CONSEQUENCES TO HEALTH; GASTRO-OESOPHAGEAL REFLUX IN THE CYSTIC FIBROSIS POPULATION

Gemma Louise Zeybel

Thesis submitted for the Degree of Doctor of Philosophy

Medical School

Institute for Cell and Molecular Biosciences

Newcastle University

14th September 2014

Abstract

The work described in this thesis relates to gastro-oesophageal reflux (GOR) and extra-oesophageal reflux (EOR) in the cystic fibrosis (CF) population. This thesis describes the characteristics of GOR and EOR and their association with pulmonary function, gastric aspiration and inflammation in the airway. In particular the study explores the increasing prevalence of overweight/obesity in CF and its association with GOR/EOR. In a sub group of patients (n=12) a longitudinal investigation took place to observe the effects of a new CFTR potentiator (Ivacaftor 150 mg/12h) on GOR/EOR symptoms.

72 CF adults recruited from a CF outpatient clinic consented to the study (39M/33F; median age 21 (16-60) years) and completed questionnaires to characterise symptoms of GOR (DeMeester score 0-9; < 1 normal) and EOR (Reflux Symptom Index (RSI) score 0-45; < 13 normal). Patients were measured for BMI kg/m^2 and grouped according to the following BMI categories: underweight <18.5 kg/m^2, normal weight 18.5-25.0 kg/m^2, overweight 25.1-30.0 kg/m^2 and obese >30.0 kg/m^2. An expectorated sputum sample was provided and analysed for biomarkers of reflux (pepsin n=69 ELISA) and inflammation (IL-6 n=62 and IL-8 n=64 ELISA). Pulmonary function (FEV_1 and FVC % predicted) and genotype were recorded at the time of data collection. Statistical relationships were assessed using the Kruskal-Wallis statistical test followed by the Mann Whitney U test.

GOR symptoms (DeMeester) were identified in 42% of patients and EOR symptoms (RSI) in 63% of patients. Pepsin was detected in 48 (70%) patient samples (median: 330ng/ml; range 80-1150ng/ml) and not correlated with GOR/EOR symptoms. GOR/EOR symptoms and gastric aspiration did not associate with pulmonary function, nor was GOR associated with inflammation of the airways. CF patients can be overweight/obese (16.7%), and this was associated with better lung function; they have less reflux. Obesity didn't show a relationship to F508del/F508del status or to gender. Ivacaftor treatment was associated with reduced symptoms of GOR and EOR (6 weeks to 12 month post medication) accompanied by positive effects on pulmonary function.

GOR is common in CF patients and EOR symptoms are very prevalent in CF and more so than GOR. Microaspiration of gastric content into the airway was not correlated with pulmonary function and occurred across the spectrum of disease severity. Overweight/obese patients experience less EOR symptoms. The CFTR potentiator Ivacaftor reduced GOR and EOR symptoms after 6 weeks of treatment.

Acknowledgements

Firstly, I would like to thank my supervisors Professor Jeff Pearson and Dr Chris Ward for their continuous support, motivation and direction throughout the four year PhD project. I give sincere thanks to Mr Amaran Krishnan for receiving ethical approval for the study and the support given in the first year of research. The project would not have been possible without the chest clinic in the Royal Victoria Infirmary for the access to cystic fibrosis patients and allowing for sample/data collection to take place. I would like to thank Dr Stephen Bourke, Dr Simon Doe, Alan Anderson, Marie Caraher and Alison Gurney for their guidance during patient recruitment and sample collection. I am thankful for the help of Mrs Gail Johnson and Mr Kasim Jiwa at the Freeman hospital during sputum processing and cytology. I would like to thank Kim Pearce for statistical support during my PhD and her motivational discussions.

I would like to thank Professor Peter Dettmar, Dr Vicki Strugala, Andrew Woodcock and Jeanine Fisher for their motivation and support during my industrial placement at Technosics Ltd. I would like to thank Professor Alyn Morice for clinical shadowing during cough clinics, sample collection and valuable knowledge shared during the grand round and the hospital ward round. This experience was most interesting during my industrial placement. Thank you to Mr Warren Jackson, Miss Jennifer Bourke and Miss Annika Rasijeff for being such a valuable part of chronic cough sample collection and patient recruitment.

I would like to thank everybody in the laboratory and office, Shruti, Pete, Mark, Matt, Berns, JoJo, Vicki, Git, Lorna, Laura, Salam, Dave, Livingstone, Adil, Hafez, Su and Mody for your friendship and motivational support.

Especially, I would like to thank my husband who is my teacher and my best friend, providing constant support and guidance as well as a wonderful life outside of work. Michenzo for his loyalty, love and always being my shadow. Also, my family, who have steered me in the right direction and supported me during the early days of education, I would like to thank them all for cheering me on at every hurdle and offering support when it was needed.

Declaration

This thesis is based on research performed in the Institute for Cell and Molecular Biosciences, Newcastle University. I performed all the work and analysis of the results with the exception of the aspects of the clinical data outlined below.

- Total Bile salt analysis
- Pulmonary function tests
- Sweat chloride tests

Publications and reviewed abstracts

M. J. McDonnell., R. Jones, J., **G. Crossfield**, S. Bourke, I. Forrest, J. Simpson, R.M. Rutherford, M.Griffin, J. Pearson and C. Ward (2014) 'Using Questionnaires To Measure The Impact Of Gastro-Esophageal Reflux In Chronic Lung Disease', [ABSTRACT A58]. *PULMONARY REHABILITATION: SYMPTOMS AND DISEASE MANAGEMENT*. American Thoracic Society, American Journal of Respiratory and Critical Care Medicine 189;2014 pp. A1914-A1914.

Gemma L Crossfield, Warren Jackson, Jennifer Burke, Andrew D Woodcock, Vicki Strugala, Chris Ward, Jeffrey P Pearson, Peter W Dettmar, Alyn H Morice
Pepsin detection despite the use of acid suppressant medication in patients with airway reflux related chronic cough. [ABSTRACT S31] Thorax 2013;**68**:Suppl 3 A19 doi:10.1136/thoraxjnl-2013-204457.38
Oral presentation delivered at: British Thoracic Society Winter Meeting 2013, London, UK.

G.L.Crossfield, A. Krishnan, J. Lordan, S. Bourke, A. Anderson, P.W. Dettmar, I.A. Brownlee, C. Ward, J.P. Pearson
WS3.1 Gastric aspiration into the CF lung – relationship with reflux symptoms and lung function. [ABSTRACT WS3.1] *Journal of Cystic Fibrosis, Volume 12, Supplement 1, June 2013, Page S5*
Oral presentation delivered at: 36th European Cystic Fibrosis Society Conference (ECFS) 2013, Lisbon, Portugal.

G. L. Crossfield, A. Krishnan, S. Bourke, A. Anderson, A. Gurney, P. W. Dettmar, I. Brownlee, C. Ward, J. P. Pearson Overweight and obesity challenges in the CF population – a warrant for revision in nutritional advice?
Oral presentation delivered at:

1. North East Postgraduate Conference 2013, Newcastle University, Newcastle upon Tyne, UK.
2. Nutrition Society Postgraduate Meeting 2013, Kings College, London, UK

G.L.Crossfield, A. Krishnan, J. Lordan, S. Bourke, A. Anderson, P.W. Dettmar, I.A. Brownlee, C. Ward, J.P. Pearson Diet and reflux – consequences for the cystic fibrosis population [ABSTRACT]

Poster presentation at: Nutrition Society Postgraduate Meeting 2012, Newcastle University, Newcastle upon Tyne, UK.

Contents

List of Figures

List of Tables

Abbreviations

ABTS	sodium dodecyl sulphate 2,2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid
ATP	Adenosine triphosphate
BA	Bile acid
BAL	Bronchioalveolar lavage
BALF	Bronchioalveolar lavage fluid
BE	Barretts oesophagus
BMI	Body mass index
BSA	Bovine Albumin
CA	Cholic acid
CC	Chronic cough
CF	Cystic Fibrosis
CFQ-R	Cystic Fibrosis Questionnaire Revised
CFTR	Cystic Fibrosis Transmembrane Regulator
CI	Confidence Interval
DGER	duodeno-gastro-oesophageal reflux exposure
DH2O	Deionised water
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DPX	Distrene-80, Plasticizer, Xylene
DTT	Dithiothreitol
ECB	Exhaled breath condensate
ELISA	Enzyme Linked Immunosorbent Assay
EO	Erosive oesophagitis
EOR	Extra-oesophageal reflux
FEV1	Forced expiratory volume in 1 second
FVC	Forced Vital Capacity
G DHC	glycodeoxycholate
G THC	glycocholate
G.P	General Practitioner
GI	Gastro-intestinal
GIQLI	Gastrointestinal Quality of LIFE Index
GOR	Gastro-oesophageal reflux
GORD	Gastro-oesophageal reflux disease
H2-RA	H2 Receptor Agonists
HARQ	Hull Airway Reflux Questionaire
HCL	Hydrochloric acid
HRP	Horseradish peroxide
HRP	Horseradish Peroxidase
IL	Interlukin
IL-6	Iterleukin 6
IL-8	Interleukin 8
LCA	Litocholic acid

LFD	Lateral flow device
LLMI	Lipid Laden Macrophage Index
LOS	Lower Oesophageal Sphincter
LOS	Lower Oesophageal Sphincter
LPR	Lanryngo-pharygeal reflux
LPR	Laryngopharyngeal Reflux
LT	Lung transplant
n	number
NERD	Non-erosive reflux disease
NW	Normal weight 18.5-24.9 kg/m2
OB	Obese BMI >30.0kg/m2
OD	Optical Density
OR	Odds Ratio
OW	Overweight BMI >25.0kg/m2
PBS	Phosphate Buffered Saline
PKA	Protein kinase cAMP-dependent
PPI	Proton Pump Inhibitor
r	Goodness of fit of linear regression
r.p.m	rotations per minute
RDA	Recommended daily allowance
RNI	Recommended nutrient intake
RSI	Reflux Symptom Index
RVI	Royal Victoria Infirmary
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
T DHC	Taurodeoxycholate
T THC	Taurocholate
TBA	Total Bile Acid
T-CA	Taurocholic acid
TLOSR	Transistant Lower oesophageal sphincter relaxation
TMB	3,3′,5,5′-Tetramethylbenzidine
U.K	United Kingdom
USA	United States of America
UW	Underweight <18.4kg/m2
v/v	Volume for volume
w/v	Weight for volume
w/w	Weight for weight
WHO	World health organisation

Chapter 1.0

General introduction

1.1 **Cystic Fibrosis from the beginning**

Cystic Fibrosis (CF) was first described in 1938 by Dr Dorothy Andersen who was working at the New York Babies Hospital as a pathologist (Orenstein *et al.*, 2000). It was known that salty skin was problematic for babies for many years but it was Paul di Sant'Agnese who discovered in 1953 that babies with CF had increased salt content in their sweat. This knowledge lead to the invention of the diagnostic tool of non-invasive sweat testing (di Sant'Agnese *et al.*, 1953). The name 'cystic fibrosis' came about due to the changes to the pancreas caused by the disease whereby the damaged pancreas becomes filled with fluid filled spaces (cysts) and scar tissue (fibrosis) (Thomson and Harris, 2008). The 1960's brought further prospect for the future of CF research with the opening of the Cystic Fibrosis Research Foundation Trust charity. This was one of many charities raising funds and awareness of the disease (Hodson *et al.*, 2007). In 1985 Professor Lap-Chi Tsui, Dr Francis Collins and Professor Jack Riordan identified the specific gene responsible for CF found on chromosome 7 and called it the cystic fibrosis transmembrane conductance regulator (CFTR) (Tsui *et al.*, 1985). The 20th century witnessed historical key scientific mile stones in CF research resulting in improved life expectancy and quality of life for CF patients (and their families) today and for the future (Simmonds, 2013).

1.2 Epidemiology of Cystic Fibrosis

CF is the most common fatal autosomal recessive disease in the Caucasian population (Wilson and Pencharz, 1998), one of the most common life-shortening diseases in white populations (Orenstein *et al.*, 2000) with expected life expectancy of 40 or 50 years today (Thomson and Harris, 2008; Salvatore *et al.*, 2012; Simmonds, 2013). About 1 in 25 people are a carrier of the recessive mutated CFTR gene with an estimated 1 in every 2500 live births affected by a mother's and father's mutated CFTR gene being carried onto their child (Bourke and Burns, 2011).

Seventy percent of babies born with CF when it was first described in 1938 were not expected to live beyond the age of 1 year (Orenstein *et al.*, 2000; Simmonds *et al.*, 2009). In the 1960's survival rates had increased to 14 years of age due to careful clinical

observation and comprehensive treatment programs of diagnostic advances and antibiotic treatment (Orenstein *et al.*, 2000; Simmonds, 2013). Reports from the Cystic Fibrosis Foundation in 2005 reported median survival to be 36.8 years. A study in 2009 reported that n=112 CF patients at their clinic in London were over 40 years of age without lung transplantation (median 43.1; range 40-71) (Simmonds *et al.*, 2009). Research supports the predicted median survival age of 50 years for patients born in the year 2000 through the observations of continued improvement in the survival of CF patients (Dodge *et al.*, 2007).

1.3 Genetic defects of Cystic Fibrosis

CF is caused by mutations on both alleles of the CF transmembrane conductance regulator (CFTR) gene that codes for the CFTR protein and there are approximately 2000 CFTR mutations described to date (Bell *et al., 2014*; Kotha and Clancy, 2013). CFTR is a chloride channel and the role is to regulate salt, fluid and pH balance in multiple organs (Van Goor *et al.*, 2013). Protein Kinase A (PKA) phosphorylates CFTR to allow ATP binding and hydrolysis which in turn opens and closes the channel across cell membranes (channel gating) to allow chloride, and bicarbonate ions to be transported. CFTR mutations result in abnormal chloride and bicarbonate ion transport. There are 5 classes of CFTR mutations, classes I-III are non-functional CFTR proteins whereas class IV-V CFTR proteins do have some function (Figure 1.1). The most common mutation affecting 80% of patients is the F508del in class II. Class II mutations bring about improper protein folding and the CFTR protein remains in the endoplasmic reticulum instead of the plasma membrane therefore there is minimal CFTR protein at the cell surface (trafficking). Class III mutations differ to class II because the CFTR protein is present at the cell surface but displays a defective channel gating resulting in minimal chloride transport. Class III mutations occur in approximately 4-5% of patients, the most common mutation in this class is G551D (Kotha and Clancy, 2013; Flume, et al., 2012; Yu, et al., 2012). The loss of chloride transport leads to elevated sweat chloride concentration, the accumulation of thick, sticky mucus in the bronchi, loss of pancreatic function, impaired intestinal absorption and reproductive dysfunction (Van Goor, et al., 2013; Yu, et al., 2012). The CFTR protein is predominantly expressed in exocrine tissues. These include the airways, intestines, pancreatic ducts and sweat glands (Van Goor *et al.*, 2009; 2013; Kotha and Clancy, 2013).

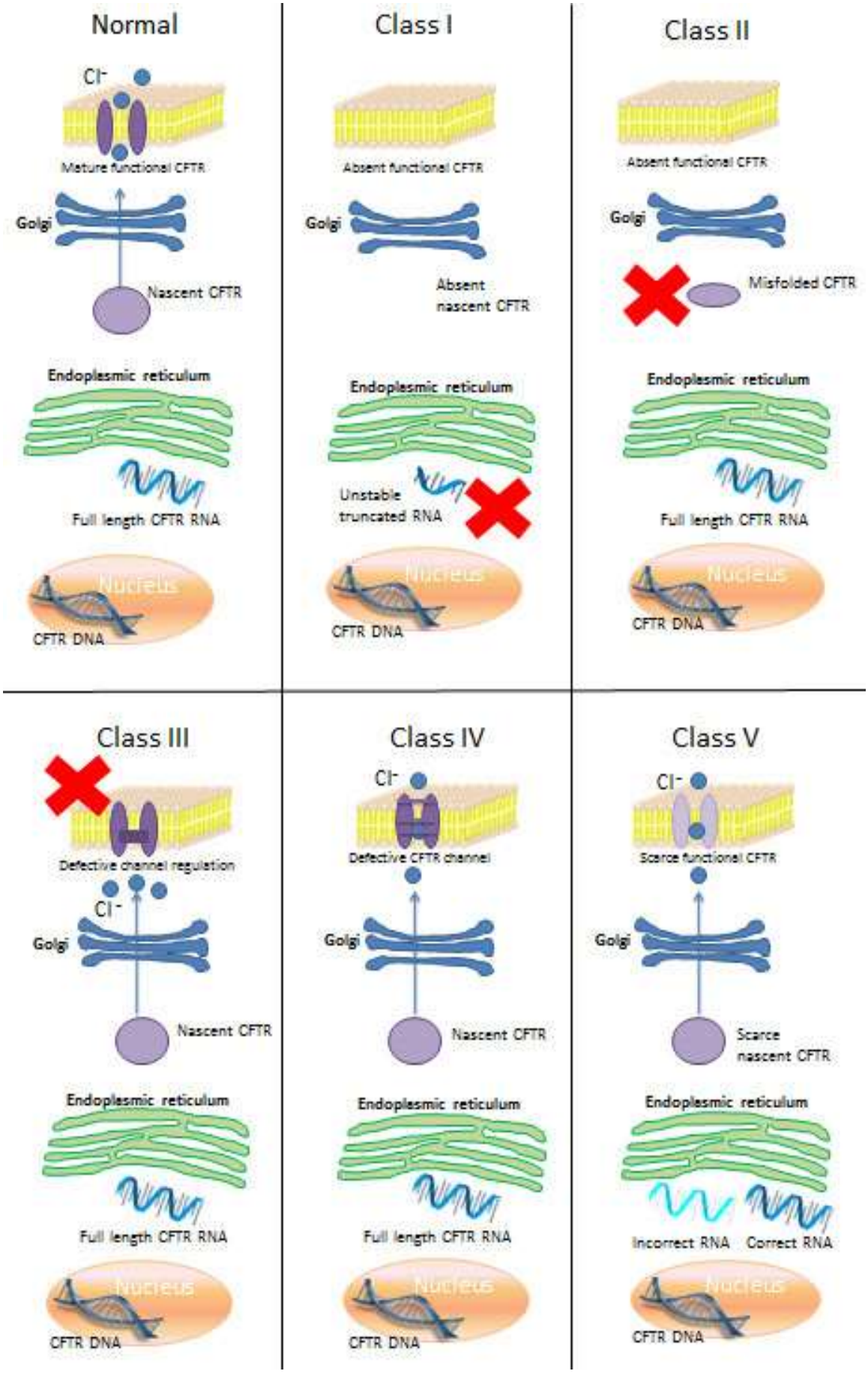

Normal
Cl-
Mature functional CFTR
Golgi
Nascent CFTR
Endoplasmic reticulum
Full length CFTR RNA
Nucleus
CFTR DNA
Class I
Absent functional CFTR
Golgi
Absent nascent CFTR
Endoplasmic reticulum
Unstable truncated RNA
Nucleus
CFTR DNA
Class II
Absent functional CFTR
Golgi
Misfolded CFTR
Endoplasmic reticulum
Full length CFTR RNA
Nucleus
CFTR DNA
Class III
Defective channel regulation
Cl-
Golgi
Nascent CFTR
Endoplasmic reticulum
Full length CFTR RNA
Nucleus
CFTR DNA
Class IV
Cl-
Defective CFTR channel
Golgi
Nascent CFTR
Endoplasmic reticulum
Full length CFTR RNA
Nucleus
CFTR DNA
Class V
Cl-
Scarce functional CFTR
Golgi
Scarce nascent CFTR
Endoplasmic reticulum
Incorrect RNA
Correct RNA
Nucleus
CFTR DNA

Figure 1.1 The classification of CFTR mutations. The following illustration represents the location of the mutated CFTR in comparison to the normal CFTR (cell at the top left) showing the mutation class and a description of the nature of the defect. The normal mature CFTR protein (top left) is present at the cell surface and allows for the transportation of chloride ions. The class I mutation results in an unstable truncated CFTR mRNA leading to total or partial absence in CFTR protein production. The class II mutation causes misfolding of the CFTR protein and the protein remains inside of the endoplasmic reticulum of the cell leading to an absent functioning CFTR at the cell surface. The class III mutation results in the CFTR protein to be defective in channel regulation, it is present at the cell surface but the protein is resistant to activation. The CFTR class mutation I, II and III result in a non-functional CFTR channel. The class IV mutation results in a CFTR channel present at the cell surface but altered conduction of the CFTR protein reduces activity. The class V mutation leads to scarce CFTR protein at the cell surface caused by the splicing of the CFTR m-RNA. The class IV and V mutations cause a reduction in function of the CFTR protein. (Boyle and De Boeck, 2013; Fanen *et al.*, 2014).

1.4 Diagnosis of Cystic Fibrosis

A classical feature in CF is salty skin which results from a high salt content in the sweat (di Sant'Agnese *et al.*, 1953). This is due to the failure of the ion channel to reabsorb chloride ions from the sweat and results in a sweat sample containing high amounts of chloride and sodium concentrations. CF patients show abnormal sweat chloride levels (CF unlikely <39mmol/L; CF possible 40-59mmol/L; CF likely >60mmol/L) (Cystic Fibrosis Foundation, 2011). A non-invasive sweat test is the gold standard biomarker to diagnose CF disease used to diagnose CF in association with typical clinical features including recurrent chest infections and pancreatic insufficiency (Veeze, 1995; Bourke and Burns, 2011). For reliable sweat testing the Gibson and Cooke method is advised (Gibson and Cooke, 1959).

Due to advances in genetic testing the CF mutation can be defined by confirming two CF mutations on each allele from approximately 1900 mutations (Kotha and Clancy, 2013) but it can be difficult to exclude CF from rare mutations. The advances in CFTR mutation discovery has highlighted patients with CFTR mutations accompanied by mild clinical features, thus less severely affected patients. These patients are classed as atypical or non-classic CF patients. They do not experience pancreatic insufficiency or recurrent pulmonary infection (Bourke and Burns, 2011).

1.5 Pulmonary complications in Cystic Fibrosis

The CF population have poor respiratory function due to thickened mucus in the lungs. Reduced chloride secretion and increased sodium reabsorption causes an increased risk of pathogen colonisation (Orenstein *et al.*, 2000). Mucus builds up on the outside of the cells expressing CFTR caused by the faulty chloride channel at the surface of the cell (Thomas and Bishop, 2007). The abundance of resulting mucus in the airways creates an environment suitable for bacteria to colonise and grow causing infections within the airway. The epithelial surface of the lungs normally have antimicrobial defences but the high salt content of the airway surface fluid causes an unnatural environment and results in inactivation of antimicrobial defences. Impaired internalization of the CFTR occurs when the CFTR is not present at the apical membrane. The defective internalization of respiratory pathogens limits bacterium clearing (Pier *et al.*, 1996). Furthermore, the CF surface of airway epithelium possesses increased apical receptors such as asialylated gangliosides receptors to allow bacteria to adhere and proliferate (Saiman and Prince,

1993; de Bentzmann *et al.*, 1996). Thus, mechanistically defective CFTR leads to repeated, cyclical chest infection reoccurrence and this leads to further lung problems and progressive lung disease with associated morbidity and mortality. (Navarro *et al.*, 2001; Steinkamp and Wiedemann, 2002; Konstan *et al.*, 2007; Thomas and Bishop, 2007; Bourke and Burns, 2011). The impaired mucociliary clearance of sputum within the airways and bacterial infection lead to pulmonary exacerbations which may lead to hospitalisation and required intravenous antibiotics (Banner *et al.*, 2009). It is lung disease which leads to premature mortality in most patients with CF.

The inflammatory system responds to the bacterial infection by producing inflammatory mediators at the site of infection. The mediators recruit neutrophils to attack the infection in order to eliminate it. The inflammatory response in the CF airway does not function effectively and cannot clear recurrent infections within the airways. Inflammation develops in the airways excessively in relation to the bacteria present and continues as the infection cannot be cleared (Bourke and Burns, 2011). This leads to a vicious cycle of excessive inflammation influx in the CF airway resulting in airway damage. Viral infections within the CF lung may potentially damage the epithelium (Banner *et al.*, 2009). Progressive pulmonary disease is a leading cause of mortality for the CF population (Anthony et al., 1999; Sagel *et al.*, 2007).

1.6 Gastrointestinal complications in cystic fibrosis

A consequence of CF is the blockage and obstruction of the pancreatic ductules by thickened mucus produced by abnormal ion transport leading to the gland being progressively destroyed. This leads to the inhibition of the pancreatic enzyme lipase into the small intestine and results in fat malabsorption, steatorrhoea and failure to gain weight. This creates further problems involving the metabolism of fat soluble nutrients, vitamin A,D,E and K (Bourke and Burns., 2011; Thomas and Bishop, 2007; Orenstein *et al.*, 2000). This is treated with pancreatic enzyme replacement therapy (PERT) taken with all food and drink to provide the patient with artificial pancreatic enzymes which breaks down dietary fat in the intestines to achieve optimum growth and nutritional status (Littlewood, 1992; Anthony, *et al.*, 1999; Morton, *et al.*, 2009). Patients with this complication are termed pancreatic insufficient and these patients have been accompanied with worse respiratory function. This was put down as a consequence of the pancreatic insufficiency

but now thought to be attributed to the mutation severity of the CFTR (Anthony, *et al.*, 1995).

It is well documented that gastro-oesophageal reflux (GOR) characterised by the retrograde movement of gastric contents up the oesophagus (Armstrong, *et al.*, 2005; Chen, *et al.*, 2010) is a problem in the CF population. GOR leads to symptoms such as heartburn and/or acid regurgitation, in some cases GOR may cause oesophageal mucosal damage, which may lead to longer term complications. Reflux reaching the proximal oesophagus and extra-oesophageal areas termed extra oesophageal reflux (EOR) has been shown to be implicated in multiple respiratory disorders and may result in coughing, sore throat, excessive clearing of the throat, post nasal drip, chest wheezing and tightness (Button *et al.*, 2005). These symptoms have been shown to reduce quality of life (Bendig *et al.*, 1982). It is reported that GOR adversely impacts lung function in CF due to the action of potent agents in refluxate (Gustafsson, *et al.*,1991; Navarro, *et al.*, 2001; Blondeau, *et al.*, 2008*a*; Pauwels *et al.*, 2013).

1.7 New treatments used in cystic fibrosis - CFTR therapeutic agents

The pace of clinical and scientific advances in CF research in the late 20th Century has been outstanding. During the first 14 years of the 21st century we already observe further scientific advances that have the potential to repair the loss of chloride transport by targeting the class defect of the CFTR. A group of CFTR modulators have been studied, these are pharmacological therapeutic strategy agents that repair the CFTR defect to restore function. On January 31st 2012 the approval of the first CFTR potentiator (Ivacaftor) took place after successful clinical trial outcomes in the class III G551D mutation. This CFTR modulator is a CFTR potentiator which induces channel opening to enable chloride ions to be transported through the CFTR present at the cell surface (Van Goor, *et al.*, 2009; Kotha and Clancy, 2013). Administration of 150 mg every 12 hours during clinical trials lowered the CFTR biomarker sweat chloride concentrations to below the CF diagnostic threshold (60mmol/L). Significant improvements were observed in pulmonary function and weight and interestingly pulmonary exacerbations and patient hospitalisation were reduced as a result of Ivacaftor treatment compared with placebo controls (Ramsey, *et al.*, 2011; Accurso, *et al.*, 2013). Ivacaftor treatment has been shown to continue its unprecedented success for the G551D CF patient population in CF clinics around the world (Reznikov, *et al.2014*; Davies, *et al.*, 2013; Harrison, *et al.*, 2013;

Hebestreit, *et al.*, 2013; Polenakovik and Sanville, 2013; Barry *et al.*, 2014). In excitable anticipation the CF community await further CFTR modulators for other CF patient populations with primary or secondary gating defects. More work is needed before we are likely to see the same results for the most common mutation, class II F508del defect that requires a CFTR corrector agent. The CFTR corrector increases the delivery of CFTR protein to the cell surface therefore increasing the flow of chloride ions. A combination approach of a CFTR corrector (Lumacaftor) with Ivacaftor for patients with the phe508del *CFTR* has shown encouraging results in clinical trials illustrating modest decreases in sweat chloride, and these studies are being consolidated and extended in ongoing trials (Boyle, *et al.*, 2014; Eckford *et al.*, 2014; Quintana-Gallego *et al.*, 2014).

1.8 The future of CF and arising concerns

The CF population are living longer due to advances in health care, clinical treatment and continuing scientific research. The numbers of CF children are now being outnumbered by the increasing number of adults in many clinics (Quon and Aitken, 2012; Simmonds, *et al.*, 2013) and it has been reported that CF patients are living into their 50's (Simmonds, *et al.*, 2013). Furthermore, those that are born today with the CFTR mutations are predicted to have normal life expectancy due to future therapies that treat the basic defect (O'Sullivan and Freedman, 2009). Positivity surrounds the increase in life expectancy for the CF aging population, although the increasing survival statistics brings challenges with it that are now forming a focus for study. The concern of CF age associated co-morbidities and age related chronic diseases of the general population is an arising challenge (Dennersten, *et al.*, 2009; Simmonds, *et al.*, 2009; Quon and Aitken, 2012; Simmonds, *et al.*, 2013; Lerín, *et al.*, 2014). GORD has been observed to be higher in the adult CF population (Ledson *et al.*, 1998; Blondeau *et al.*, 2008; Sabati *et al.*, 2010; Phitidis *et al.*, 2014).

It was reported that CF BMI status has increased beyond an ideal status (BMI 25.0kg/m^2) and this could be attributed to nutritional interventions of a high fat and calorie diet. There is an increasing prevalence of CF overweight and obese BMI status (Kastner-Cole *et al.*, 2005; Stack *et al.*, 2007; Munck *et al.*, 2007; Coderre *et al.*, 2012; Panagopoulou *et al.*, 2014); Stephenson *et al.*, 2013; Kochavi *et al.*, 2014) which has been associated with increased risks of GORD in the general population (El-Serag, *et al.*, 2005, Dore *et al.*, 2008; Hampel, *et al.*, 2005).

1.9 Overall aims of the thesis

The importance of long-term health is increasingly recognised for the aging CF population, but further research is required. This thesis focused on the prevalence of GOR and EOR in the CF population at the Royal Victoria Infirmary chest clinic, Newcastle upon Tyne.

The characteristics of GOR and EOR were explored and each was determined using validated questionnaires. Gastric aspiration of reflux contents were determined by analysing GOR biomarkers in the expectorated sputum of CF patients. The association with pulmonary function was explored. In addition, the presence of inflammation within the airway was measured in expectorated sputum to assess the role gastric aspiration may play in the inflammatory response.

Assessment was made of the role a CFTR potentiator, Ivacaftor, may have on the presence of reflux (symptoms and gastric aspiration). This was measured by the longitudinal investigations over a 12 month period in 12 patients with the G551D mutation receiving Ivacaftor for the first time.

The prevalence of overweight and obesity was investigated and its association with GOR in the CF population and their association with gender and genotype were assessed.

Chapter 2.0

Methodology

2.1 Project specific materials

Dulbecco A Phosphate buffered saline, pH 7.3, Oxoid Limited, Basingstoke, Hampshire, England.

TWEEN® 20, SIGMA life Science, SIGMA-ALDRICH Co, 3050 Spruce Street, St Louis, MO 63103, USA.

Sputolysin® - Dithiothreitol (DTT), Calbiochem, Merck KGaA, Darmstadt, Germany.

Maxisorp NUNC-Immuno 96 well plate, Thermo Scientific NUNC™ F96, Kamstrupvej, 90 P.O.BOX 280, DK-4000, Roskilde, Denmark.

Pre-cut transparent microplate sealers, Grelner Bio-One Limited, Brunel way, Stroudwater Business Park, Stunehouse, Glossier UK.

Sodium Dodecyl Sulphate, BDH Laboratory Supplies, Poole, UK.

Bovine Serum Albumin, Cohn Fraction V Powder pH 7.0, VWR International Ltd, Hunter Boulevard, Magna Park, Lutlerworth, Leicestershire, UK.

Pepsin, Porcine gastric mucosa, SIGMA life Science, SIGMA-ALDRICH Co, 3050 Spruce Street, St Louis, MO 63103, USA.

Pepsin primary antibody monoclonal anti-pepsin antibody, W59117G, Biodesign Int. US/AMS Biotechnology, UK.

Secondary horseradish peroxidise conjugated polyclonal anti-goat/sheep antibody, Sigma, UK.

R&D systems human and recombinant IL-8 and IL-6 kit (catalog number: DY208 &DY206) containing:

Capture antibody mouse anti-human IL-8 and IL-6

Detection antibody biotinylated goat anti-human IL-8 and IL-6

Standard recombinant human IL-8 and IL-6

Streptavidin conjugated to horseradish peroxidase (HRP)

(R&D Systems Europe, Ltd. Abingdon, UK)

2.2 Ethics and consent

Full ethical approval was granted for this study by the County Durham & Tees Valley 2 Research Ethics Committee (REC NO: 10/H0908/8) (Appendix 8.1). The ethical application process of this study was carried out by Mr Amaran Krishnan. Each patient recruited signed the consent form to agree to take part in the study.

2.3 Patient Recruitment

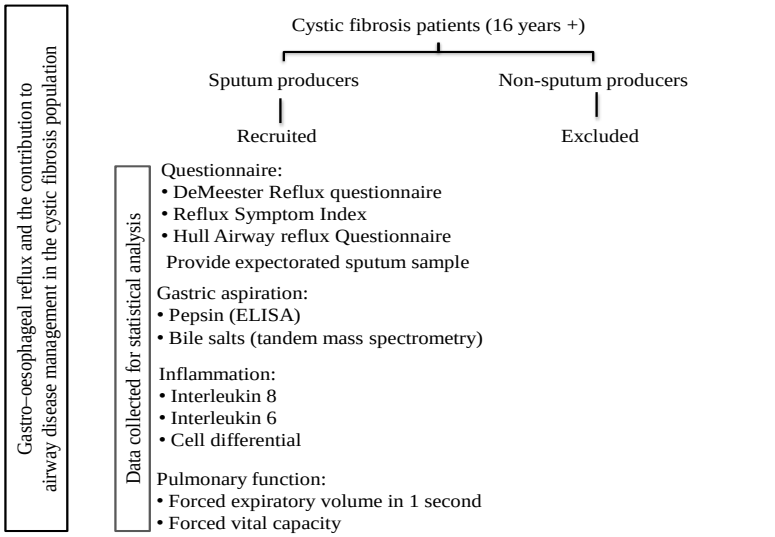
Seventy two patients 16 years or older with clinical cystic fibrosis undergoing routine clinical appointments at the outpatient chest clinic in the Royal Victoria Infirmary (RVI) were recruited to participate in the study. Eleven patients were recruited by telephone call and attended scheduled appointments with Mr Amaran Krishnan for oesophageal studies in the endoscopy clinic at the RVI. Each patient (n=72) received details about the study and had the opportunity to read the study information sheet. Once they agreed to take part in the study the patient was asked to listen to the researcher talk through the consent form and each point was ticked to say that they agreed. The consent form was signed by the patient and the person taking consent in which the printed name, signed name and the date was provided. A copy of the consent form was stored at the research facility and also the patient received a copy along with the study information sheet (Appendix 8.2). The following criteria were taking into consideration when recruiting patients (Table 2.1 and Figure 2.1).

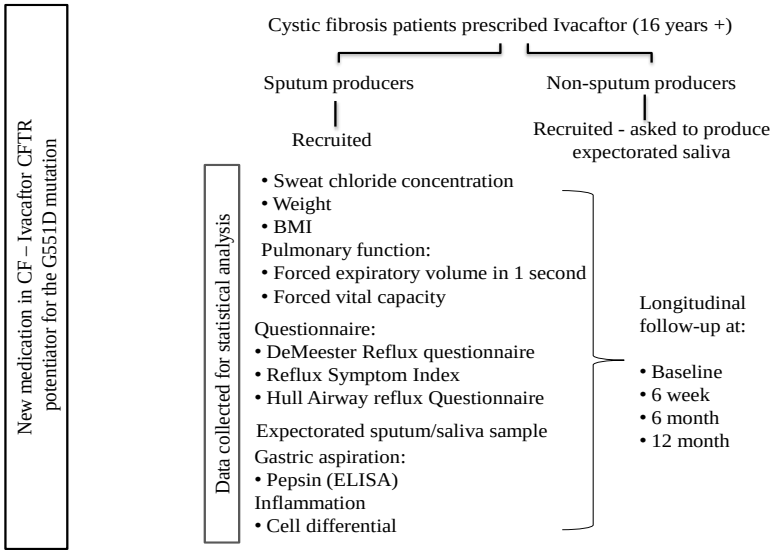
Inclusion	**Exclusion**
16 years and above	None sputum providers
Cystic fibrosis patient	Under 16 years of age
Attending appointment at Royal Victoria Infirmary; Freeman hospital, James Cook.	tube fed only
Sputum producers upon request (accept for Ivacaftor patient cohort/ all recruited)	

Table 2.1 Subject inclusion and exclusion criteria

In an attempt to increase subject numbers an amendment to the ethical approval was applied for and granted to recruit CF patients from two other hospitals. CF patients were

recruited from a pre-lung transplant assessment clinic at the Freeman Hospital (Newcastle) and the monthly outreach chest clinic at James Cook Hospital in Middlesbrough.


Gastro–oesophageal reflux and the contribution to airway disease management in the cystic fibrosis population
Cystic fibrosis patients (16 years +)
Sputum producers
Non-sputum producers
Recruited
Excluded
Data collected for statistical analysis
Questionnaire:
• DeMeester Reflux questionnaire
• Reflux Symptom Index
• Hull Airway reflux Questionnaire
Provide expectorated sputum sample
Gastric aspiration:
• Pepsin (ELISA)
• Bile salts (tandem mass spectrometry)
Inflammation:
• Interleukin 8
• Interleukin 6
• Cell differential
Pulmonary function:
• Forced expiratory volume in 1 second
• Forced vital capacity


New medication in CF – Ivacaftor CFTR potentiator for the G551D mutation
Cystic fibrosis patients prescribed Ivacaftor (16 years +)
Sputum producers
Non-sputum producers
Recruited
Recruited - asked to produce expectorated saliva
Data collected for statistical analysis
• Sweat chloride concentration
• Weight
• BMI
Pulmonary function:
• Forced expiratory volume in 1 second
• Forced vital capacity
Questionnaire:
• DeMeester Reflux questionnaire
• Reflux Symptom Index
• Hull Airway reflux Questionnaire
Expectorated sputum/saliva sample
Gastric aspiration:
• Pepsin (ELISA)
Inflammation
• Cell differential
Longitudinal follow-up at:
• Baseline
• 6 week
• 6 month
• 12 month

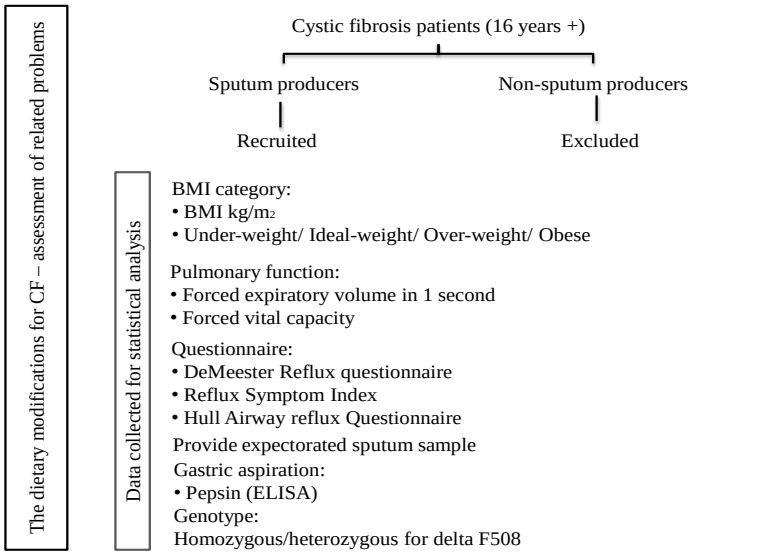

The dietary modifications for CF – assessment of related problems
Cystic fibrosis patients (16 years +)
Sputum producers
Non-sputum producers
Recruited
Excluded
Data collected for statistical analysis
BMI category:
• BMI kg/m2
• Under-weight/ Ideal-weight/ Over-weight/ Obese
Pulmonary function:
• Forced expiratory volume in 1 second
• Forced vital capacity
Questionnaire:
• DeMeester Reflux questionnaire
• Reflux Symptom Index
• Hull Airway reflux Questionnaire
Provide expectorated sputum sample
Gastric aspiration:
• Pepsin (ELISA)
Genotype:
Homozygous/heterozygous for delta F508

Figure 2.1 Summary of patient recruitment and experimental strategy for each result chapter. The summary illustrates the title of each chapter and the data collected for the analysis.

2.4 Patient attendance

A total of 72 patients fulfilled all the study requirements and were enrolled onto the study. Attempts were made to increase the study number over the recruitment period; a further 24 were approached but were unable to produce a sputum sample. Two of these patients provided all the study requirements and failed to produce sputum after many attempts so they were excluded from the study.

2.5 Sputum collection

Expectorated sputum sample were requested from each patient (accept for the assessment of Ivacaftor treatment as expectorated saliva was used to replace sputum for the non-producers). The patient was provided with a sputum pot and was given time to produce a sample in the consulting room that they were directed to by the nurse upon arrival to the RVI, within the contamination regulations of the chest clinic. The patients were asked whether they would prefer privacy for this activity and everyone was requested to leave the room if required. The expectorated sample was then stored on ice in a secure sample collection box until processing within 2 hours of collection in a class 2 cabinet in the laboratory. Recruitment usually took place during the hours of 14.00 to 17.00. These appointment times were made available for the CF patients to ensure that the consulting rooms were cleaned and prepared for each patient depending on the bacteria infection within their airways to avoid cross contamination. The cross contamination rules for the outpatient clinic times set by the respiratory unit in the RVI aimed to set a clinic date depending on colonization of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

2.6 Sputum processing

In a ventilated class 2 cabinet the sputum plugs were separated from the surrounding saliva, The plugs were transferred to a pre-weighed centrifuge tube and the sputum weight determined. Dulbeccos phosphate buffered saline (DPBS) was added to the sputum (8x initial sputum weight) and allowed to disperse in a centrifuge tube rocking on ice for 15

minutes. The sample was centrifuged at 2500rpm for 10 minutes at 4°C, the supernatant was removed and further centrifuged at 2500rpm for 10 minutes and the resulting supernatant was aliquotted in preparation for further analysis. The aliquots were labelled and stored at -70°C.

The sputum pellet consisting of mucus and cells were further processed to determine total cell counts using a hemocytometer and to then analyse cell differentials (macrophages, neutrophils, eosinophils, lymphocytes and squamous cells). A 0.2% preparation of sputolysin (Dithiothreitol (DTT) in deionised water (DH_2O)) was used to break down the sulphide bonds within the sputum sample. Sputolysin was added (x4 initial sputum weight) to the pellet and allowed to disperse in a centrifuge tube rocking on ice for 10 minutes. PBS was added to the dispersed pellet (4x initial sputum weight). The dispersed pellet solution was filtered through a 48µm nylon gauze into a centrifuge tube and centrifuged at 2000rpm for 10 minutes at 4°C. After centrifugation the supernatant with DTT was not used for any experimental analysis due to DTT interference. The DTT supernatant was discarded. The pellet was re-suspended in PBS and a 1:1 mix of 20µl trypan blue and cell suspension was modulated for cell count analysis. The cells were counted using an improved Neubauer haemocytometer. The remaining cells were washed with Dulbeccos phosphate buffered saline (D-PBS) and centrifuged at 800rpm for 10 minutes at 4°C. The supernatant was discarded and D-PBS was added to the pellet to adjust the cell concentration to about 0.5x 10^6 cells per ml. Twelve cytospins were prepared using 80µl of the cell suspension and spun at 450 rpm for 6 minutes in a cytospin centrifuge. The cytospins were air dried for 2 minutes and stored for staining.

2.7 Cell Staining

2.7.1 Geimsa 2 (Romanovsky) Stain

The stock solution of Geimsa consisted of Azure B thiocyanate dissolved in Dimethyl sulfoxide (DMSO) (1.25%) slowly added to Eosin Y dissolved in Methanol (0.16%) and stored at 18ºC. The working dilute solution of Geimsa consisted of stock solution diluted 1:1000 with PBS (pH 7.4) containing 0.05% tween (PBST). One cytospin per patient was fixed in 100% acetone for 10 minutes at room temperature and allowed to air dry. The slides were stained with the working dilution of Geimsa for 10 minutes. They were dispersed and rinsed in dH_2O for 1 minute. Once air dried the slides were mounted with a glass cover slip with the addition of DPX (Distrene-80, Plasticizer, Xylene). The principle

of the Geimsa stain is to stain the nucleus of all types of inflammatory cells. A differential count of the cell types was performed. The percentage of neutrophils, macrophages, eosinophils, lymphocyte and squamous as well as the total number of cells per ml of solution were determined.

2.7.2 Oil Red O

The principle of the Oil Red O stain is to specifically stain the lipid contained in the cells because this has been proposed as a useful reflux diagnostic tool (Hopkins et al., 2010; Hayes *et al.,* 2013). The stock solution consisted of Oil Red O dissolved in isopropanol (0.5%) and stored at 18ºC. The working solution consisted of stock Oil Red O solution (30ml) diluted in dH_20 (20ml) and allowed to stand for 10 minutes. The solution was then filtered and covered with parafilm. 1 cytospin per patient was fixed in 100% formalin for 10 minutes at room temperature, and then rinsed in dH_2O and again in isopropanol. The slides were stained with freshly prepared working solution Oil Red O for 15 minutes and then were rinsed in isopropanol for 10 seconds and again in dH_2O. The slides were counterstained with Harris Haematoxylin for 1 minute to stain the nucleus of each cell. They were rinsed in dH_2O until the stain appears blue in colour. The slides were mounted with a glass cover slip with the addition of aqueous mountant (Glycerin Jelly: Gelatin 10g, dH_20 60ml, Glycerol 70ml, Phenol 0.25g). The slides were ready for counting and each cell was analysed for lipid laden macrophage content straight after staining. The Oil Red O stain is clearly visible for 24 hours and cannot be stored for later analysis. The lipid laden macrophage index was used to score each patient's lipid laden macrophage content.

