your reflux disease. For each question, please choose the answer that is most appropriate to you. Please answer each question by ticking **one** box per row.



1. Thinking about your symptoms over the past 7 days, how often have you had the following?

	Have not had	1 day	2 days	3-4 days	5-6 days	Daily
a. A burning feeling behind your breastbone	☐	☐	☐	☐	☐	☐
b. Pain behind your breastbone	☐	☐	☐	☐	☐	☐
c. A burning feeling in the centre of the upper stomach	☐	☐	☐	☐	☐	☐
d. Pain in the centre of the upper stomach	☐	☐	☐	☐	☐	☐
e. An acid taste in your mouth	☐	☐	☐	☐	☐	☐
f. Unpleasant movement of material upwards from the stomach	☐	☐	☐	☐	☐	☐
g. Burping (gas coming from the stomach through the mouth)	☐	☐	☐	☐	☐	☐
h. Hoarseness	☐	☐	☐	☐	☐	☐
i. Coughing	☐	☐	☐	☐	☐	☐
j. Difficulty swallowing	☐	☐	☐	☐	☐	☐
k. A bitter taste in your mouth	☐	☐	☐	☐	☐	☐
l. Stomach contents (liquid or food) moving upwards to your throat or mouth	☐	☐	☐	☐	☐	☐
m. Heartburn	☐	☐	☐	☐	☐	☐

RESQ-7 - UK-English – 7 day recall - paper. 6 March 2009 1(2)

2. Thinking about your symptoms over the past 7 days, how would you rate the intensity of the following?

	Did not have	Very mild	Mild	Moderate	Moderately severe	Severe
a. The burning feeling behind your breastbone	☐	☐	☐	☐	☐	☐
b. Pain behind your breastbone	☐	☐	☐	☐	☐	☐
c. The burning feeling in the centre of the upper stomach	☐	☐	☐	☐	☐	☐
d. Pain in the centre of the upper stomach	☐	☐	☐	☐	☐	☐
e. Acid taste in your mouth	☐	☐	☐	☐	☐	☐
f. Unpleasant movement of material upwards from the stomach	☐	☐	☐	☐	☐	☐
g. Burping (gas coming from the stomach through the mouth)	☐	☐	☐	☐	☐	☐
h. Hoarseness	☐	☐	☐	☐	☐	☐
i. Coughing	☐	☐	☐	☐	☐	☐
j. Swallowing difficulty	☐	☐	☐	☐	☐	☐
k. The bitter taste in your mouth	☐	☐	☐	☐	☐	☐
l. Stomach contents (liquid or food) moving upwards to your throat or mouth	☐	☐	☐	☐	☐	☐
m. Heartburn	☐	☐	☐	☐	☐	☐

Appendix 8 IBS-SSS

IBS-SSS (v1 01/04/07)

IRAS number 177609

INSTRUCTIONS

This form is designed to enable us to record and monitor the severity of your IBS. It is to be expected that your symptoms might vary over time, so please try and answer the questions based on how you currently feel (ie over the last 10 days or so). All information will be kept in strict confidence.

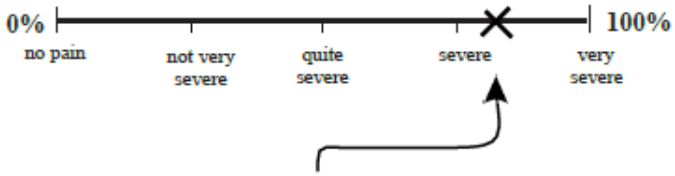
1. For questions where a number of different responses are a possibility please circle the response appropriate to you.