The lipid index is the sum of the intensity staining scores for 100 consecutive macrophages based on a semi quantitative scale of 0 (no staining) to 4 (maximum staining). Thus, the possible range is 0 - 400. Under a microscope each macrophage is given a score of 0 to 4 depending on the visible red droplets within the cytoplasm and the nucleus. The table below illustrates each score. Once 100 macrophages have been scored the total score can be calculated for each patient (Table 2.2).

Score	Example	Details
0		0: absence of staining
1		1: one or a few lipid droplets
2		2: many distinct droplets
3		3: many droplets with visible nucleus
4		4: many droplets completely covering the cytoplasm and obscuring the nucleus

Table 2.2 The Lipid Laden Macrophage Index scoring system

2.8 Questionnaire

2.8.1 Identification of GOR symptoms

All patients were evaluated by the validated DeMeester reflux questionnaire (Appendix 8.3.1) to assess the occurrence of typical GOR symptoms (heartburn, regurgitation and dysphagia). Each symptom was given a score of 0 to 3 (0: no symptoms; 1: occasional episodes, 2: reason for medical visit, 3: interference with daily activities) and each score was totalled to give an overall score with a maximum score possible of 9. If a patient score was <1 they were classed as none symptomatic for GOR and if they had a total score >2 they were classed as GOR symptomatic (DeMeester *et al.,* 1980; DeMeester *et al.,* 1981). The questionnaire was validated using 393 patients with suspected oesophageal disease that underwent interview with the author of the questionnaire to complete the DeMeester reflux questionnaire. The patients underwent 24 hour pH oesophageal monitoring to determine abnormal reflux episodes. They underwent a fiberoptic esophagoscopy to determine the presence of endoscopic eosophagitis. They also underwent a biopsy of the oesophageal mucosa to determine the presence of inflammatory infiltrate within the mucosa to consider evidence of esophagitis. This was deemed abnormal if the total reflux score sum was above the upper limit of a healthy volunteer cohort determined previously in 15 healthy volunteers of an age range similar to that of the GOR patients (Johnson and DeMeester, 1974). The 24 hour pH test score correlated with the recorded typical symptoms of heartburn, regurgitation and dysphagia. 73% patients with an abnormal 24 hour acid reflux test and 64% of patients with esophagitis experienced typical reflux symptoms. There were only 3% of patients with esophagitis and a negative 24 hour pH test that did not experience classical reflux symptoms. In addition 90% of patients with typical symptoms of reflux and esophagitis had a positive 24 hour pH test result highlighting the validity of the DeMeester reflux questionnaire to determine GOR.

2.8.2 Identification of EOR symptoms

The Reflux Symptom Index (RSI) questionnaire (Appendix 8.3.2) was used to assess EOR/atypical GOR symptoms. The questionnaire was first used to assess laryngopharyngeal reflux but has been validated to assess EOR in many studies. The questionnaire consists of a series of questions about characteristics of EOR including the following: hoarseness, clearing of the throat, postnasal drip, difficulty swallowing, coughing, breathing difficulties, golbus and GOR symptoms. The questionnaire required an answer from 0 to 5 where 0 is no problem at all to 5 being a severe problem. The scores

were totalled and the patient classed as EOR symptomatic if they received a score of 13 and above (a score <12 was classed as none EOR symptomatic) (Belafsky *et al.*, 2002). The questionnaire was validated using 25 patients with laryngopharyngeal reflux and 25 age-matched and gender-matched healthy controls with no symptoms or evidence of laryngopharyngeal reflux. The patients completed the RSI score before and after the administration of proton pump inhibitors, the median score pretreatment was 21 and the median score post-treatment was 13. The median score for the healthy volunteers was 13 and therefore this was used as the normal range of symptoms, any score above that was deemed abnormal.

The questionnaires above have been used as validated questionnaires in studies assessing GOR and EOR in a cohort of patients receiving anti-reflux surgery (Robertson *et al.*, 2012) and EOR symptoms of patients with sleep apnea causes by GOR (Laohasiriwong *et al.*, 2013).

2.8.3 Airway reflux cough

The Hull Airway Reflux Questionnaire (HARQ) (Appendix 8.3.3) was used to assess cough related EOR. This validated questionnaire assessed symptoms similar to the RSI with additional cough questions and asked if the patient experienced a metallic taste in the mouth. The questionnaire required the patient to score the symptom severity from 0 (no symptoms) to 5 which is a severe problem. The scores were totalled and the patients classed as cough related EOR symptomatic if they received a score of 13 and above (a score <12 was classed as none cough EOR symptomatic) (Morice *et al.*, 2011). There were 185 patients experiencing chronic cough for 8 weeks or more that had an objectively proven diagnosis of significant acid gastro-oesophageal reflux on 24-h pH assessment. There were 70 healthy controls included in the validity of the HARQ questionnaire. The scores of the questionnaire were compared to identify a marked difference between healthy controls and chronic cough patient HARQ scores with little overlap. The analysis of direct estimation using the lower limit of normal to be 0, a 95% reference interval was calculated to give a 95% reference range of 0-13, with the distribution giving a mean score of 4.3 with the standard deviation to be 12.7. The upper limit of the HARQ normal score was therefore 13 and any score above that was deemed abnormal. There were 10 chronic cough patients with a score below 13 which is 6% of the patient population giving the HARQ sensitivity for detecting cough hypersensitivity of 94.1% and specificity 95%.

2.9 Pepsin identification and quantification using indirect ELISA

The immunoassay reported herein is an indirect enzyme-linked immunosorbent assay (ELISA) developed in-house from the laboratory of Jeffrey Pearson (Stovold *et al.*, 2007). The protocol was optimised for the analysis of sputum supernatant by increasing antibody concentrations and introducing shaking at 500-700 rpm during incubation steps. Maxisorp NUNC-immuno 96-well plate were used for the pepsin ELISA.

- Standard and sample coating

The standard of pepsin from porcine gastric mucosa (sigma) was diluted in PBS ranging from 0 to 100 ng/ml, 100µl was coated onto each well. The sputum processing resulted in a 1 in 8 (wt/vol) dilution and no further dilutions were necessary. In duplicate 100µl of each diluted sputum sample was coated onto the well. A negative control (in duplicate) was added for both the standard and samples, the sample in the negative control was treated in every step excluding the primary antibody and incubating in antibody diluent instead. The plate was covered with pre-cut transparent microplate sealers and incubated for 12-16 hours at room temperature with shaking at 700rpm.

- Blocking

After the 12-16 hour incubation the residue was discarded and the plate was dried thoroughly. The wells were blocked with 300µl of 1% bovine albumin (BSA) for 2 hours at room temperature with shaking at 500rpm to block unspecific antibody binding sites.

- Primary antibody

The blocking buffer was discarded and the wells were dried by blotting thoroughly. 100µl of primary anti-pepsin antibody (anti-pepsin, Biodesign, Saco, MN, USA) was added to duplicate wells at a dilution of 1:100 in 0.1% BSA/PBS solution. 100µl of 0.1% bovine albumin BSA solution was added to negative wells serving as a background well. The plate was incubated with shaking at 700rpm at room temperature for 2 hours.

- Secondary antibody

The solution was discarded and wells washed twice with 300µl 0.05% Tween20 in PBS, and twice with PBS, then thoroughly dried by blotting. 100µl of the secondary antibody (horseradish peroxidise (HRP)-conjugated anti-sheep/goat, Sigma, UK) was added to each well (positive and negative) diluted 1:1000 in 0.1% BSA and incubated with shaking at 700rpm at room temperature for 1.5-2 hours.

- Development

After incubation the solution was discarded and the wash step was repeated with a thorough blot to dry. The addition of 100µl of substrate solution (2,2'Azino-bis 3 ethylbenzothiazoline 6 sulfonic acid (ABTS) from Sigma) was added to each well and developed for 20 minutes at room temperature. The reaction was stopped by the addition of 50µl 1% sodium dodecyl sulphate (SDS) in DH_2O.

- Analysis

The colour development in each well of the plate was analysed on the plate reader Infinite 200Pro at 405nm. The data was captured using the Tecan i-control software (1.10.4.0 serial number 1301007217). Figure 2.2 describes the principle of the indirect ELISA.

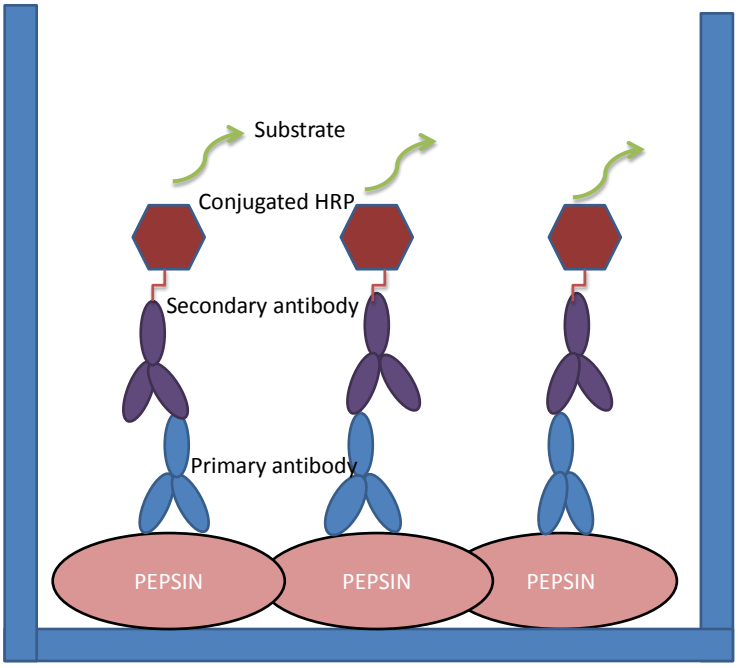


Figure 2.2 Schematic of the indirect pepsin ELISA. The pepsin antigen is added to the well and immobilised by direct absorption to the bottom of the plate. The primary unlabelled antibody binds to the pepsin antigen which is further detected by the secondary antibody which is conjugated with horse-radish peroxidise (HRP). ABTS is added as a substrate which reacts with HRP to produce a green colour which is proportional to the amount of pepsin in the well and this colour is measured at 405nm.

2.10 Bile salt identification and quantification using tandem mass spectrometry

Twenty-four sputum supernatant samples were analysed for total bile acids by tandem mass spectrometry in Sheffield, UK. Bile salts were analysed by identifying taurine and glycine conjugates of cholate and deoxycholate and their isomers. These account for 95% of the total bile acids because the minor sulphated components are not measured using this method (Pearson and Parikh, 2011). The bile acids were calculated by adding the

individual bile salt values. They were measured by using a Waters Acquity TQ Detector tandem mass spectrometer with direct flow injection able to measure a lower detection limit of 0.01μmol/L. The samples were pre-extracted using 500mg SPE cartridge (Supelco LC-18) and then washed with 10ml methanol, followed by washing with 10ml distilled H_2O. The sample and standard were loaded in amounts of 400μl of sample/standard in 10ml distilled H_2O with 0.15 nmol of d4-taurocholic acid. The column was washed with 10ml of distilled H_2O and 2 ml hexane. The addition of 20 ml ethanol was used to elute the bile salts and then they were reduced to dry matter by the process of rotary evaporation. The dry extract from each sample was reconstituted in 1ml of acetonitrile prior to centrifugation at 13000 rpm for 5 minutes. The supernatant of each sample was injected into the mass spectrometer. The analysis of the samples was carried out by electrospray ionisation in negative ion mode using mass spectrometry (collision gas argon) in multiple reaction mode for conjugated bile acids.

2.11 Interleukin 8 (IL-8) identification and quantification using capture ELISA

To measure human Interleukin 8 (IL-8) a DuoSet ELISA kit was purchased from R&D systems (catalogue number: DY208). This kit provides a sandwich ELISA with a sensitivity to detect 31pg/ml and measures up to 2000pg/ml. The assay was carried out as detailed in the kit protocol with provided materials and is described below.

- Coating with capture antibody

100µl mouse anti-human IL-8 diluted to 4µg/ml in PBS was added to each well on a 96 well microplate and sealed with an adhesive plate cover. The plate was incubated for 16 hours at 18^{o}C.

- Wash step

The contents of the plate were discarded and each well washed with wash buffer three times. The plate was dried by inversion and by patting the plate with force on paper towels.

- Blocking step

100µl of 1% BSA was added to each well and incubated at 18^{o}C for 1 hour.

- Standards and samples

After washing as in step 2, 100µl of sputum supernatant was added in duplicate to assigned wells. 100µl of recombinant human IL-8 (0-2000pg/ml in a 7 point 2-fold serial dilution) was added to 8 wells in duplicate. The plate was sealed and incubated for 2 hours at 18^{o}C.

- Detection antibody

After washing as in step 2, 100µl of biotinylated goat anti-human IL-8 diluted to 20ng/ml in reagent diluent (0.1% BSA, 0.05% tween 20 in Tris-buffered saline (0 mM Trizma Base, 150 mM NaCl) at pH 7.2 – 7.4 and filtered through 0.2 µm filter paper) was added to each well. The plate was sealed and incubated at 18^{o}C for 2 hours.

- Development

After washing, 100µl of streptavidin HRP at a dilution of 1:200 in reagent diluent was added to each well and incubated for 20 minutes out of direct light at 18^{o}C. The contents were discarded and washed and 100µl of TMB (1:1 H_2O_2 and tetramethylbenzidine) was added to each well then incubated for a further 20 minutes out of direct sunlight at 18^{o}C. The addition of 50ul stop solution (2N H_2 SO_4) was required for each well and the plate was placed on the plate shaker for 1 minute at 100rpm.

- Analysis

The optical density (OD) of the contents of each well was immediately determined using a microplate reader set at 450nm and corrected at 570nm (OD@570nm-OD@450nm) to correct for optical imperfections.

- Determination of IL-8 in sample from standard

The average OD value was derived for the standard and sample wells, the zero IL-8 standard OD value was subtracted from each mean value. GraphPad (version 5) was used to create a standard curve with a 4 parameter logistic curve fit. The sample values were interpolated from the standard curve.

2.12 Interleukin 6 (IL-6) identification and quantification using capture ELISA

To measure natural human Interleukin 6 (IL-6) a DuoSet ELISA kit was purchased from R&D systems (catalogue number: DY206). This kit provides a sandwich ELISA with a sensitivity to detect 9pg/ml and measures up to 600pg/ml. The assay was carried out as detailed in the kit protocol with provided materials as is described below.

- Coating with capture antibody

100µl mouse anti-human IL-6 diluted to 2µg/ml in PBS was added to each well on a 96 well microplate and sealed with an adhesive plate cover. The plate was incubated for 16 hours at 18^{o}C.

- Wash step

The contents of the plate were discarded and each well washed with wash buffer three times. The plate was dried by inversion and patting the plate with force on paper towels.

- Blocking step

100µl of 1% BSA was added to each well and incubated at 18^{o}C for 1 hour.

- Standards and samples

After washing as in step 2, 100ul of sputum supernatant was added in duplicates to assigned wells. 100µl of recombinant human IL-6 (0-600pg/ml in a 7 point 2-fold serial dilution) was added to 8 wells in duplicate. The plate was sealed and incubated for 2 hours at 18^{o}C.

- Detection antibody

After washing as in step 2, 100µl of biotinylated goat anti-human IL-6 diluted to 50ng/ml in reagent diluent (1% BSA at pH 7.2 – 7.4) was added to each well. The plate was sealed and incubated at 18^{o}C for 2 hours.

- Development

After washing, 100µl of streptavidin HRP at a dilution of 1:200 in reagent diluent was added to each well and incubated for 20 minutes out of direct light at 18^{o}C. The contents were discarded and washed and 100µl of TMB was added to each well then incubated for a further 20 minutes out of direct sunlight at 18^{o}C. The addition of 50µl stop solution (2N H_2 SO_4) was required for each well and the plate was placed on the plate shaker for 1 minute at 100rpm.

- Analysis

The optical density of the contents of each well was immediately determined using a microplate reader set at 450nm and corrected at 570nm (OD@570nm-OD@450nm) to correct for optical imperfections.

- Determination of IL-6 in sample from standard

The average OD value was derived for the standard and sample wells, the zero IL-6 standard OD value was subtracted from each mean value. GraphPad (version 5) was used to create a standard curve with a 4 parameter logistic curve fit. The sample values were interpolated from the standard curve.

The figure 2.3 describes the principle of a sandwich ELISA used from R&D systems specific for IL-8 and IL-6.

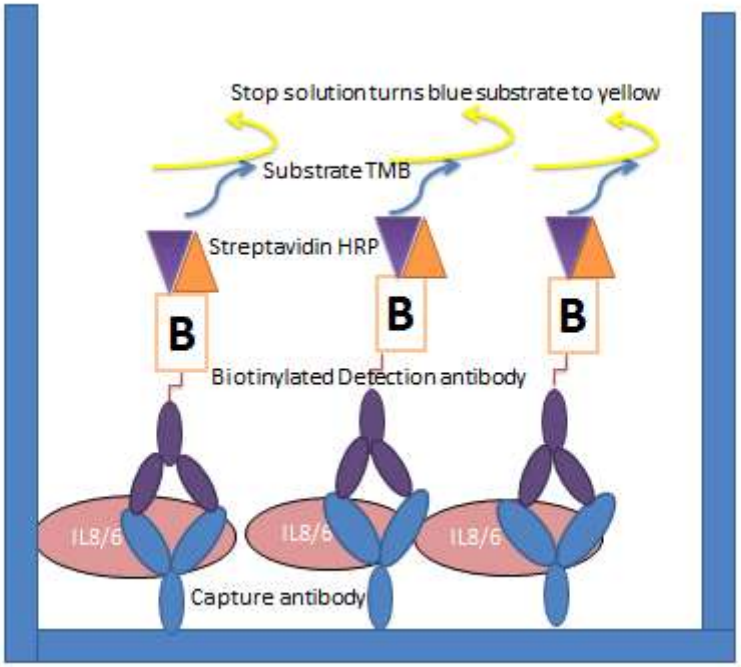


Figure 2.3 Schematic of the sandwich IL-8 and IL-6 ELISA
The capture antibody was coated onto the surface of the plate and absorbed through overnight incubation and any remainder is washed away. The standard (IL-8/IL-6) and samples bind to the antigen specific immobilised capture antibody followed by the biotinylated detection antibody. The streptavidin horseradish peroxidase binds to the detection antibody which binds to the tetramethylbenzidine (TMB) substrate solution to result in a blue colour development in proportion to the amount of IL-8/IL-6 analyte present. The colour development is then stopped after 20 minutes by adding stop solution and the yellow colouration is absorbed at 450nm.

2.13 Pulmonary function

Forced expiratory volume in 1 second (FEV_1) and Forced vital capacity (FVC) were recorded at routine appointments by clinically trained staff. The predicted values for these measurements were corrected according to age, gender and height reference ranges using European standards ECCS (Laszlo *et al.*, 2006). The results were gathered and provided for the study by Mr Alan Anderson (CF Nurse Specialised) under the authority of Dr Stephen Bourke (Respiratory Consultant, RVI).

2.14 Genotype

The patients CFTR genotype was collected by Mr Alan Anderson from the patient's record file. Patients were classed as severe if they were homozygous for deltaF508 genotype and less severe if they were heterozygous for deltaF508 genotype.

2.15 G551D treatment with Ivacaftor

In February 2013 twelve patients receiving Ivacaftor medication were asked to consent to the following research study. Each patient gave informed consent to be interviewed, the interview included questions regarding demographics, 24 hour dietary recall and completing three reflux questionnaires (DeMeester Reflux questionnaire, Reflux Symptom Index questionnaire (RSI), Hull Airway Reflux Questionnaire (HARQ)). Patients were also asked to provide a biological sample for the purpose of a research project evaluating the effects of Ivacaftor medication to benefit others in the future. The patients were asked to provide expectorated sputum but if they could not produce this they were asked to produce expectorated saliva. The patients were informed that interviews and sample collection would take place on four occasions, before medication; 6 weeks after first taking medication; 6 month after first taking medication and 12 months after first taking medication. The sputum and saliva samples were processed as described above and the samples were analysed for pepsin as described above.

2.16 Statistical analysis

GraphPad Prism version 5 (GraphPad Software Inc, La Jolla, California, USA) was used to perform statistical tests on the data collected. Non-parametric tests were used and there were no assumption of specific distribution of data sets. MiniTab (version 16) was used to analyse data for the Pearson correlation. The data was regarded as statistically significant if the P value was equal to or less than 0.05 and the P values were stated for each significant result.

Chapter 3.0

Gastro-oesophageal reflux and the contribution to airway disease management in the cystic fibrosis population

3.1 Introduction

3.1.1 The definition of Gastro Oesophageal Reflux (GOR) Disease (D)

Gastro-oesophageal reflux (GOR) is characterised by the retrograde movement of gastric contents up the oesophagus (Armstrong and Sifrim, 2010; Chen *et al.*, 2010). The reflux of gastric contents into the oesophagus is a normal physiological event; Zerbib, *et al.*, (2005) found that oesophageal investigations over a 24 hour period of 72 healthy adults presented reflux events that were a combination of acid (<4pH), weakly acidic (pH 4-6), liquid and gas. These data illustrate that the reflux of gastric contents is a frequent physiological event that occurs in the healthy population. If oesophageal reflux occurs at abnormal frequency (at least once a week) and often related to extended clearance time, it is termed Gastro-oesophageal reflux disease (GORD). The Montreal consensus group defined GORD as a condition, which develops when the reflux of stomach contents causes troublesome symptoms and/or complications (Vakil *et al.*, 2010). The term GORD characterises multiple complications with varying levels of injury to the oesophagus. The term covers a wide spectrum of symptoms, complications and features of tissue damage. There are three main classical symptoms of GORD: The first being heartburn, it is the description for a burning sensation of the substernal chest area. Number two, the regurgitation of stomach contents is experienced and these may reach the pharynx, larynx and oral cavity leading to an acidic taste in the mouth. The third classical symptom is regurgitation, this may be provoked by bending over or on position of strain in severe cases (Klauser *et al.*, 1990; Chen *et al.*, 2010). Dysphagia is the feeling of food sticking to the retrosternal area and difficulties swallowing the food which related to the reflux related oesophageal spasm and disordered peristalsis (Gillen and McColl, 2011).

The definition of GORD covers a spectrum of complications causing macroscopic tissue damage to the oesophagus which can be identified under inspection of upper gastrointestinal endoscopy such as oesophageal mucosal damage, oesophagitis and oesophageal erosions (Moayyedi and Talley, 2006; Gillen and McColl, 2011).

GORD complications are caused by mucosal damage and may be identified during endoscopy, these are peptic stricture, Barrett's oesophagus (secondary to inflammation)

and, in severe cases, are adenocarcinoma of the oesophagus (Meining and Classen, 2000; Kulig *et al.*, 2004). These complications of GORD have been reported to be a burden to health on a global scale and impairs the quality of life of those who experience it (Dent *et al.*, 2005; Armstrong and Sifrim, 2010).

An epidemiological analysis of 12 studies described the prevalence of GORD in the general population defined as at least weekly heartburn and/or acid regurgitation to be approximately 10–20% in the western world and 5% in Asia (Dent *et al.*, 2005). The incidence in Britain was determined to be 4.5 per 1000 person years (95% confidence interval (CI) 4.4–4.7) (Ruigómez *et al.*,2004). However, the data collected for the prevalence and incidence of GORD was collected over 10 years ago.

3.1.2 The definition of Extra Oesophageal Reflux (EOR)

Since the 1960's GOR has been associated with extra oesophageal reflux (EOR). The term EOR refers to the symptoms that reflux of gastrointestinal contents have on the upper gastrointestinal tract (Jaspersen *et al.*, 2006). These are namely the pharynx, larynx, nasal cavity (Vakil, *et al.*, 2010). The refluxate has been associated with tooth decay (Moazzez, *et al.*, 2004) , Otitis media (Tasker, *et al.*,2002) and identified in the airways (Stovold, *et al.*, 2007; Blondeau, *et al.*, 2008). Symptoms include chronic cough and asthma that have been associated with pulmonary complication (Kennedy, *et al.*, 1962). Other symptoms include hoarseness of the voice, clearing of the throat, post nasal drip, golbus, epigastric burning, and nausea (Armstrong, 2005; Klauser *et al.*, 1990; Jaspersen *et al.*, 2006; Ruigomez, *et al.*, 2008).

3.1.3 Anatomy and physiology

The oesophagus is a long muscular tube connecting the pharynx to the stomach. Food and liquid travels down the oesophagus via peristaltic muscle movements of the oesophagus which takes the bolus into the stomach. The lower oesophageal sphincter is a circular muscle layer at the distal oesophagus with 90% basal pressure and functions as an anti-reflux barrier (along with the crural diaphragm) to prevent the retrograde movement of the stomach contents (Figure 3.i). On swallows or belches the muscle of the lower oesophageal sphincter normally relaxes to allow food pass into the stomach or intragastric air to pass out. The relaxations that are not induced by swallowing or eructation are termed transient lower oesophageal sphincter relaxations. (Boeckxstaens, *et al.*, 2005; Waugh *et al.*, 2006). The anti-reflux mechanism of the lower oesophageal sphincter may fail leading

to GOR of the stomach contents and exposing the oesophagus to gastric acid and other damaging contents of refluxate (Sifrim and Holloway, 2001; Farré, *et al.*, 2013).

The lining of the oesophagus is squamous epithelium consisting of three layers, the mucosa, the submucosa and the circular muscle layer (Figure 3.b). There are two mucus gel layers on the inside of the stomach in a continuous two part surface layer to protect the stomach against gastric secretions including pepsin and acid. The two independent gel mucus layers in the oesophagus each serve a different protective purpose. The first firm adherent mucus layer protects the underlying epithelial cells against pepsin and acid as they would otherwise be destroyed. These epithelial cells secrete bicarbonate into this gel layer to neutralise the acid to give an environment of around pH 7 at the surface. The overlaying mucus gel layer forms a viscous liquid when mixed with consumed food to reduce the shear forces between the food and the mucosal cells (Pearson and Brownlee, 2005). The gastric mucus layer stops at the gastroesophageal junction thus the oesophagus requires a different protective mechanism against acid and pepsin. The mucus within the oesophageal musosa is present in small quantities and it is secreted by the salivary glands. The oesophagus may be able to produce a small amount of watery bicarbonate secretion and it secretes carbonic anhydrase, and heat shock protein expression. but does not have mucus or goblet cells present like the mucus in the stomach. The mucus can only withstand acidic environment for a short period of time thus serves little protection against reflux of the stomach contents (Figure 3.a and b). Saliva in the oesophagus may provide some protection. The oesophageal surface layer can shed a layer of dead cells which can protect the viable cells from acid exposure (Dixon *et al.*, 2001; Johnston *et al.*, 2003; Pearson and Brownlee, 2005; Pearson and Parikh, 2010). There is also no protection against gastric aspiration of such gastric components to the airway epithelia.

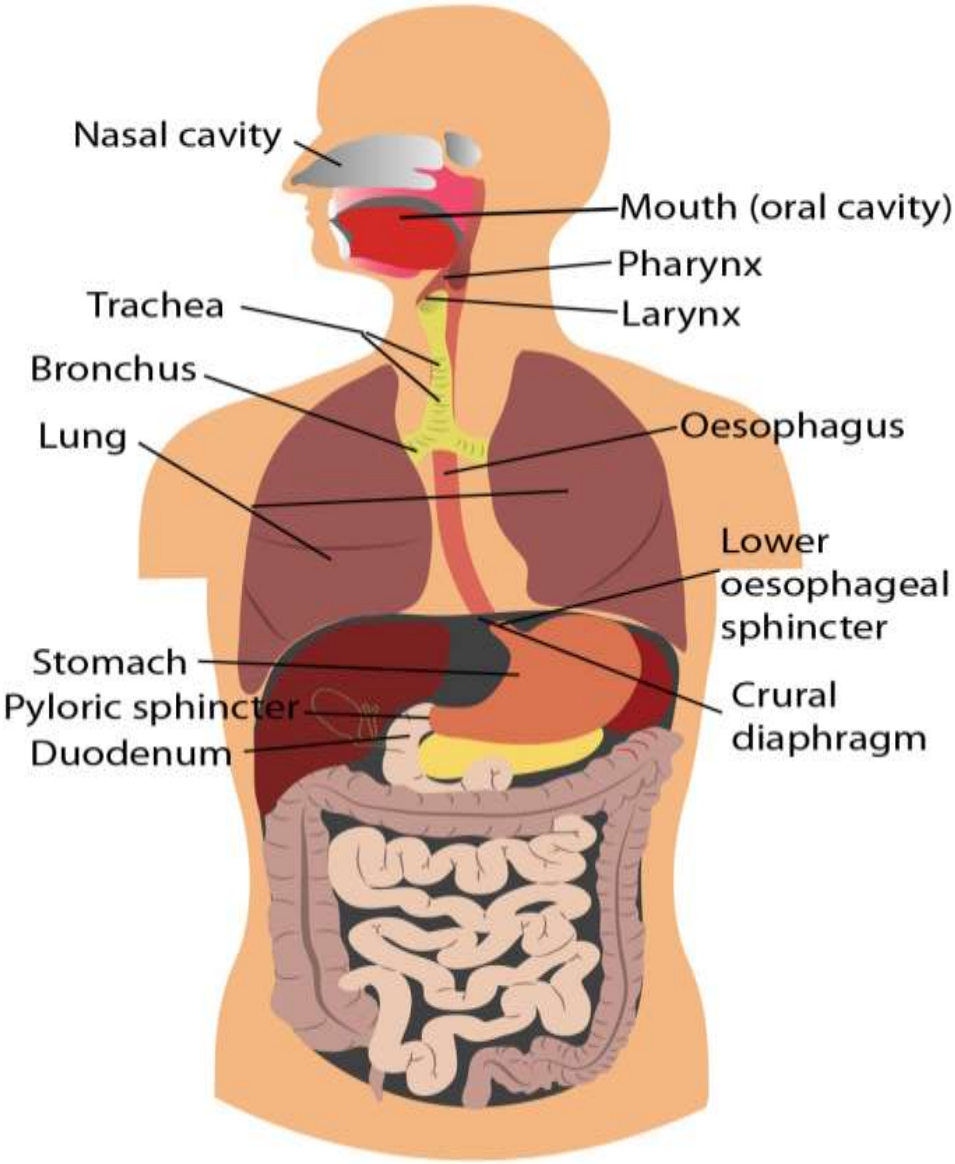


Figure 3.a The schematic of the anatomy of the digestive system and airways. The illustration describes the components of the digestive and respiratory system. The illustration was purchased from canstockphotos and information modified from Anatomy and Physiology in Disease, Chapter 12, The digestive system (Boeckxstaens, 2005) (Waugh *et al.*, 2006).

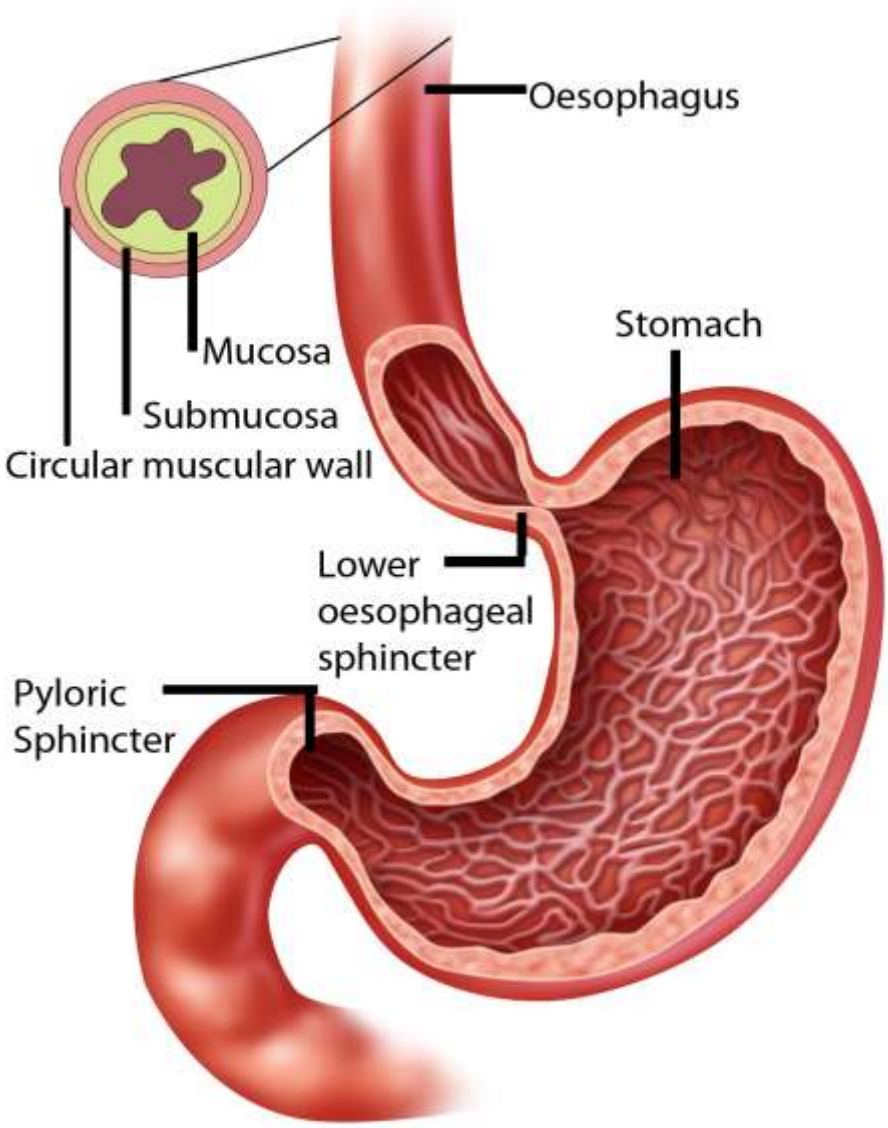


Figure 3.b schematic of the structure of the oesophagus and the stomach. The oesophagus is a long muscular tube with a squamous epithelium consisting of three layers; outer circular muscular wall, submucosa and mucosa. Food passes down the oesophagus and into the stomach. The lower oesophageal sphincter is a circular muscle layer at the distal oesophagus and functions as an anti-reflux barrier to prevent the retrograde movement of stomach contents. On swallows or belches the muscle relaxes to allow food to pass into the stomach or intragastric air to pass. The illustration was purchased from canstockphoto and information modified from Anatomy and Physiology in Disease, Chapter 12, The digestive system (Boeckxstaens, 2005; Waugh *et al.*, 2006).

3.1.4 Damaging components of refluxate

Refluxate contains aggressive agents such as acid, pepsin and bile salts (Pearson and Parikh, 2010). The reflux of the stomach and small intestinal duodenum contents are damaging to the oesophagus. The stomach's function is to liquefy consumed food and it does this with the aid of acid, pepsin and strong rhythmic muscle contractions (Pearson and Brownlee, 2005). The cells in the mucosa of the stomach produce gastric secretions; they are arranged in structures called gastric pits made up of several gastric glands. The chief (peptic) cells synthesise, store and secrete the inactive enzyme precursor pepsinogen, and the parietal cells secrete hydrochloric acid (HCL) to activate pepsinogen into its active form of pepsin. Once the inactive enzyme pepsinogen is activated into pepsin by HCL in the stomach it becomes a stable molecule and active up to a pH of 6.0. The major secretion of the surface epithelial cells is mucus. Mucus forms a continuous two part surface layer to protect the stomach against gastric secretions including pepsin and acid. (Pearson and Brownlee, 2005).

Pepsin(s) by definition are acidic proteases and there are eight different pepsin isoforms that have been identified to date. These different pepsins have maximal activities against proteins such as haemoglobin which vary between pH 1.9 and 3.6 (Kageyama *et al.*, 2002). Pepsin 3 encompasses 70.3 (± 2.6) % of the total pepsins in refluxate, it has an optimum pH of 2.4 to 2.8 against haemoglobin. Interestingly it retains <10% activity against purified protein at pH 4.0 and 5% - 10% at pH 5, and holds a measurable activity at pH 6 (Pearson & Parikh, 2011). It has been shown that the epithelium of the oesophagus and larynx are essentially resistant to damage at pH 4.0, but are damaged when pepsin is present. This illustrates that in laryngopharyngeal reflux (LPR) pH 4.0 or above refluxate would only be damaging if it contained pepsin (Bulmer *et al.*, 2010). Acid with a pH below 4 can remain without causing damage in the stomach but would cause damage to the oesophagus (Johnston *et al.*, 2003).

The reflux of duodenal fluid secretions of bile and pancreatic enzymes into the stomach is termed duodenogastric reflux (Figure 3.b). The muscle of the pyloric sphincter may relax to release duodenal fluid into the stomach. Bile has an alkaline pH of 8. Bile salts are synthesised in the liver and function to emulsify lipids and cholesterol in the small intestine to be absorbed in the intestinal wall promoting cholesterol elimination. The process enables fat soluble vitamins, A, D, E, and K to be readily absorbed in the intestines (Waugh *et al.*, 2006). Bile acids are conjugated with taurine and glycine amino acids within the liver to form bile salts. Primary bile acids are those synthesised in the

liver (glycodeoxycholate (G-DHC); glycocholate (G-THC); taurodeoxycholate (T-DHC) and taurocholate (T-THC) and they make up about 85% of the total acids. The secondary bile acids produced from bacterial action in the intestine is called free lithocholate (Hofmann, 1999). Bile acids have been shown to be damaging to the oesophageal mucosa of Barrett's oesophagus patients due to its association with the upregulation of cyclooxygenase-2 expression and cell proliferation properties. Exposure to oesophageal epithelium has been shown to result in DNA damage (Jolly *et al.*, 2004).

3.1.5 Aetiology of GOR

GOR occurs due to the failure of physiological protective mechanisms but the physiological parameters pertaining to levels of reflux have been poorly characterised. The majority of GOR occurs due to impairment of the anti-reflux mechanism whereby the resting tone of the lower oesophageal sphincter becomes relaxed allowing the gastric contents to be released into the oesophagus (Meining and Classen, 2000; Armstrong, 2005). An inappropriate transient lower oesophageal sphincter relaxation (TLOSR's) causes most reflux episodes (Dent *et al.*, 1980). This frequently occurs post-prandially that are not induced by swallowing (Cucchiara *et al.*, 1991). It has been reported that the duration of lower oesophageal sphincter relaxations are longer in GOR patients compared to healthy controls (Holloway, *et al.*, 2000). The primary mechanisms described in the literature for transient lower oesophageal sphincter relaxation are reduced pressure of the lower oesophageal sphincter (Ledson *et al.*, 1998); delayed gastric emptying (Couturier *et al.*, 2004) and impaired fundic relaxation. Other reasons have been mentioned to affect transient lower oesophageal sphincter relaxations including the diet; obesity; high levels of oestrogen during pregnancy; and certain medications (Nandurker *et al.*, 2004; Armstrong, 2005). Across the general population the severity of GORD encompasses a large spectrum and the disease is multifactorial. The reason for differences in disease severity is unknown but Armstrong, (2005) has suggested it could be a result of individual differences in oesophageal protection. Epithelial bicarbonate secretion in the oesophagus or salivary bicarbonate production could be impaired in the patients that suffer with oesophageal lesions and an increased protective mechanism could be present in non-erosive reflux disease (NERD) patients such as increased bicarbonate secretion (Farré, *et al.*, 2013).

3.1.6 Diagnosis of GOR

The diagnosis of GOR can be made through the assessment of symptoms. Questionnaire assessment has been designed to characterise symptoms of both GOR and

EOR. The tools are designed to determine whether the patient is experiencing typical symptoms and validated questionnaires may be used to define abnormal symptoms.

The DeMeester reflux Questionnaire assesses the classical symptoms of GOR with the assessment of heartburn, regurgitation and dysphagia in a 3-point questionnaire with a severity score of 0 to 3 (Figure 3c). This questionnaire is a widely used tool to assess GOR dating from the 1980's (DeMeester *et al.,* 1981; DeMeester *et al.,* 1980). To assess EOR symptoms a questionnaire designed for laryngopharyngeal reflux assessing hoarseness, vocal changes and fatigue, excessive throat clearing, globus, post nasal drip, chronic cough and heartburn. The Reflux Symptom Index (RSI) was validated in 2002 and included 25 patients with laryngopharyngeal reflux and 25 age and gender matched controls. The questionnaire has 9 questions that are each scored for severity from 0 to 5, a total score of 13 or above suggests abnormal symptoms and deemed positive for laryngopharyngeal reflux (Figure 3.d) (Belafsky *et al.,* 2002). Chronic cough has been associated with reflux and shares a relationship with symptoms of EOR such as asthma like symptoms and post nasal drip (Irwin *et al.,* 1984; Mandal *et al.,* 2013). Chronic cough has been reported to be a consequence of the aspiration of reflux contents and causing hypersensitivity to the upper airways. (Avidan *et al.,* 2001). Morice and colleagues developed a questionnaire to characterise chronic cough due to the hypersensitivity of the upper airways caused by GOR. The term airway reflux defines the extra-oesophageal symptoms of the airways caused by reflux (hoarseness, chronic coughing, globus, and wheezing). The 14 point questionnaire has been validated and is a diagnostic instrument for the assessment of cough hypersensitivity caused by airway reflux (Morice *et al.,* 2011) (Figure 3.e).

Oesophageal reflux can be confirmed by upper gastrointestinal endoscopy, oesophageal manometry and pH metry. These measure different aspects of how the oesophagus and stomach function, if the refluxate is liquid or gas, and the pH of the refluxate. This invasive procedure allows for the measurement of liquid and gas traveling up the oesophagus and can detect the pH of the liquid. Evidence of acid (pH<4) and weakly acidic (pH4-6) exposure through 24 hour pH monitoring will verify GORD when compared to normal values (Zerbib *et al.,* 2005). Upper gastrointestinal endoscopy is the gold standard of determining GOR because the endoscopic evidence of oesophageal erosions can be used to document the damage to the mucosa and identify carcinoma. However, this procedure is not useful for assessing refluxate traveling beyond the oesophagus into the aerodigestive tract or airways. Assessing gastric aspiration with non-

invasive methods can be achieved by reflux biomarker analysis of sputum supernatant (Stovold *et al.,* 2007) and lipid laden macrophage index (LLMI) of sputum cells (Colombo *et al.,* 1987; Hopkins *et al.,* 2010).

There are non-invasive methods to measure gastric aspiration by the analysis of reflux biomarkers such as pepsin and bile acids in the samples collected from the airways or pharynx (expectorated sputum, saliva or Bronchoalveolar lavage). For research purposes an enzyme-linked immunosorbent assay (ELISA), based on a monoclonal antibody to porcine pepsin has been locally developed. It has measured both pepsin and total pepsinogens, henceforth referred to as "pepsin" in human Bronchoalveolar lavage and sputum samples. This ELISA provides a non-invasive alternative to identify and quantify reflux markers (Stovold *et al.,* 2007). Stovold *et al.* (2007) assessed BAL samples of 57 subjects using this ELISA to detect pepsin a biomarker of gastric aspiration. The study found that significant amounts of pepsin was present in lung transplant patients compared to controls ($p<0.004$). The Peptest^{TM} is a lateral flow device specific for human pepsin 3, the pepsin found in the stomach. Pepsin can be detected from saliva and sputum samples as low as 25ng/ml (Strugala, *et al.,* 2007). It has been shown to be a useful tool for testing reflux in a clinical setting due to the rapid and inexpensive procedure. It was shown that the diagnostic utility of the Peptest to measure pepsin in expectorated saliva in patients with chronic cough was very useful showing pepsin positive tests in at least one of three samples in 64% of chronic cough patients and 36% of healthy volunteers (n=300) with no GOR symptoms result in a pepsin positive test (Hayat *et al.,* 2013). Bile acids can be measured using methods such as tandem mass spectrometry and diagnostic enzymatic kits designed to measure bile salts in serum (Blondeau *et al.,* 2008; Aseeri *et al.,* 2012; Savarino *et al.,* 2013). It has been reported that the preferred method to analyse bile acids in the samples of airways is tandem mass spectrometry because the limit of detection was 0.01 µmol/L which is lower than the sensitivity of the enzymatic kits at 5µmol/L (Parikh, *et al.,* 2013).

Symptoms of gastroesophageal reflux

Symptom	*Grade*	*Description*
Heartburn		
None	-0-	No heartburn
Minimal	-1-	Occasional episodes
Moderate	-2-	Reason for medical visit
Severe	-3-	Interference with daily activities
Regurgitation		
None	-0-	No regurgitation
Minimal	-1-	Occasional episodes
Moderate	-2-	Predictable on position or straining
Severe	-3-	Episodes of pulmonary aspiration manifested by chronic nocturnal cough or recurrent pneumonias
Dysphagia		
None	-0-	No dysphagia
Minimal	-1-	Occasional episodes
Moderate	-2-	Required liquids to clear
Severe	-3-	Episode of meat impaction requiring medical treatment

Figure 3.c The DeMeester Reflux Questionnaire to assess gastro-oesophageal reflux (GOR) classical symptoms. The questionnaire is a 3 point questionnaire for symptoms of heartburn, regurgitation and dysphagia with a maximum score of 9. (DeMeester *et al.*, 1981; DeMeester *et al.*, 1980).

The Reflux Symptom Index (RSI)						
Within the last month, how did the following problems affect you?	0 = No Problem					5 = Severe Problem
Circle the appropriate response.						
1.Hoarseness or a problem with your voice	0	1	2	3	4	5
2. Clearing your throat	0	1	2	3	4	5
3. Excess throat mucus or postnasal drip	0	1	2	3	4	5
4. Difficulty swallowing food, liquids, or pills	0	1	2	3	4	5
5. Coughing after you ate or after lying down	0	1	2	3	4	5
6. Breathing difficulties or choking episodes	0	1	2	3	4	5
7. Troublesome or annoying cough	0	1	2	3	4	5
8. Sensations of something sticking in your throat or a lump in your throat	0	1	2	3	4	5
9. Heartburn, chest pain, indigestion, or stomach acid coming up	0	1	2	3	4	5
	TOTAL RSI SCORE					

Figure 3.d The Reflux Symptoms Index (RSI) questionnaire to assess laryngopharyngeal reflux. The self-administered nine-item reflux symptom index (RSI) for the assessment of symptoms in patients with laryngopharyngeal reflux. The scale for each individual item ranges from 0 (no problem) to 5 (severe problem), with a maximum total score of 45. A score of 13 or above indicates an abnormal RSI score (Belafsky *et al.*, 2002).

The Hull Airway Reflux Questionnaire Within the last month, how did the following problems affect you?	(0 = no problem and 5 = severe/frequent problem)					
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
Excess mucus in the throat, or drip down the back of your nose	0	1	2	3	4	5
Retching or vomiting when you cough	0	1	2	3	4	5
Cough on first lying down or bending over	0	1	2	3	4	5
Chest tightness or wheeze when coughing	0	1	2	3	4	5
Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5)	0	1	2	3	4	5
tickle in your throat, or a lump in your throat	0	1	2	3	4	5
Cough with eating (during or soon after meals)	0	1	2	3	4	5
Cough with certain foods	0	1	2	3	4	5
Cough when you get out of bed in the morning	0	1	2	3	4	5
Cough brought on by singing or speaking (for example, on the telephone)	0	1	2	3	4	5
Coughing during the day rather than night	0	1	2	3	4	5
A strange taste in your mouth	0	1	2	3	4	5
	Total score out of 70					

Figure 3.e The Hull Airway Reflux Questionnaire (HARQ) to assess airway reflux cough hypersensitivity. The HARQ is a validated 14 point questionnaire to assess the present of cough due to airway reflux. This is self-administered and has 14 items. Responses to each question can vary from 0 to 5 to maximum of 70. A score of 13 or over is abnormal and is used to determine airway reflux (Morice, *et al.*, 2011).

3.1.7 GOR in the CF population

Reflux is common in the CF population both in paediatric and the adult patients. The first observation of GOR in CF was identified in 1975 by a French researcher at a time when the age of survival was around 10 years of age. The study uncovered the problem of GOR that has now become a major concern in the CF community (Feigelson and Sauvegrain, 1975). Reported high prevalence of GOR in infant CF patients started a research topic of interest at various CF paediatric clinics. Early investigations of GOR in CF observed that seven paediatric CF patients experienced abdominal pain, peptic oesophagitis, upper GI haemorrhage and oesophageal stricture. This raised the awareness of GOR and highlighted the importance of detection and treatment to prevent GOR associated morbidity (Scott *et al.* 1985; Brodzicki *et al.,* 2002). The classical symptoms heartburn, regurgitation and vomiting were observed in paediatric CF patients with a reported prevalence of 55%. Evidence that GOR contents were reaching the airways was illustrated by the association with recurrent pneumonia (Scott *et al.,* 1985; Vinocur *et al.,* 1985). Interestingly, the association of GOR and poor nutritional status was observed with many GOR patients failing to thrive (Vinocur *et al.,* 1985; Malfroot and Dab, 1991). The availability of genotype analysis allowed researchers to study the role of CFTR mutations and prevalence of GOR. Researchers reported that the CFTR mutation did not affect the incidence of GOR (Brodzicki *et al.,* 2002).

As CF survival time has increased, studies assessing GOR symptoms in the adult CF population (16-50 years) proceeded for the first time in 1998. At this time the prevalence of heartburn was reported to be 80% in CF adults, regurgitation and dysphagia was experienced in 52% (Ledson *et al.,* 1998). Other studies have reported even higher prevalence of classical GOR symptoms at 85% (Blondeau, *et al.,* 2008). The prevalence of EOR symptoms has been shown to affect 94% of CF patients (Blondeau, *et al.,* 2008). Recently, Sabati, *et al.,* (2010) used validated questionnaires to determine GOR and this prospective cross sectional study of CF patients in the USA found that 63% of CF patients reported GOR symptoms with 24% experiencing symptoms at least weekly. Interestingly, there were 66% symptomatic patients using PPI therapy illustrating that PPI therapy does not alleviate classical GOR symptoms for all patients (Sabati *et al.,* 2010). The prevalence of GOR in CF has been shown to be up to 85% and EOR 95% in the adult CF population and the differences in prevalence may be due to the variety of research tools used to define the presence such as questionnaires, pH impedance and manometry, reflux biomarker identification and clinical features.

3.1.8 The role of GOR in the pathogenesis of CF pulmonary disease

Several observations have demonstrated that GOR may be associated with increased pulmonary disease. It has been observed that cystic fibrosis patients with GOR have poorer lung function than those without abnormal GOR. This was defined by a persistent decline in lung function measured by forced expiratory volume in 1 second (FEV_1) (Navarro *et al.,* 2001; Palm, Salwicki and Rosen, 2012). To support this, Gustafsson and colleagues (1991) examined pulmonary function and chest radiographs of twelve CF patients to reveal that patients with better respiratory results (n=4) had fewer signs and symptoms of oesophageal dysfunction and GOR. Controversial evidence emerged in 1996 by Escobar and colleagues who found that their research of 10 infants with abnormal reflux index was not related to decline in spirometry data. Although the study number was low and this data cannot be used to represent the general CF population. Evidence for GOR leading to a decrease in pulmonary function was observed in paediatric patients in the Netherlands in a longitudinal study comparing GOR in CF (Van der Doef *et al.,* 2009). Acid and non-acid GOR may have an effect on lung disease (Blondeau, *et al.,* 2008). The acidic contents of the stomach was shown to reach the upper oesophagus and the trachea, and could be prevalent in around 35% of CF patients (Ledson, Tran and Wilshaw, 1998; Pauwels, *et al.,* 2011). It is reported that gastric aspiration may participate in further deterioration of lung function. However, the true potential damage that GOR and aspiration may cause is yet to be fully understood. Reflux reaching the proximal oesophagus and extra-oesophageal areas have been shown to be implicated in multiple respiratory disorders. For the first time it was reported that CF patients experience duodeno-gastric reflux of bile acid (n=5) and bilirubin (n=8 p=0.003) in a study comparing the contents of gastric juice of CF with healthy volunteers (n=10) (Hallberg, Fandriks and Strandvik, 2004). This refluxate has been identified in the airways by pepsin and BA detection in sputum, BAL and tracheal washings (Blondeau *et al.,* 2010). Reflux biomarkers have been shown to be a useful method of assessment in the samples collected from the airways (sputum, saliva, tracheal washings and BAL). Pepsin and BA have been the most widely studied biomarkers with some work on, trypsin, and bilirubin. Interestingly, GOR has been associated with *Pseudomonas aeruginosa* and *Staphylococcus aureus* by a number of studies (Van der Doef *et al.,* 2009; Palm, Salwicki and Rosen, 2012). This finding suggests that refluxate leads to poorer lung function which

is either a primary cause of gastric content damage to the epithelium or a secondary effect of increased pulmonary exacerbations potentially related to GOR.

The CF population tend to live longer now, this brings about complications that were not previously seen in paediatrics. There have been cases of oesophageal malignancies reported in adult CF patients which are attributed to GOR. This highlights the importance of clinical care surrounding GOR in CF. Whether the care is to increase quality of life, prevent GOR diseases or to prevent gastric aspiration it is important to treat patients and research this area. It was documented in 1993 that Barretts oesophagus was observed in two CF adolescents with GOR and this had not been previously described and esophagitis was prevalent in 7 patients with GOR (Hassal, 1993; Brodzicki, Trawinska-Bartnicka and Korzon, 2002)

3.1.9 Mechanisms of GOR in the CF population

Understanding the mechanism of GOR in CF may lead to a better understanding of the problem. Chronic cough is a common problem for CF patients (Fathi, *et al.*, 2008), Bendig (1982) explained that chronic cough in CF may result in GOR by increased abdominal pressure and a depressed diaphragm. This was supported by Blondeau *et al.*, (2008) who observed a positive association between oesophageal acid exposure and the cough symptom association probability ($p=0.03$). However, researchers in Hull (Fathi *et al.*, 2009) argued that the sequence of GOR and cough can work both ways and speculate that it is GOR causing chronic cough in CF. The authors reported that patients with chronic cough that was untreatable with conventional cough medicine underwent laparscopic Nissen Fundoplication to result in a significant improvement of cough symptoms ($p=0.01$). Interestingly, pulmonary function significantly increased post operatively with a 50% reduction of pulmonary exacerbations suggesting GOR leading to gastric aspiration could be a contributor to pulmonary disease.

In 1991, Cucchiara and colleagues found that GOR in CF could be due to an inappropriate relaxation of the lower gastro-oesophageal sphincter. This was supported by findings by other researchers that found the LOS pressure lower than 5mmHg was significantly correlated with a higher DeMeester score determined by oesophageal investigation (mean 81.0, range 47.9-128.8) compared to patients with a normal LOS pressure (mean 26.9, range 8.7-56.5) (Ledson, Tran and Wilshaw, 1998).

The mechanisms of increased GOR in the CF population could be multifactorial. Pauwels et al., (2011) found multiple defective mechanisms in CF but could not associate them with GOR. Delayed gastric emptying was prevalent in 33% of 33 patients but this did not correlate to reflux episodes assessed by pH impedance and there were significantly lower LOS pressure observed in CF (n=12) when compared to healthy controls (n=11)(p=0.04). A later study observed that reflux caused by transient lower oesophageal sphincter relaxations was not found to be different in CF compared to healthy controls (Pauwels *et al.,* 2012).

3.1.10 The treatment of GOR in the CF population

Drug therapy for general GORD patients include H_2 receptor antagonists (H_2-RA) and proton pump inhibitors (PPI) to lower the volume of the refluxate and increase the pH of the refluxate. PPI treatment (lansoprazole and Omeprazole) is primary treatment for GOR. Research has stated that PPI therapy is far superior to H_2-RAs (Chiba, *et al.,* 2003; van Pinxteren, *et al.,* 2003; Armstrong, 2005). However, 5-20% of patients do not respond to current therapy, PPI therapy is not effective at 20mg/day for many GOR patients and the medication may be effective if increased to 40mg/day (Armstrong, 2005). PPI therapy will reduce the volume and time of acid exposure in GOR patients but the acid exposure will still tend to be above normal (Gerson, *et al.,* 2005; Gerson, *et al.,* 2004). In America, 50% of CF patients are currently treated with PPI medication to reduce the acidity in the stomach. This is used for both GOR treatment and to increase efficiency of pancreatic enzyme replacement therapy (Smyth, *et al.,* 2014). Despite the benefits of PPI or H_2RA treatment lowering refluxate pH, the refluxate will still contain pepsin, bacteria and many other damaging agents; it will also be weakly acidic (pH 5-7). Therefore, PPI therapy will not prevent reflux episodes and refluxate reaching the oesophagus can still cause damage, but lessen the symptoms of reflux (Pearson & Parikh, 2011). A previous study found that bile acids were present in the saliva samples of 38 CF patients and 11 of these patients were on PPI therapy (Blondeu *et al.,* 2008) illustrating that gastric contents reach the pharynx regardless of PPI therapy. However, it has been reported that the method of bile acid detection used in this study was not sensitive enough to detect the low bile acid levels reported (below 5mmol/L) (Parikh, *et al.,* 2013). In addition, it has recently been shown that 65% of patients on acid suppression medication reported continued classical GOR symptoms (Sabati *et al.,* 2010).

Surgical management of GOR has been shown to slow down the decline in pulmonary function, increase weight and reduce exacerbations (Sheikh, *et al.,* 2013). If patients do not respond to medicated treatment they can be considered for Nissen Fundoplication. The first published reports of surgery outcomes in CF children at a Childrens medical centre in the USA reported 28% discontinued reflux medication post-surgery and there were no surgical mortalities. Interestingly, surgery significantly improved lung function by 8.6% per year ($p=0.004$) in children with a starting FEV_1 below 60% predicted (Boesch and Acton, 2007). Furthermore, surgical intervention (laparoscopic fundoplication) was found to be highly effective in controlling reflux in a small selection of CF patients that did not respond to medication (Fathi, *et al.,* 2009). Hayes and colleagues reported a reduction in lipid laden macrophages post laparoscopic fundoplication suggesting decreases in aspiration of gastric contents (Hayes, *et al.,* 2013).

GOR is a recognised problem in the CF community. A recent publication by the European Cystic Fibrosis Society highlighted the awareness of GOR prevalence in the CF centre and suggest that patients experiencing symptoms should be diagnosed and subsequently treated for GOR (Smyth, *et al.,* 2014).

3.1.11 Inflammation in the CF airways and the association with pulmonary disease

A major characteristic of CF disease is the susceptibility to chronic pulmonary infection caused by inflammation responding to the bacterial infection. They have a negative impact on health reducing the quality of life and survival therefore antibiotic treatment is prescribed upon identification of bacterial infection. Progressive pulmonary disease is a leading cause of mortality for the CF population (Sagel *et al.*, 2007; Anthony *et al.,* 1999). The clinical indicators of a pulmonary infection vary and there are many indicators such as chronic cough, sputum production, shortness of breath, chest pain, decreased exercise ability and decreased appetite (Smyth *et al.,* 2008). A new symptom, change in symptoms or worsening of existing symptoms is termed exacerbations (Bhatt *et al.,* 2013).

Upon bacterial infection (such as *Pseudomonas aeruginosa*), the inflammatory system responds to the bacterial infection by producing inflammatory mediators at the site of infection. (Segal *et al.,* 2007). The epithelium of the airway produce cytokines and chemokines to initiate the immune response via Natural Killer cells intracellular signalling. Interleukin 8 (IL-8) and Interleukin 6 (IL-6) are proinflammatory cytokines released by the airway epithelium cells to mediate inflammatory response. The mediators recruit

neutrophils to attack the infection in order to eliminate it. Neutrophils are phagocytes with a function to engulf bacteria to eliminate invading infection from the site. The inflammatory response in the CF airway does not function effectively (as described in chapter 1) and cannot clear recurrent infections within the airways (Shanks *et al.,* 2010). Inflammation develops in the airways excessively in relation to the presence of bacteria and continues as the infection cannot be cleared (Bourke and Burns, 2011). This leads to a vicious cycle of excessive inflammatory cell/metabolite influx in the CF airway resulting in airway damage. Viral infections within the CF lung may potentially damage the epithelium (Banner *et al.,* 2009). IL-8 has been shown to be one of the most potent of chemoattractants to activate neutrophils. Studies have shown that samples taken from the CF lung such as sputum (Bergin, *et al.,* 2013) and BAL (Bonfield *et al.,* 1995) have significantly elevated concentrations of IL-8 and IL-6 compared to healthy controls and this has a negative correlation with pulmonary function (Colombo, *et al.,* 2011).

The measurement of inflammatory mediators in the sputum of CF patients has been used to identify disease activity in CF lung disease. Inflammatory response has been demonstrated in the CF airway by counts of abundant neutrophils dominating other cells in sputum and BAL samples taken from the CF lung. These samples have also been shown to contain high levels of inflammatory mediators such as IL-8 and IL-6. The levels of IL-8 and IL-8 in CF sputum have been reported to be released in excess by the airway epithelium and this is thought to be due to the absence of normal function highlighting further consequences of CFTR dysfunction in addition to channel gating dysfunction (Segel *et al.,* 2007). Respiratory tract epithelium damage caused by defective cytokine production is one theory to the enhanced inflammatory response in the airway but not other sites of CF epithelium. Starosta et al., (2007) found that Interleukin-8 (IL-8) concentrations and numbers of neutrophils were correlated to the number of proximal reflux events measured by 24h eosophageal pH measurements. McNally *et al.,* (2011) supported this finding and produced data to illustrate a higher IL-8 concentration in CF patients with raised pepsin levels in BAL samples suggesting increased lung inflammation due to gastric aspiration. Neutrophil elastase is a marker of airway inflammation and can be quantified in the CF lung. Evidence suggests that bile acids contained in the lung significantly associated with neutrophil elastase ($p=0.05$) (Pauwels *et al.,* 2012).

3.1.12 Aims and objectives of the following chapter

Due to a wide range of prevalence of GOR reported in the CF population the following study aimed to investigate the prevalence of GOR and EOR in CF adults at the out-patient clinic, Newcastle upon Tyne allowing for the assessment of a range of CF disease severity and easy patient access. During my study the characteristics of GOR and EOR were assessed using validated questionnaires specific for GOR and laryngopharyngeal reflux termed herein as EOR. Gastric aspiration was determined by the analysis of reflux biomarkers present (pepsin and bile acids) in expectorated sputum samples collected during out-patient clinic appointments. The lipid laden macrophage index was determined by staining the cells of sputum samples. This has been used as a reflux diagnostic tool assessing the lipid contained in macrophages of sputum cells.

The association between GOR and pulmonary function was assessed to investigate the relationship between reflux in the CF population and pulmonary function based on GOR/EOR symptoms and gastric aspiration. The spirometry measurements (FEV_1 and FVC) were calculated according to the percentage predicted based on the ERS guidelines.

The inflammation within the airways was explored by methods of expectorated sputum from within the airway. The relationship between gastric aspiration and pulmonary inflammation was explored.

3.2 Results

3.2.1 Study population and sampling

The Ethical approval for this study was granted by the County Durham & Tees Valley 2 Research Ethics Committee (REC NO: 10/H0908/8). There were 72 adult CF patients (range 16-60 years, median 21) recruited onto the study from their routine clinical appointments at the RVI, Newcastle Upon Tyne between 2011 and 2014. There were 39 males and 33 females with a median weight of 57.8kg (range 34.1-87.0) and median BMI of 21.5 (range 14.1-30.9). (See figure 3.1, 3.2, 3.3, 3.4 and 3.5 and see appendix 8.7 for individual patient data).

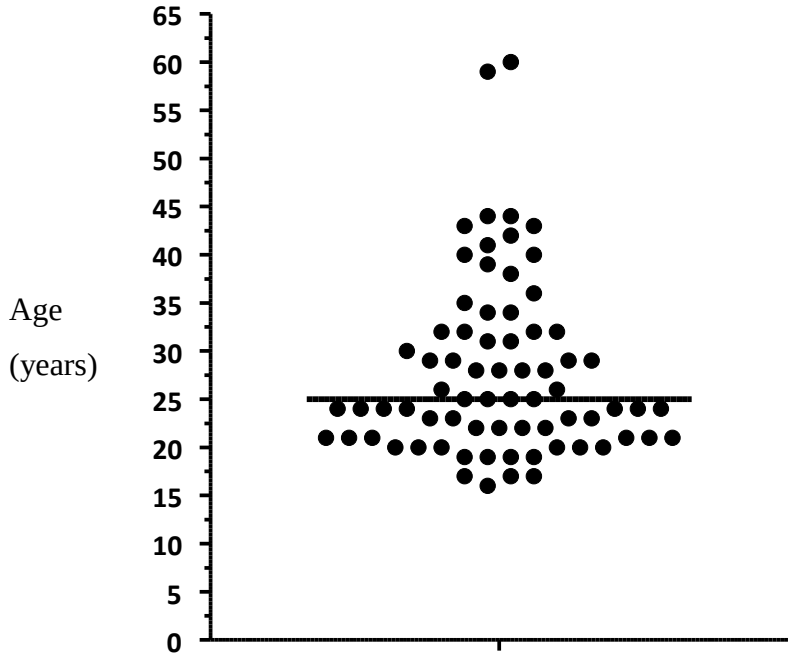


Figure 3.1 Patient age (n=72, range 16-60, median 21 years)

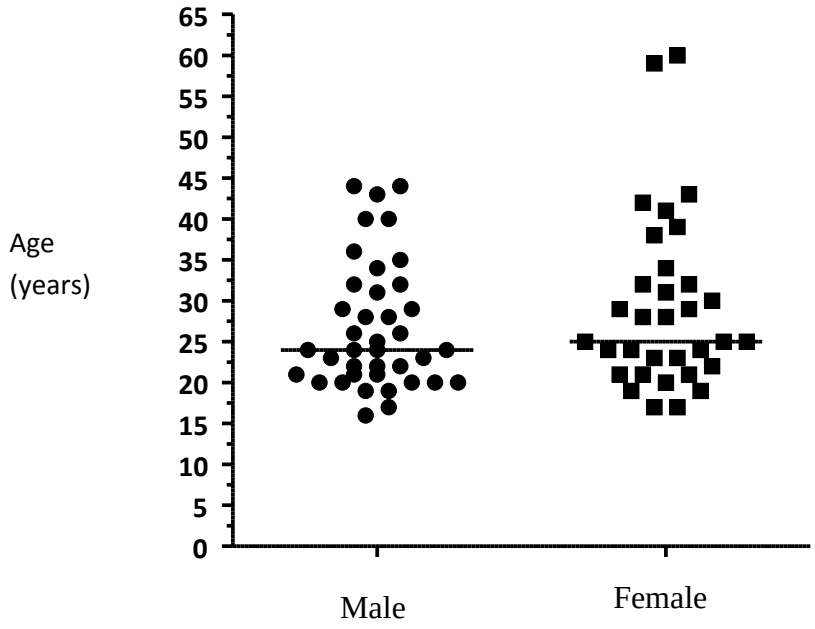


Figure 3.2 The age of male (n=39) and female (n=33) patients (male range 16-44, median 24 years; female range 17-60, median 25)

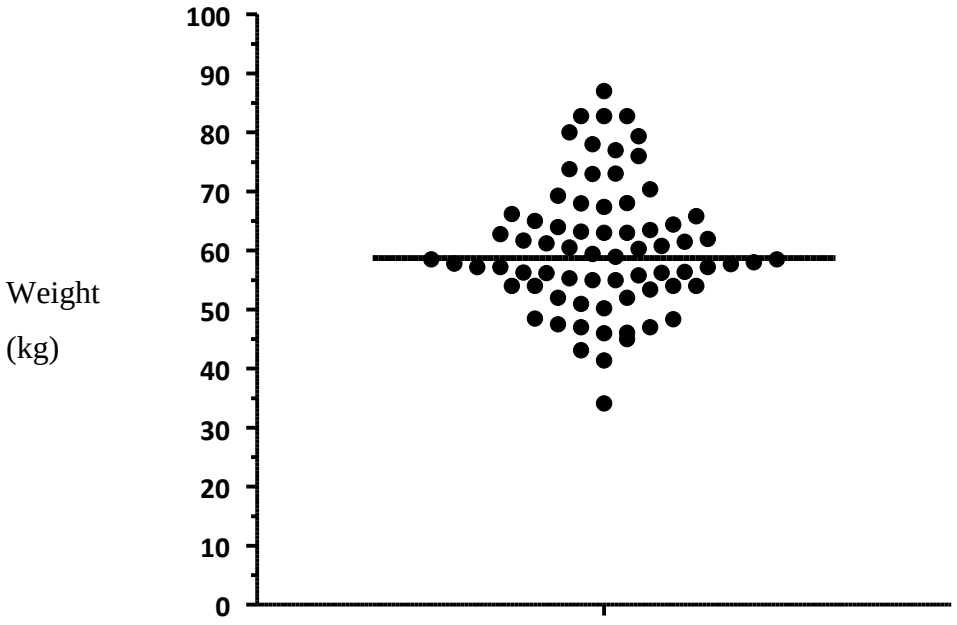


Figure 3.3 Patient weight (kg) (n=72; range 34.1 -87.0 kg, median 58.7 kg)

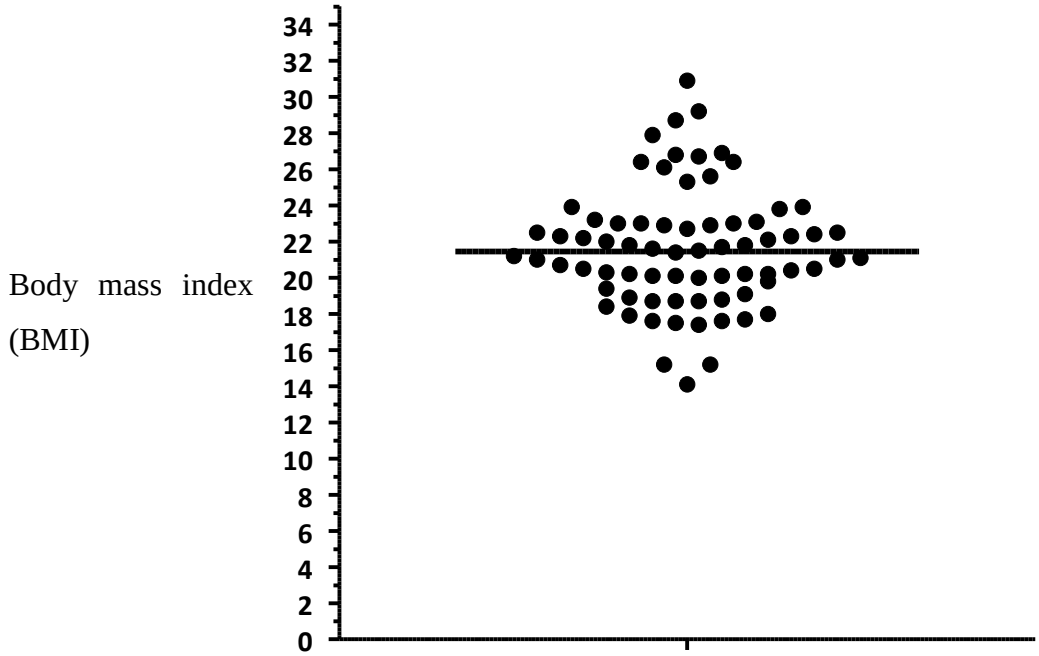


Figure 3.4 Patient Body Mass Index (BMI kg/m^2) (n=72; range 14.1-30.9, median 21.5)

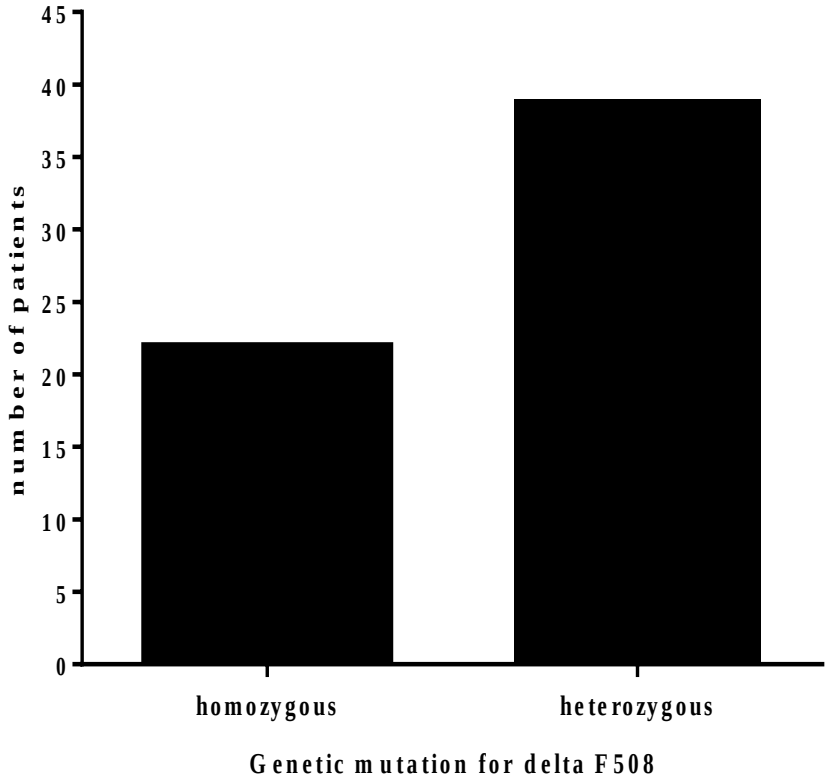


Figure 3.5 The prevalence of delta F508 in the 61 patients recorded was n=22 homozygous and n=39 heterozygous.

3.2.2 The investigation into the prevalence of reflux symptoms in the CF population.

The symptoms of reflux were assessed by questionnaire. GOR symptoms were determined by the DeMeester Reflux Questionnaire (n=72), EOR symptoms were determined by the Reflux Symptom Index (RSI) (n=72) and the assessment of airway reflux cough was determined by the Hull Airway Reflux Cough Questionnaire (HARQ), This was introduced at a later stage in the study following feedback from presentation of my work at a national meeting and so had a lower number of results (n=22). Of the 72 patients recruited the median DeMeester score was 1 (range 0-9, normal = 1 or below). 30 patients (42%) scored >2 presenting GOR symptoms (median 2, range 2-7) (Figure 3.6). The median RSI score was 16 (n=72, range 0-43) and more than half of patients 45 (63%) were EOR symptomatic, with an RSI score >13 (median 19, range 13-43) (Figure 3.7). Reflux associated cough was determined by the Hull Airway Reflux Questionnaire (HARQ). The HARQ questionnaire was introduced in January 2013 therefore n= 22 patients completed the HARQ from the total patients recruited (n=72). The median score of the 22 patients who completed the HARQ questionnaire was 15 and there were 11 patients (50%) symptomatic of airway reflux cough (median 31, range 15-44) (Figure 3.8). The questionnaire data illustated that 42% of the patients that were recruited experienced GOR symptoms, 63% experienced EOR symptoms and 50% of 22 patients experienced airway reflux cough. This level of symtomatic burden was present despite the fact that 46 patients were treated with proton pump inhibitors (PPI) and 3 treated with Histamine$_2$ Receptor Antagonists (H_2RA) for acid reflux. There were 20 patients that were not taking any medication to treat acid reflux. Of the 30 patients that experienced GOR symptoms 63% (n=19) were on PPI medication. There were 69% (n=31) of patients on PPI medication who experienced EOR symptoms. Of the 3 patients taking H_2RA's there were 2 patients who experienced GOR symptoms and EOR symptoms. Similarly there were 7 patients on PPI medication that scored an abnormal HARQ score (n=11) (Figure 3.9).

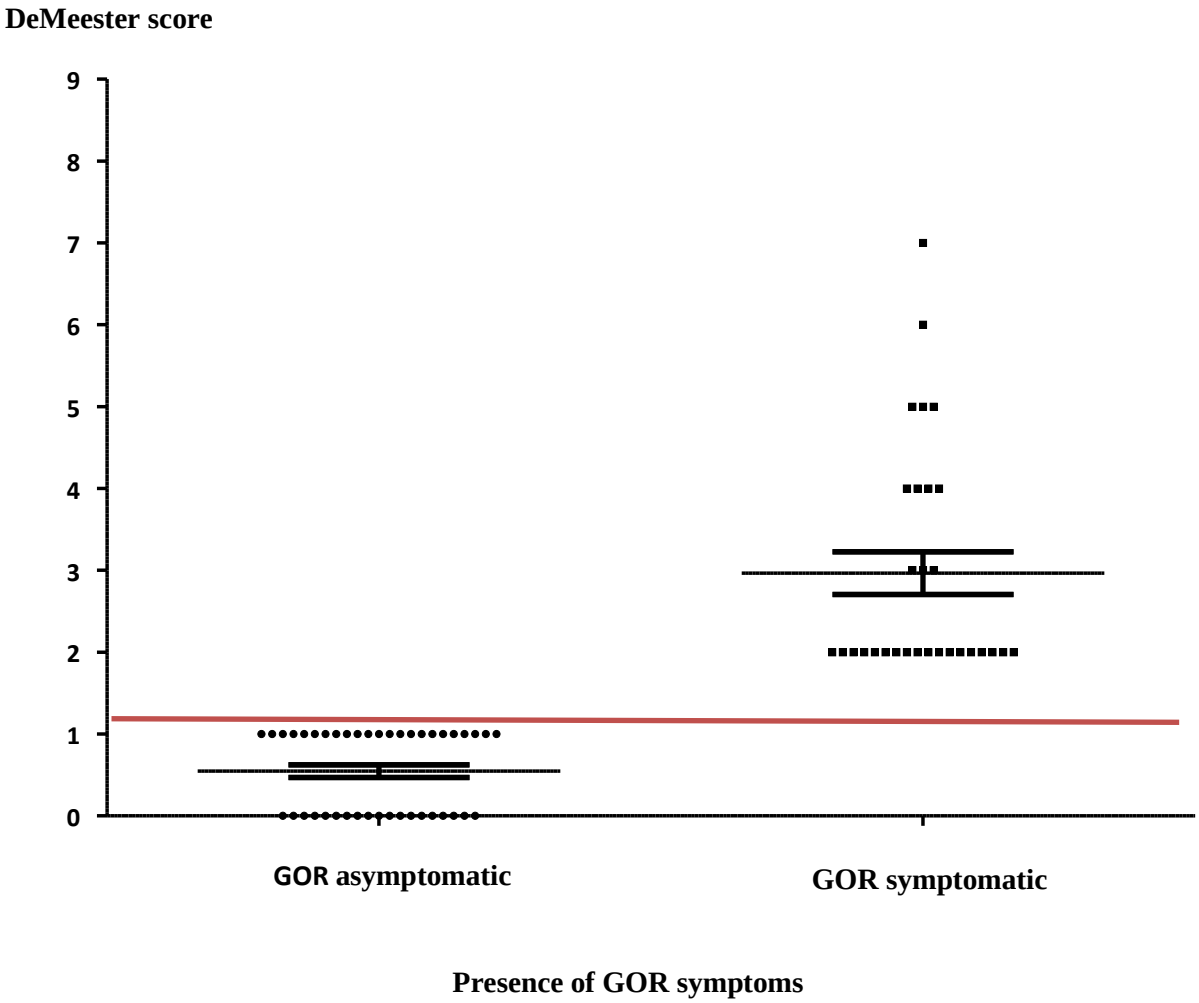


Figure 3.6 Presence of GOR symptoms determined by the DeMeester reflux questionnsire maximum score 9; normal score <1 represented by the red line. There were 42 patients asymptomatic of GOR determined by a DeMeesterscore of 1 or below (58%) (range 0-1, median 1. 30 (42%) patients were GOR symptomatic (range 2-7, median 2) determined by a DeMeester score of 2 or above. The graph illustrates individual patient DeMeester scores with Mean and SEM.

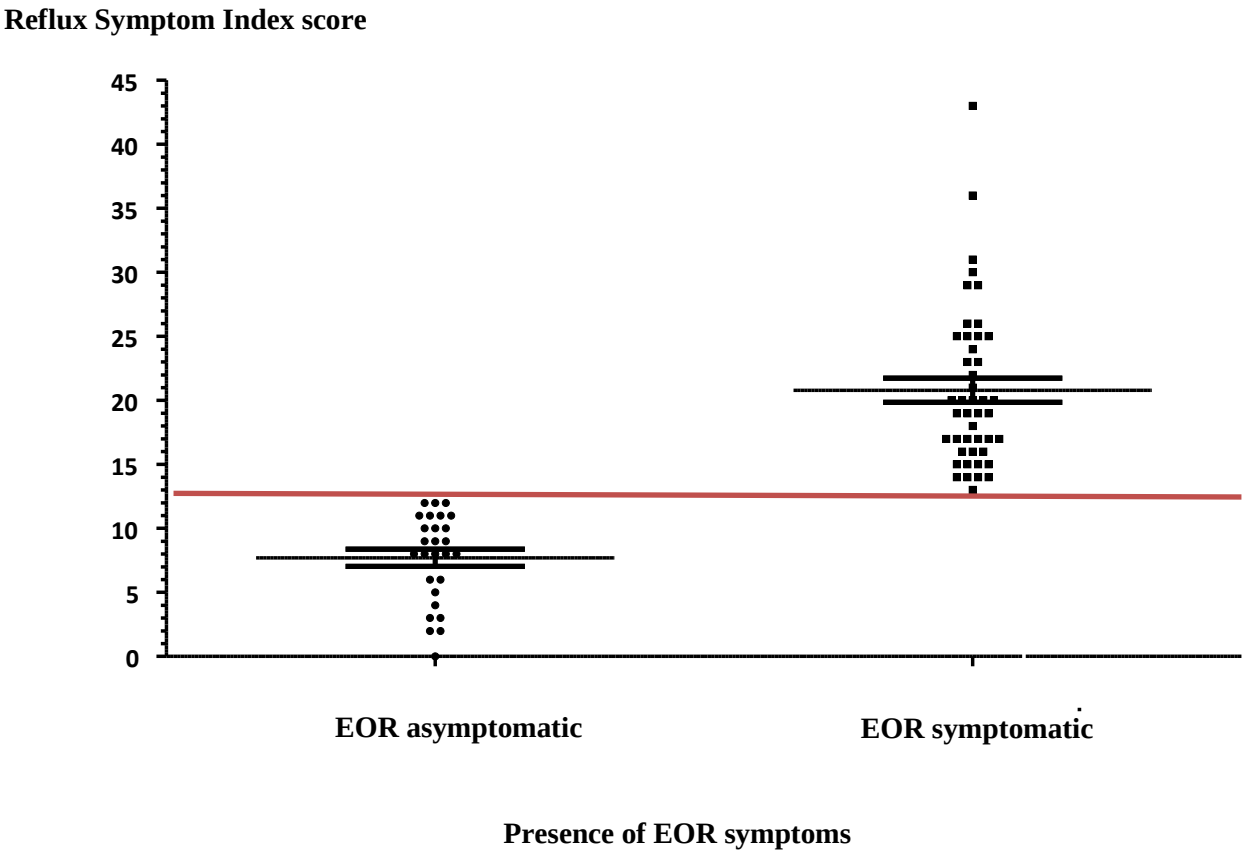


Figure 3.7 Presence of EOR symptoms determined by the Reflux Symptom Index (RSI) Questionnsire maximum score 45; normal score <12 represented by the red line. There were 27 (37%) patients asymptomatic for EOR determined by a RSI score of 12 or below (range 0-12, median 8). There were 45 (63%) patients that were EOR symptomatic determined by an RSI score of 13 or above (range 13-43, median 19). The graph illustrates individual patient RSI scores with Mean and SEM.

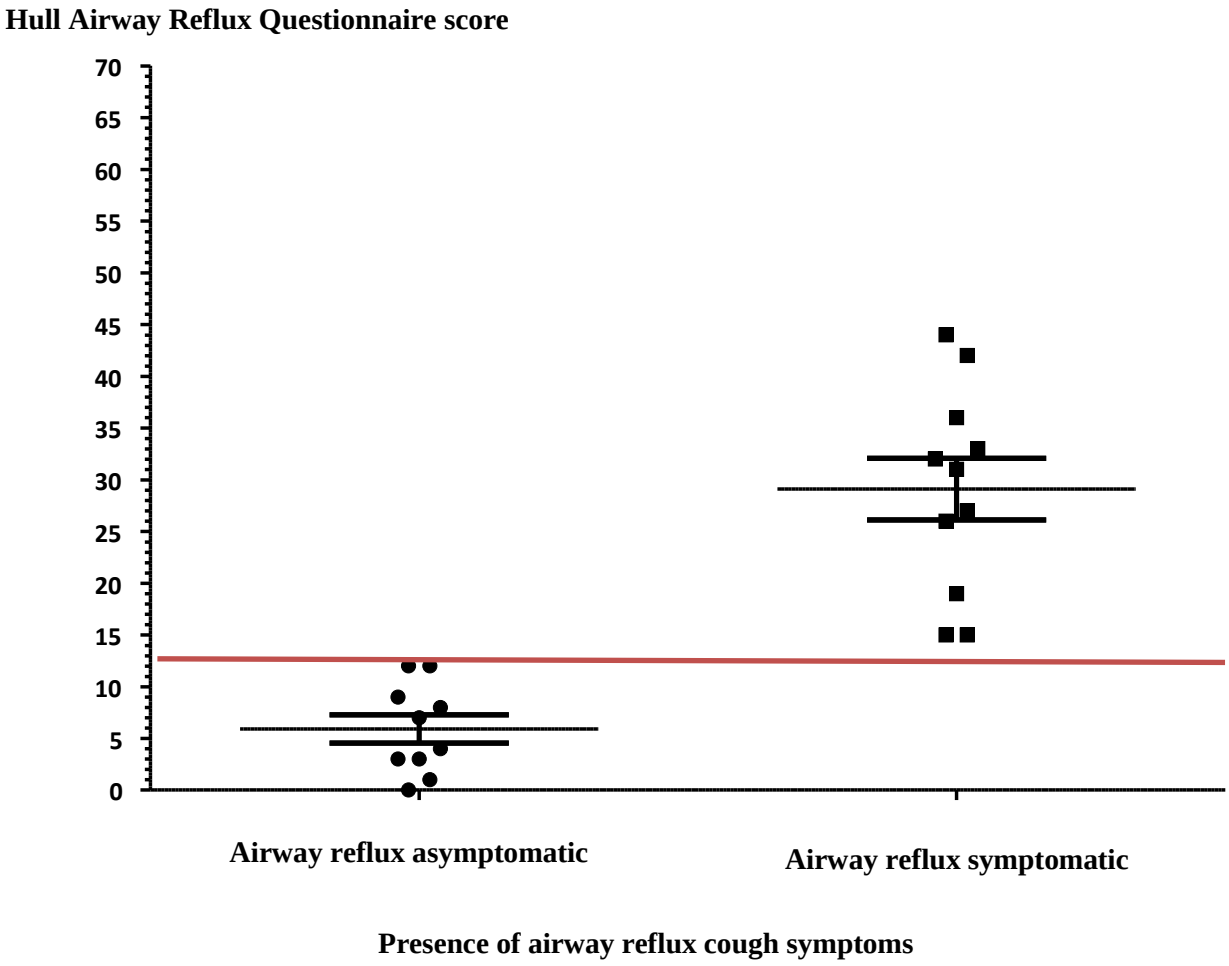


Figure 3.8 Presence of airway reflux cough (ARC) symptoms determined by the Hull Airway Reflux Questionnaire (HARQ) n=22; maximum score 70; normal <12 represented by the red line. There were 11 (50%) patients that were asymptomatic of airway reflux cough symptoms determined by a HARQ score of 12 or below (range 0-12, median 6). There were 11 (50%) patients symptomatic for airway reflux cough determined by a HARQ score of 13 and above (range 15-44, median 31). The graph illustrates individual patient HARQ scores with Mean and SEM.

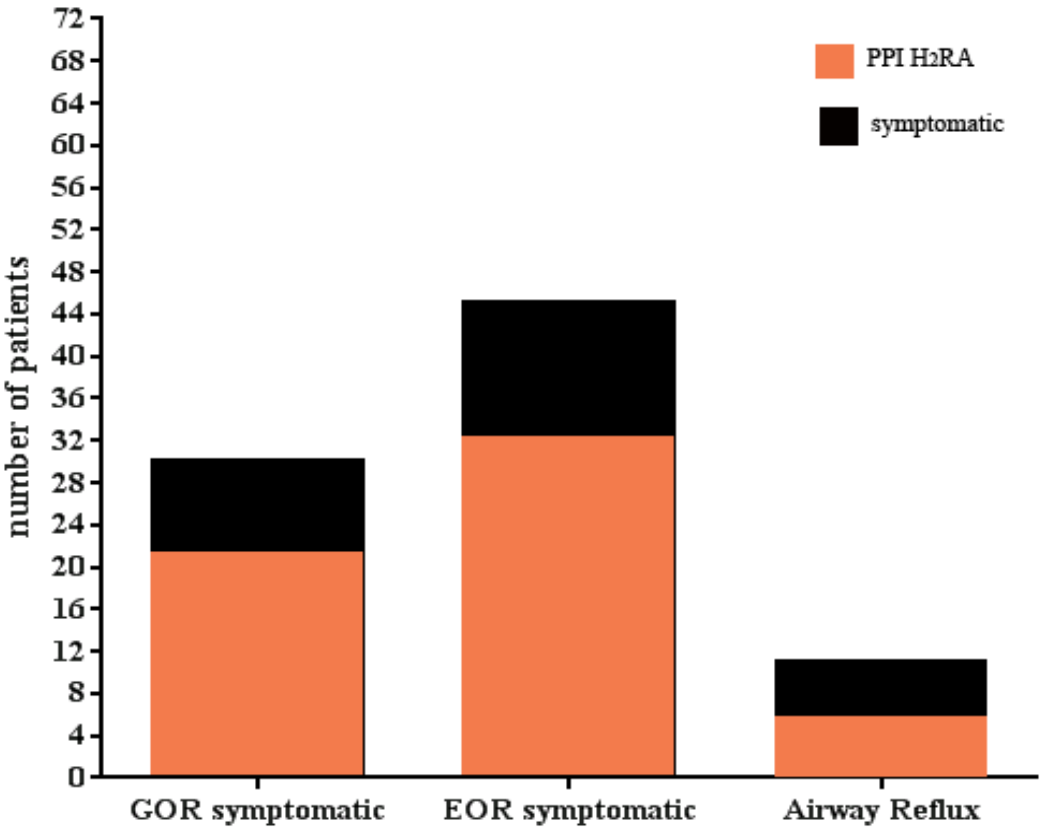


Figure 3.9 The number of symptomatic patients using PPI and H_2RA medication. There were 30 patients symptomatic for GOR determined by the DeMeester reflux questionnaire (score of 2 or above), of which 19 were on PPI medication and 2 were on H_2RA medication. 45 patients experienced EOR symptoms determined by the RSI score (score of 13 or above) of which 31 patients were on PPI medication and 2 were on H_2RA medication. Of the 22 patients that completed the HARQ questionnaire to assess airway reflux 11 were symptomatic and 7 were on PPI medication.