2. Some questions will require you to write in an appropriate response.

3. Some questions require you to put a cross on a line which enables us to judge the severity of a particular problem.

For example:

How severe was your pain?



this answer would indicate that pain is approximately 80% severe

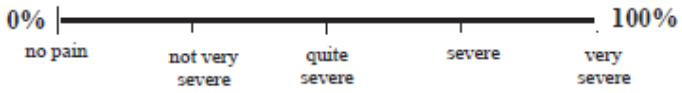
PART 1 : SEVERITY SCORE

1. a) *Do you currently suffer from abdominal (tummy) pain?* **YES** **NO** *Circle appropriate box*

For office use only

SCORE

b) *If yes, how severe is your abdominal (tummy) pain?*

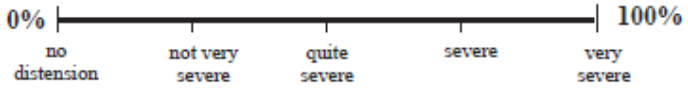


c) *Please enter the number of days that you get the pain in every 10 days.*
For example if you enter 4 it means that you get pain 4 out of 10 days. If you get pain every day enter 10

Number of days with pain ____ x10

2. a) *Do you currently suffer from abdominal distension* (bloating, swollen or tight tummy)*
*(*women, please ignore distension related to your periods)* **YES** **NO** *Circle appropriate box*

b) *If yes, how severe is your abdominal distension/tightness*



3. *How satisfied are you with your bowel habit?*

4. *Please indicate with a cross on the line below how much your Irritable Bowel Syndrome is affecting or interfering with your life in general*

IBS SEVERITY SCORE:

Appendix 9 Sample preparation for MS

First stage

- Prepare 200ml Phosphate-buffered saline (PBS) in HPLC grade water (see bottle of PBS tablets for instructions)
- Prepare a 0.1% Dithiothreitol (DTT) solution in PBS
    - Weigh out 50mg DTT and add 50ml PBS to make a 0.1% solution
- Zero a 15ml test tube on the balance and then add sputum/saliva sample – record weight of sample
- Add 4 x weight of 0.1% DTT solution (weight/volume equivalent)
- Vortex vigorously for 30s before incubating 30min on roller mixer at room temp – at 10min intervals vortex the tube
- Filter sample through a 100um cell strainer into a 50ml tube. May need to touch strainer to the side of the tube to encourage sample to filter.
- Pipette liquid into a new 15ml falcon and centrifuge 450xg 4^{o}C 10min
- Transfer the liquid fraction to low binding microfuge tubes and re-centrifuge 16000xg 30min 4^{o}C
- Remove and combine liquid fractions for each sample – aliquot and freeze material at -20^{o}C in low bind tubes

Second stage

- Take 2ug of sample (if concentration is 0.2ug/ul then take 10ul) in a 0.5ml low bind tube.
- Add 150ul Ammonium Bicarbonate (AmBic) to make 160ul digest starting volume
- Add 10ul Rapigest – heat 80^{o}C for 10mins
- Add 10ul AmBic
- Add 10ul of a diluted 3.3mg/ml concentration of trifluoroacetic acid (TFA) – incubate at room temperature in the dark for 30min
- Add 10ul trypsin – incubate overnight at 37^{o}C with 450rpm shaking

Appendix 10 Permissions for use of copyrighted images

- The New England Journal of Medicine and Molecular & Cellular Proteomics do not require written permission for use of copyrighted images within a PhD thesis. As such they are acknowledged as requested for figure 3.1 and 11.3.

- For Figures 6.1, 6.2 and 6.3 see below:

Dear Robert Lord:

Thank you for your interest in Journal of Neurogatroenterology and Motility (JNM).
We are sorry for the delayed response.
You can download the article "Reflux Evaluation of Esophageal Motor Function with High-resolution Manometry" on JNM website http://www.jnmjournal.org.
You can use the figures (figure 1, figure 4, figure 5 and figure 7) with citation.
Thank you.

Sincerely,

The Editorial Office of JNM

- For figure 11.2 see below:

Dear Dr. Robert Lord,
Thank you for placing your order through Copyright Clearance Center's RightsLink® service

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