3.2.3 The investigation of gastric aspiration in the CF population

3.2.3i Pepsin as a biomarker for gastric aspiration

The initial experiments carried out to identify pepsin in sputum supernatant involved optimisation of an in house pepsin indirect ELISA. The optimisation of the blocking solution was assessed to prevent nonspecific binding to the plate. It was determined by blocking a plate with three pepsin standard curves with a range of 0 to 100ng/ml using 1% BSA (R^2=0.99), 1% milk powder (R^2=0.72) and 5% milk powder (R^2=0.71). The standard curve which was blocked with 1% BSA was deemed the superior blocking agent compared to milk powder therefore it was used in the pepsin ELISA protocol of further experiments. The standard curve used to determine pepsin concentration was linear between 0 and 100ng/ml. The pepsin ELISA linear standard curve measured an absorbance value up to 0.79 optical density units (Figure 3.10).

The standard operating procedure for sputum processing required a sputum sample to be homogenised in DPBS at 8 times the sputum plug weight (1:8 dilution) (see Chapter 2 Methods: subheading 2.7). Prior to sample collection and processing the analysis of pepsin recovery in sputum supernatant was investigated. To ensure pepsin could be detected from a 1:8 sputum supernatant a experimental sputum plug was collected and processed as directed. The 1:8 sputum supernatant samples was spiked with known concentrations of porcine pepsin in order to recover the known concentration through the pepsin ELISA procedure. Pepsin was recovered from the spiked sputum supernatant sample to a lower detection limit of 10ng/ml (1ng/100µl). Pepsin was not recovered at 0.0ng 0.3ng, 0.5ng. Sputum spiked with 1.0 ng, 1.5 ng 3.0 ng and 5.0 ng of porcine pepsin resulted in full recovery. Therefore samples could be collected and processed as directed using the standard dilution.

Sputum samples were processed according to the standard protocol and therefore were diluted 1:8 in DPBS to homogenize the sputum plugs. The samples were aliquotted, labelled and stored at -70°C for later analysis. The question was raised about the correct dilution factor of sputum supernatant of CF patients to ensure any pepsin that was to be detected fell within the standard curve of 0-100ng/ml of porcine pepsin. Thus, various dilution factors of sputum supernatant were assessed (1:8, 1:200, 1:1000) to ensure the pepsin detected would fall within the standard curve range of 0-100ng/ml dilution and determining the correct dilution factor for future sample analysis. From the same sputum supernatant sample the concentration of pepsin recovered from the 1:8 dilution was

5ng/100µl (50ng/ml); 1:200 dilution 0ng/100µl; 1:1000 dilution 0ng/100µl. The dilution factor of sputum supernatant suitable for the pepsin ELISA to fit onto a standard curve of 0-100ng/ml was the 1:8. The prepared sputum supernatant samples were used at the dilution they were stored at and there were no further dilution necessary.

Sputum supernatant samples were analysed for pepsin (n=69) using the indirect ELISA and the overall range of pepsin was 0 to 1150ng/ml with the median of 230ng/ml. Of the 69 patient samples analysed there were 48 (70%) patient samples with detectable levels of pepsin with a median of 330ng/ml ranging from 80 to 1150ng/ml. There were 21 (30%) patient samples that did not contain pepsin above the lowest level of accurate quantification of pepsin at 1ng in 100µl (100ng/ml). Figure 3.10 shows a typical standard curve and figure 3.11 displays the individual patient pepsin values measured in sputum supernatant samples.

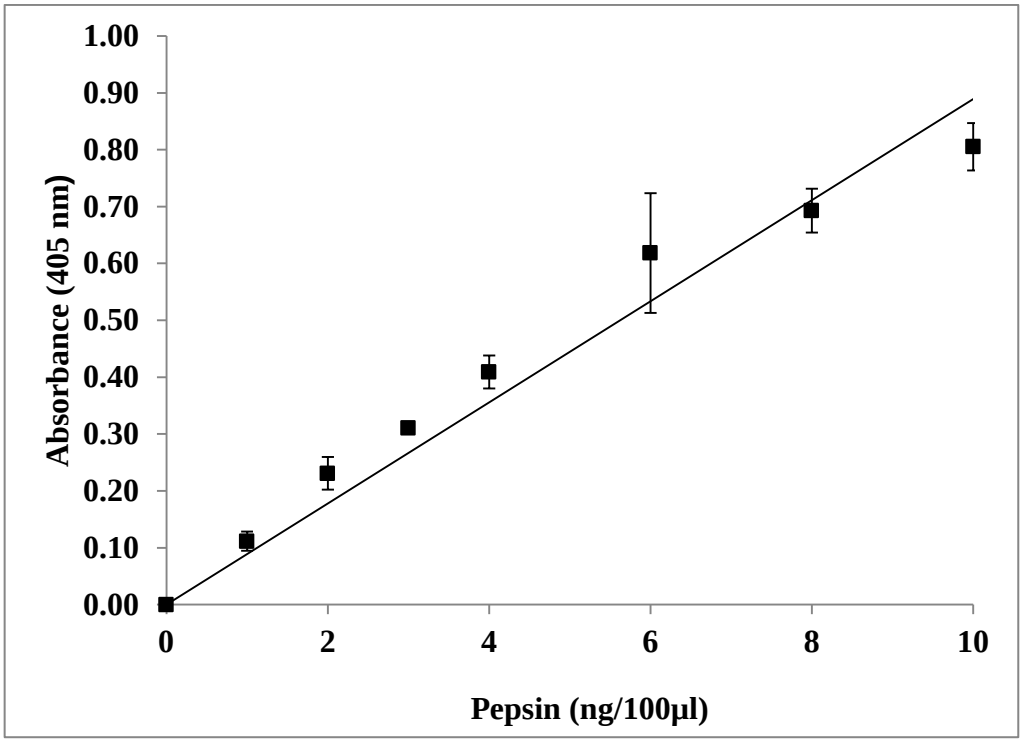


Figure 3.10 Porcine pepsin standard curve measured with in-house in-direct ELISA using goat monoclonal primary antibody at 1:100 followed by monoclonal secondary to goat monoclonal raised in mouse (peroxidase conjugated) at 1:1000 (mean ± SEM, n=3).

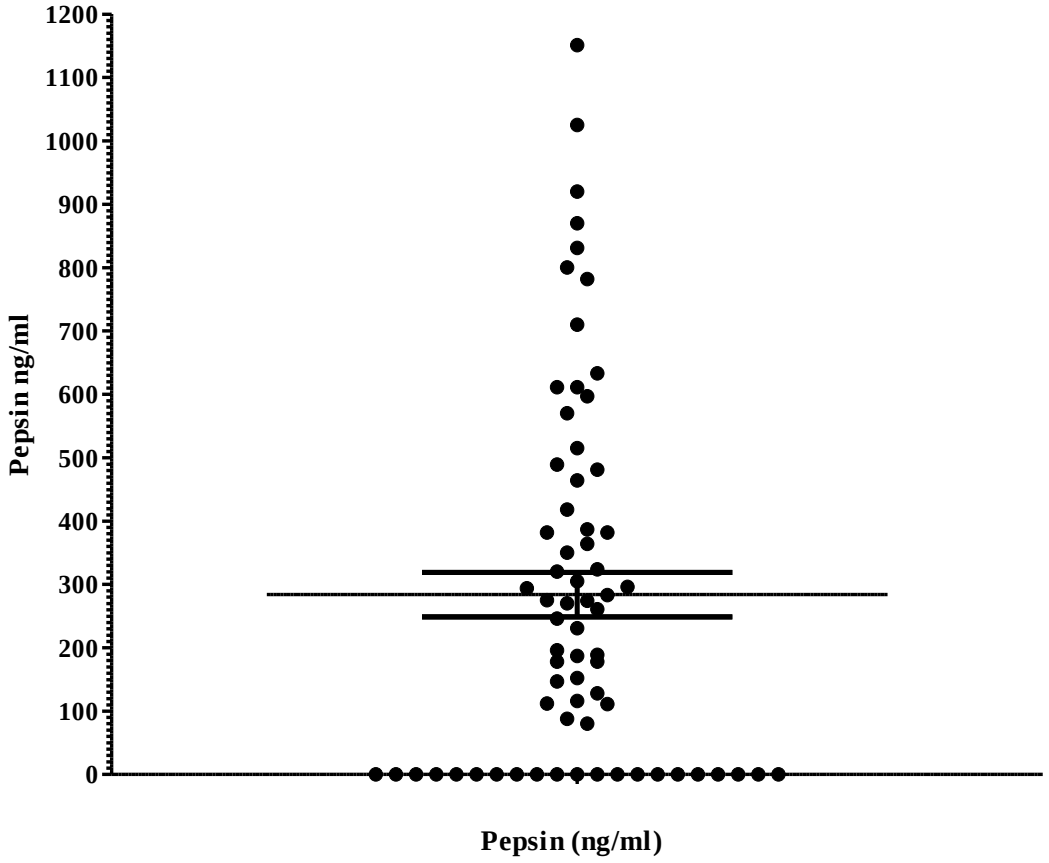


Figure 3.11 The analysis of sputum supernatant pepsin levels (ng/ml) in 69 patients measured with in-house ELISA using goat monoclonal primary antibody at 1:100 followed by monoclonal secondary to goat monoclonal raised in mouse (peroxide conjugated) at 1:1000 (mean with SEM). The range of pepsin was 0 to 1150ng/ml, median 230, mean 280, SEM 35.

3.2.3ii Total Bile Acid as a biomarker for gastric aspiration

Taurine and glycine conjugates of cholate and deoxycholate and their isomers were measured using a mass spectrometry Acquity TQ Detector (Waters Corp, Milford, MA). The Taurine conjugates make up around 25% of the total bile acids and the glycine conjugates make up 75%. Although trace free bile acids are present and these make up to 5% of the total bile acids (J. P. Pearson and S. Parikh, 2011). The term Total Bile Acids (TBA) herein describes the Taurine and glycine conjugates which equates for 90-100% of TBA. The individual bile acids were detected to a sensitivity of 0.01µMol/L and then these levels were added together to give the TBA concentration. The concentrations were assessed in 24 sputum supernatant patient samples at Sheffield Childrens Hospital, UK. The median level of TBA's was 0.22 µmol/L and ranged from 0.00 to 3.17 (figure 3.12 and 3.13). Glycine and Taurine conjugates were compared to find that there were no median differences between the two conjugates. Median Glycine conjugates were 0.08 µMol/L (range 0.00-2.24) and Taurine conjugates were 0.08µMol/L (range 0.00-0.96). Reported TBA in human serum are reported to be up to 10µmol/L and therefore used as a lower detection limit when assessing abnormal levels (Parikh *et al.*, 2013).

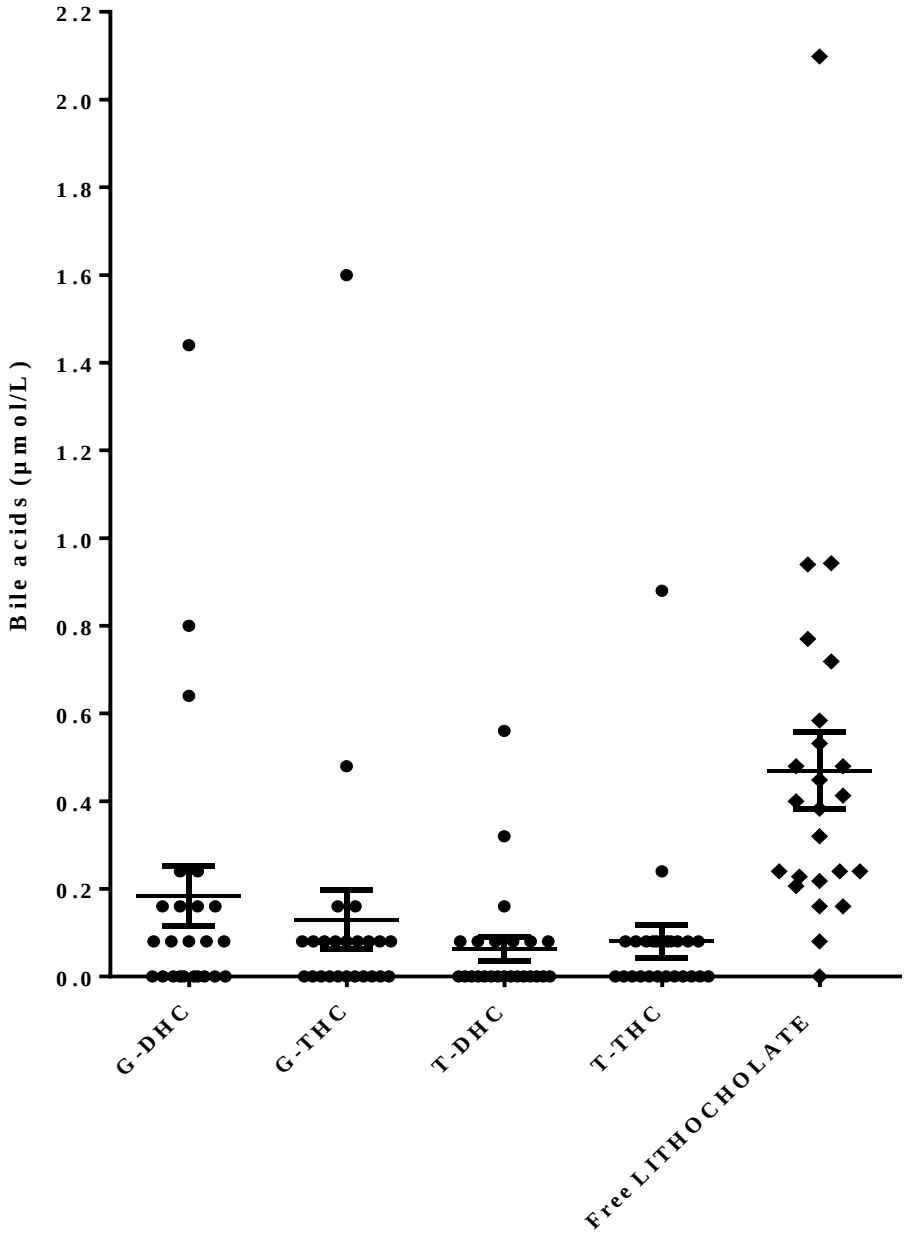


Figure 3.12 Taurine and glycine conjugates of cholate and deoxycholate and their isomers in sputum supernatant samples were measured using an Acquity TQ Detector (Waters Corp, Milford, MA). Individual bile acid levels of sputum supernatant (n=24) samples are presented measured by mass spectrometry sensitive to 0.01µMol/L and multiplied by dilution factor (1:8). Median levels of glycodeoxycholate (G-DHC) were 0.08 (range 0-1.44), glycocholate (G-THC) 0.08 (range 0-1.60), taurodeoxycholate (T-DHC) 0.00 (range 0-0.56), (taurocholate (T-THC) 0.04 (range 0-0.88) and free lithocholate 0.39 (range 0-2.10).

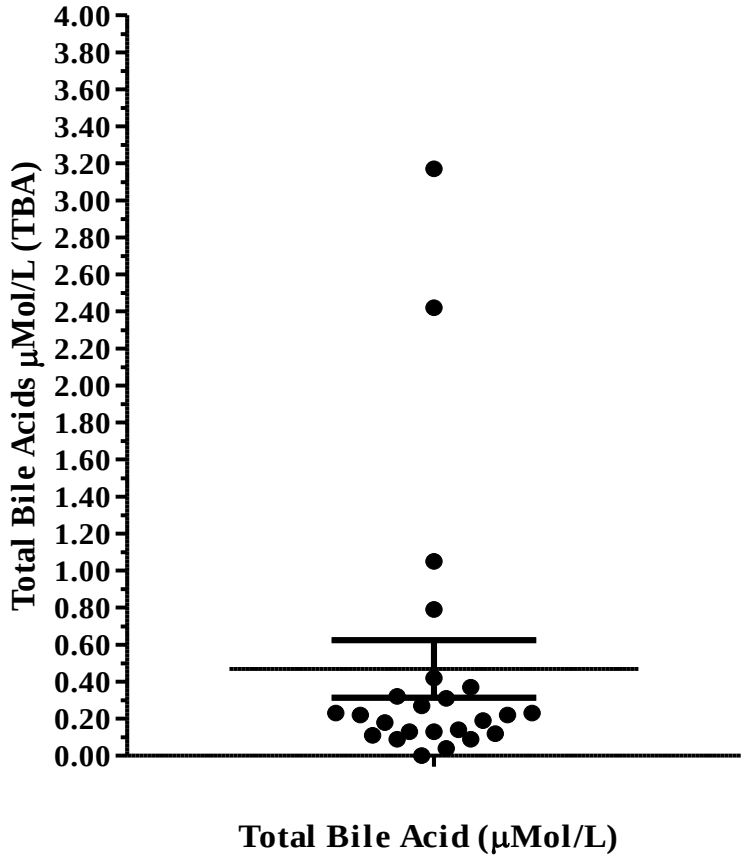


Figure 3.13 Sputum supernatant TBA levels measured by mass spectrometry to a sensitivity of 0.01µMol/L. Median TBA levels were 0.22 (range 0-3.17, mean 0.47, SEM 0.16 µMol/L).

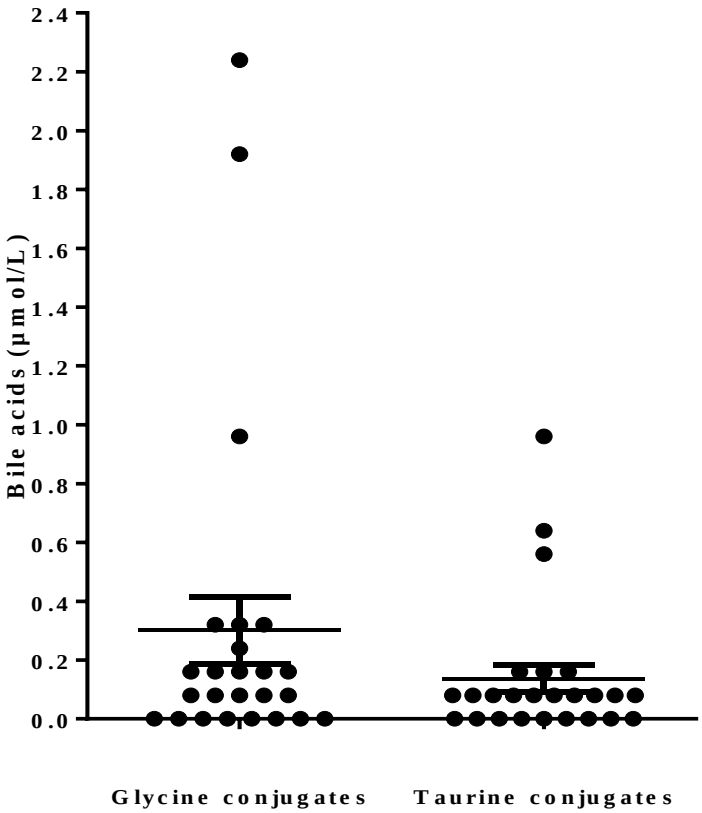


Figure 3.14 Sputum supernatant Glycine conjugates and Taurine conjugates concentration measured by mass spectrometry to a sensitivity of 0.01µMol/L. Median Glycine conjugates were 0.08 µMol/L (range 0.00-2.24) and Taurine conjugates were 0.08µMol/L (range 0.00-0.96).

3.2.3iii The Lipid Laden Macrophage Index Score as a diagnostic tool for gastric aspiration

The oil red O stain was used to determine lipid laden macrophage index score (LLMIS) from sputum samples that were processed and prepared to $0.5x10^6$/ml cells on a glass slide and stained with Oil Red O/Sudan Red followed by a counter stain to identify the nucleus of cells with Harris Haematoxylin. The cells were differentiated into neutrophils, eosinophils, macrophages and lymphocytes by counting a total of 500 cells and calculating the percentage of each cell type based on characteristic morphology. The LLMI score requires the assessment of 100 consecutive macrophages upon the visible identification of red lipid droplets contained within the cell. The cell differential of the sputum supernatant samples analysed (n=36) showed that there were a median 0% macrophages (range 0 to 0.8%) present and therefore prevented identification of lipid laden macrophages (Table 1 and Figure 3.15).

Cell type	Median %	Range %	Mean %	SEM %
Neutrophil	100	97 – 100	99	0.09
Eosinophil	0	0 – 0.8	0	0.03
Macrophage	0	0- 2	0	0.05
Lympocyte	0	0-1	0	0.05

Table 3.1 The cell differential of expectorated sputum of CF patients. Sputum samples were processed to provide a cell cytospin of 500,000 cells per ml on a glass slide. Each cell sample was stained to identify the cell differential count. The Giemsa stain identified that in 36 patient samples analysed the median neurophil counts were 100% (97.2-100); median eosinophil counts were 0% (0.4-0.8); median macrophage counts were 0% (0-2); median lymphocytes counts were 0% (0-1.2)

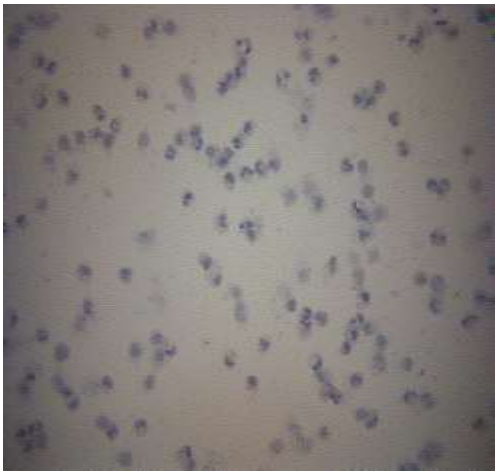
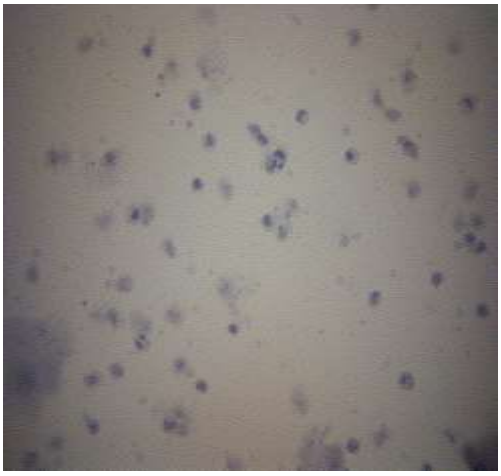

a)

b)

Figure 3.15 A representative image of the Oil Red O stained sputum supernatant samples illustrating the presence of neutrophil nuclei and no red droplets visible. (a and b represent two different patients with no macrophages present, thus a lipid laden macrophage score of 0)

3.2.4 The relationship between aspiration of gastric juice and symptoms of GOR/EOR

The DeMeester questionnaire score was used to determine the presence of GOR symptoms in 72 patients. The patients were grouped into those that were symptomatic of GOR scoring 2 or above on the questionnaire (GOR) and those that were asymptomatic (no GOR) of GOR scoring 1 or below. The pepsin concentration detected in each group was assessed using a non-parametric two-tailed Mann-Whitney U test to show that there were no statistical differences of pepsin concentration detected in sputum samples and the presence of GOR symptoms (p=0.11) although patients asymptomatic of GOR had higher concentrations compared to symptomatic patients. The median level of pepsin detected in the GOR symptomatic group was 140ng/ml (n=30, range 0-800 ng/ml) and median levels in the asymptomatic group were 270ng/ml (n=39, range 0-1150 ng/ml) (Figure 3.16). The RSI questionnaire score was used to determine the presence of EOR symptoms in the aero digestive tract in 72 patients. The patients were grouped into those that were symptomatic of EOR scoring 13 or above on the questionnaire (EOR) and those that were asymptomatic of EOR scoring 12 or below. The pepsin concentrations detected in each group was assessed using a non-parametric two-tailed Mann-Whitney U statistical test to show that there were no statistical differences of pepsin levels detected in sputum samples and the presence of EOR symptoms (p=0.23). The median level of pepsin detected in the EOR symptomatic group was 180 ng/ml (n=47, range 0-1020 ng/ml) and median levels in the asymptomatic group were higher at a median concentration of 290 ng/ml (n=22, range 0-1150 ng/ml) (Figure 3.17). Of the 24 patient sputum samples that were analysed for BA's there were 16 patients that were symptomatic for GOR symptoms with median TBA concentration of 0.3 µmol/L (range 0.0 to 3.2) and there were 8 patients that were asymptomatic of GOR symptoms with a median TBA level of 0.2 µmol/L (range 0.0 to 2.0). There were 20 patients that were symptomatic of EOR symptoms with median TBA levels of 0.2 µmol/L (range 0.0 to 3.2) and 4 patients that were asymptomatic of EOR symptoms with a median of 0.2µmol/L (range 0.2-1.2). There were no statistical difference between TBA levels and symptoms of EOR (P=0.56). or GOR (p=0.07) but the concentrations were higher in the symptomatic group (Figure 3.18 and 3.19).

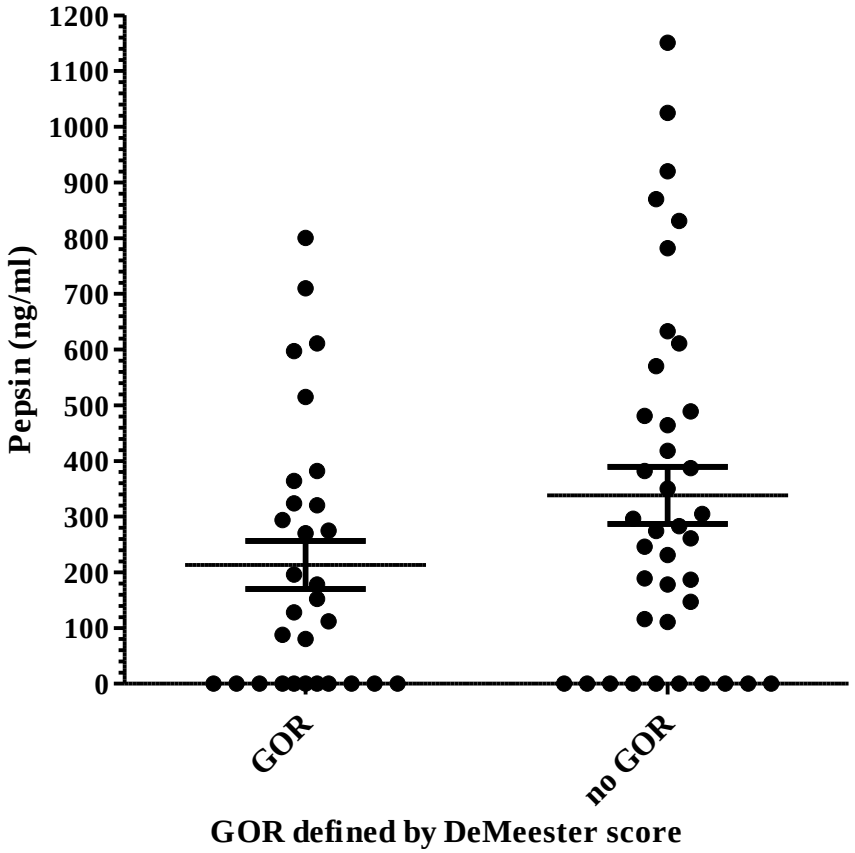


Figure 3.16 Sputum supernatant pepsin levels in symptomatic GOR patients (n=30) defined by DeMeester Score >2 (range 0-800ng/ml, median 140, mean 210, SEM 43) and asymptomatic GOR patients (n=39) defined by DeMeester score <1 (range 0-1150ng/ml, median 270, mean 330, SEM 51). There was no statistical difference between pepsin levels and the symptoms of GOR (P=0.11) assessed by Mann Whitney U Test and Gaussian approximation.

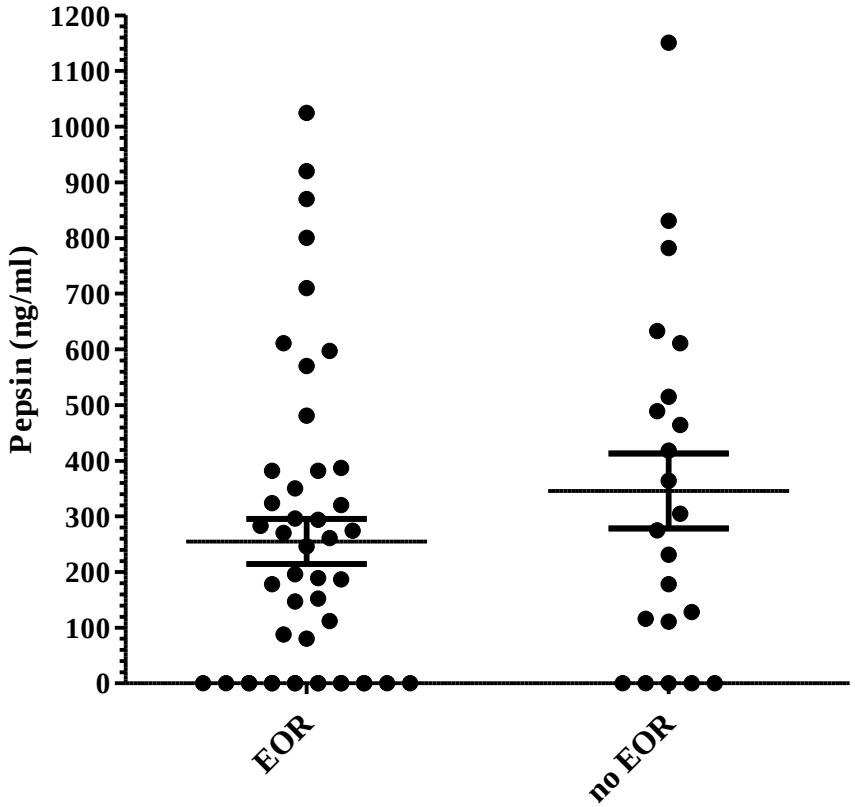


Figure 3.17 Sputum supernatant pepsin levels in symptomatic EOR patients (n=47) defined by RSI Score >13 (range 0-1020ng/ml, median 180, mean 280, SEM 41) and asymptomatic EOR patients (n=22) defined by RSI score <12 (range 0-1150ng/ml, median 290, mean 340, SEM 60). There was no statistical difference between pepsin levels and the symptoms of EOR (P=0.23) assessed by Mann Whitney U Test and Gaussian approximation.

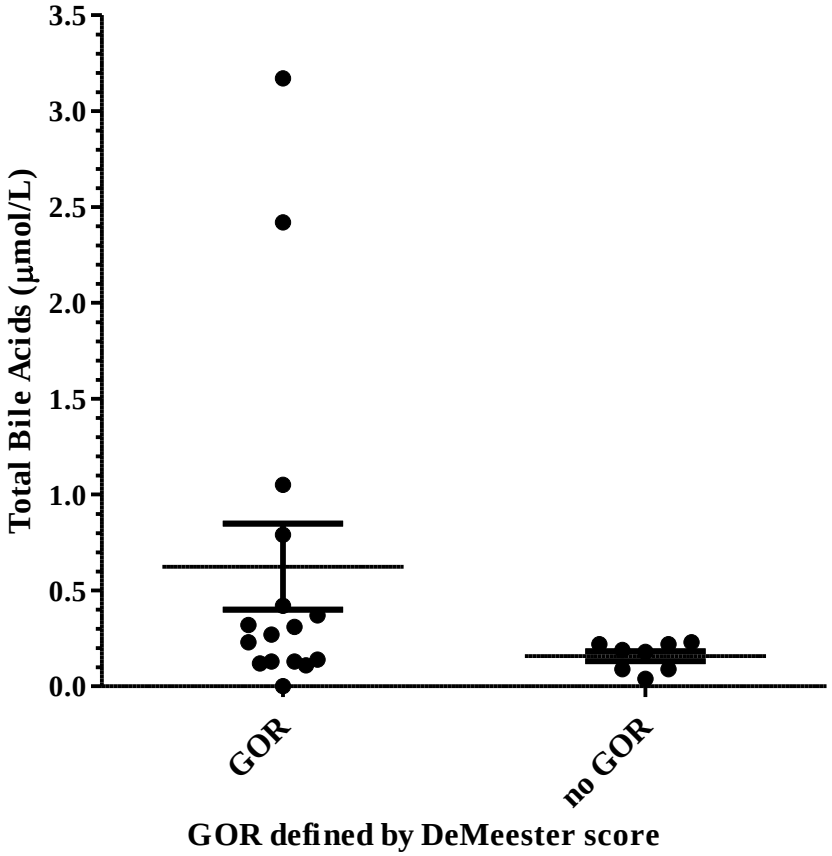


Figure 3.18 Sputum supernatant TBA levels in symptomatic GOR patients (n=16) defined by DeMeester Score >2 (range 0.0-3.2 µmol/L, median 0.3, mean 0.6, SEM 0.2) and asymptomatic GOR patients (n=8) defined by DeMeester score <1 (range 0.0-2.0 µmol/L, median 0.2, mean 0.2, SEM 0.0). There was no statistical difference between pepsin levels and the symptoms of GOR (P=0.07) assessed by Mann Whitney U Test and Gaussian approximation.

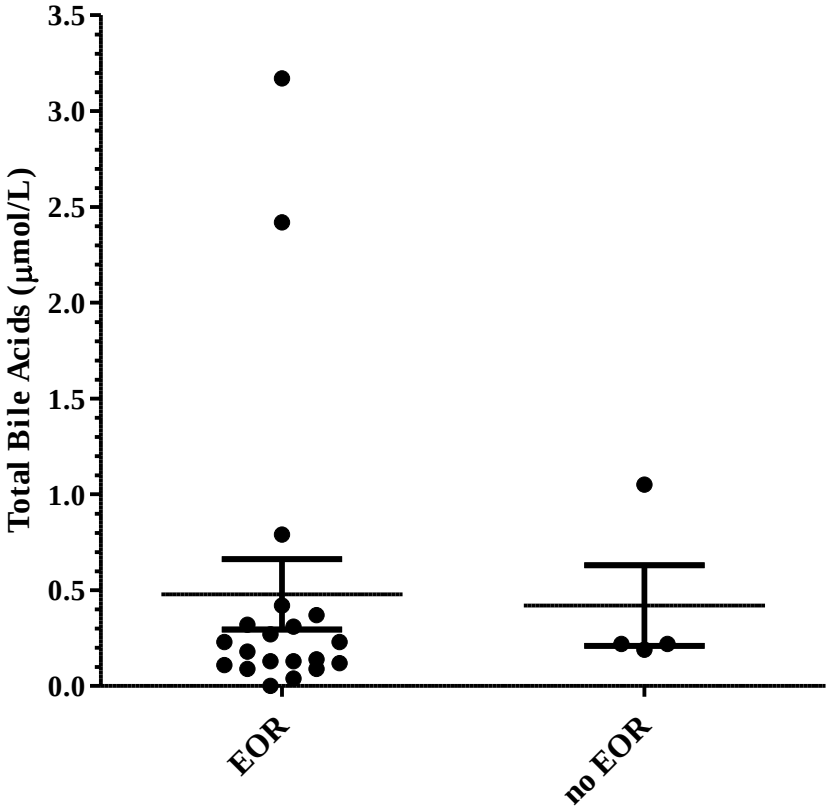


Figure 3.19 Sputum supernatant TBA levels in symptomatic EOR patients (n=20) defined by RSI Score >13 (range 0.0-3.2 µmol/L, median 0.2, mean 0.5, SEM 0.2) and asymptomatic GOR patients (n=4) defined by DeMeester score <1 (range 0.2-1.2 µmol/L, median 0.2, mean 0.4, SEM 0.2). There was no statistical difference between pepsin levels and the symptoms of GOR (P=0.56) assessed by Mann Whitney U Test and Gaussian approximation.

3.2.5 The investigation into the association between reflux and CF pulmonary function

Pulmonary function was routinely assessed during the clinical appointment and the results of pulmonary function (L) were expressed relative to predicted values. Predicted values were calculated based on gender, height and age and observed values calculated as a percentage of the predicted value. Of the 61 patient pulmonary function scores collected the median FEV_1% was 47% (range 12-110%) of the predicted value and the median FVC% predicted was 66% (range 11-119%) (Figure 3.21 and 3.22). These values were consistent with airway obstruction. To assess whether the presence of gastric aspiration into the airways was associated with a decrease in pulmonary function, the patients were grouped into pepsin positive and pepsin negative sub groups and the pulmonary function scores of each group were compared with a non-parametric two tailed statistical test. There was no statistical difference between the median pulmonary function scores and presence of gastric aspiration in the airways determined by the presence of pepsin (p=0.44) but the trend of increased pulmonary function with decreased pepsin concentrations is illustrated in Figure 3.23. The relationship between TBA levels measured in 22 patients and pulmonary function was assessed to show that there was no correlation (TBA and FEV_1% P=0.675; TBA and FVC% P=0.696). There were 2 patient sputum supernatant samples containing high levels of TBA, as shown on figure 3.24 and 3.25, patient A sample contained 3.16µmol/L with a low FEV_1% of 30% and a low FVC% of 34%. Patient B as shown on figure 3.24 and 3.25 sputum supernatant sample contained 2.42µmol/L TBA and had a high pulmonary function of FEV_1% 80 and FVC% 93. The two patients illustrate that duodenal GOR demonstrated by high levels of TBA in sputum supernatant was inconclusive regarding the relationship between GOR and decreased pulmonary function.

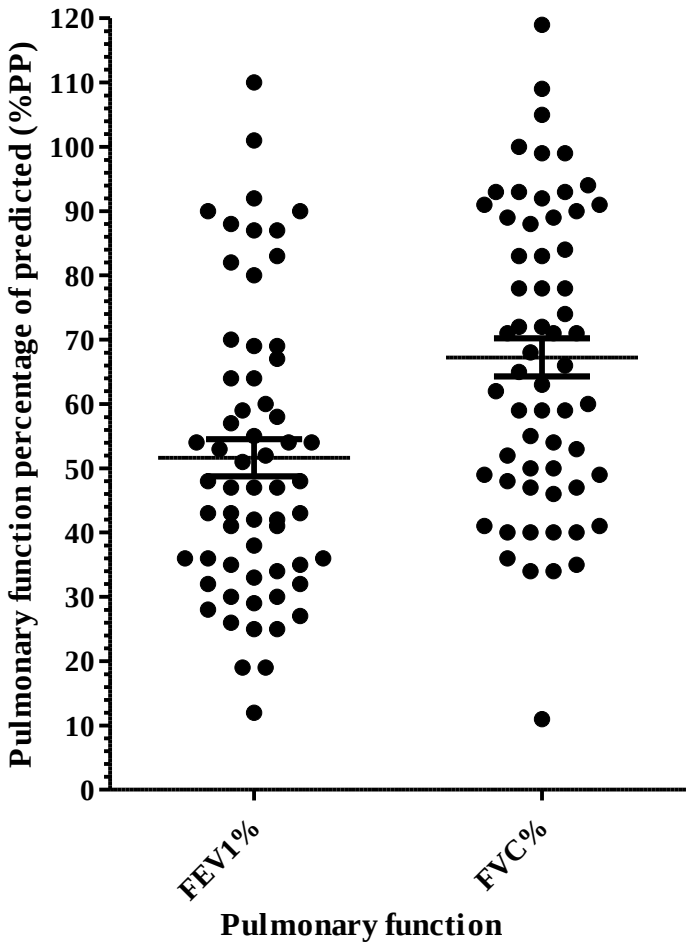


Figure 3.21 The pulmonary function of CF patients measured by FEV_1 and FVC percentage predicted. n=61 patients presented in the percentage of predicted pulmonary function. The median FEV_1% predicted was 47% (range 12-110%, SEM 2.9) and the median FVC% predicted was 66% (range 11-119%, SEM 3.0).

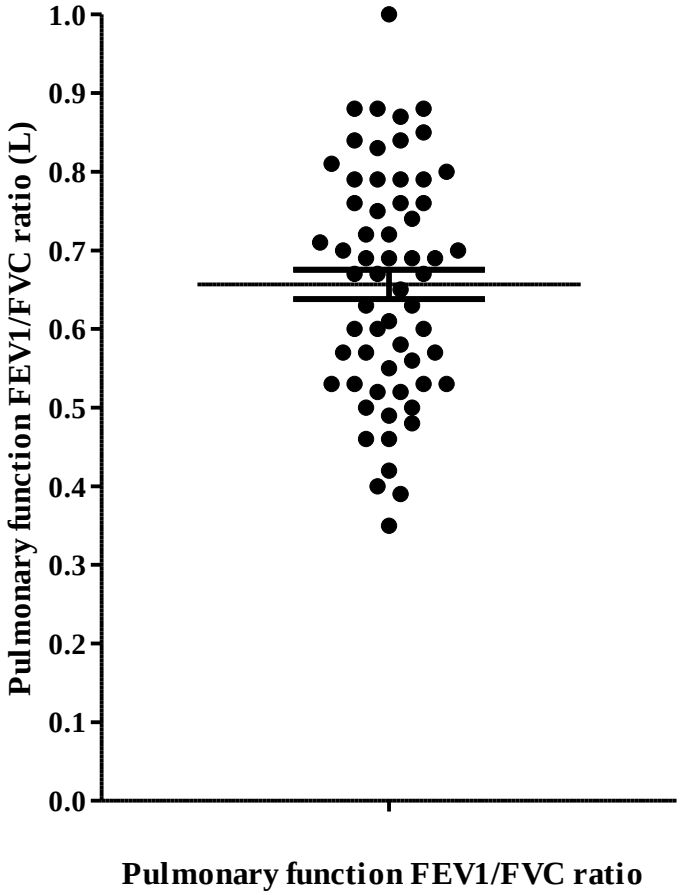


Figure 3.22 The FEV_1 and FVC ratio (L) of CF patients n=61. The range of FEV_1/FVC ration was between 0.35 and 1.0 with the median 0.67, (mean 0.66; SEM 0.02).

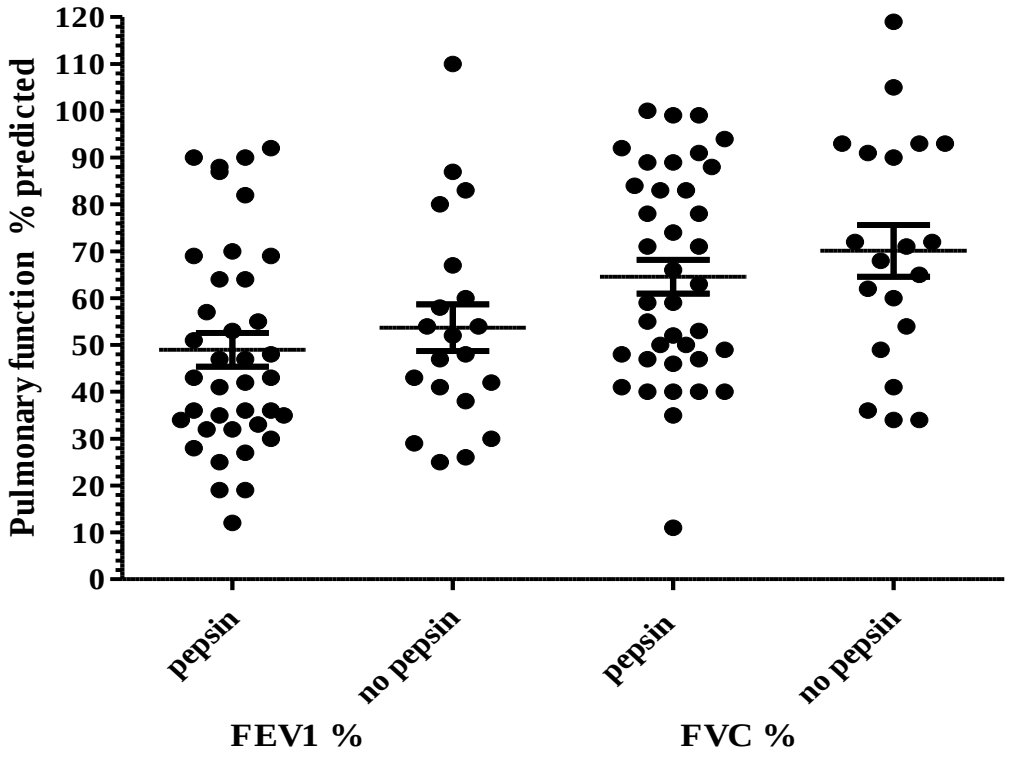


Figure 3.23 Pulmonary function test results (% predicted) of patients that experience gastric aspiration measured by pepsin ELISA (n=38) (FEV_1% range 12-92%, median 43, mean 49, SEM 4; FVC% range 11-100, median 61, mean 65, SEM 4) and those that did not experience gastric aspiration (n=20) (FEV_1% range 25-110, median 50, mean 54, SEM 5; FVC% range 34-119, median 70, mean 70, SEM 6). There was no statistical difference in pulmonary function in patients with gastric aspiration (FEV_1% P=0.46) (FVC% P= 0.44) analysed by Mann Whitney U test with Gaussian approximation.

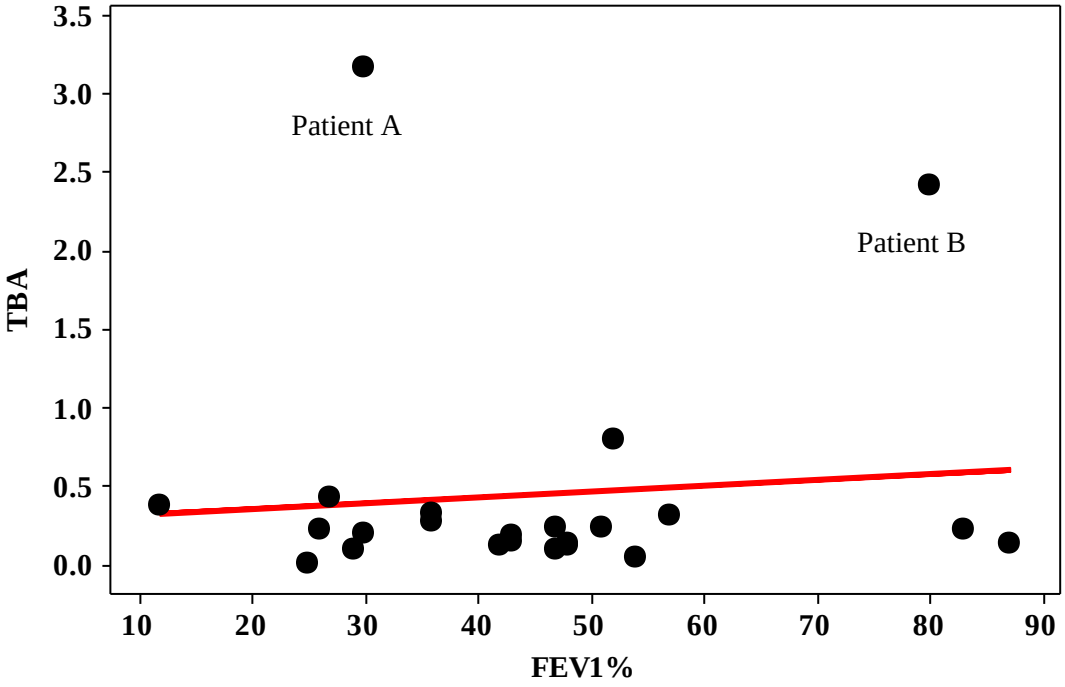


Figure 3.24 Sputum supernatant samples TBA levels compared to FEV_1% (n=22) on a XY scatter graph. The Pearson correlation test was used to analyse the statistical relationship between levels of TBA in sputum supernatant samples and the FEV_1% score of patients. There was no statistically difference between levels of TBA and FEV_1% (P=0.675; Pearson correlation of TBA and FEV_1% = -0.092).

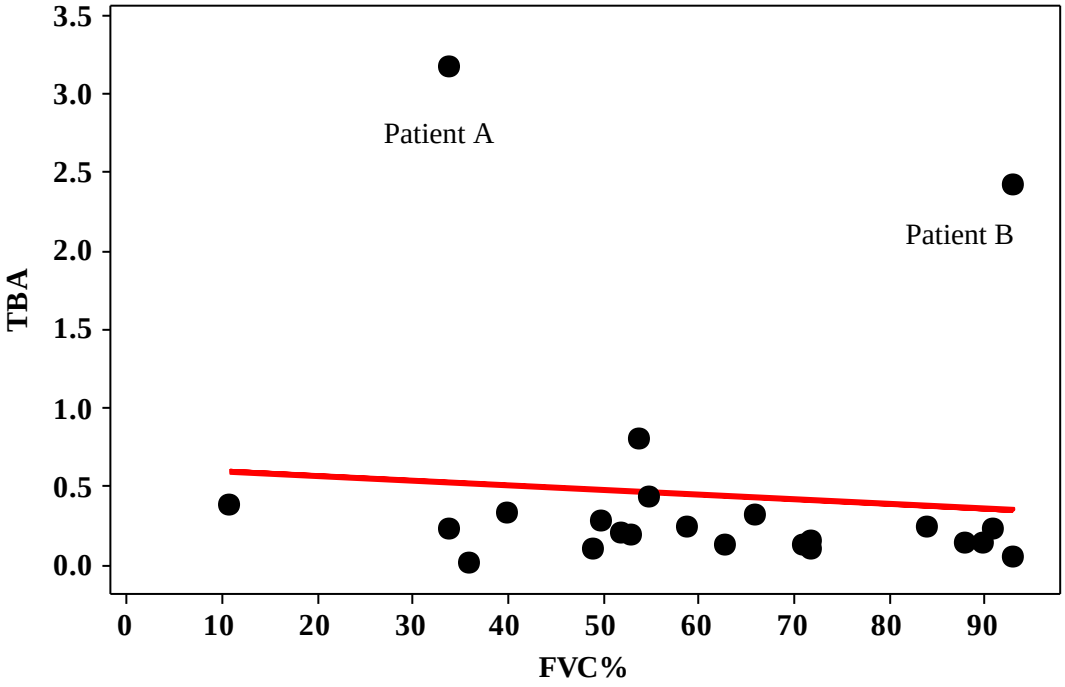


Figure 3.25 Sputum supernatant samples TBA levels compared to FVC% (n=22) on a XY scatter graph. The Pearson correlation test was used to analyse the statistical relationship between levels of TBA in sputum supernatant samples and the FVC% score of patients. There was no statistically difference between levels of TBA and FVC% (P=0.696; Pearson correlation of TBA and FVC%% = -0.086).

3.2.6 Neutrophilic inflammation and increased cytokines in sputum from cystic fibrosis population

The processing of sputum samples was carried out as directed by standard operating procedures of the cytology laboratory at the William Leech centre, Freeman Hospital. The viscous sputum plugs were selected from expectorate sample to reduce squamous cell contamination from saliva contained in the expectorated sputum sample. The contamination of squamous cells was required to be below 20% to ensure the sample was a valid representation of the airway and so inflammatory cell counting was quicker, easier, and reproducible (Efthimiadis *et al.*, 2002). There were 36 expectorated sputum samples processed and a cell differential was performed under a light microscope at X400. 500 inflammatory cells were counted and the percentage of cell types was calculated. There was a median of 100% neutrophils (range 97.2 to 100, mean 99.8, SEM 0.09) contained in the CF sputum samples (P= 0.0001). There was a median of 0.6% eosinophils (range 0.4 to 0.8, mean 0.6, SEM 0.12), a median of 1.3% macrophages (range 1 to 2, mean 0.4, SEM 0.30), and a median of 0.6% lymphocytes (range 0.2 to 1.2, mean 0.4, SEM 0.14) present in expectorated CF sputum.

The resulting supernatant of 64 sputum samples was analysed for IL-8 levels using the R&D systems IL-8 capture ELISA for recombinant and human IL-8. The disregard of saliva of expectorated sputum samples results in the analyte in sputum supernatant to be unaffected or diluted by the confounding influence of saliva. The analyte concentration was corrected for by the dilution factor (Efthimiadis *et al.*, 2002). The kit measured IL-8 concentrations between 32 pg/ml to 2000 pg/ml. Figure 3.26 shows a shows a typical standard curve of the IL-8 with an R^2 value of 0.99. The overall range of IL-8 was 1 to 35ng/ml with the median of 9ng/ml. Of the 64 patient samples analysed all the patient samples contained detectable levels of IL-8. Figure 3.27 displays the individual patient IL-8 values measured in sputum supernatant samples.

The resulting supernatant of 62 processed sputum samples was analysed for IL-6 concentrations using the R&D systems IL-6 capture ELISA for recombinant and human IL-6. The kit measured IL-6 to a level between 10 pg/ml and 600 pg/ml. The sputum supernatant was used at an x8 dilution introduced during the sputum processing procedure and no further dilution was required for the IL-6 analysis. Figure 3.28 shows a typical standard curve of the IL-6 standard with a R^2 value of 0.99. The overall range of IL-6 was 0 to 700pg/ml with the median of 0ng/ml. Of the 62 patient samples analysed there were

16 patient samples with detectable levels of IL-6 with a median of 100pg/ml ranging from 30 to 700pg/ml. There were 46 patient samples that did not contain IL-6 above the lowest level of accurate quantification of IL-6 at 10pg/ml. Figure 3.29 displays the individual patient IL-6 values measured in sputum supernatant samples.

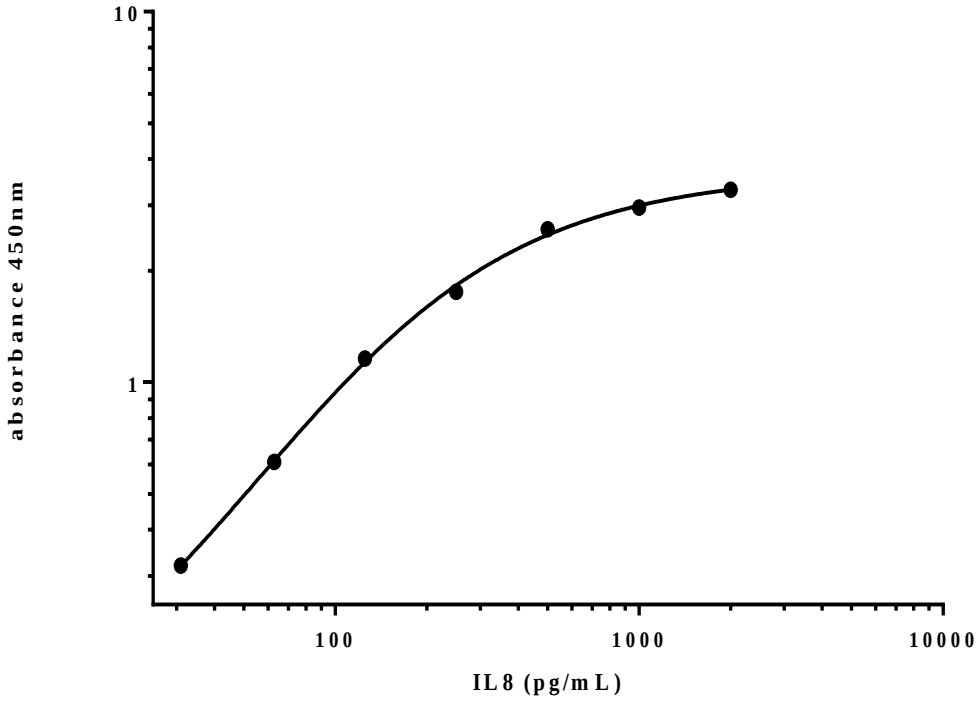


Figure 3.26 IL-8 standard curve measured with R&D systems kit for capture ELISA using mouse anti-human IL-8 capture antibody at 4µg/ml followed by biotinylated goat anti-human IL-8 at 20ng/ml (mean ± SD). The detection range of the ELISA was 32pg/ml to 2000pg/ml. The four parameter regression curve fit gave a R^2 value of 0.99.

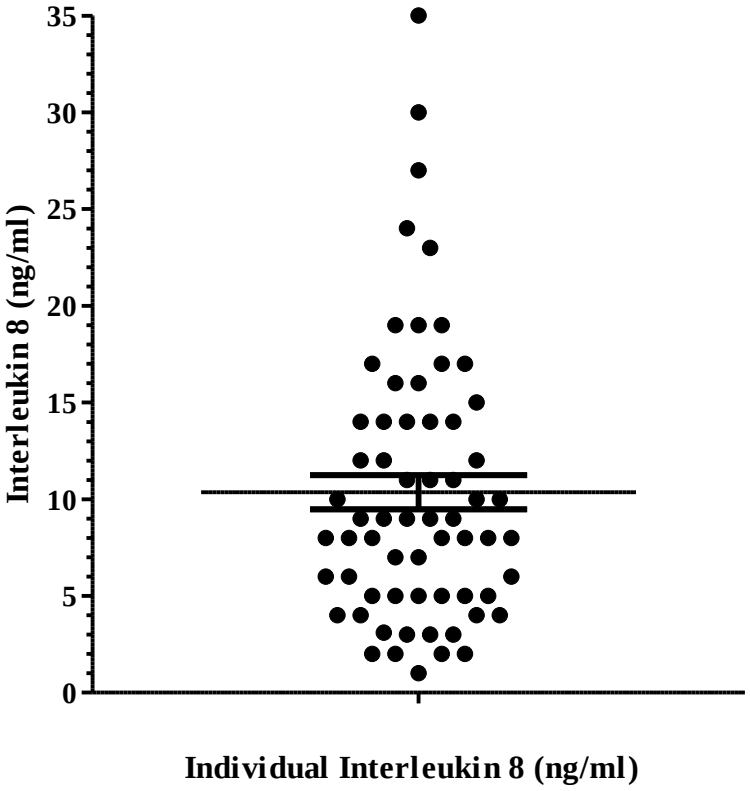


Figure 3.27 The analysis of sputum supernatant IL-8 concentration (ng/ml) in 64 CF patients measured with the R&D Systems IL-8 kit for capture ELISA using mouse anti-human IL-8 capture antibody at 4µg/ml followed by biotinylated goat anti-human IL-8 at 20ng/ml. The IL-8 concentration ranged from 1 to 35 ng/ml, with a median of 9ng/ml; a mean of 10ng/ml, and SEM 1ng/ml.

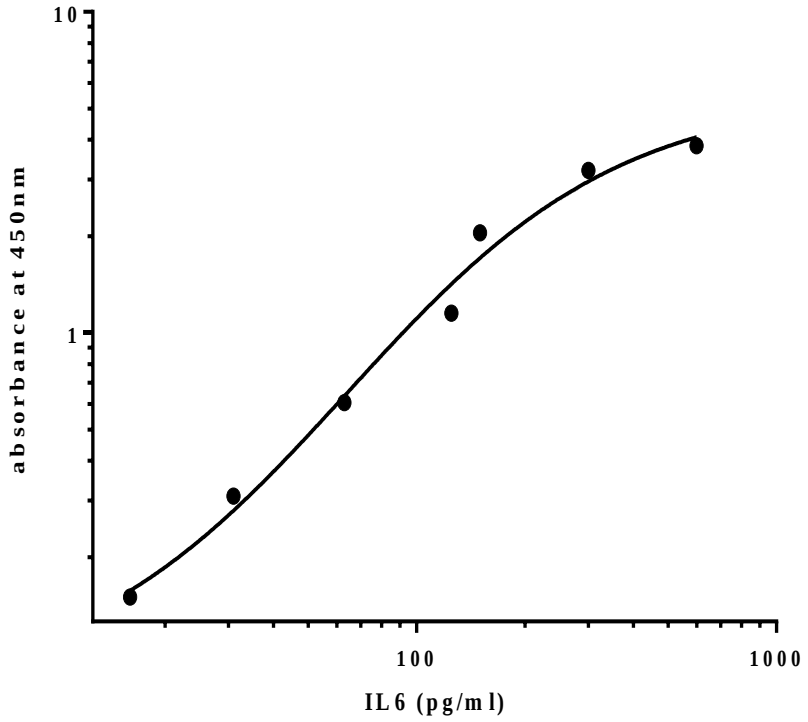


Figure 3.28 IL-6 standard curve measured with R&D systems kit for capture ELISA using mouse anti-human IL-6 capture antibody at 2µg/ml followed by biotinylated goat anti-human IL-6 at 50ng/ml (mean ± SD). The detection limit of the ELISA was 10pg/ml to 600pg/ml. Variable slope of four parameters gave an, R^2 value of 0.99.

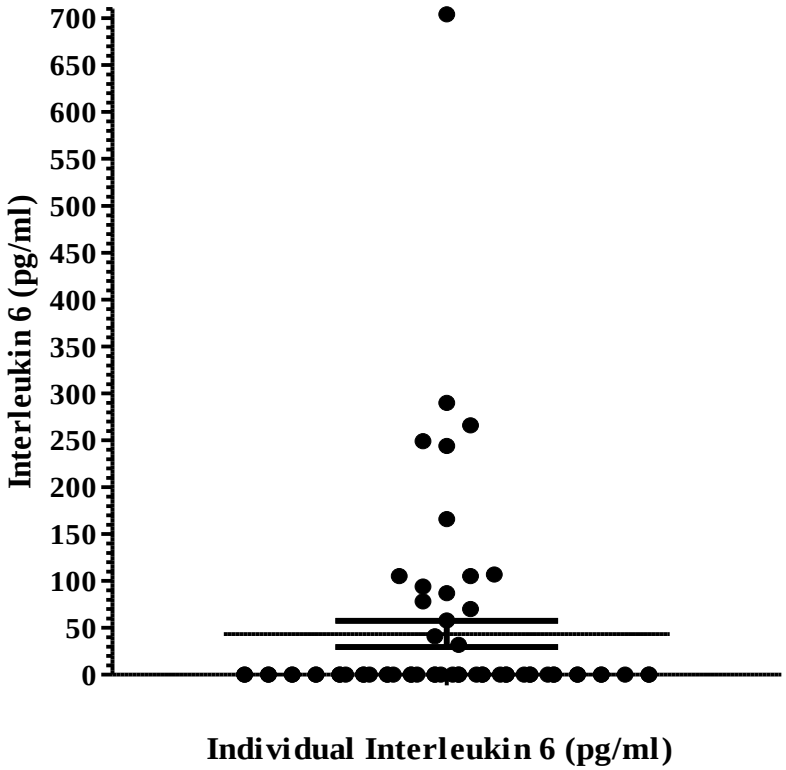


Figure 3.29 The analysis of sputum supernatant IL-6 concentration (pg/ml) in 62 CF patients measured with the R&D Systems IL-6 kit for capture ELISA using mouse anti-human IL-6 capture antibody at 2µg/ml followed by biotinylated goat anti-human IL-6 at 50ng/ml. The IL-6 concentration ranged from 0 to 700pg/ml, with a median of 0pg/ml; a mean of 43pg/ml, and SEM 10pg/ml.

3.2.7 Correlation between cytokine production and the CFTR mutation in the CF population

The concentrations of IL-8 and IL-6 cytokine production in the airways of CF patients were assessed in patients homozygous and heterozygous for delta F508. The median levels do not differ significantly (IL-8 homozygous median was 5ng/ml and heterozygous median 10ng/ml; P=0.46; IL-6 homozygous median was 0pg/ml and heterozygous median 0pg/ml; P=0.16) but the range of IL-8 and IL-6 are higher in the heterozygous group of patients (IL-8 range 1 to 30ng/ml; IL-6 0 to 700pg/ml) than the homozygous range (IL-8 range 2 to 23ng/ml; IL-6 range 0 to 166pg/ml) as illustrated in figure 3.30 and 3.31.

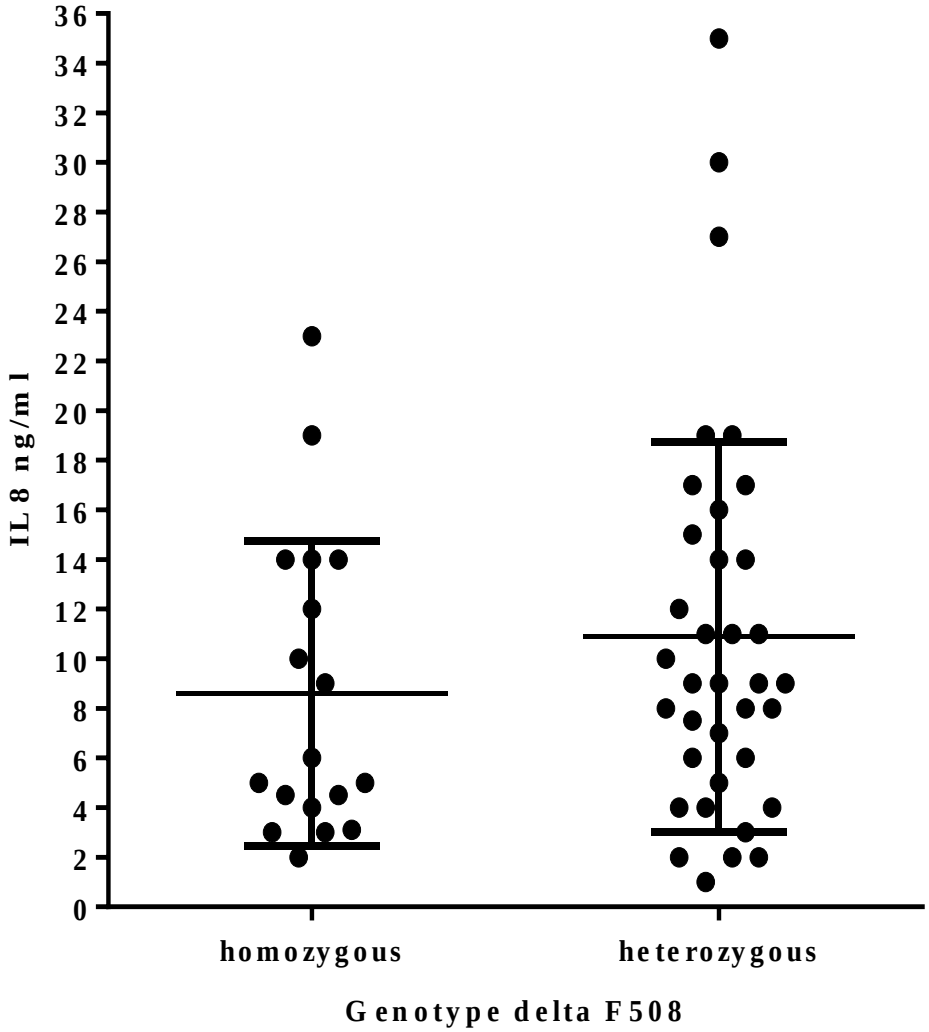


Figure 3.30 IL-8 concentrations of patients depending on genotype. Homozygous IL8 median levels were 5ng/ml ranging from 2 to 20ng/ml (n=18, n=4 missing data, mean 8.6, SEM 1.45) and heterozygous IL-8 median levels were 10ng/ml ranging from 1 to 35 ng/ml (n=36, n=3 missing data, mean 11, SEM 1.31). Nonparametric Two tailed T-test (Mann-Whitney test with Gaussian approximation) show no significant difference between the IL-8 levels of patient who are homozygous or heterozygous for delta F 508 (P=0.46).

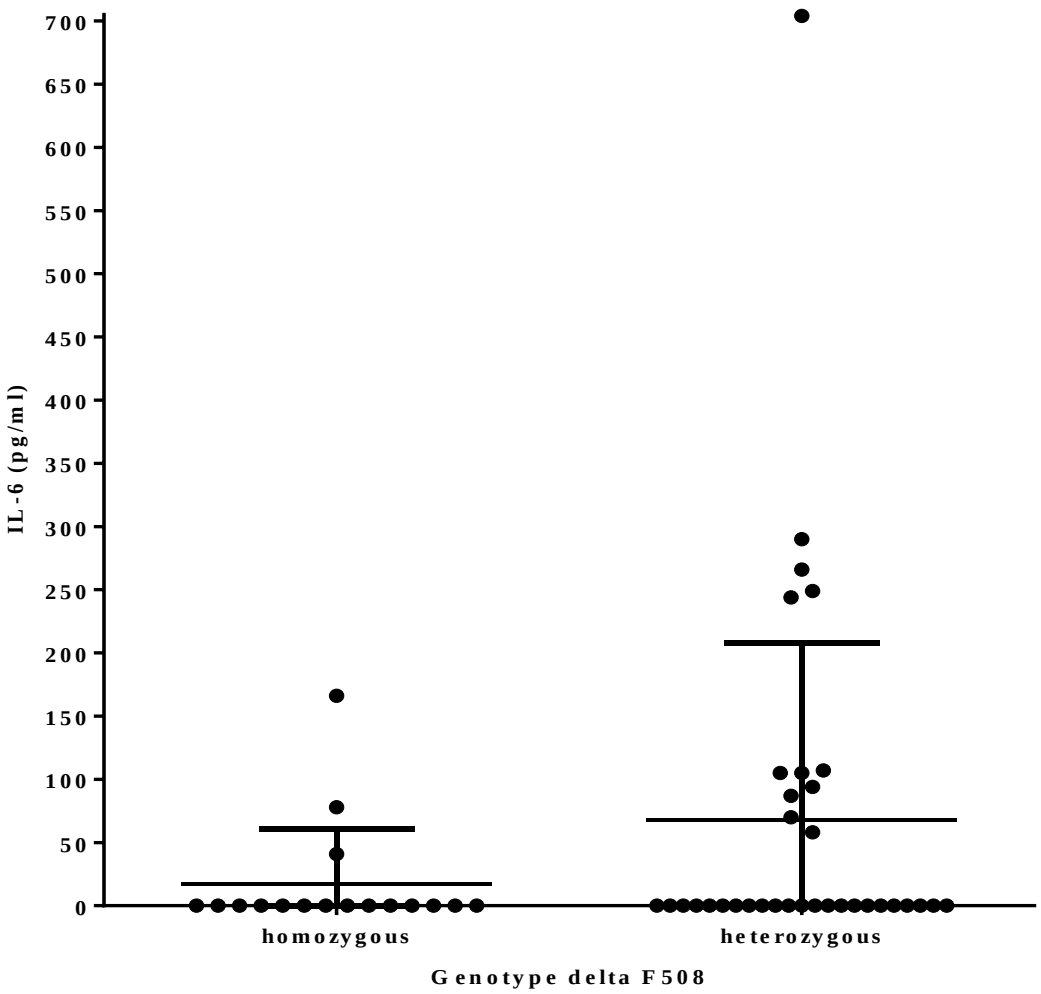


Figure 3.31 IL-6 concentrations of patients depending on genotype Homozygous IL-6 median levels were 0pg/ml ranging from 0 to 166pg/ml (n=17, n=5 missing data, mean 17, SEM 10.6) and heterozygous IL-6 median levels were 0pg/ml ranging from 0 to 704pg/ml (n=35, n=4 missing data, mean 68, SEM 23.7). Nonparametric Two tailed T-test (Mann-Whitney test with Gaussian approximation) show no significant difference between the IL-6 levels of patient who are homozygous or heterozygous for delta F 508 (P=0.16).

3.2.8 Correlation between cytokine production and GOR/EOR symptoms in the CF population

The DeMeester questionnaire score was used to determine patients symptomatic of GOR. The patients were grouped into those that were symptomatic of GOR scoring 2 or above on the questionnaire (GOR) and those that were asymptomatic (no GOR) of GOR scoring 1 or below. The IL-8 levels detected in each group was compared using a non-parametric two-tailed statistical test which showed that there were no statistical differences of IL-8 levels detected in sputum samples and the presence of GOR symptoms (p=0.38). The median level of IL-8 detected in the GOR symptomatic group was 10ng/ml (n=29, range 1-30ng/ml) and median levels in the asymptomatic group were 8ng/ml (n=35, range 1-35ng/ml) (Figure 3.32). The same analysis was used to identify a relationship of IL-6 levels and GOR. There was no statistical difference of IL-6 levels and GOR symptoms (P=0.12). The median IL-6 levels in the GOR symptomatic group were 0pg/ml (n=29, range 0-270pg/ml) and the asymptomatic group median IL-6 scores were 0pg/ml (n=33, range 0-700pg/ml) as shown in figure 3.33.

The RSI questionnaire score was used to determine the presence of EOR symptoms in the aero digestive tract and used to identify EOR symptomatic patients. The patients were grouped into those that were symptomatic of EOR scoring 13 and above on the questionnaire (EOR) and those that were asymptomatic (no EOR) of EOR scoring 12 or below. The IL-8 levels detected in each group was compared using a non-parametric two-tailed statistical test to show that there were no statistical differences of IL-8 levels detected in sputum samples and the presence of EOR symptoms (p=0.72). The median level of IL-8 detected in the EOR symptomatic group was 8ng/ml (n=43, range 1-35ng/ml) and median levels in the asymptomatic group were 9ng/ml (n=21, range 2-30ng/ml) (Figure 3.34). The same analysis was used to identify a relationship of IL-6 levels and EOR. There was no statistical difference of IL-6 levels and EOR symptoms (P=0.63). The median IL-6 levels in the EOR symptomatic group were 0pg/ml (n=41, range 0-290pg/ml) and the asymptomatic group median IL-6 scores were 0pg/ml (n=21, range 0-700pg/ml as shown in Figure 3.35.

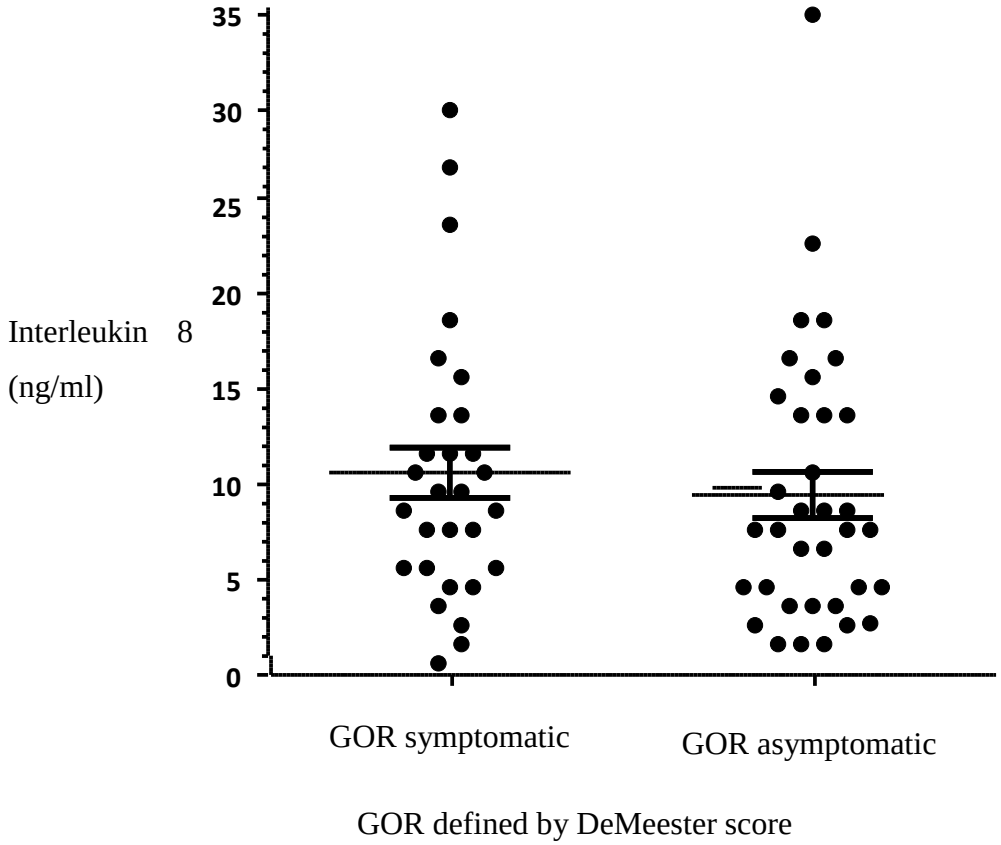


Figure 3.32 Sputum supernatant IL-8 levels as measured by R&D Systems capture ELISA in GOR symptomatic patients defined as having a DeMeester score 2 or above (n=29, range 1-30ng/ml, median 10, mean 11, SEM 1.3) and asymptomatic patients defined as having a DeMeester score of 1 or less (n=35, range 2-35ng/ml, median 8, mean 10, SEM 1.2). There was no statistical difference of IL-8 levels between symptomatic and asymptomatic GOR patients (P=0.38) analysed by Mann Whitney U test with Gaussian approximation.

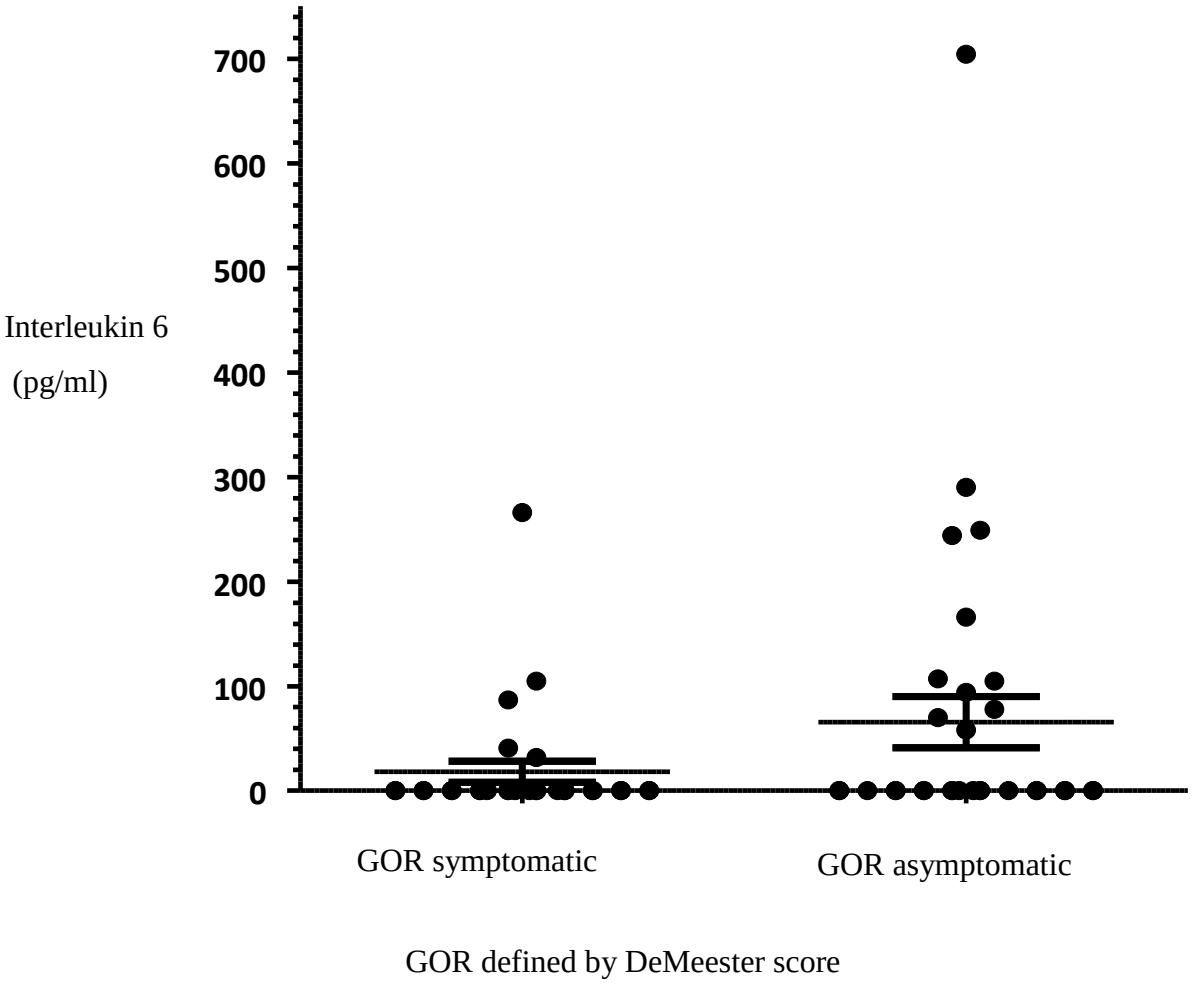


Figure 3.33 Sputum supernatant IL-6 levels as measured by R&D Systems capture ELISA in GOR symptomatic patients defined as having a DeMeester score 2 or above (n=29, range 0-266pg/ml, median 0, mean 18, SEM 10) and asymptomatic GOR patients defined as having a DeMeester score of 1 or less (n=33, range 0-704pg/ml, median 0, mean 66, SEM 24). There was no statistical difference of IL-6 levels between symptomatic and asymptomatic GOR patients (P=0.12) analysed by Mann Whitney U test with Gaussian approximation.

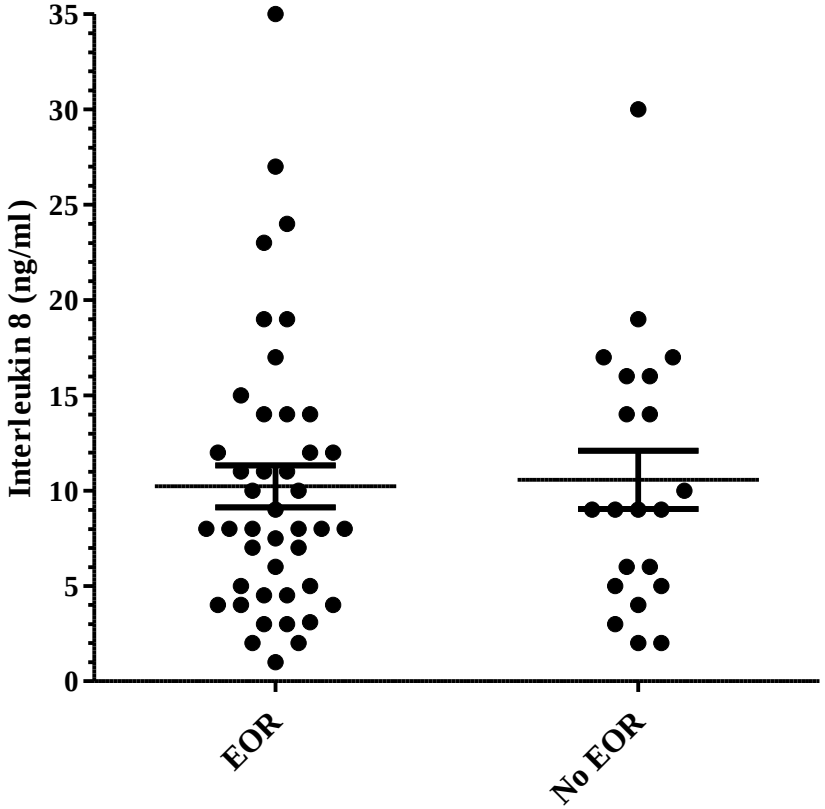


Figure 3.34 Sputum supernatant IL-8 levels as measured by R&D Systems capture ELISA in EOR symptomatic patients defined as having a RSI score 13 or above (n=43, range 1-35ng/ml, median 8, mean 10, SEM 1) and asymptomatic EOR patients defined as having a RSI score 12 or below (n=21, range 2-30ng/ml, median 9, mean 11, SEM 2). There was no statistical difference of IL-8 levels between symptomatic and asymptomatic EOR patients (P=0.72) analysed by Mann Whitney U test with Gaussian approximation.

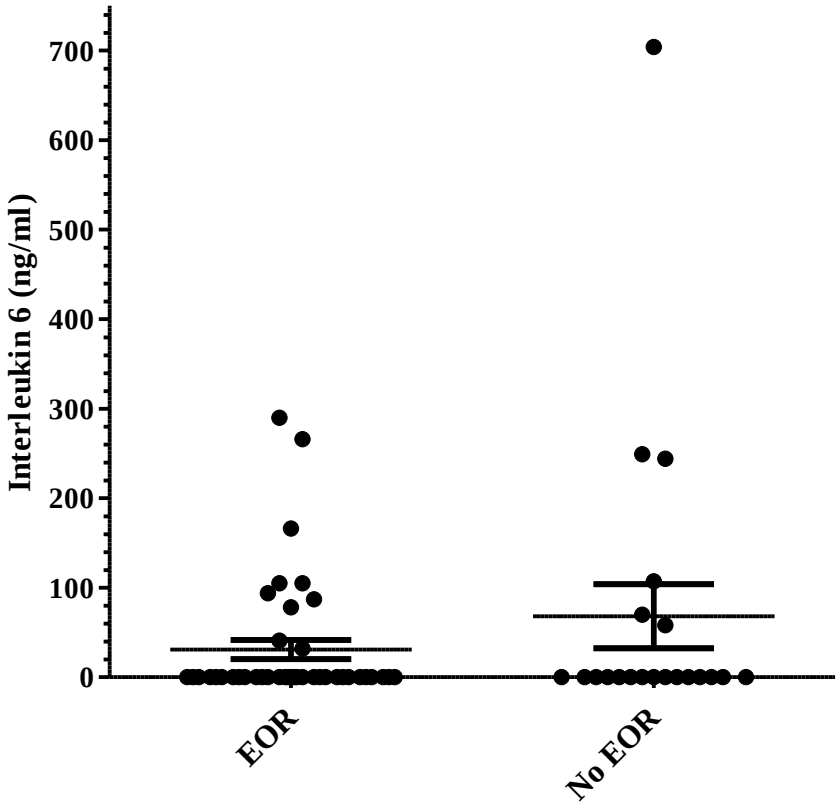


Figure 3.35 Sputum supernatant IL-6 levels as measured by R&D Systems capture ELISA in EOR symptomatic patients defined as having a RSI score 13 or above (n=41, range 0-290pg/ml, median 0, mean 31, SEM 11) and asymptomatic EOR patients defined as having a RSI score 12 or below (n=21, range 0-704pg/ml, median 0, mean 68, SEM 36). There was no statistical difference of IL-6 levels between symptomatic and asymptomatic EOR patients (P=0.63) analysed by Mann Whitney U test with Gaussian approximation.

3.2.9 Cytokine production and gastric aspiration in the CF population

Gastric aspiration was investigated by identifying pepsin and total bile acids (TBA) levels in sputum supernatant samples. Firstly, the investigation into the relationship between gastric aspiration determined by the presence of pepsin and its effects on cytokine levels in the airways was assessed. The patients were grouped into those that had measurable amounts of pepsin levels (above 1ng/100µl) in sputum supernatant and those that did not have measurable pepsin levels identified above the lower detection limit of 1ng/100µl assayed on the in-house indirect pepsin ELISA. The levels of IL-8 and IL-6 in each group were compared to identify any effects of gastric aspiration on the production of these cytokines. The median level of IL-8 detected in the pepsin positive group was 8ng/ml (n=46, range 2-36ng/ml) and median level in the group that did not have pepsin detected was 10ng/ml (n=18, range 1-28ng/ml) (Figure 3.36). There was no statistical difference between levels of IL-8 in the sputum samples from the sputa of CF patients and the presence of gastric aspiration confirmed by pepsin identification (P=0.33). The relationship between levels of IL-6 and gastric aspiration detected by pepsin identification was not statistically significant (P=0.70). The median IL-6 levels in each group was 0pg/ml (pepsin positive n=44, range 0-290; pepsin undetectable n=18, range 0.700) but interestingly one patient that did not have pepsin present in the sputum supernatant had a very high level of 700pg/ml IL-6 (Figure 3.37).

The relationship between the levels of TBA detected in the sputum supernatant of CF patients and the production of IL-8 and IL-6 cytokines was explored. Of the 24 patient sputum samples that were analysed for TBA's there were 22 patient samples analysed for IL-8 and IL-6 due to 2 patient samples being too small for multiple analysis. A scatter graph was produced of TBA levels along the y axis and IL-8 (Figure 3.38) and IL-6 (Figure 3.39) levels on the x axis because TBA's were present in all patient samples and therefore the patients could not be divided into groups of gastric aspiration present or absence on TBA detection. A linear regression fit illustrated the relationship of increasing TBA levels with increasing cytokine levels. There were no statistical relationship between TBA levels in the sputum supernatant of patients and cytokine production of IL-8 (P=1.00) and IL-6 (P=0.487). Interestingly, two patients with the highest TBA levels of 3.17 and 0.80µmol/L had no IL-6 detected within the sputa in the airway collected by expectorated sputum sample (median TBA result of patients with no detectable IL-6 was 0.23µmol/L; range 0.09 to 3.17) compared to 7 patients with IL-6 production that had low levels of

TBA below 0.27µmol/L (median 0.19µmol/L; range 0.00 to 1.27) illustrated in Figure 3.39.

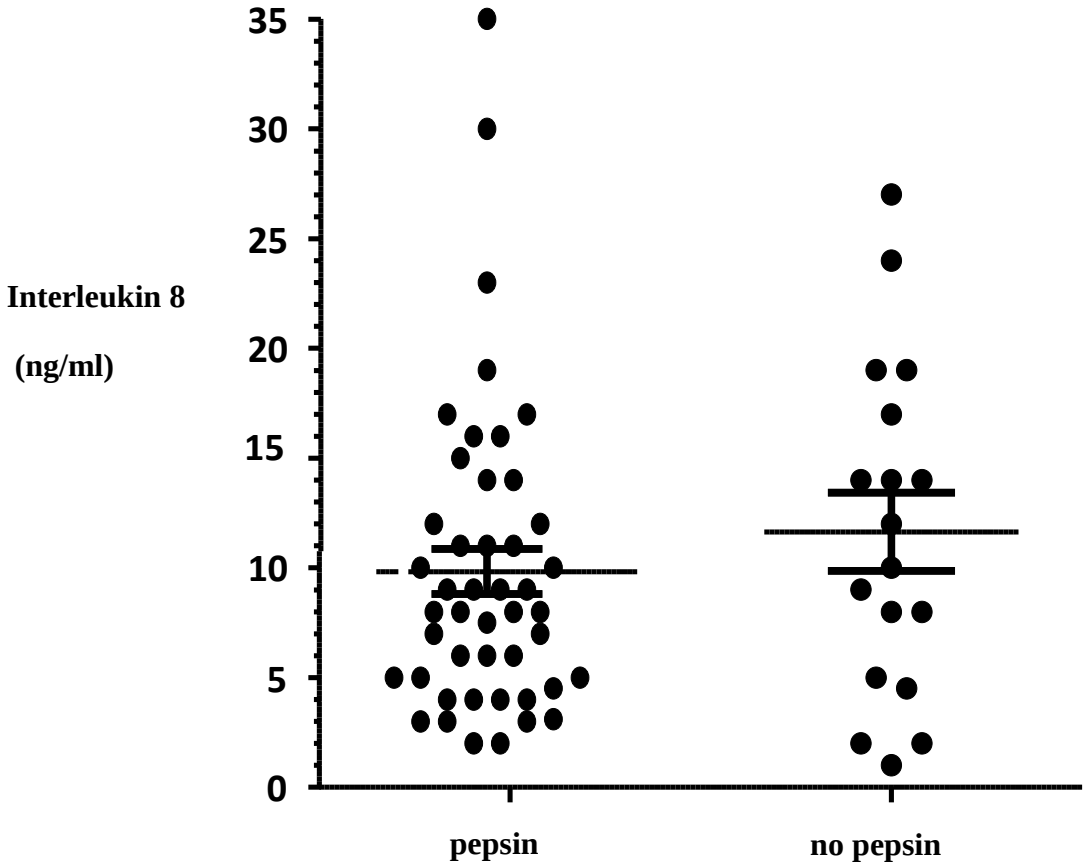


Figure 3.36 Sputum supernatant samples IL-8 levels in patients that have gastric aspiration measured by pepsin ELISA (n=46, range 2-35ng/ml, median 8, mean 10, SEM 1) and those that did not have gastric aspiration (n=18, range 1-28, median 11, mean 12, SEM 2). There was no statistical difference of IL-8 levels and gastric aspiration (P=0.33) analysed by Mann Whitney U test with Gaussian approximation.

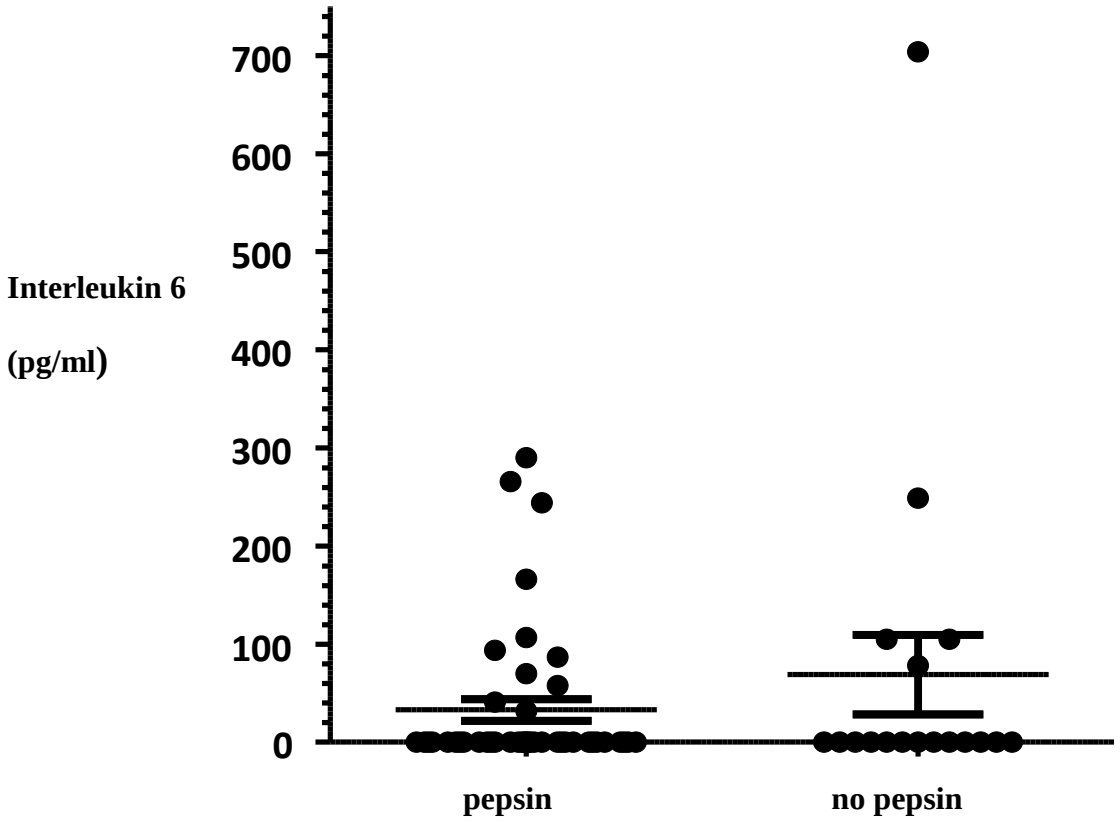


Figure 3.37 Sputum supernatant samples IL-6 levels in patients that have gastric aspiration measured by pepsin ELISA (n=44, range 0-290, median 0, mean 32, SEM 11) and those that did not have gastric aspiration (n=18, range 0-700, median 0, mean 70, SEM 40). There was no statistical difference of IL-6 levels and gastric aspiration (P=0.70) analysed by Mann Whitney U test with Gaussian approximation.

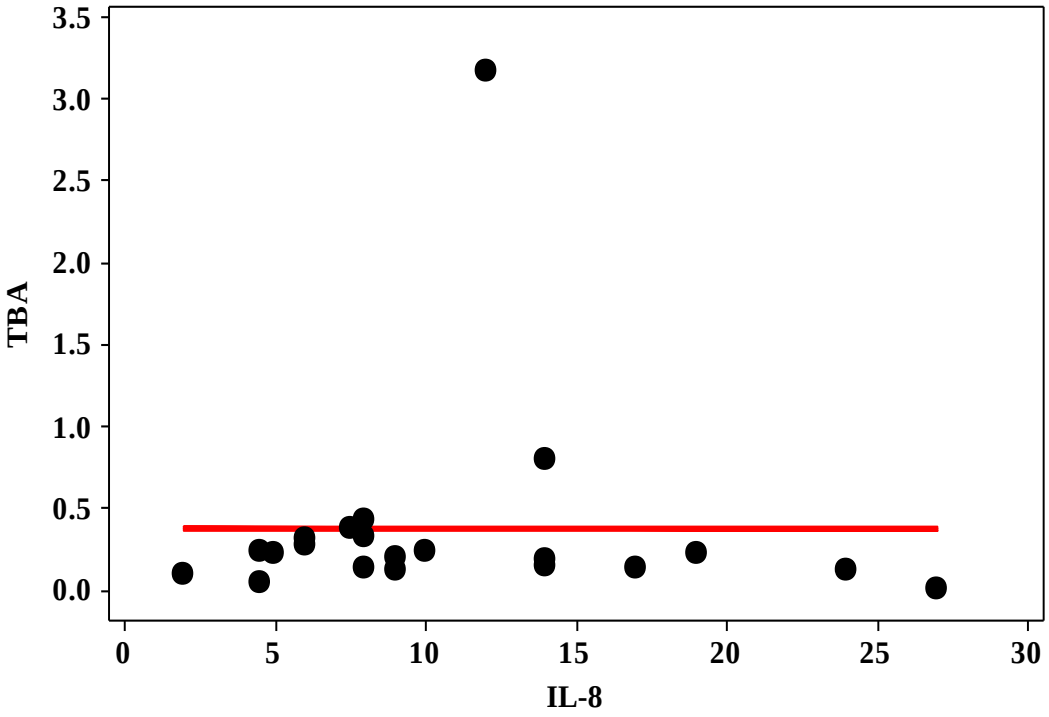


Figure 3.38 Sputum supernatant samples IL-8 levels compared to TBA levels (n=22) on a XY scatter graph. The Pearson correlation test was used to analyse the statistical relationship between levels of IL-8 and TBA in sputum supernatant samples. There was no statistical difference between the two analytes either positive or negative (R^2=0.0%, n=22, P=1.00; Pearson correlation of TBA and IL-8 = -0.000).

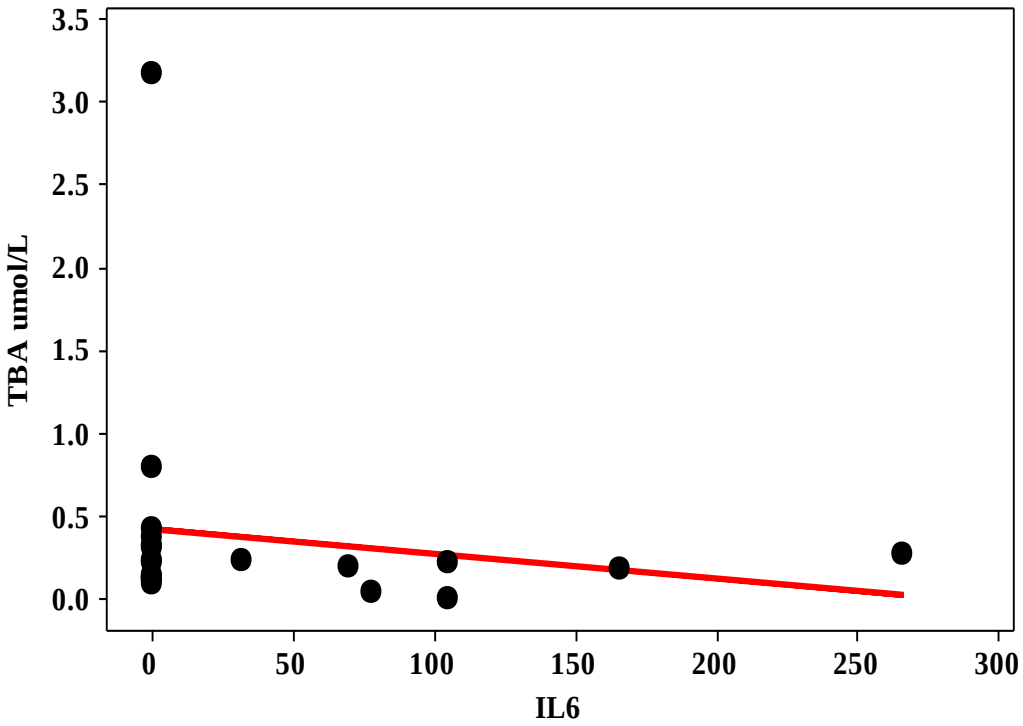
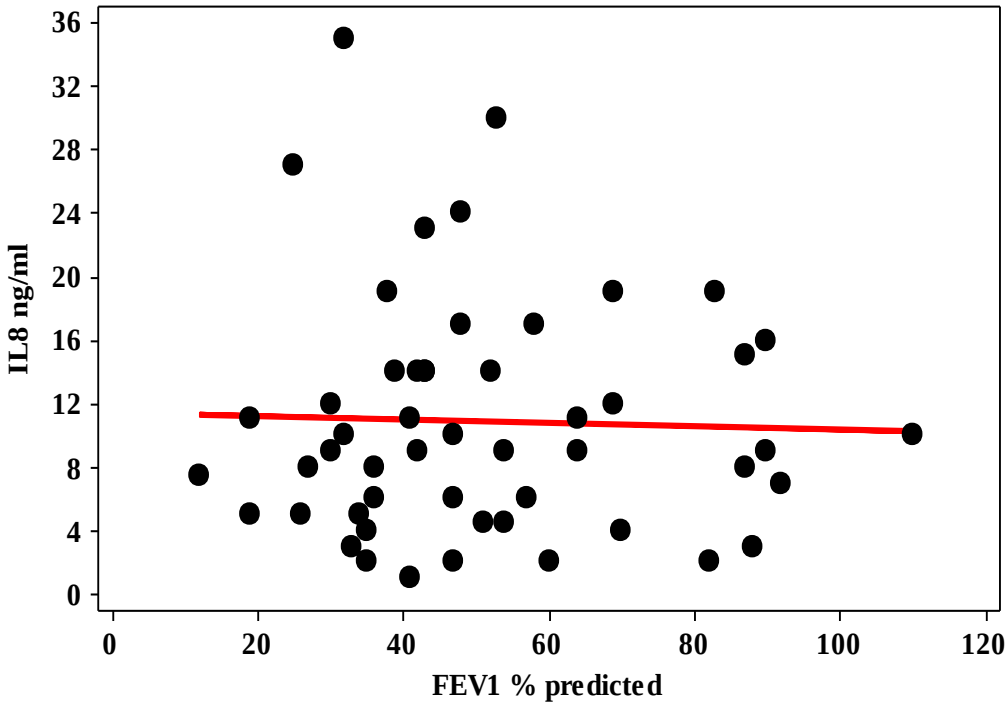


Figure 3.39 Sputum supernatant samples IL-6 levels compared to TBA levels (n=22) on a XY scatter graph. The Pearson correlation statistical test was used to analyse the statistical relationship between levels of IL-6 and TBA in sputum supernatant samples. There was no statistical difference between the two analytes either positive or negative in sputum supernatant samples (R^2=2.6%, n=22; P-Value = 0.487; Pearson correlation of TBA and IL-6 = -0.161).

3.2.10 Cytokine levels and pulmonary function in the CF population

The levels of cytokine production (IL-8 and IL-6) in the airways was assessed by capture ELISA (R&D systems). The production of cytokines (levels of IL-8 and IL-6) and their association with pulmonary function was evaluated by plotting the data points of each cytokine on the Y axis and plotting the pulmonary function (FEV_1 and FVC % predicted) along the X axis of a scatterplot. A linear regression curve was fitted to identify the relationship of increasing cytokines and pulmonary function. The production of IL-8 levels (FEV_1% n=60, P=0.811, R^2 0.1%; FVC% n=60, P=0.493, $R^2$0.9%) and IL-6 (FEV_1% n=60, P=0.265, R^2 2.5%; FVC% n=60, P=0.126, R^2 4.6%) were not statistically related to pulmonary function.

A



B

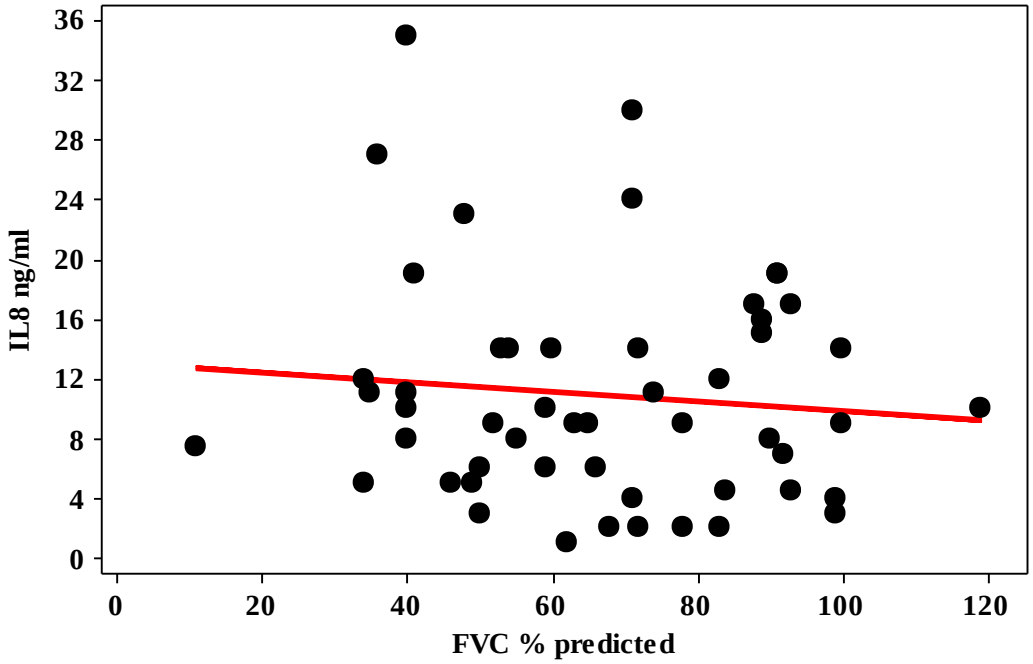


Figure 3.40 The presented data illustrates the XY scatterplot of patient IL-8 levels on the y axis and patient FEV_1% (A) (n=60; R^2 0.1%) and FVC% (B) (n=60; R^2 0.9%) predicted scores along the X axis. The Pearson correlation statistical test was used to analyse the relationship between levels of IL-8 and pulmonary function (FEV_1% R^2=0.1%,P=0.811; FVC% R^2=0.9%,P=0.493).

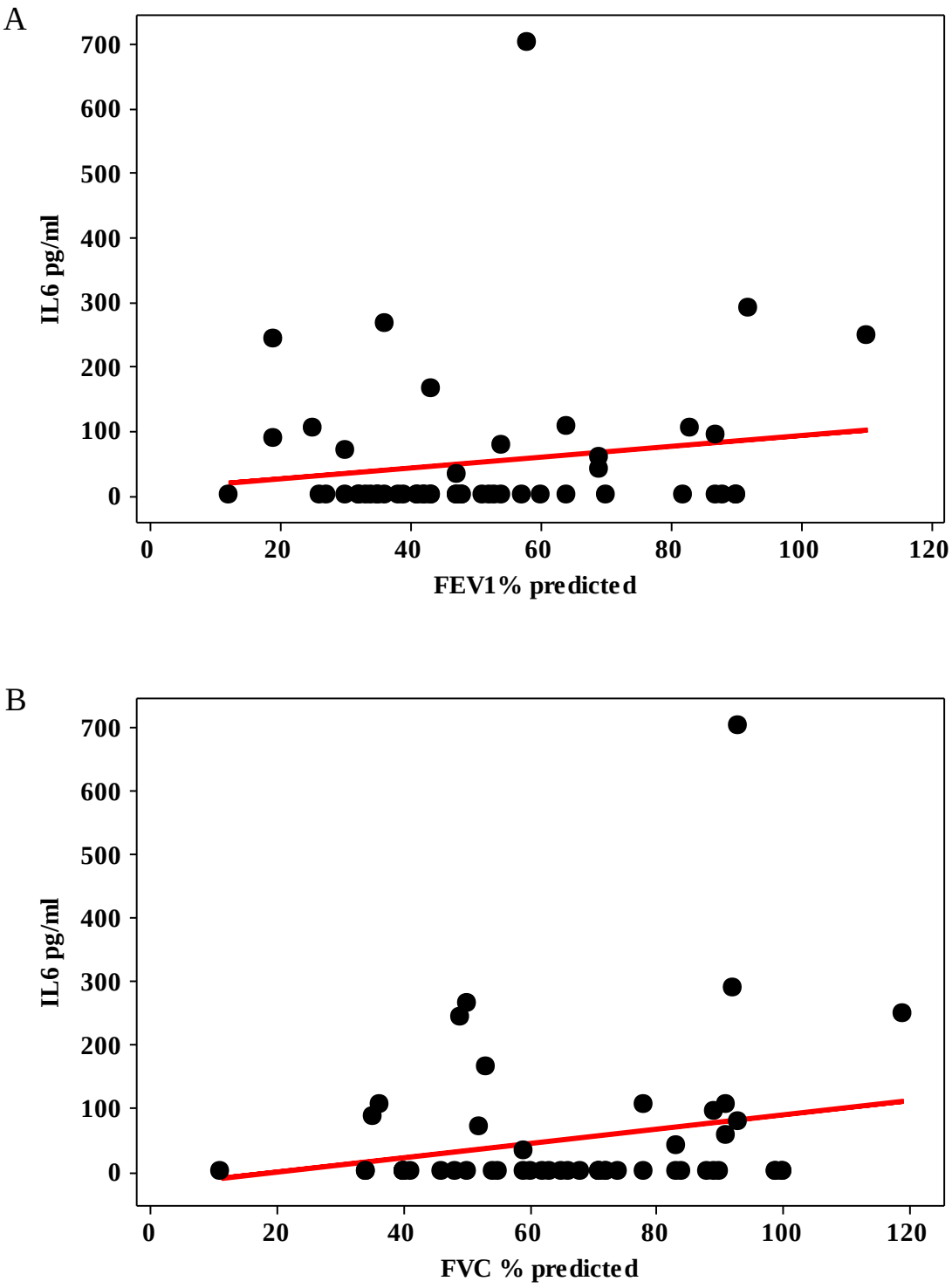


Figure 3.41 The presented data illustrates the XY scatterplot of patient IL-6 levels on the y axis and patient FEV_1% (A) (n=60; R^2 2.5%) and FVC% (B) (n=60; R^2 4.6%) predicted scores along the X axis. The Pearson correlation statistical test was used to analyse the relationship between levels of IL-6 and pulmonary function (FEV_1% R^2=2.5%, P=0.265; FVC% R^2=4.6%, P=0.126).

3.3 Discussion

3.3.1 The symptoms of GOR and EOR in the study population

It is a normal occurrence for the healthy population to experience oesophageal reflux episodes. These episodes entering the oesophagus are a mixture of liquid and gas leaving the stomach, pH and impedance illustrated that these can be non-acidic, acidic or weakly acidic (Sifrim, *et al.,* 2001; Zerbib, *et al.,* 2005). The common belch, termed eructation is the escape of air released from the oesophagus into the pharynx. Air swallowed into the stomach during eating is intragastric air and subsequently re-enters the oesophagus by the transient lower oesophageal sphincter relaxation to prevent this air entering the pylorus and intestines. It has been demonstrated that these can occur 25 to 30 times per day in the normal population (Bredenoord, *et al.,* 2013). However, these normal physiological events may become of increased frequency (Mittal *et al.*, 1995), more acidic (Sifrim, *et al.,* 2001), of larger volume and of increased oesophageal exposure time (Dent *et al.*, 1980; Weusten *et al.*, 1995). These abnormal reflux episodes result in a multitude of potential complications affecting the oesophagus, pharynx, nasal cavity and the airways termed GORD. It also results in injury to the epithelium of the oesophagus causing erosions, inflammation, and in severe cases carcinoma. It is reported that the prevalence of GORD in the general population is 10–20% in the Western world and 5% in Asia. GORD is demonstrated to decrease the quality of life and also lead to mortality (Dent *et al.*, 2005; Armstrong, *et al.,* 2010).

The prevalence figures from the general population highlight the high degree of GOR prevalence in the CF population at 63-80% and EOR prevalence even higher at 94% (Ledson *et al.*, 1998). The following study aimed to assess the prevalence and characteristics of reflux in 72 patients recruited from the RVI, Newcastle upon Tyne. The present study found that the prevalence of classical GOR symptoms (heartburn, regurgitation and dysphagia) was 42% which is twice that found in the general population, however it was lower than the CF prevalence data reported in the literature. An early study describing the prevalence of classical GOR symptoms at 80% did not mention if the patients were taking acid suppression medication to alleviate symptoms (Ledson *et al.*, 1998) which is one reason the present data differ. One recent study in 2010, reported the prevalence of 63% and over half of those were on PPI medication (Sabati *et al.*, 2010). The prevalence in our current is similar to this and we report that of the n=30 GOR

symptomatic patients 70% were on PPI or H_2RA. We also note that EOR symptoms were reported in 63% of the whole study population and specifically airway reflux cough present in 50% of the 22 patients who completed the HARQ questionnaire. Of the 45 patients that were EOR symptomatic 73% were on acid suppression medication. And over half the patients with airway reflux demonstrated by the HARQ were on PPI. This illustrates that PPI medication does not alleviate upper airways symptoms in many of the CF patients.

Many studies have explored oesophageal reflux in the CF population using pH metry and intraluminal impedance. It has been reported that 85% (28/33) CF patients presented GOR that was mostly acidic (Blondeau *et al.,* 2008a). In a small number of 11 patients with classical GOR symtoms, Ledson and colleagues (1998) demonstrated with impedance studies that 8 had GOR. Interestingly, ambulatory tracheal investigation demonstrated that 4 patients experienced tracheal acidification accompanied by GOR episodes. The oesophageal studies used small numbers but these were appropriate to confirm reflux episodes and explore the pH and the distance it reaches up the oesophagus.

The advantage of the questionnaire tool within this study was high patient compliance, as evidenced by the collection of GOR and EOR symptoms in all the patients recruited. The questionnaires are validated tools and they are inexpensive, easy to use and produce 100% patient data collection. Sabati *et al.,* (2010) recruited 201 patients and assessed GOR with two validated questionnaires. Whereas, invasive pH and oesophageal impedance studies have a much smaller study number due to the invasive nature of the research tool. For example Blondeau *et al.* (2008) recruited 33 CF patients to explore GOR and Ledson *et al.,* (1998) recruited 11 CF patients. However, these research tools do not allow for the investigation of gastric aspiration.

3.3.2 Evidence of pepsin and bile acid aspiration in the CF population

It has been demonstrated that gastric contents may reach the proximal oesophagus, the trachea via saliva from the pharynx and the bronchioles identified via sputa from the larger airways. The production of sputum on the outside of airway epithelium is a common characteristic in CF, the patients are encouraged to expectorate the sputum via physiotherapy methods to clear the airways. Therefore the collection of sputum to explore gastric aspiration was an advantage of the study design. The disease in question has the characteristic of sputum production and we asked patients to expectorate random sample during recruitment in the out-patient clinic allowing for a non-invasive sample.

Unfortunately there were a small number of patients that produced small quantities of expectorated sputum and consequently resulted in missing data from analysis of the analytes pepsin (n=3), IL-8 (n=8) and IL-6 (n=10) due to insufficient sample quantity. Nebulised sputum production could have been used however this procedure would have required ethical approval amendment which would have delayed the project progression due to the procedures invasive nature. A trained nurse would have been required to accompany the patient and required to provide nebuliser equipment. The study did not include this technique but future studies could use this if the patient has difficulty to produce increased quantity of sputum and thus allow for more components in the sputum to be analysed with full n number in each. The possibility of increasing pepsin concentration due to the procedures ability to induce a GOR even would have caused false positive results.

There is evidence to support the presence of gastric contents in the airways of the CF population. The two analytes pepsin and bile have been widely used as a biomarker of aspiration in other airway diseases and are reported in the literature using various samples from the airway such as BAL and sputum. Also, saliva samples from the pharynx (Blondeau *et al.,* 2010; Grabowski *et al.,* 2011; Davis *et al.*, 2013).

Farrell *et al.,* (2006) identified pepsin in the BAL fluid of 33 children with GOR who were not diagnosed with CF using a pepsin sandwich ELISA to discover that pepsin levels were associated with proximal reflux and chronic cough (Farrell *et al.,* 2006). This was supported by another study assessing pepsin levels in the BAL of 65 children with proximal reflux also observing a positive correlation between proximal reflux and pepsin using a Pepsin assay (Starosta *et al.*, 2007). A direct Pepsin ELISA was developed in our laboratory here in Newcastle Upon Tyne in 2006. This pepsin ELISA was used as a tool to assess gastric aspiration in BAL samples collected from lung allographs and found that pepsin was significantly higher in lung transplantation recipients compared to healthy controls (Stovold *et al.*, 2007). In 2008, Blondeau and colleagues identified pepsin in the BAL fluid of patients receiving lung transplantation. It was observed that 7 CF patients included in the study had higher levels of pepsin compared to patients with other respiratory diseases such as pulmonary fibrosis, pulmonary hypertension and chronic obstructive pulmonary disease (Blondeau *et al.,* 2008b). Other authors have shown that pepsin was higher in the BAL of 31 CF patients compared to 7 healthy controls (McNally *et al.,* 2011). The sputum of GOR related chronic cough patients was analysed for pepsin and compared with the healthy controls. Pepsin was detected in both the samples of

chronic cough patients and healthy controls and it was illustrated that there was no difference observed. The interesting finding from this study was that pepsin levels were higher in the patients that were not using PPI medication suggesting that acidic reflux events activate pepsin (Grabowski *et al.*, 2011). Furthermore, the sputum samples were induced and it has been demonstrated that induced sputum could cause physiologic GOR in a healthy population resulting in increased pepsin levels (Ervine *et al.*, 2009). The present study found that approximately 70% of the samples analysed (n=48 of 69) were positive for pepsin. The pepsin ELISA was sensitive to 10ng/ml and as our sputum plugs were corrected for the 8 times dilution with PBS the final concentration lower than 80ng/ml were not reported as pepsin positive. The presence of pepsin below the lowest levels of sensitivity may derive from plasma pepsinogen. The pepsin ELISA detected a range of 80 to 1150ng/ml of pepsin in the 48 patient samples (67%). The present study used a primary antibody specific for 'Pepsin A' that makes up 86% or all pepsin found in gastric juice (Bardhan *et al.,* 2012). It is important that the primary antibody is specific to 'pepsin A' and does not bind to gastricsin (known as 'pepsin C') due to the observation of Bohman *et al.,* (2013) who found gastricsin but not 'Pepsin A' in the lower airways of 11 patients undergoing surgery that were not GOR or CF patients. The study suggests that assays not specific for 'pepsin A' should interpret the results with caution as gastricsin could be expressed from pneumocytes of the airways (Bohman *et al.*, 2013). Bulmer and colleagues found that pepsin exposure to the porcine laryngeal mucosa damaged the respiratory epithelium and membrane bound mucins measured by the release of DNA (Bulmer *et al.*, 2010). This finding illustrates the potential damage that could be caused to the epithelium of the aero-digestive tract and airways of patients in this study with pepsin positive sputum samples.

Pauwels *et al.,* (2012) identified bile acids in 56% of sputum samples collect from CF patients and 13% in healthy volunteers. However, the sputum was nebulised and may not be a good comparison to CF expectorated sputum (Ervine *et al.*, 2009) and the number of CF patients were much higher (n=41) than the number of healthy controls (n=15) (Pauwels, *et al.*, 2012). Blondeau and colleagues (2010) identified bile acid in the saliva of 23 of a total 65 CF patients and reported that healthy volunteers had no bile acid detected (Blondeau *et al.*, 2010). However, saliva originates from the oral cavity and drains back into the pharyngeal area thus it is coughed from the pharynx and is not representative of the fluid contained in the airway. In addition, the studies reported bile acid levels as low as 0.2µmol/L and this is below the detection limit of the enzymatic ELISA claimed by the

company at 1mmol/L. This finding becomes important when patients are diagnosed and treated according to bile acid content of physiological samples. Parikh and colleagues explored the methods used for bile acid detection within research and clinical facilities and found that the enzymatic methods used in the previous studies mentioned report levels of bile acids that are below the detection limits of the kit (Parikh *et al.*, 2013). It was reported in 2012 that bile acids were measured in the explanted lungs of CF patients undergoing a lung transplant. A sample of the total 30ml airway lavage was measured for taurine and glycine conjugates of cholate and isomers and taurine and glycine conjugates of deoxycholate and isomers by tandem mass spectrometry. This method allows for sensitive lower detection limits of 0.01 µmol/L. The study suggested that future research into the reflux of duodeno gastric reflux contents and micro aspiration should use this method (Aseeri *et al.*, 2012). Due to reports of the enzymatic method used previously creating an uncertainty about the results and given the sensitivity of the tandem mass spectrometry method the following study used the latter studies method. The present study detected median total bile acids (>80% of total bile acids) to be 0.22 µmol/L and ranging from 0.00 to 3.17 µmol/L. Our findings are supported by Aseeri *et al.,* (2012) demonstrating TBA content of CF BAL samples to be a median of 0.19µmol/L by the tandem mass spectrometry method. In addition, Pauwels, and colleagues demonstrated bile acids in CF sputum to be a median of 1.5 µmol/L by the enzymatic assay. This data has been suggested that CF patients may aspirate duodeno-gastric contents into the airways, however the normal values for bile acids in the plasma has been demonstrated to be <10 µmol/L and therefore used as a lower detection limit in the literature (Pearson and Parikh, 2011; Parikh, et al., 2013). Due to the low concentrations of TBA (below 10µmol/L) detected in 22 CF sputum supernatant samples we could not assume that the patients experienced aspiration of duodenal-gastro-oesophageal refluxate. The present study did not continue with the analysis of TBA in further samples beyond n=22. The detection of pepsin in samples of the airway has been shown to be the best method to explore gastric aspiration due to the sensitive and inexpensive methods of analysis available. Previous reports regarding bile salts to show that duodenal reflux associated with GOR can be questionable depending of the method of analysis and the concentrations reported are not above the maximum normal levels found in the serum, thus their origin cannot be certain. The measurement of taurine and glycine conjugates of cholate, taurine and glycine conjugates of deoxycholate and isomers by tandem mass spectrometry is praised by its sensitivity, although it is an expensive procedure.

Hallberg *et al.,* (2004) identified duodenogastric reflux of the duodenal contents through the pylorus and into the stomach in CF patients with significantly higher levels of bilirubin and bile acids identified in gastric perfusate compared to healthy controls (Hallberg *et al.*, 2004). Early studies into the effects of bile salts on the rabbit oesophagus investigated whether increased concentration of bile salts (up to 5mmol/L) or increased exposure time damaged the oesophageal muscoa (Harmon *et al.*, 1981). Kivilaakso and colleagues suggest that bile salts and pepsin with the presence of gastric acid are components of refluxate that cause the greatest damage to oesophageal musoca (Kivilaakso *et al.*, 1980). In human studies, the presence of gastric acid and duodenogastric reflux has been shown to be associated with severe oesophagitis in children and the presence of isolated gastric acid or gastric reflux is associated with mild oesophagitis (Orel and Markovic, 2003).

Interestingly, the present study observed GOR and EOR symptoms in those patients taking acid suppression medication (PPI and H_2RA). It was illustrated that 63% patients experiencing GOR symptoms were on acid suppression which illustrates the medication is not alleviating the symptoms caused by acidic refluxate. There were 73% of patients using acid suppression that were symptomatic of EOR. It has been shown previously that acid suppression medication is not useful for EOR symptoms because the refluxate reaching the aero-digestive tract and beyond is not acidic, it has been suggested that pepsin and bile acids are the damaging contents which are unaffected by acid suppression medication (Bardhan *et al.*, 2012). Furthermore, Grabowski et al., (2011) observed bile acids in the sputum of chronic cough patients treated with PPI and there were no differences in bile acid contents of these patients compared to the untreated group. Pepsin was identified in the sputum of 15 chronic cough patients treated with PPI to reduce symptoms, however pepsin was higher in the patients that were untreated. The results from this study show even higher pepsin concentrations in healthy control subjects so the data should be interpreted with caution. The presence of pepsin was slightly higher in the patients that were asymptomatic of GOR and EOR, however there were no statistical differences. However, there were two patients with a high TBA content that were symptomatic for GOR and EOR. Due to the low number of patients demonstrating this effect the assumption cannot be made regarding bile acid effects associated with GOR and EOR symptoms. The Bilitec duodenogastric reflux monitoring with oesophageal monitoring may be needed to explore this finding further to illustrate the backflow of duodenal contents into the stomach followed by proximal reflux events.

The study desired to assess the presence of gastric lipid digested by alveolar macrophages by staining the lipid contained within the macrophage with Oil Red O. The lipid laded macrophage index (LLMI) score as a tool to assess gastric aspiration requires the lipid laden macrophage count of 100 consecutive macrophages present on a cytological slide (Wang *et al.*, 2010). The lipid identified within a macrophage is not specific to gastric content. They could be dietary lipids that have been aspirated during food consumption, furthermore, they could be breakdown products of cellular debris within the lung/airways mucus or could even have been absorbed by macrophages in the plasma/lymphatic fluid prior to ending up in the airways. The cellular analysis of the sputum samples demonstrated that the samples contained a high number of neutrophils with a median of 100% neutrophils therefore the lipid laden macrophage analysis was not possible. Research is contradictory regarding the fitness of the LLMI in identifying aspiration of the gastric contents into the airways. In 1987 it was reported that 85% of patients with respiratory tract disorders with GOR presented lipid laden macrophages in BAL samples compared to 19% of patients without GOR (Nessbaum *et al.,* 1987). It was suggested that the simple sighting of lipid laden macrophages demonstrates a nonspecific finding because a LLMI score below 72 out of a possible 400 was observed in children with no clinical suspicion of aspiration. The study suggested quantifying the lipid in the macrophage by the LLMI was a good test for recurrent aspiration in light of the median LLMI of above 86 was shown in patient with definite aspirators (Colombo and Hallberg, 1987). Recently, the diagnostic tools was used to assess the effectiveness of Nissen funcoplication in CF patients after receiving a lung transplant by comparing LLMI before and after surgery and comparing the scores with patients receiving lung transplant that refused Nissan fundoplication. The study demonstrated a reduction in LLMI after surgery in the patients that underwent a Nissan fundoplication compared to those that did not. This suggests that the LLMI score is a useful tool to assess gastric aspiration (Hayes *et al.*, 2013). Other authors have also confirmed the effectiveness of the LLMI tool in lung transplant patients (Hopkins *et al.*, 2010) and patients with GOR symptoms (Parameswaran *et al.*, 2000). However, many studies have found that the LLMI is not a good marker of gastric aspiration in patients with abnormal reflux index confirmed by dual channel 24 hour pH monitoring identifying proximal reflux (Kitz *et al.*, 2012). Additionally, the LLMI of sputum samples collected from diagnosed erosive oesophagitis patients were not different to the LLMI of healthy volunteers (Köksal *et al.*, 2005). Chang et al. also found the LLMI score was not abnormal in patients defined with GORD with

the presence of reflux oesophagitis (Chang *et al.*, 2005). The present study could not perform the LLMI in the sputum of CF patients, however a BAL sample may have provided enough macrophages to assess the usefulness of the aspiration assessment tool.

3.3.3 The association of gastric aspiration with pulmonary function in the CF population

Research suggests that gastric aspiration may lead to poor pulmonary function (Pauwels *et al.*, 2012) and could be a contributing factor to lung damage in CF (Aseeri *et al.*, 2012) (Mousa and Woodley, 2012; Woodley *et al.*, 2014). This association has been assumed due to the reduced pulmonary function in CF patients with increased acid GOR than those without GOR (Navarro *et al.*, 2001). Blondeau et al. reported that the FEV_1% predicted was higher in patients with bile acids absent from the sputum of CF patients (Blondeau *et al.*, 2008a). Other researchers observed that wheezing was strongly associated with a decrease in pulmonary function, a symptom of EOR (Konstan *et al.*, 2007). However, others did not find any correlation with GOR symptoms and pulmonary function measured by FEV_1 and FVC (Sabati *et al.*, 2010). The evidence provided may provide possible answers but the issue still remains inconclusive. The present study evaluated the association of gastric aspiration with the pulmonary function of CF patients. There were no association observed between the gastric aspiration of pepsin or bile acids with pulmonary function. Our data could not support the findings observed in the literature, this could be due to the difference of methods used to assess the observation. For example, Pauwels and colleagues (2012) assessed bile acids in sputum samples using the enzymatic assay previously illustrated not to be a sensitive method of assessment. Aseeri et al. assessed bile acids using the same method as the present study but analysed BAL samples as opposed to sputum. Other authors assessed proximal reflux with oesophageal pH impedance monitoring and therefore did not assess the entity of fluid from the airways (Mousa and Woodley, 2012; Woodley *et al.*, 2014).

Pauwels *et al.*, (2012) observed a relationship between the presence of bile acids and inflammation within the airway of CF patients. This study assesses the concentration of neutrophil elastase, a biomarker of inflammation in the sputum samples of CF patients. The study observed higher concentrations of neutrophil elastase in the sputum of CF patients with bile acids present compared to those that did not. The limitations of the bile acid assay used has been mentioned, however this finding illustrates that inflammation is present in the CF lung and further studies are needed regarding the effects of gastric

aspiration and inflammation. Furthermore, high IL-8 concentrations observed in the BAL fluid of CF patients have been associated with the presence of pepsin (McNally *et al.*, 2011). The present study observed high neutrophil content in the sputum of CF patients and this was accompanied by high IL-8 in all 72 CF sputum samples, however IL-6 was detected in only 16 patients samples. This is supported by finding by Fischer and colleagues, (2014) who detected median IL-8 concentration of 50ng/ml in the sputum of CF patients and low detected frequencies of IL-6 that were not high enough to report. The present study did not observe any association with inflammation in the airways of CF patients and the presence of gastric aspiration determined by pepsin. Nor did the results show any differences in pulmonary function and the presence of inflammation.

A limitation of the current study was the exclusion of CF patients that could not provide sputum. In the present study there were 24 potential patients that could have been recruited but were unable to provide expectorated sputum. Potential findings regarding the difference between GOR and EOR symptoms associated with sputum production was unexplored. The purpose of the exclusion was down to one of the major aims of the study involving to the analysis of sputum samples for biomarkers of reflux and inflammation. We could have included non-sputum producers to gather symptoms data in absence of gastric aspiration and inflammation data or included the possibility of BAL fluid collection from these patients. However, the BAL sample is taken from one section of the bronchiole, and it is not representative of the whole lung. In addition, the protocol of the BAL describes the installation of 30-180 ml infiltrated into the bronchiole and then recollected for the desired analysis. There is a possibility that concentration of pepsin or bile acids diluted in 30-180 ml is present at undetectable concentrations. Additionally, there would be a major difference in methodology to collect fluid from the airways, and the results of pepsin ad bile acids from sputum and BAL fluid may not be easily compared. The positive aspect that we have observed amongst the patients besides the common characteristic of sputum producers, is that there is a wide range of demographics, genotype, and pulmonary function. Thus, we can study GOR and EOR in a wide range of CF disease severity with varying disease characteristics. The number of CF patients in our study were high compared to similar studies, our study recruited 72 CF sputum producers, whereas Blondeau et al. (2008) recruited 33 CF patients and Pauwels et al. (2012) 41 CF patients. These two studies, like ours, only collected one sample per patient on recruitment. The sample was not specified to be provided when the patient was experiencing GOR or EOR symptoms and this may result in the varied pepsin levels because the reflux event could be

cleared and we do not know how long pepsin will remain within the airway. Due to the method of sample processing method the sputum sample was processed within 2 hours of collection to perform the cellular analysis. The ideal sputum collection for future studies would be collected from each patient on a morning, on symptoms, post prandial and before bed. However, a change of sputum protocol would be required so that it was not necessary for the samples to reach the lab within 2 hours, therefore the cellular or inflammatory biomarker analysis for all 4 samples collected would not be possible.

3.3.4 Summary of results from chapter 3

In summary, the CF population are living longer, with increased life survival rates reported to be 40-50 years. This is encouraging, but this also raises the concern of diseases observed that may have terminal outcomes other than the existing CF causes of death. The consequence of long-term GOR exposure to the oesophagus and aero digestive tract lead to unpleasant symptoms, may reduce pulmonary function and lead to fatality. As this study has highlighted, there is a high prevalence of GOR and EOR in the adult CF population with many patients aspirating gastric contents. The data did not support the previous findings in regard of this causing decreased pulmonary function or increasing inflammation. The CF airway was shown to contain high numbers of neutrophils and high concentrations of IL-8 were present but not associated with gastric aspiration, thus the study cannot show any damage caused by gastric aspiration in the CF airway.

Chapter 4.0

New medication in CF – Ivacaftor CFTR potentiator for the G551D mutation

4.1 Introduction

4.1.1 New medications for Cystic Fibrosis – Ivacaftor for the CFTR G551D mutation

The first CFTR potentiator agent has been accepted by the North American Food and drug association for patients with the G551D mutation available as an oral tablet called VX-770; Caledeco or Ivacaftor. The class III G551D mutation requires agents which induce channel opening to enable chloride ions to be transported through the CFTR present at the cell surface. This type of agent is a CFTR potentiator (Kotha and Clancy, 2013).

As mentioned in chapter 1 there are 6 classes of CFTR mutations. The CFTR G551D mutation is a class III none functional gating mutation. Class III mutations occur in approximately 4% of CF patients in the U.S, 5% in the U.K and increasing to 11% in the Republic of Ireland, the most common mutation in this class is G551D (Flume, et al., 2012; Yu, et al., 2012; Kotha and Clancy, 2013; Barry *et al.*, 2014). The CFTR protein is present at the cell surface and composed of two nucleotide-binding domains. In a normal manner, the opening and closing of the channel pore or channel gating is tightly regulated by phosphorlylation, adenosine triphosphate (ATP) binding and hydrolysis. Protein Kinase A (PKA) phosphorylates CFTR to allow ATP binding and hydrolysis which in turn opens and closes the channel across cell membranes (channel gating) to allow chloride, and bicarbonate ions to be transported. In the G551D mutation the mechanisms do not function in the normal manner and the channel does not open or close leading to poor flow of chloride ions in and out of the cell and resulting in loss of epithelial chloride transportation (Van Goor et al., 2013; Yu, at al., 2012). The Ivacaftor molecule acts as a potentiator and aims to normalise the CFTR gating channel able the transport of chloride ions. The Ivacaftor molecule was thought to modify one or more of these regulatory events and it was described in 2012 by Eckford et al., (2012) in detail. The study reported that the Ivacaftor molecule works through an ATP-independent mechanism working in a nonconventional manner. It enhances ATPase activity in the G551D mutated CFTR in absence of magnesium-ATP.

Unregulated chloride ion transport leads to CF disorders in the exocrine organs. The loss of chloride transport leads to elevated sweat chloride concentration and also causes other CF complications such as the accumulation of thick, sticky mucus in the bronchi, loss of pancreatic function, impaired intestinal absorption and reproductive dysfunction. The accumulation of thick sticky mucus in the airways leads to decreased pulmonary function, bacterial growth and exacerbations. This leads to an increase in energy demands caused by a decrease in appetite, poor fat digestion and increase in energy expenditure from breathing difficulties (Orenstein *et al.*, 2000; Simmonds, 2013). Mucus blocks the pancreatic ducts minimising the release of pancreatic enzymes and impairing intestinal absorption. As a consequence malabsorption and malnutrition occur which is corrected for by pancreatic enzyme replacement capsules with every meal to aid digestion and absorption. CF complications are multifactorial and result in a multitude of problems which reduce quality of life (Van Goor *et al.,* 2013; Yu *et al.,* 2012). Measuring the sweat chloride concentrations of CF patents is a widely used tool to diagnose CF as this method can be carried out on patients of any age, it is a relatively cheap procedure and sweat chloride changes occur early on in disease (Accurso et al., 2014). Normal sweat chloride concentrations are <39mmol/L, these concentrations would provide the clinician with the knowledge that a patient were at an unlikely risk of CF. Concentrations ranging from 40-59 mmol/L would put the patient at a possible likelihood of CF and concentrations above 60mmol/L would suggest the patient was likely to have CF (Cystic Fibrosis Foundation, 2011).

4.1.2 Ivacaftor outcome of clinical trials and the CF clinic

In 2010, investigational studies over a 28 day period started to take place on the oral bioavailable agent designed to potentiate CFTR channels that are positioned at the surface of the cell. A double blind, placebo-controlled multicentre, phase two trial was carried out to evaluate the safety and adverse effects of Ivacaftor involving 39 adult CF patients. The participation requirements set specified that patients were to be diagnosed with the CFTR G551D mutation on at least one allele with a FEV_1% predicted to be 40% or higher and aged 18 years or over. Various doses of Ivacaftor (25, 75 or 150 mg) or placebo were received every 12 hours for 28 days. Within subject improvement of CFTR ion-channel function was observed with decreases in sweat chloride. At day 28 of treatment the median sweat chloride measurements reduced by -59.5mmol/L from baseline concentrations (P=0.008) in patients receiving 150 mg (every 12 hours for 28 days).

Improvements were observed in within subject pulmonary function scores (FEV_1% predicted) illustrated by significant increases of 8.7% (P=0.008). Reports were made that the drug was safe to use and improvements in CFTR and pulmonary function were evident (Accurso, et al., 2010). This study was followed by a randomised, double blind, placebo-controlled phase three trial to assess efficacy and safety of Ivacaftor this time including younger CF patients of >12 years, diagnosed with CF with the G551D mutation on one CFTR allele, had an FEV_1 of 40-90% of their predictive value and for a longer duration of 48 weeks. The study reiterated the beneficial effects of 150mg Ivacaftor seen previously. Ivacaftor every 12h (n=83) was shown to increase pulmonary function (FEV_1% predicted) by 10.6% compared to the control group (n=78) at the 24 week follow-up. Decreases were observed in sweat chloride concentration by -48.1mmol/L compared to controls at 48 weeks follow-up. The treated patients gained an average of 2.7kg more weight than the control group at 48 weeks follow-up. The frequency of pulmonary exacerbations were reduced by 55% compared to placebo. Although 28 patients in the group receiving Ivacaftor experienced a total of 47 exacerbations this was significantly lower than the 44 placebo patients experiencing 99 exacerbations (P=0.001). The respiratory symptoms improved in the Ivacaftor group compared to placebo determined by the Cystic Fibrosis Questionnaire Revised (CFQ-R) score reduction (Ramsey *et al.,* 2011). The beneficial results of Ivacaftor were mirrored in a retrospective case control study from n=21 patients receiving Ivacaftor in the UK and Ireland. After 3 months of treatment the pulmonary function scores, weight and BMI had increased significantly from baseline (Barry *et al.*, 2014). Interestingly, the study highlighted that Ivacaftor treatment reduced the days of intravenous antibiotics received at home and in hospital. Additionally two patients were able to reduce oxygen therapy and one patient stopped nasogastric tube feeding. These positive outcomes illustrate the health benefits to the Ivacaftor treated patients. It also highlights the clinical efficiency of the expensive treatment by reducing the costs of other healthcare costs associated with severe CF pulmonary and gastrointestinal disorders. A case study of one patient homozygous for G551D that did not meet the requirements for previous study participation was reported at a CF centre in Ireland. The 19 year old patient had a baseline FEV_1 of 24% predicted value, lower than the cut off in clinical trials, which was reported to increase to 35% of predicted after 8 weeks of treatment and increasing to 40% of predicted at 12 months with an overall increase of 15% of predicted. Improvements in sweat chloride concentration resulted in normalised levels at 18mmol/L from 92mmol/L at baseline. The study illustrates the potential of Ivacaftor for not only

heterozygous but also G551D homozygous patients, and there are 8 persons with this genotype in Ireland (Harrison *et al.*, 2013). Observations of Ivacaftor use in 14 severely ill CF patients with a FEV_1% <40.0% of their predicted score were reported from a CF clinic in Germany. Unfortunately over the 13 months follow-up 3 patients died of CF complications reported not related to Ivacaftor use, and 2 patients were discontinued due to personal reasons. Reported small increases of pulmonary function were observed from baseline in this patient group of a mean 5.2% predicted over the 13 month. The increases were small compared to previous studies observations and were thought to be due to severe lung damage already present leading to a limited improvement via Ivacaftor (Hebestreit *et al.*, 2013).

4.1.3 Aims of the following chapter

As the CFTR mutation results in multiple complications in exocrine tissues there is now a strong interest in the possible clinical benefits of Ivacaftor that have not yet been investigated. CFTR is expressed in the epithelia of the gastrointestinal tract and it has been reported that up to 90% of CF patients experience gastro-oesophageal reflux (GOR) leading to extra-oesophageal reflux (EOR) problems (Blondeau *et al.*, 2008). The following chapter describes the early experience of Ivacaftor use in the NHS Newcastle hospitals. This chapter is an evaluation of gastrointestinal effects of Ivacaftor. The main aim of the study was to focus particular investigations on the effects on the presence of GOR, EOR and gastric aspiration in the G551D CF populations and evaluate any beneficial effect of Ivacaftor in reducing these symptoms.

4.2 Results

4.2.1 Patient demographics

There were a total of 12 patients recruited onto the study. There were seven males and five females with a median age of 23 years ranging from 17 to 38 years (Figure 4.1). Each patient was weighed at baseline, 6 weeks after, 6 month after and 12 months after the initial medication. The median weight at baseline was 64kg (range; 41-83Kg) increasing significantly by 2kg after 6 months (median 66, range; 43-92Kg, P=0.003) and 1.4kg (median 65.4 (range 40.7 to 90.2, P=0.07) at 12 months of treatment (no median increase after 6 weeks (median 63kg, range; 41-87Kg). Of the 12 patients recruited 7 increased weight by a mean of 2.9Kg after 6 weeks of starting Ivacaftor medication (range;0.5-8Kg) and these patients increased weight further by a mean of 4.6kg after 6 months compared to baseline (range;1.4-12.6Kg) (Table 4.1 and figure 4.2). The other 5 patients did not increase the weight 6 weeks after treatment and all but one of these 5 patients increased their weight by the 6 month follow-up appointment. BMI was calculated to assess nutritional status of each patient at baseline, 6 weeks after, 6 month after and 12 months after initial medication. The median BMI at baseline was 22.4 (range; 17.5-30.9) increasing by 0.1 at 6 week follow-up (median 22.5, range; 17.4-30.9), increasing significantly by 2.4 at 6 month follow-up (median 24.8, range; 18.1-31.1, P=0.004) and increasing by 2.2 (P=0.03) at 12 month follow-up when compared to baseline. The BMI of 7 patients of whom put on weight increased by a mean of 1.25kg/m^2 after 6 weeks of starting Ivacaftor medication (range; 0.1-3.3kg/m^2) and increasing further by a mean of 2.0kg/m^2 after 6 months compared to baseline (range; 0.7-4.3kg/m^2) (Table 4.2 and figure 4.3). At the 12 month follow-up the data of 3 patients was not recorded due to absence. Of the 12 patients there were 7 patients treated with PPI therapy and 1 with H_2RA's previous to starting Ivacaftor to reduce GOR symptoms. The medication continued throughout the study with no patients discontinued of PPI or H_2RA.

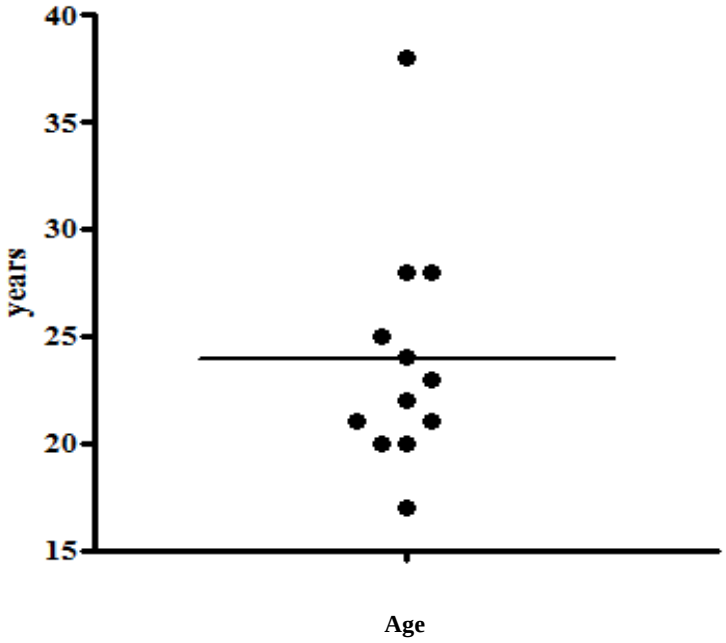


Figure 4.1. Patient age of CF patients treated with Ivacaftor (n=12, range 17-38, median 22.5) years. The graph shows the individual patient age and the median.

Patient		Weight (Kg)			
no	**sex**	**Baseline**	**6 weeks**	**6 month**	**12 month**
1	**M**	48.4	50.0	56.1	53.8
2	**M**	79.4	87.4	92.0	90.2
3	**F**	41.4	41.2	42.8	40.7
4	**F**	61.5	62.0	66.8	65.4
5	**F**	47.0	49.8	51.3	ND
6	**F**	54.0	57.0	60.4	60.6
7	**F**	65.8	65.8	64.8	63.9
8	**M**	57.8	60.4	59.5	ND
9	**M**	77.0	75.4	78.6	78.8
10	**M**	73.8	73.8	75.6	75.8
11	**M**	67.4	63.8	73.6	ND
12	**M**	82.8	84.6	84.4	82.8

Table 4.1. Summary of recorded weight (Kg) of each patient treated with Ivacaftor (1-12) at baseline, 6 week, 6 month and 12 month follow-up (ND represents no data available). The baseline median was 64.0kg (range 41.0 – 83.0); 6 week follow-up median was 65.4kg (range 40.7 0 81.0); 6 month follow-up median was 66kg (range 43-92) and 12 month follow-up median was 65.4kg (range 40.7 – 90.2).

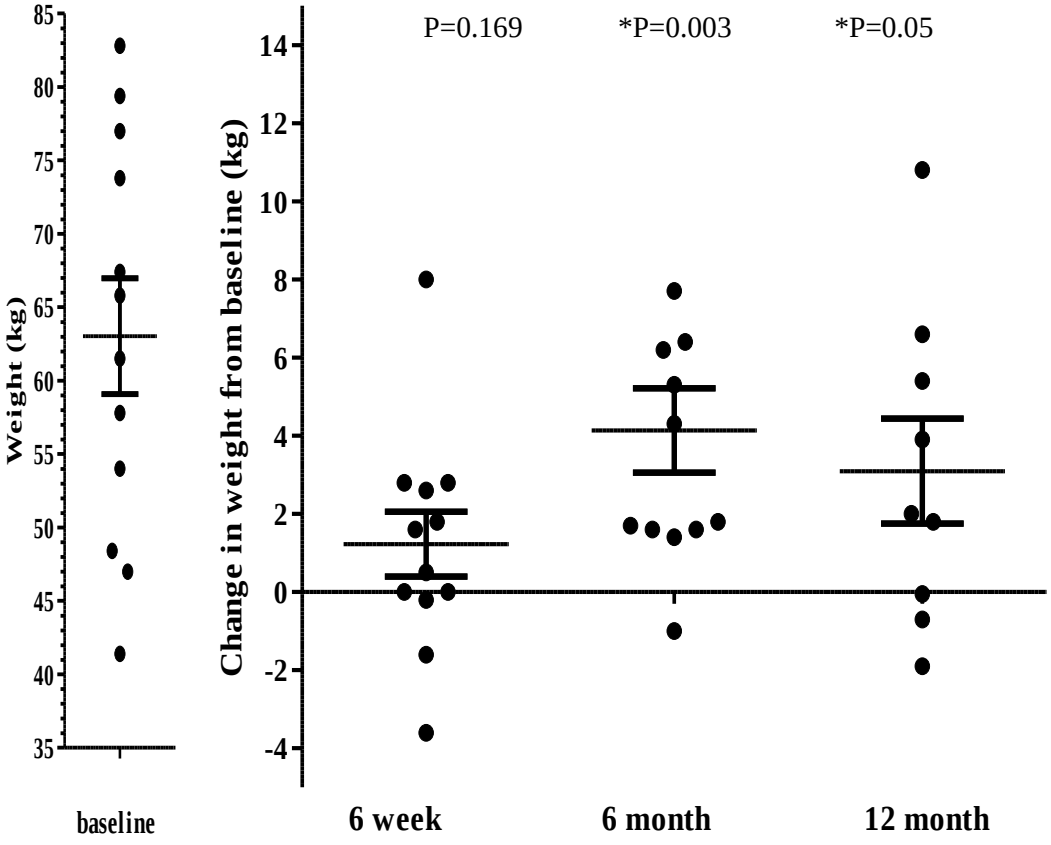


Figure 4.2. Individual dot plot of patient weight (kg) treated with Ivacaftor presented on the right as a change in weight (kg) recorded 6 weeks after initial treatment (P=0.169, 95% CI (-0.40, -2.70)), 6 month after initial treatment (p=0.003, 95% CI (-6.50, -1.76)) and 12 months n=9 (P=0.05, 95% CI) after treatment from baseline weight displayed on the left (no change from baseline = 0). Statistical analysis performed using one sample Wilcoxon signed rank test.

Patient	BMI			
	Baseline	**6 weeks**	**6 month**	**12 month**
1	21.2	22.2	24.9	23.6
2	26.8	29.5	31.1	30.2
3	17.5	17.4	18.1	17.1
4	22.2	22.5	24.2	24.0
5	20.9	22.1	22.8	ND
6	21.3	22.5	23.9	24.0
7	30.9	30.9	30.4	30.1
8	19.8	20.7	20.4	ND
9	26.1	25.5	26.6	27.7
10	25.3	25.5	26.2	26.2
11	22.5	21.3	24.6	ND
12	26.9	27.6	27.6	26.9

Table 4.2. Body Mass Index measurements of each patient treated with Ivacaftor recorded at baseline, 6 week, 6 month and 12 month follow-up (ND represents no data available). The baseline median was 22.4kg/m^2 (range 17.5 – 30.9); 6 week follow-up median was 22.5kg/m^2 (range 17.4 – 30.9); 6 month follow-up median was 24.8kg (range 18.1 – 31.1) and 12 month follow-up median was26.2kg/m^2 (range 17.1 – 30.2).

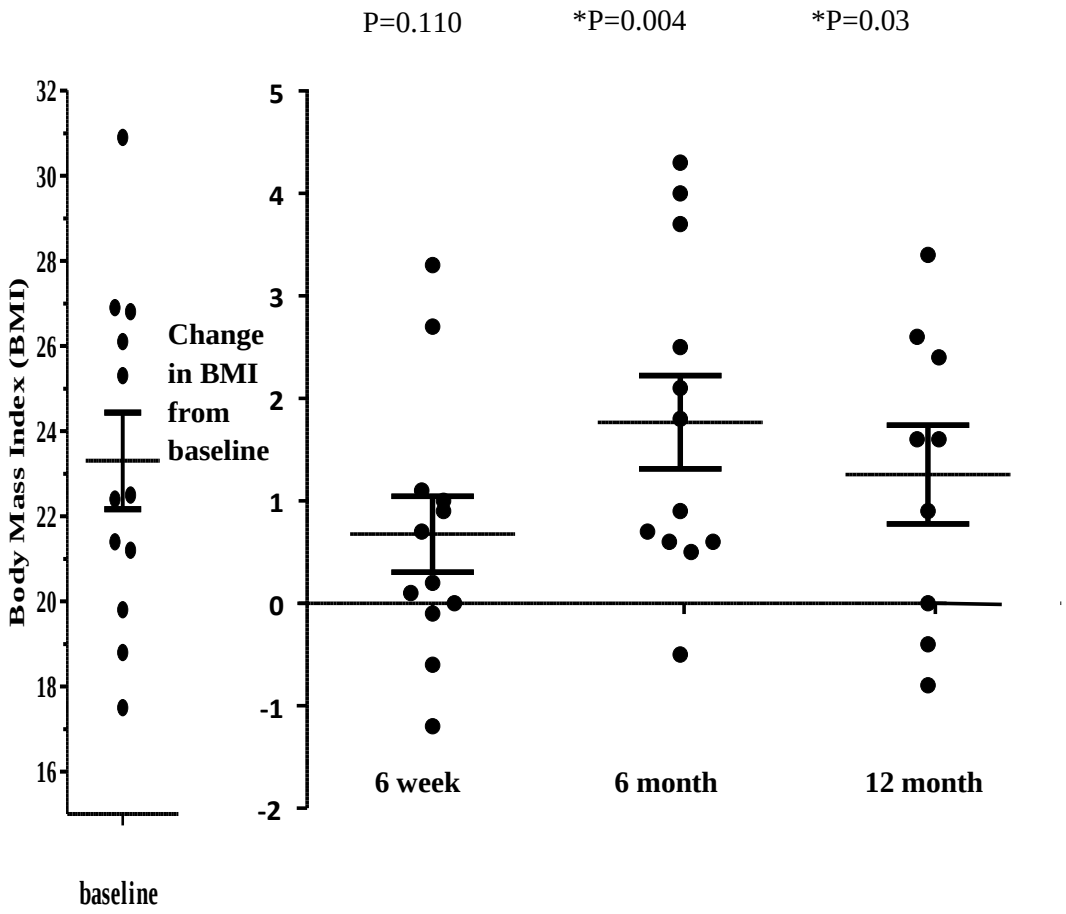


Figure 4.3. Individual dot plot of patient BMI treated with Ivacaftor presented as a change in BMI status recorded 6 weeks after initial treatment (P=0.110, 95% CI (-0.10, -1.60), 6 month after initial treatment (P=0.004, 95% CI (0.65, 2.75) and 12 month after initial treatment (P=0.03 95% CI (-2.4, -0.16) from baseline (no change from baseline = 0). Statistical analysis performed using one sample Wilcoxon signed rank test.

4.2.2 Clinical assessment of sweat chloride levels to monitor Ivacaftor performance

At each clinical appointment the sweat chloride test of each patient was performed by a trained clinical biochemist and assessed in the laboratory. The results were shared amongst clinicians and researchers involved in the present study. Normal sweat chloride concentrations are <39mmol/L, these concentrations would provide the clinician with the knowledge that a patient were at an unlikely risk of CF. Concentrations ranging from 40-59 mmol/L would mean the patient has a possible likelihood of CF and concentrations above 60mmol/L would suggest the patient was likely to have CF (Cystic Fibrosis Foundation, 2011). A patients baseline sweat chloride concentration falling below 60mmol/L or by >30% determines response to treatment. In cases of a baseline <60mmol/L the concentration must fall by 30% (NHS Commissioning Board, 2013).

The median sweat chloride concentration was 109 mmol/L (range; 61-131) at baseline significantly decreasing (P=0.001) to a median of 51 mmol/L (range 27-94) at 6 week follow-up. Two patients sweat chloride concentrations fell below 39mmol/L at 6 weeks follow-up meaning that they had normal concentrations. The concentrations remained significantly decreased at a median of 45 mmol/L (range; 20-91) with 6 patients resulting in normal sweat chloride concentrations <39mmol/L (Table 4.3 and Figure 4.4).

Patient	Sweat chloride concentration mmol/L		
	Baseline	**6 weeks**	**6 month**
1	61	**30**	**20**
2	116	62	59
3	101	40	**34**
4	114	42	**37**
5	109	60	56
6	108	59	53
7	107	66	65
8	95	**27**	**30**
9	131	94	91
10	112	50	66
11	110	47	**37**
12	108	52	**36**

Table 4.3. Measurements of sweat chloride (mmol/L) at baseline, 6 weeks and 6 months follow-up. The median sweat chloride concentration at baseline was 109 mmol/L (range; 61-131); a median of 51 mmol/L (range 27-94) at 6 week follow-up and a median of 45 mmol/L (range; 20-91) at 6 month follow-up. The green font indicates where patients sweat chloride concentrations has returned to the normal range of below 39mmol/L. A trained professional perfomed the sweat test which involved transdermal administration of pilocarpine by iontophoresis to stimulate sweat gland secretion. The resulting sweat is collected onto a gauze/filter paper/Macroduct coil and quantified for chloride concentration in a laboratory (Farrell *et al.*, 2008).

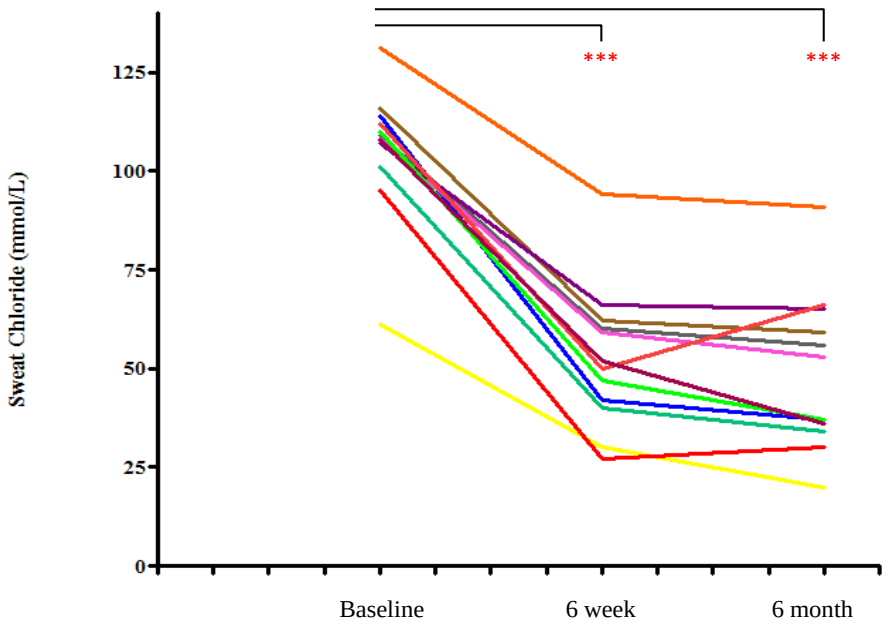


Figure 4.4. Individual patient sweat chloride level treated with Ivacaftor (mmol/L) measured at baseline, 6 weeks after medication and 6 month after medication. The median sweat chloride concentration at baseline was 109 mmol/L (range; 61-131); a median of 51 mmol/L (range 27-94) at 6 week follow-up and a median of 45 mmol/L (range; 20-91) at 6 month follow-up. Statistical analysis was performed using the Kruskal-Wallis test and Dunn's Multiple Comparison Test. *** statistically significant (P<0.001).

4.2.3 Clinical assessment of pulmonary function to monitor Ivacaftor performance

Pulmonary function of each patient was measured by FEV_1 and FVC. These were then calculated as a percentage of predicted values by a trained nurse at the out-patient clinic, RVI, Newcastle according to European Respiratory Society guidelines (Pellegrino *et al.*, 2005; Laszlo, 2006). The measurements were recorded at baseline and each follow-up appointment (6 weeks, 6 months and 12 months post Ivacaftor treatment). The median FEV_1% predicted was 79% (range; 19-110%) at baseline, increasing to a median of 101% (range; 35-132%) at 6 week follow-up, 93% (range; 32-124%) at 6 month follow-up and 96% (range: 32-114) at 12 month follow-up. All twelve patients increased FEV_1% predicted after 6 weeks of Ivacaftor treatment. The mean FEV_1% predicted significantly increased by 15% (range; 9-37%) (P=0.003) after 6 weeks of treatment and remained at 15% (range; 4-32) above baseline after 6 month (P=0.004) and 12 months (P=0.06) of treatment. The median FVC% predicted was 89% at baseline (range; 35-119) increasing to a median of 100% at 6 weeks (range; 60-125), 100% at 6 months (range; 53-124) and 102% at 12 months (range; 54-116) follow-up appointments. The mean FVC significantly increased by 13% (range 2 to 29) after 6 weeks of treatment (P=0.003) and remained at 13% higher than baseline %, (range 1 to 28) (P=0.003) after 6 months of treatment. The mean FVC% decreased to 5% change at 12 months compared to baseline, range 0 to 27 (P=0.36) (Table 4.4 and Figure 4.5 and 4.6).

pt	Baseline		6 weeks		6 months		12 months	
	FEV_1%	**FVC%**	**FEV_1%**	**FVC%**	**FEV_1%**	**FVC%**	**FEV_1%**	**FVC%**
	p	**p**	**p**	**p**	**p**	**p**	**p**	**p**
1	90	92	101	99	106	103	104	102
2	110	119	132	125	124	124	96	105
3	28	47	40	61	32	53	32	54
4	90	103	100	105	104	108	107	110
5	87	89	105	118	110	114	-	-
6	35	78	44	89	44	93	38	64
7	90	89	102	100	90	90	98	97
8	19	35	35	60	35	63	-	-
9	88	99	103	113	109	111	114	116
10	70	99	75	106	83	106	83	106
11	64	74	101	95	96	97	-	-
12	64	78	79	90	84	93	85	95

Table 4.4 FEV_1% predicted and FVC % predicted of CF patients treated with Ivacaftor recorded at baseline, 6 week, 6 month and 12 months (- no data available). At baseline the median FEV_1% was 79% (range 19 to 110, mean 70, SEM 8) and the median FVC% was 89% (range 35 to 119, mean 84, SEM 7). At 6 weeks follow-up the median FEV_1% was 101% (range 35 to 132, mean 85, SEM 9) and FVC% was 100 (range 60 to 125, mean 97, SEM 6). At 6 months follow-up the median FEV_1% was 93% (range 32 to 124, mean 85, SEM 9) and FVC% 100% (range 53 to 124, mean 97, SEM 6). At 12 month follow-up (n=9) the median FEV_1% was 96% (range 32 to 114, mean 84, SEM 10) and FVC 102% (range 54 to 116, mean 94, SEM 7)

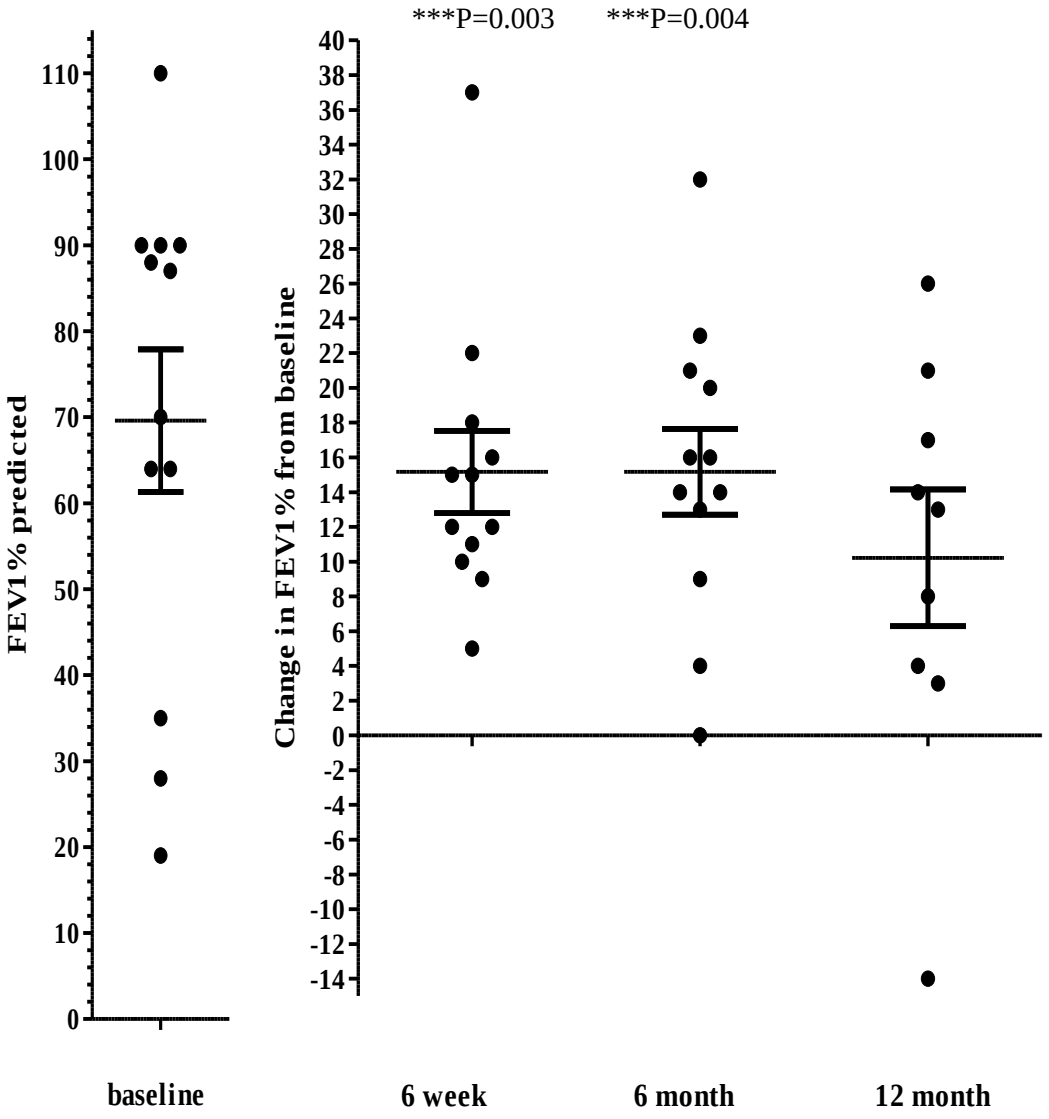


Figure 4.5 Change in % predicted of forced expiratory volume in 1 second (FEV_1 % predicted) at 6 weeks (mean 15%, range 9 to 37) (P=0.003, 95% CI (10.50, 19.00), 6 month from baseline (mean 15%, range 4 to 32) (P=0.004, 95% CI (10.00, 20.5) and 12 month from base (mean 10%, range -1 to 27) (P=0.06) (0= no change from baseline). Statistical analysis performed using one sample Wilcoxon signed rank test.

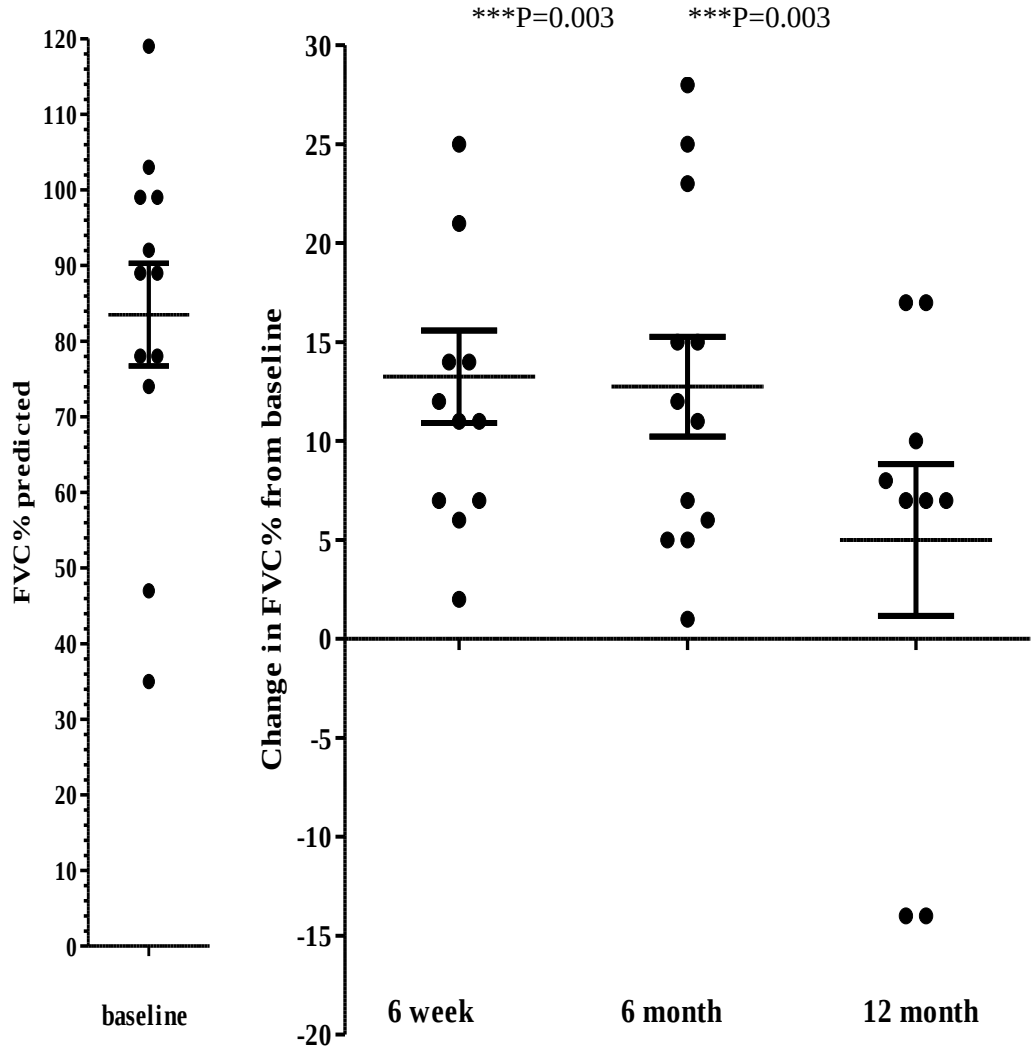


Figure 4.6 Change in % predicted of forced vital capacity (FVC % predicted) at 6 weeks (mean 13%, range 2 to 29) (P=0.003, 95% CI (8.0, 18.00), 6 month (mean 13%, range 1 to 28) (P=0.003, 95% CI (6.5, 18.5) and 12 month (mean 5, range 0 to 27) (P=0.36) follow-up from baseline (0= no change from baseline). Statistical analysis performed using one sample Wilcoxon signed rank test.

4.2.4 Gastro-oesophageal reflux in the G551D CF population; effects of Ivacaftor

GOR was present in three patients (median score 1, range; 0-5) at baseline as determined by the DeMeester reflux questionnaire and was defined by having a DeMeester score <2, this decreased to one GOR symptomatic patient (median score 0, range; 0-5) at the 6 week follow-up and to two GOR symptomatic patients (median score 0, range; 0-5) at the 6 month follow-up. Of the 12 patients studied there were 6 patients that experienced a fall in GOR symptoms after 6 weeks of Ivacaftor treatment and this was mirrored at 6 month follow-up when compared to baseline scores. There were 8 patients interviewed at the 12 month follow-up and with 1 patient presenting GOR symptoms. There was a trend for a significant change in DeMeester score 6 weeks after initial treatment (P=0.06) (Table 4.5 and figure 4.7). EOR was present in six of nine patients at baseline determined by the RSI questionnaire (RSI score >13) and decreasing to two patients after 6 weeks of Ivacaftor treatment. There were three EOR symptomatic patients determined at the 6 month follow-up. There is a significant reduction in RSI scores post medication with all but one patient showing a continuous reduction in RSI score during follow-up (P=0.008) (Table 5.6 and Figure 4.8). The Hull airways reflux questionnaire (HARQ) is another tool designed to assess EOR. Using this questionnaire there were 5 of the 6 patients mentioned above presenting EOR symptoms at baseline showing that both tools detected EOR in 5 symptomatic patients. The number of symptomatic patients decreased during Ivacaftor treatment (n=2 at 6 weeks follow-up and n=3 at 6 and 12 month follow-up). There was a significant decrease of EOR determined by the HARQ score at 6 weeks post treatment (p=0.031) (Table 4.7 and Figure 4.9).

DeMeester reflux Score (GOR determined by score >1 max 7)				
patient	**baseline**	**6 week**	**6 month**	**12 month**
1	1	0	1	1
2	1	1	0	1
3	1	0	0	ND
4	0	0	0	0
5	1	1	0	0
6	**2**	0	**2**	1
7	**5**	**5**	**5**	**6**
8	**2**	1	0	ND
9	0	0	0	0
10	1	0	0	0
11	0	1	1	ND
12	1	0	0	ND

Table 4.5. DeMeester score of patients treated with Ivacaftor at baseline 6 week, 6 month and 12 month (The numbers highlighted in red represent patients presenting GOR symptoms scoring >2; ND represents no data available). Median DeMeester score at baseline was 1, ranging from 0 to 5 and there were 3 patients with a DeMeester score over >1 to determine GOR symptoms. Median DeMeester score at 6 weeks follow-up was 0, ranging from 0 to 5 and there was 1 patient with a DeMeester score over >1 to determine GOR symptoms. Median DeMeester score at 6 month follow-up was 0, ranging from 0 to 5 and there were 2 patients with a DeMeester score over >1 to determine GOR symptoms. There were 8 patients that attended appointments at the 12 month follow-up and median scores were 0 (range 0-6) with one patient presenting GOR symptoms.

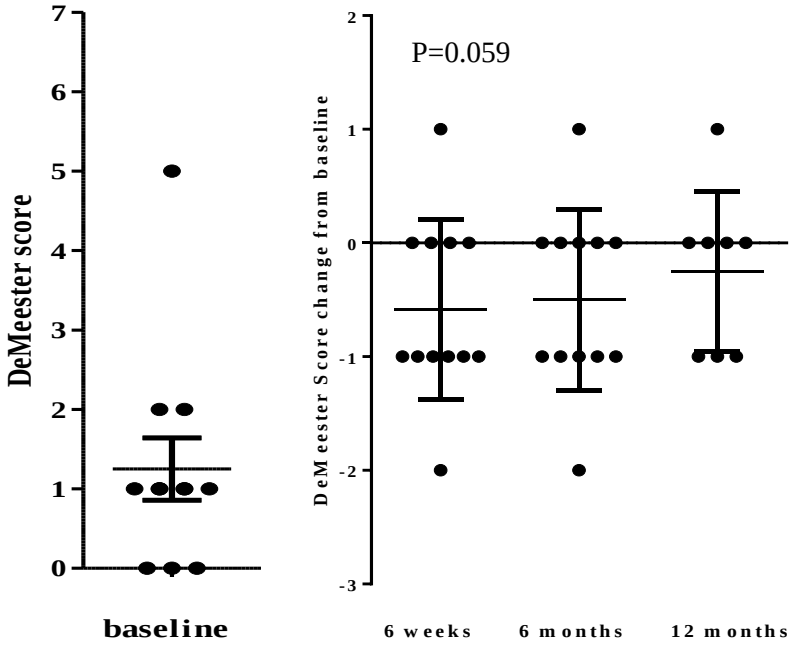


Figure 4.7. Change in DeMeester reflux score of patients treated with Ivacaftor at 6 weeks (P=0.059), 6 months (P=0.091) and 12 months (P=0.62) from baseline (0= no change from baseline). Statistical analysis performed using one sample Wilcoxon signed rank test and the decrease in DeMeester score was significantly reduced 6 weeks after medication (P=0.059).

Reflux Symptom Index Score (EOR determined by score >13 max 45)				
RSI	**baseline**	**6 week**	**6 month**	**12 month**
1	14	0	1	2
2	2	3	3	11
3	23	10	16	ND
4	11	9	6	2
5	17	12	3	15
6	29	23	16	16
7	12	14	16	14
8	15	11	7	ND
9	3	2	2	0
10	4	1	2	0
11	15	5	5	ND
12	8	3	1	ND

Table 4.6. Reflux Symptom Index (RSI) score (maximum score 45) of patients treated with Ivacaftor at baseline 6 week, 6 month and 12 month (The numbers highlighted in red represent patients presenting EOR symptoms scoring RSI >13; ND represents no data available). Median RSI score at baseline was 13, ranging from 2 to 29 and there were 6 patients with a RSI score over >13 to determine EOR symptoms. Median RSI score at 6 weeks follow-up was 7, ranging from 0 to 23 and there was 2 patients with a RSI score over >13 to determine EOR symptoms. Median RSI score at 6 month follow-up was 4, ranging from 1 to 16 and there were 3 patients with a RSI score over >13 to determine EOR symptoms. There were 8 patients that attended appointments at the 12 month follow-up and median scores were 7 (range 0-16) with 3 patient presenting EOR symptoms.

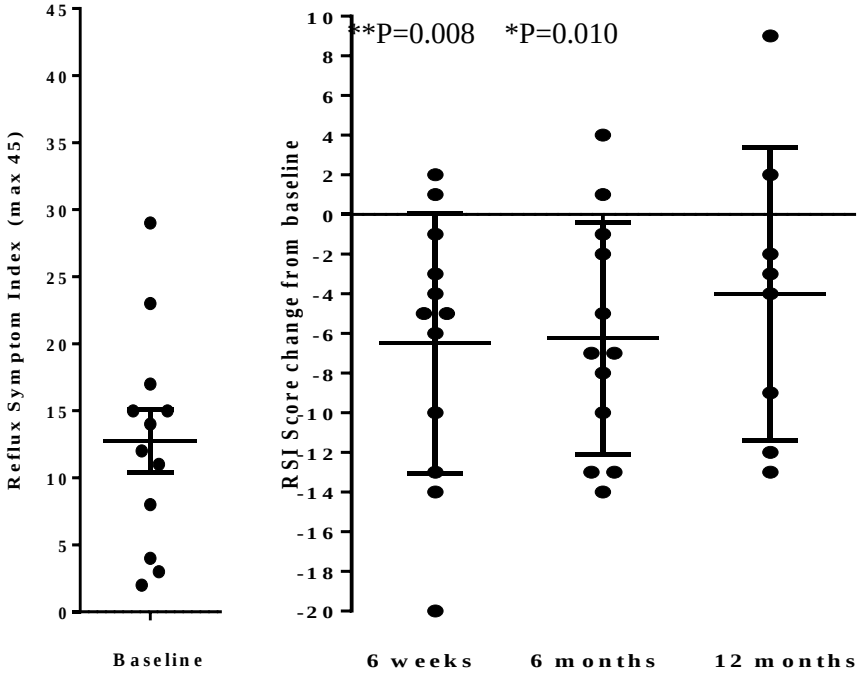


Figure 4.8. Change in Reflux Symptom Index score (maximum score 45) of patients treated with Ivacaftor at 6 weeks (P=0.008, 95% CI (10.5, 2.00)), 6 month (P=0.010, 95% CI (10.00, 2.00)) and 12 month (P=0.16) from baseline (0= no change from baseline). Statistical analysis performed using one sample Wilcoxon signed rank test (mean ± SEM).

Hull Airways Reflux Questionnaire score (EOR determined by score >13 max 70)				
HARQ	**Baseline**	**6 week**	**6 month**	**12 month**
1	12	5	5	2
2	3	2	3	10
3	31	11	16	ND
4	7	6	4	2
5	19	16	17	13
6	33	27	24	16
7	12	15	18	16
8	15	11	12	ND
9	3	1	1	0
10	4	8	2	1
11	27	8	7	ND
12	8	3	1	ND

Table 4.7. Hull Airway Reflux Questionnaire (HARQ) score (maximum score 70) at of patients treated with Ivacaftor baseline 6 week, 6 month and 12 month (The numbers highlighted in red represent patients presenting EOR symptoms scoring HARQ >13; ND represents no data available). The individual patient HARQ measured at baseline are shown with a median score of 12 (n=12, range 3-33, mean 14.5, SEM 3.1) and 5 patients presented EOR symptoms. The median score at the 6 weeks follow up was 8 (n=12, range 1-27, mean 9.4, SEM 2.1) with 3 patients presenting EOR symptoms, 6 month after medication the median score was 6 with 4 patients presenting EOR symptoms (n=12, range 1-24, mean 9.2, SEM 2.3) and 12 month after medication the median score was 6 with 3 patients presenting EOR symptoms (n=8, range 0-16, median 6, mean 7.5, SEM 2.5).

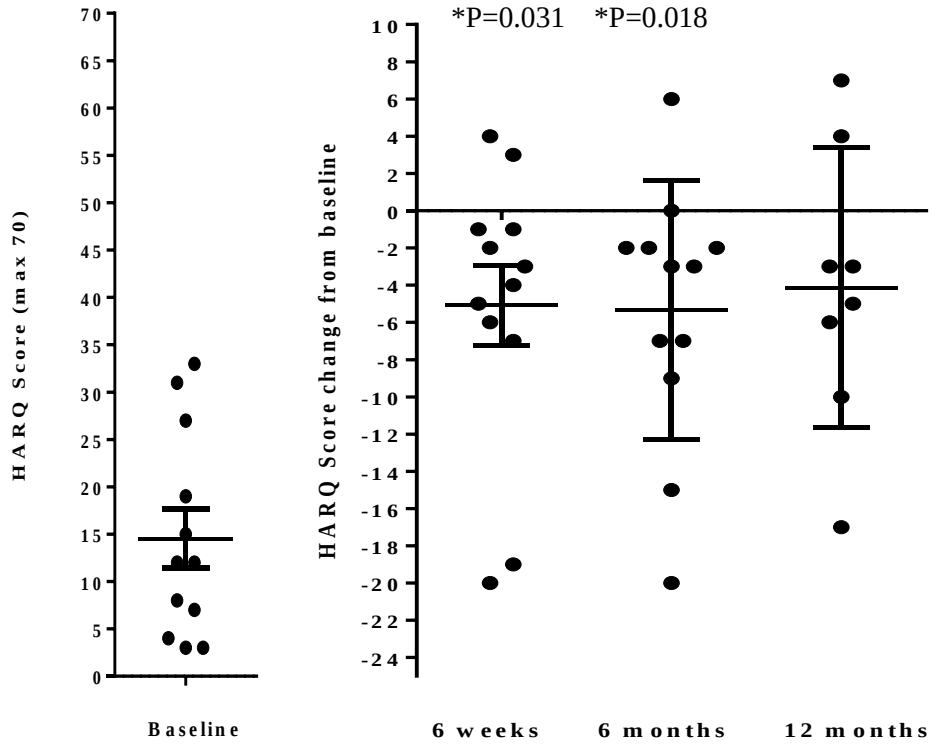


Figure 4.9. Change in Hull Airways Reflux score (maximum score 70) of patients treated with Ivacaftor at 6 weeks (P=0.031, 95% CI (10.50, 1.00)), 6 month (P=0.018, 95% CI (10.00, 1.50)) and 12 (P=0.17, 95% CI (-2.17, 10.42) month from baseline (0= no change from baseline). Statistical analysis performed using paired t-test (CI=95%).

4.2.5 The association of extra-oesophageal reflux symptoms with pulmonary function

The pulmonary function of EOR symptomatic and none-symptomatic patients (RSI and HARQ score; EOR symptomatic patients scored >13 and none EOR symptomatic scored <12) was evaluated. The mean FEV_1 % predicted of EOR symptomatic patients determined by the RSI score was 54% at baseline (range; 19-90) and significantly higher at 85% for the non-symptomatic group (range; 64-110) (P=0.037). FEV_1 % of predicted was higher in the patients that did not experience EOR symptoms compared to those of symptomatic patients. The 6 week follow-up showed that the mean FEV_1% predicted of patients not symptomatic for EOR was 73% (range; 44-102%) which was higher than the EOR symptomatic patients post medication (mean 55%, range; 32-90%) and were similar during the rest of the study period. The mean FEV_1% predicted of EOR symptomatic patients determined by the HARQ score were 47% (range; 19-87%) at baseline whereas none-symptomatic patients had a significantly higher mean FEV_1% predicted of 86% (range; 64-110%) (P=0.02). FEV_1 % predicted was higher in the none-symptomatic group mean 85% (range; 35-132), 6 month mean 92% (range; 35-124) compared to the EOR symptomatic group (6 week mean 84% (range; 44-105), 6 month mean 69 (range 32-110) after treatment. At the 12 month follow-up there were 8 patients interviewed and who provided a score, 2 of whom were EOR symptomatic and had FEV_1% scores of 38 and 98%. There were 6 non-symptomatic patients with a median FEV_1% of 103 (range 83-114). The data was not statistically analysed because of the low number of patients in the EOR symptomatic group. (Figures 4.10 and 4.11).

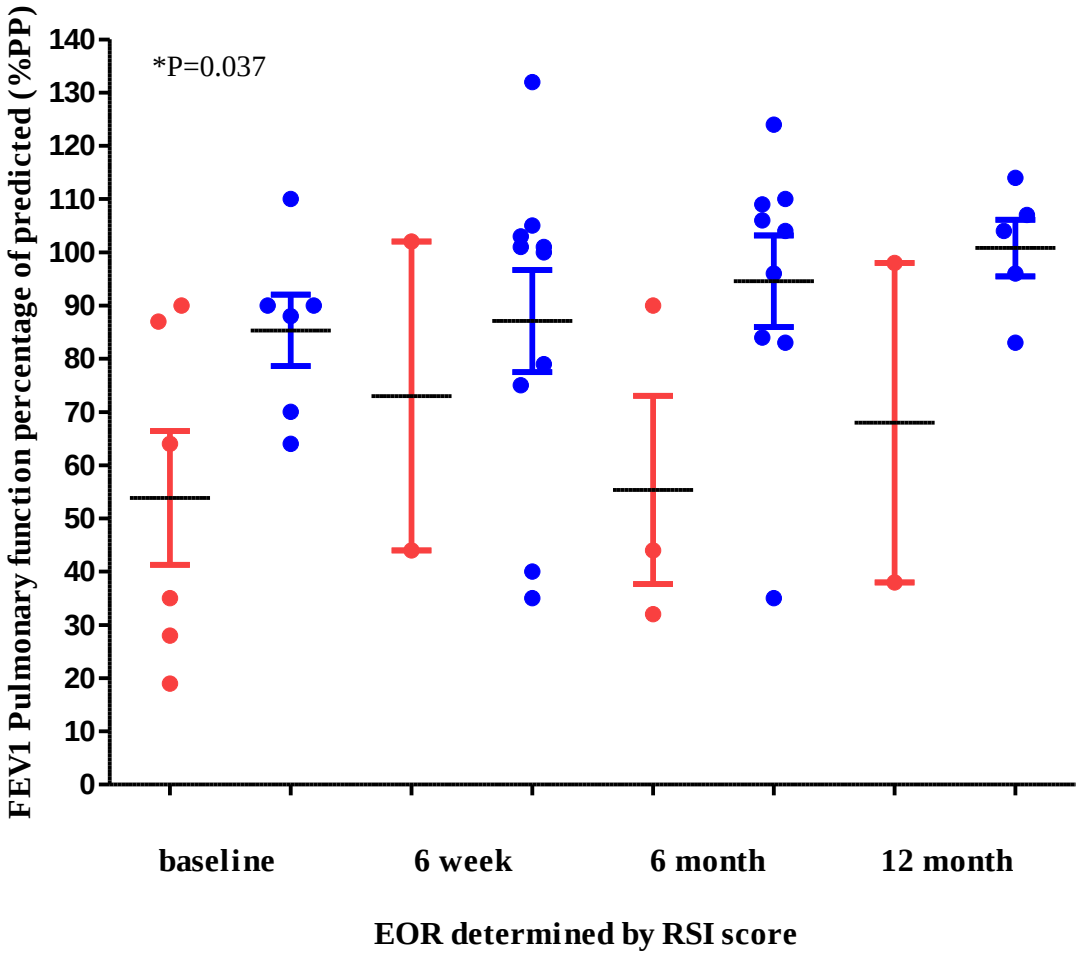


Figure 4.10. Forced expiratory volume in 1 second % predicted in reflux symptomatic patients (red) treated with Ivacaftor and none symptomatic (blue) CF patients determined by the RSI score in a longitudinal follow-up before medication (P=0.037), 6 weeks after initial medication, 6 month after initial medication (P=0.10) and 12 month after initial medication. Statistical analysis performed using Mann-Whitney Test (CI=95%) for groups with 3 or more data points (baseline and 6 month).

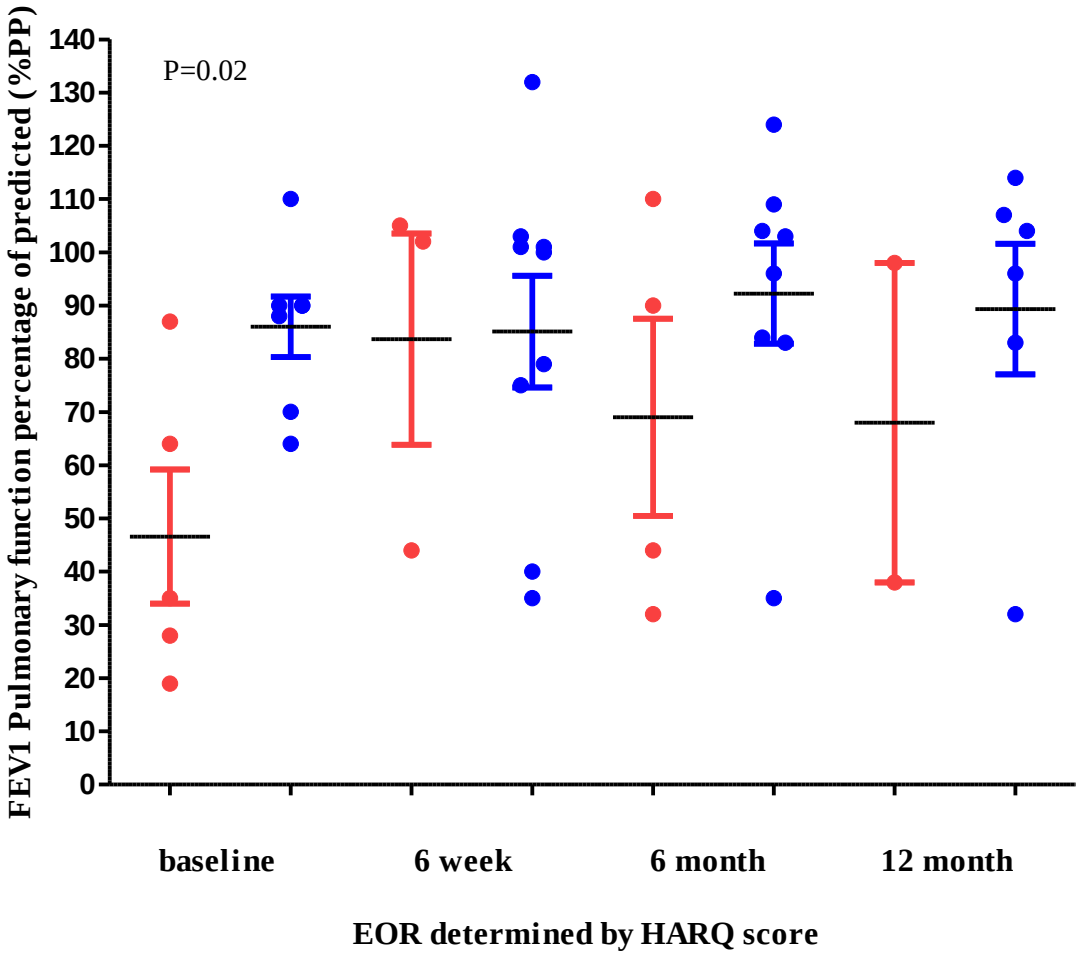


Figure 4.11. Forced expiratory volume in 1 second % predicted in reflux symptomatic patients (red) and none symptomatic (blue) CF patients determined by the HARQ score in a longitudinal follow-up before medication (symptomatic median 47%, range 19-87; none-symptomatic median 86%, range 64-110) (P=0.02), 6 weeks after initial medication (symptomatic median 84%, range 44-105; none-symptomatic median 85%, range 35-132) (P=0.58), 6 month after initial medication (symptomatic median 69%, range 32-110; none-symptomatic median 92, range 35-124) (P=0.46) and 12 month (symptomatic median 68, range 36-133; none symptomatic median 89, range 35-139) after initial medication. Statistical analysis performed using Mann-Whitney Test (CI=95%) for groups with 3 or more data points (baseline and 6 month).

4.2.6 The collection and analysis of sputum samples and expectorated saliva

Each patient was asked to produce an expectorated sputum sample at baseline and each follow-up; if sputum could not be produced the patient was advised to produce expectorated saliva. There were 8 sputum samples and 2 saliva samples collected at baseline from 12 patients with 2 patients unable to provide samples. The number of patients who produced Sputa reduced post treatment, there were 5 sputum samples and 3 saliva samples collected at 6 weeks follow-up; 5 sputum and 6 saliva samples collected at 6 months follow up and 3 saliva samples collected at 12 month follow-up but no sputum produced (Table 4.8). The samples were analysed for pepsin identification and quantity using the in-house pepsin ELISA. At baseline the median pepsin levels was 481ng/ml (range 110 to 1150), at 6 weeks the median was 400ng/ml (range 0 to 820), at 6 month the median was 230 (range 0 to 1502). There was no pepsin detected in the 2 saliva samples collected at the 12 month follow-up. There was no statistical difference of pepsin levels at baseline compared to post medication (baseline median 480ng/ml; range 110-710, 6 week median 400ng/ml; range 0 – 820, 6 month median 230ng/ml; range 0 – 610 and 12 month follow-up only two saliva samples analysed with no pepsin present. (P=0.33) (Table 4.9 and Figure 4.12). A cell differential confirmed that the sputum samples analysed at baseline were 100% neutrophilic. In contrast there were two sputum samples at the 6 week follow-up containing 0.4 and 32% macrophages and 2 samples at 6 month follow-up containing 6 and 2% macrophages. A lipid laden macrophage score was performed for all sputum samples. There was lipid present in two samples contained in the macrophages, the LLMI score was not performed due to insufficient number of macrophages. (Tables 4.10 and 4.11 and Figure 4.13).

Sputum and expectorated saliva sample collection				
Patient no	**baseline**	**6 weeks**	**6 months**	**12 months**
1	sputum	sputum	saliva	saliva
2	sputum	No sample produced	sputum	No sample produced
3	sputum	sputum	sputum	ND
4	sputum	No sample produced	sputum	saliva
5	sputum	saliva	saliva	No sample produced
6	No sample produced	No sample produced	saliva	No sample produced
7	No sample produced	saliva	saliva	No sample produced
8	sputum	sputum	saliva	ND
9	saliva	sputum	sputum	No sample produced
10	saliva	saliva	saliva	saliva
11	sputum	No sample produced	No sample produced	ND
12	sputum	sputum	sputum	ND

Table 4.8. Sputum and expectorated saliva sample collection from patients treated with Ivacaftor at baseline, 6 week, 6 month and 12 month follow-up. There were 8 sputum samples and 2 expectorated saliva samples collected at baseline, 5 sputum samples and 3 expectorated saliva samples collected at 6 week follow-up, 5 sputum samples and 6 expectorated saliva samples collected at 6 month follow-up and 3 expectorated saliva samples collected at 12 month follow-up. (ND represents no data available).

Pepsin concentration (ng/ml) measure in sputum and expectorated saliva				
Patient no	**Baseline**	**6 weeks**	**6 months**	**12 months**
1	261	464	0	0
2	-	-	644	-
3	870	543	704	ND
4	1151	-	1502	-
5	238	0	0	-
6	-	-	165	-
7	-	0	0	-
8	710	820	230	ND
9	116	347	405	-
10	150	352	0	0
11	481	-	-	ND
12	611	696	611	ND

Table 4.9. Pepsin levels measured by in-house indirect pepsin ELISA in sputum and expectorated saliva samples at baseline (median 480 ng/ml; range 110-710), 6 week (median 400 ng/ml; range 0 – 820), 6 month (median 230 ng/ml; range 0 – 610) and 12 month follow-up only two saliva samples analysed with no pepsin present (- no sample analysed). (ND represents no data available).

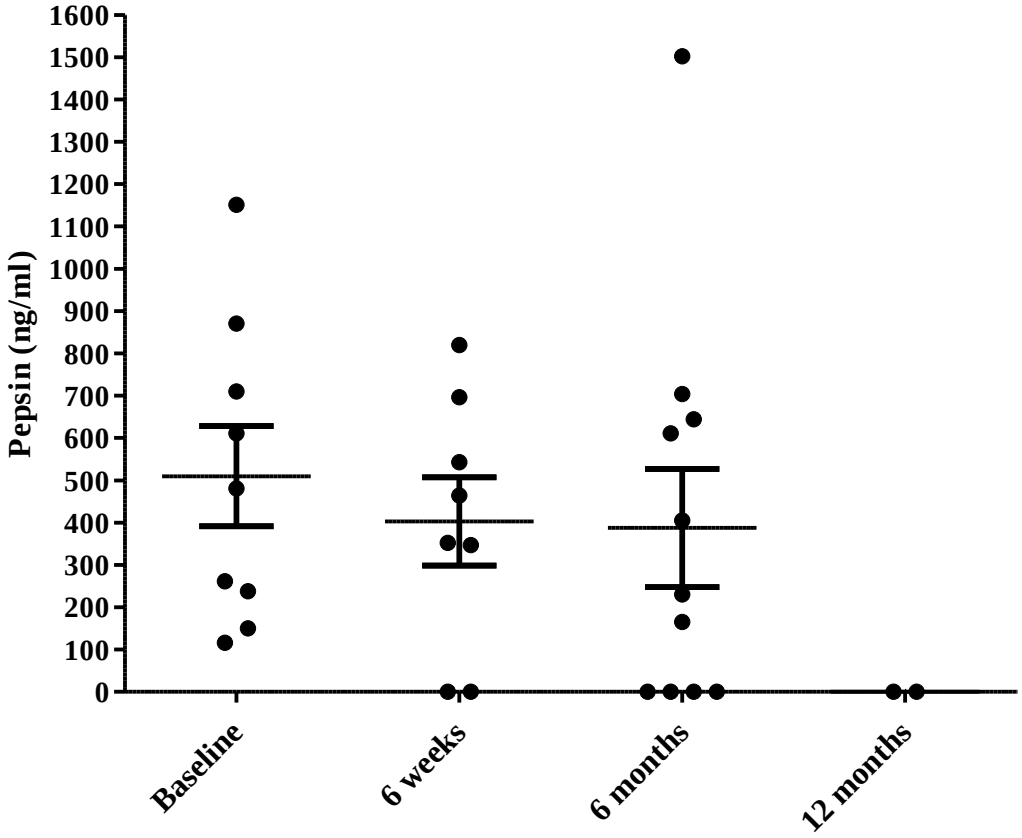


Figure 4.12. The analysis of sputum supernatant and expectorated saliva pepsin levels (ng/ml) in patients treated with Ivacaftor at baseline (n=9), 6 weeks (n=8), 6 month (n=11) and 12 month (n=2) follow-up measured with in-house ELISA using goat monoclonal primary antibody at 1:100 followed by monoclonal secondary to goat monoclonal raised in mouse (peroxide conjugated) at 1:1000 (mean with SEM). The range of pepsin at baseline was 116 to 1151ng/ml, median 481, mean 510, SEM 118. The range of pepsin at 6 weeks was 0 to 820ng/ml, median 408, mean 402, SEM 105. The range of pepsin at 6 months was 0 to 1502ng/ml, median 230, mean 387, SEM 139. There was no pepsin detected in the 2 saliva samples collected at 12 month follow-up. No statistical difference between groups (P=0.33) using the Kruskal-Wallis statistical test.

Cell differential of sputum sample (Giemsa stain)															
Sub no	**Macrophages %**			**Neurophils %**			**Lymphocytes %**			**Eosinophils %**			**Squamous %**		
	BL	**6W**	**6M**	**BL**	**6W**	**6M**	**BL**	**6W**	**6M**	**BL**	**6W**	**6M**	**BL**	**6W**	**6M**
1	0	0	…..	100	100	…..	0	0	…..	0	0	0	0	0	…..
2	…...	…..	0	…..	…..	100	…..	…..	0	…..	…..	0	430	…..	100
3	0	0	2	100	100	98	0	0	0	0	0	0	0	0	20
4	0	…..	0	100	…..	100	0	…..	0	0	…..	0	5	…..	60
5	0	…..	…..	100	…..	…..	0	…..	…..	0	…..	…..	1	…..	…..
6	….	…...	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..
7	0	…...	…..	100	…..	…..	0	…..	…..	0	…..	…..	2	…..	…..
8	0	0	…..	100	100	…..	0	0	…..	0	0	…..	5	0	…..
9	…..	0.4	6	…..	99.6	94	…..	0	…..	…..	0	…..	…..	2	0
10	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..
11	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..	…..
12	0	32.4	0	100	67.6	100	0	0	0	0	0	0	0	25	0.1

Table 4.10. Cell differential of cytological slide preparation of sputum sample (n=12) from patients treated with Ivacaftor stained with Giemsa. 10/12 (83%) samples contain 100% neutrophils. Two samples contain macrophages, sample number 9 at 6 weeks follow-up contained 0.4% and sample number 12 contained 32% at 6 week follow-up.

Lipid laden Macrophage Score (Oil red O staining of cell preparation)			
Patient number	**Oil Red O**		
	BL	**6W**	**6M**
1	0	0	…..
2	0	…..	0
3	0	0	0
4	0	…..	0
5	0	…..	…..
6	…..	…..	…..
7	0	…..	…..
8	0	0	…..
9	…..	0	0 (ORO)
10	…..	….	…..
11	0	….	…..
12	0	0(ORO)	0

Table 4.11. Lipid laden macrophage score for each cytological slide of patients treated with Ivacaftor was performed by staining with Oil Red O (Sudan red). The LLMI was not performed due to insufficient macrophage total (100 needed to perform the score). Two samples contained lipid laden macrophages; patient 9 at 6 month and patient 12 at 6 week follow-up represented as ORO.

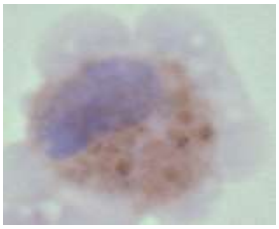
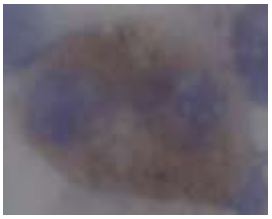
Photography of Lipid laden macrophage		
Patient	**Follow-up**	**Lipid laden macrophage**
9	**6 month**	
12	**6 week**	

Figure 4.13 The photography of a Lipid laden macrophage under light microscopy (×40) from patient treated with Ivacaftor 9 at 6 month follow-up and patient 12 at 6 week follow-up.

4.3 Discussion

The CFTR potentiator Ivacaftor was made available by the FDA for CF patients with the G551D CFTR mutation on January 31st 2012 (Polenakovik and Sanville, 2013). This potentiator aims to normalise the gating channel of the CFTR to able the transport of chloride ions. This can be tested by measuring sweat chloride levels as a way of measuring activity of the drug, and testing compliance with the drug (De Boeck *et al.*, 2013; Accurso *et al.*, 2014). The present study observed the longitudinal effects of the G551D CFTR mutated recipients prescribed with Ivacaftor medication attending routine clinical appointments at the Newcastle upon Tyne RVI chest clinic. The following study presents the recorded measurements taken at baseline, and during the 6 week, 6 month and 12 month follow-up appointments of patients receiving Ivacaftor treatment (150mg/12h). The outcome measurements to assess the effects of Ivacaftor were measurement of sweat chloride concentrations, weight gain or loss, and pulmonary function (FEV_1% FVC% predicted).

In the G551D mutation the CFTR protein is present at the cell surface but the channel does not open or close in a normal manner leading to poor flow of chloride ions in and out of the cell and resulting in loss of epithelial chloride conductance (Yu, at al., 2012). CF patients with this mutation have in abnormal sweat chloride levels and this is used as a gold standard biomarker to diagnose CF disease (normal concentration levels <39mmol/L; possible chance of CF 40-59mmol/L; likely chance of CF >60mmol/L) (Cystic Fibrosis Foundation, 2011). Continuation of Ivacaftor treatment requires a sweat chloride fall below 60mmol/L or 30% lower than baseline concentrations (NHSCommissioningBoard, 2013). Sweat chloride concentrations were measured at baseline and then repeated at 6 weeks and 6 month follow-up. The present study found that the use of Ivacaftor in the Newcastle Upon Tyne G551D CF cohort significantly decreased sweat chloride levels (P=0.001) at 6 weeks follow-up at a median of -58mmol/L and this reduction remained at 6 months follow-up with a median of -64mmol/L from baseline concentrations. The sweat chloride concentrations measured above 60mmol/L at baseline illustrating existing CFTR abnormalities and continuation requirements were met by all patients. Interestingly, at 6 weeks follow-up 7 patients observed a fall in sweat chloride below 60mmol/L and 2 patients observed a fall below 39mmol/L meaning normal concentrations were met. The 3 remaining patients observed a fall of more than 30% of their baseline sweat chloride

concentrations. At the 6 month follow-up a further fall in sweat chloride was observed with 6 patients now having normalised concentrations. These results indicate that the administration of prescribed Ivacaftor (150mg/12h), a CFTR potentiator, results in increased chloride flux. This is supported by Ramsey *et al.,* (2011) who found that treated patients reduced sweat chloride by -48mmol/L at 48weeks from baseline when compared to controls. Accurso *et al.,* (2010) reported a sweat chloride reduction of -59.5mmol/L when assesses 4 weeks after the start of treatment (P=0.008). However, Barry *et al.,* (2013) questions the sweat chloride measurement as a marker of clinical response. Accordingly, it was observed that in 4 patients treated with Ivacaftor the improvement in pulmonary function and BMI were significant after 1 month, but at 8 weeks follow-up the sweat chloride concentrations did not fall below 60mmol/L or by 30% from baseline and these patients failed to meet continuation criteria. The sweat chloride tests were repeated in all 4 patients to observe 2 patients had now met the continuation criteria. One patient admitted to missing one dose of Ivacaftor treatment. The two patients that did not observe the required sweat chloride reductions were discontinued from Ivacaftor despite an increase of 15 and 17% FEV1% predicted from baseline. Barry *et al.,* (2013) questioned the sensitivity of the sweat chloride test as a measure of clinical response. Additionally the data highlight the importance of commitment to medication to observe consistent reduced sweat chloride concentrations.

CFTR proteins are expressed in the gastrointestinal tract leading to an excess of thick and sticky mucus blocking the pancreatic ducts. This minimises the release of pancreatic enzymes which impairs intestinal absorption resulting in malabsorption and malnutrition (Van Goor *et al.,* 2013; Yu *et al.,* 2012). Factors contributing to poor weight gain complications are multifactorial and result in a multitude of problems which reduce quality of life (Thomas and Bishop, 2007; Bourke and Burns, 2011). Ivacaftor may affect gastrointestinal epithelia which may increase nutrient absorption resulting in a possible improved pancreatic enzyme release thus increasing weight. The present study found that the median weight of all patients did not increase at 6 weeks from baseline supporting the issue that CF weight gain is multifactorial and could be affected by increased caloric needs due to exacerbations, diabetes and anorexia. However, seven of the 12 patients increased weight by an average of 2.9kg at the 6 weeks follow-up appointment. The success of weight gain was not associated with severity of lung disease. The overall median weight measured at 6 months significantly increased by 2kg from baseline (P=0.003) and further increasing to 4.6kg at 12 months from baseline. Our data is supported by recent literature

by Barry *et al.*, (2013) who reported a small increase of 0.6kg/m^2 observed over a 1 month follow-up period. It was observed that in a study of Ivacaftor patients with a FEV_1% of predicted of above 40% gained an average of 3.1kg after 48weeks of treatment (Ramsey *et al.*, 2011). Patients with a FEV_1% predicted below 40% were reported to gain an average of 2.1kg over a 12 month period (Hebestreit *et al.*, 2013).

Unregulated chloride ion transport leads to CF disorders in the exocrine organs. The accumulation of thick sticky mucus in the airways leads to decreased pulmonary function, bacterial growth and exacerbations resulting in increased energy demands (Thomas and Bishop, 2007; Bourke and Burns, 2011). The literature has shown that the effects of normalised CFTR channel gating by the use of Ivacaftor has led to significant increases in pulmonary function (Accurso *et al.,* 2010; Ramsey *et al.,* 2011). The present study mirrored findings that were found in clinical trials and showed that Ivacaftor led to a significant increase of median pulmonary function measurements determined by FEV_1 and FVC % predicted (<P=0.004). At baseline the median FEV_1% was 79% of the predicted value based on age, sex and height in accordance with the ERS guidelines (Laszlo, 2006). The median pulmonary function (FEV_1% predicted) measurements increased the most at the 6 week follow-up representing a relative 21% increase from the baseline measurements and remained consistent up to 12 month follow-up. Three of the patients had a FEV_1% predicted below 40% which was the cut off for participation in the previous clinical trials. These patients responded to treatment and increases in pulmonary function were observed resulting in a FEV_1% predicted over 40% for two patients at 6 weeks follow-up. This was supported by findings from the clinical trial published in 2011 by Ramsey *et al.,* (2011) who reported an average increase of 17.2% in FEV_1% at 6 month from baseline. In addition Accurso *et al.,* (2010) reported an increase of 8.7% at 4 weeks from baseline (P=0.008). Barry *et al.,* (2013) observed an increase in FEV_1% predicted after 1 month of Ivacaftor treatment from a median of 64.3% to 73.4% (Barry *et al.*, 2014). Spirometry has been used as a gold standard to measure declining pulmonary function in CF but the measurement has now been regarded inadequate enough to detect early signs of progressive lung disease because FEV_1% is reduced slowly and any falls in measurements mean that the lungs have begun the decline caused by infections and inflammatory responses that are difficult to reverse. To date no other measurement of pulmonary function has taken over FEV_1 measurements but the Lung Clearance Index holds prospect of fulfilling criteria of early detection and sensitivity of lung function decline. It consists of multiple breath washouts to measure ventilation homogeneity (Davies *et al.*, 2008).

Interestingly, Davies *et al.*, (2013) compared lung clearance index in a phase 2 multicentre placebo controlled, double-blind 2x2 crossover study. Ivacaftor, 150mg x2 daily led to significantly improved lung clearance compared to placebo. It was also added that lung clearance methods was a sensitive method to detect a response to Ivacaftor (Davies *et al.*, 2013). The measurement may not be as highly regarded but the wide use of FEV_1% predicted allows studies to compare results and in our case response to treatment. The clinical trials into the efficiency of Ivacaftor used the spirometry measurements and thus this allows our study to compare findings. The lung clearance index informs us about abnormalities in the small airways and it is suggested that increased lung clearance index scores suggest airway narrowing caused by inflammation or sputum plugs (Davies *et al.*, 2008). The present study observed that less sputum was produced post Ivacaftor treatment. There were 8 patients who were able to expectorate sputum on recruitment at baseline and this decreased to 5 patients during 6 week and 6 month follow-up. Interestingly, at the 12 month follow-up there was no sputum expectorated by 8 patients that attended appointments and 4 patients failed to attend their arranged appointments leading us to believe they were feeling well and healthy thus not requiring hospital appointments. On a negative note the study was unable to compare biomarkers of reflux and inflammation from all patients during the study. On a positive note, the patients were unable to expectorate sputum, in some cases provided saliva indicating the positive effects of Ivacaftor on the CFTR epithelium in the airways. The lung clearance index would have provided more information about the inflammation and sputum present in the small airways but this is a new method and Davies *et al.*, (2008) report only few studies have reported lung clearance index scores to date due to complex and expensive technologies needed to analyse samples. This method may be used for research purposes but not for clinical follow-up. This may be related to findings of Reznikov *et al.*, (2014) who investigated the antibacterial properties of Ivacaftor on strains of *Streptococcus pneumoniae* and *Staphylococcus aureus* which are two common pathogens of the CF airway. The antibacterial properties of Ivacaftor were observed by a dose dependant reduction in *Streptococcus pneumonia* determined by bioluminescence. There were only mild decreases of *Staphylococcus aureus* and this was thought to be due to the outer membrane of these bacteria to be a barrier to the Ivacaftor molecule. These data suggest that Ivacaftor may reduce the bacteria colonization within the CF airways which may therefore reduce the inflammatory response. In essence, the reduction of sputa may be related to a reduction of the vicious cycle surrounding infection and inflammation in the

CF airway by Ivacaftor treatment. A reduction in pulmonary exacerbations is a common observation seen in CF patients post Ivacaftor treatment. The phase III trial conducted in 83 patients observed that 67% of patients were free of pulmonary exacerbations by week 48 of the trial Ramsey *et al.*, (2011). Further analysis into these observed decreases show that less patients reported coughing and sputum production in the treated group compared to the placebo group (Flume *et al.*, 2013) . A case report highlighted the significant improvement in the health of a severely ill CF patient of the G551D/deltaF508 mutation with a FEV_1% of 24% of the predicted value following Ivacaftor treatment. The case report illustrated that the hospitalised patient improved pulmonary function, discontinued from supplementary oxygen and didn't experience a pulmonary exacerbation in the 7 month Ivacaftor treatment (Polenakovik and Sanville, 2013).

The benefits of Ivacaftor on the epithelia containing CFTR expression have highlighted the efficiency of this molecule to restore CFTR function. To date, no reports have been made regarding the effects of Ivacaftor on CFTR function in gastrointestinal epithelia. GOR is highly prevalent in CF (Ledson *et al.*, 1998; Blondeau *et al.*, 2008; Sabati *et al.*, 2010) but the effects of Ivacaftor on GOR and EOR symptoms have not been investigated to date. GOR occurs in up to 90% of CF patients resulting in the administration of proton pump inhibitors (PPI) (Blondeau *et al.*, 2008). The present study aimed particular evaluations on the effect of Ivacaftor on gastro-oesophageal reflux symptoms. The aim of the study was to firstly evaluate the effects of Ivacaftor medication on the symptoms of GOR and EOR in these patients. Patients were interviewed regarding their gastro-oesophageal symptoms using three validated questionnaires (DeMeester Reflux Questionnaire; RSI and HARQ); these were then assessed against pulmonary function. This study observed that GOR was present in 3 patients at baseline, the patients experienced classical GOR symptoms. Following Ivacaftor treatment two of these patients reported less GOR symptoms and the DeMeester score reduced below the cut off for GOR (<1) at the 6 week follow-up. It was illustrated that GOR symptoms were not highly prevalent in the study population due to 9 patients not experiencing symptoms. One patient continued to experience GOR symptoms over the 12 month follow-up, it was reported that this patient had a hiatus hernia and thus suffered with GOR. At baseline 6 patients were experiencing EOR symptoms determined by the RSI and HARQ score (>13) and following 6 weeks of Ivacaftor treatment the symptoms decreased to a normalised score in 5 patients (determined by RSI score <12). This was supported by a second questionnaire assessing EOR (HARQ) which illustrated a decrease in EOR symptoms post

treatment. Interestingly, pulmonary function is significantly higher in the non-EOR symptomatic patient group when compared to the EOR symptomatic group at baseline (P=0.037). This finding was observed throughout the study from baseline to 12 months suggesting that the decrease in pulmonary function seen in patients experiencing EOR Symptoms (determined by RSI and HARQ score) may be due to the complications caused by EOR. The numbers of patients positive for EOR symptoms post treatment were low (n=2-3) and therefore unable to argue effect of treatment. The treatment of Ivacaftor illustrated that EOR symptoms decrease but we were not able to investigate a relationship between EOR symptoms and increased pulmonary function due to low numbers. Interestingly, Flume *et al.,* (2013) illustrated that there were less coughing reported by patients when treated with Ivacaftor compared to those in the control group of a phase III trial of Ivacaftor. The presence of reflux biomarkers were assesses in sputum and saliva samples provided. There was no relationship observed between gastric aspiration and the treatment of Ivacaftor due to sample inconsistency over the follow-up period. The biomarkers used as a tool to diagnose gastric aspiration was presence of pepsin in the sputum supernatant and presence of lipid laden macrophages in the cells of the airways. It was observed that patients experiencing GOR symptoms throughout the study treated with PPI therapy and H_2RA's continued to experience symptoms. There were 4 patients EOR symptomatic taking PPI and H_2RA medication. This suggests that the medication did not alleviate classical (GOR) or atypical (EOR) symptoms of reflux by evidence of persisting symptoms. Our data has illustrated these findings previously in the CF patients that are not treated with Ivacaftor and are these patients are discussed in chapter 3 (Crossfield *et al.,* 2013).

Cytological slide preparations were stained with Oil Red O to identify lipid laden macrophages. This stain has been used as a diagnosis tool for microaspiration of stomach contents into the airways. Macrophages (n=100) are identified under a microscope and each given a score from 0-4 depending on presence of red (lipid) pigmentation. This microaspiration tool was not useful to determine possible reflux events in the present study population due to macrophages present in only 3 patients determined by the a cell differential (Giemsa cell stain). Only one patient sample contained >100 macrophages and it was therefore possible to score for lipid laden macrophages. Of the patient sputum samples collected the cell differential confirmed that they were abundant in neutrophils ranging from 67 to 100 %. The sputum of CF patients was neutrophilic at baseline and a lipid laden macrophage score was unable to be produced. The identification of

macrophages within the sputum of 4 samples post medication resulted in an observation of lipid laden macrophages. This illustrates that less neutrophil influx into the lungs post medication and that gastric aspiration may still be present.

In summary, the present study supports the literature published to date and confirms that patients receiving Ivacaftor treatment improve CFTR protein function resulting in decreased sweat chloride concentrations, increased pulmonary function and weight gain. This study presents a novel finding that has not been investigated to date. Ivacaftor treatment reduced the symptoms of GOR and EOR which have an effect on pulmonary function in the CF population.

5.0 Chapter 5

The dietary modifications for Cystic Fibrosis – assessment of related problems

5.1 Introduction

5.1.1 Dietary recommendations for the CF patient

CF is associated with malnutrition and weight loss as a result of the basic defect of the disease. In the 1930's one of the early signs that was observed in babies and infants with CF was the failure to gain weight despite eating well accompanied by foul smelling, oily stool known as steatorrhoea (Thomson and Harris, 2008). This is due to deficient CFTR expression in the cells of the pancreatic ducts, which become blocked with mucus leading to insufficient pancreatic enzyme secretion. The diet cannot be efficiently digested in the absence of the pancreatic enzymes lipase, amylase, trypsin and chymotrypsin, thus leading to fat, carbohydrate and protein malabsorption and as a result the fat is passed in the stool. It has been reported that 85-90% of CF patients have pancreatic insufficiency resulting in the patient having difficulty gaining weight and malabsorption of fat soluble vitamins A,D,E and K (Cohen *et al.*, 2005). The CFTR expression in cells of the airways are also affected leading to increased mucus secretion in the airways. The production of sputum within the lungs requires nitrogen, when increasing sputum production occurs the patient becoming deficient of amino acids. Sputum blocks the airways resulting in symptoms of chronic cough and heavy breathing, and in turn increased energy expenditure in the work of breathing. Bacteria and fungi populate within the CF airways and the patient suffers with chronic lung infections resulting in poor eating habits and decreased appetite (Orenstein *et al.*, 2000; Thomson and Harris, 2008). The past 50 years have seen advances in CF clinical nutrition and highlighted the significant importance of nutritional intervention within the CF clinic. The first serious discussions of the importance of nutritional status in CF was prompted in 1974 by Douglas Crozier who started the CF clinic in Toronto. The CF children were advised to have a diet of high saturated fat foods including whole milk, butter, eggs and animal fats along with 60 to 100 pancreatic enzyme tablets per day. This resulted in a higher survival rate when compared to CF patients at other Canadian and the Boston CF clinic, who adhered to the conventional low fat diet to reduce steatorrhoea (Crozier, 1974; Hodson *et al.*, 2007). This was supported by

observations published in 1978 demonstrating the inverse relationship between underweight CF patients and survival (Kraemer *et al.*, 1978). In addition, findings by Gaskin *et al.,* (1982) indicated significantly higher FEV_1% of predicted and lower mortality in patients with patients that were able to absorb fat compared to those with steatorrhoea (Gaskin *et al.*, 1982). Furthermore, it was highlighted that patients at the Toronto CF clinic maintaining a normal growth rate and nutritional status had longer survival rates compared those at the Boston CF clinic that showed deficient nutrition and growth (Corey *et al.*, 1988). Later studies assessing nutrition in CF found that long-term nutritional support to improve weight-gain led to better nutritional status, better lung function and associated increased survival (Jelalian *et al.*, 1998). These significant developments in dietetic care generated a European consensus in 2002 on the nutrition advice for CF patients to increase fat and calories accompanied by Pancreatic enzyme replacement therapy (Sinaasappel *et al.*, 2002).

Routine dietetic care and nutritional management have remarkably overcome problems of malnutrition in CF. The dietitian monitors growth and nutritional needs. They provide dietetic advice, meal planning, suggest food and supplements and adjust pancreatic enzyme replacement therapy (PERT). The diet of a CF patient is modified to meet the requirements of nutrients needed despite the complications caused by the disease manifestations. The main modifications are explained further focusing on energy, fat, enzyme replacement, protein and additional supplements.

The CF patient requires increased energy in the diet. The requirements vary and depend on individual age, gender, nutritional status, pancreatic insufficiency and pulmonary function although the guidelines are set at 120-150% of the estimated average requirement for energy (White *et al.*, 2004; Hodson *et al.*, 2007). These requirements are met by advising the liberal use of high fat food such as high-fat snacks, fried food, full fat products, and adding butter, cheese, oil and cream to meals (Hodson *et al.*, 2007; Webster-Gandy *et al.*, 2012).

Pancreatic enzyme replacement therapy (PERT) is a method used to aid in the absorption of fat, carbohydrate and protein to prevent malabsorption, malnutrition and growth failure as a complication of CF. The United Kingdom Committee of the Safety of Medicines advise that CF patients receive a daily upper limit of 10,000 Units of lipase per kilogram of body weight (Committee of the Safety of Medicine, 1995). PERT capsules are coated with a pH sensitive microsphers designed to be resistant to the acidic conditions of the stomach. The capsules are dispersed with chyme at a pH of 5.5 to 6.0, thus available to

metabolise fat in the small intestine. Agents that increase the pH of the stomach environment including H2 antagonists and proton pump inhibitors have been shown to potentiate (increase effect of PERT) the benefits of PERT (Robinson, Smith and Sly, 1990; Heiherman, 1992).

Dietary fat is increased to provide higher energy per gram of food (fat has 9 Kcal per gram compared to carbohydrate at 4 Kcal per gram). The % energy from fat in a normal diet is around 28% and this is increased to a recommended 40% of energy from fat for a CF patient. The management is individualised to calculate the ideal weight of the patient based on their height and body composition. PERT is prescribed for pancreatic insufficient patients to aid the metabolism of fat. The recommended units are modified to each individual patient and dependant on fat intake e.g. two PERT capsules (Creon 10,000, Solvay) of 10,000 units of lipase, 600 units of protease and 8,000 units of amylase with each meal and one with each snack containing fat, protein or starchy carbohydrate. It is advised for the capsule to be taken just before or with each meal and snack and avoiding mixing it with hot food or drinks to minimise the risk of deactivating the enzymes (Webster-Gandy *et al.,* 2006; Thomas and Bishop, 2006). Oral food supplements have been recommended for patients that have poor weight gain in the form of juices, ready drinks or powder to mix with milk and contain high carbohydrate or fat in a small serving (Thomson and Harris, 2008).

A diet high in protein is recommended to promote growth and development due to poor nutrition intake and nitrogen loss during sputum production. The amino acids are present in abundance in sputum, it was reported that CF sputum specimens contained higher amino-acid content than non-CF samples. There were various amino acids identified in the sputum specimens such as leucine, isoleucine, phenylalanine, tyrosine, alanine, serine and methionine or valine or both (Barth *et al.,* 1996). Due to the increased protein uptake during sputum production the protein intake is advised to be increased by 20% of the reference nutrient intake (Webster-Gandy *et al.,* 2012). In practice, this is achieved because the protein intake is likely to be increased if the patient adheres to the advised high energy intakes (Hodson *et al.*, 2007) by increasing the amount of food per se.

The supplementation of fat soluble vitamins A,D,E and K are recommended for pancreatic insufficient patients as deficiency is common due to the inadequate vitamin absorption co-absorbed with fat. The risk of vitamin toxicity is also a concern if vitamins are absorbed from both the diet and supplements so patients should receive annual vitamin serum level measurement to monitor the deficiency/toxicity of A,D,E and K. The vitamin

levels should be adjusted to meet the requirements of each patient. The suggested doses of fat soluble vitamins A, D, E and K for an adult are 1200-3000µg; 20-50µg; 100-200mg and 10mg respectively (Yankaskas *et al.*, 2004; Hodson *et al.*, 2007).

5.1.2 Prevalence of overweight and obesity in CF

During the past 50 years researchers, clinicians and dietitians have consistently shown that increasing CF patient's weight to achieve normal growth and avoidance of under-nutrition brings significant positive impact (Simon *et al.*, 2009; Stephenson *et al.*, 2013). Due to these advances in knowledge regarding the importance of nutritional status in CF we observe CF patients surviving longer with a median age of death to be 40 - 50 today (Simmonds, 2013). It has been reported that those that are born today with the CFTR mutations will have normal life expectancy due to future therapies that treat the basic defect (O'Sullivan and Freedman, 2009).

The positive outcome of increasing BMI status for CF patients over the years has led to BMI increasing beyond what is classified by the WHO as an ideal weight (BMI >25.0). BMI classifications were redefined by the WHO in 1998 as underweight (kg/m^2 <18.5); ideal weight (kg/m^2 18.5-24.9); pre-obese/over-weight (kg/m^2 25.0 – 29.9) and obese (kg/m^2 >30.0). The report defined over-weight and obesity to identify individuals or groups at risk of morbidity and mortality who can be prioritised for intervention. The report highlighted that a BMI over $25kg/m^2$ increased the risk of co-morbidities (such as cardiovascular disease and certain types of cancer) and a BMI over $30kg/m^2$ increased the risk to a moderate level (WHO, 1998). An emphasis on characterising the prevalence of overweight and especially obesity in CF is becoming increasingly prioritised to assess the potential health risks a population with increasing life expectancy.

In 1979 the first cases of over-weight male patients with CF were identified in America (n=22) (di Sant'Agnese and Davis, 1979). A study reporting overweight/obese BMI from 1985 to present day in Toronto described significant increases in BMI over a 25 year analysis. The over-weight and obese BMI scores increased significantly over 2.5 decades (P=0.001). There was a reported 7.0% of adult CF patients with a BMI over 25.0 kg/m^2 from records in 1985 to 1989; increasing to 15.8% in 1990 to 1999 and increasing further to 18.4% in the decade 2000 to 2011 (Stephenson *et al.*, 2013). Review of the literature published over the past decade has reported increasing percentages of over-weight BMI status in CF ranging from 4.3% to 18.4% and obesity ranging from 0.7% to 5.9%

(Kastner-Cole *et al.*, 2005; Stack *et al.*, 2007; Munck *et al.*, 2007; Coderre *et al.*, 2012; Stephenson *et al.*, 2013; Smith et al, 2014; Panagopoulou *et al.*, 2014; Kochavi, 2014). Interestingly, Ireland reported the highest percentage of obesity among children in their paediatric CF clinic at 5.9% in 2007 (Stack *et al.*, 2007) followed by 3.8% in an adult CF clinic in Toronto at a similar time (2000-2011) (Stephenson *et al.*, 2013).

The characteristics of patients with a BMI over 25.0kg/m^2 have been reported highlighting that there were less homozygous deltaF508 patients and higher numbers of pancreatic sufficient patients, with increased pulmonary function when compared to under-weight and ideal weight patients ((Munck *et al.*, 2007; Coderre *et al.*, 2012; Panagopoulou *et al.*, 2014). Researchers in Canada observed the average BMI increased by 3.8% per year over a 25 year period for pancreatic sufficient patients compared to an increase of 0.4% per year for pancreatic insufficient patients (P=0.001) (Stephenson *et al.*, 2013). However, one research team identified 9% of over-weight patients and 1% obese patients in a large cohort (n=1118) of adult CF patients homozygous for delta F508 in the U.K. The study revealed that increasing FEV_1% was positively associated with increasing BMI although only significant up to a BMI of 23.0kg/m^2 (Kastner-Cole *et al.*, 2005).

Beneficial effects have been illustrated with increasing BMI of over-weight and obese classifications but the literature has failed to provide evidence for potential risks of increasing BMI in the ever-aging CF population. It has been described that CF patients are living longer now, they may have a normal life expectancy in the future, thus it is a major priority to determine the risks presented to them in older age. Kastner-Cole *et al.*, (2005) proposed the need for caloric counselling for adult CF patients with a BMI above 23 kg/m^2 because they observed only a small benefit of obesity on pulmonary function increases (Kastner-Cole *et al.*, 2005). In support of this, Stephenson et al (2013) suggested that the traditional high fat and high calorie diet maybe disadvantageous to CF patients heterozygous for delta F508 and in particular those pancreatic sufficient in view of small increases seen in FEV_1% and higher prevalence of BMI >25.0 for these patients (Stephenson *et al.*, 2013). Both of the studies witnessed an increase in pulmonary function with increasing BMI despite the increase not being significant, and as this is a predictor of survival the increase becomes of value to the patient and their family however small the increase may be. The authors highlight their concern of over-weight and obesity for the future of the CF patients in view of chronic diseases associated with obesity in the general population (i.e. heart disease, high blood pressure). However, less co-morbid conditions such as diabetes and cirrhosis were observed in a French CF cohort of over-weight and

obese CF patients than the under-weight and ideal weight patients. Regrettably, 1.7% of patients with a BMI below 24.9kg/m^2 were on the lung-transplant list and 1% deceased (Munck *et al.*, 2007). Furthermore, (Panagopoulou *et al.*, 2014) documented no characteristics of metabolic syndrome in the overweight/obese patients with only positive findings of the associated of increased pulmonary function relating to a higher BMI.

The issue of modified nutritional advice for over-weight and obese patients with CF is yet to be resolved. Further study has been recommended to assess the problems that may occur with over-weight/obesity in CF as the population's life expectancy increases (Kastner-Cole *et al.*, 2005; Stephenson *et al.*, 2013). So far, however, there has been no discussion about the potential negative effects that the traditional high fat/calorie CF diet may have on the existing high prevalence of GOR. As the CF life expectancy increases the CF over-weight and obese population may also increase. High fat diets and increased BMI are predictors of GOR in the general population and thus an important area to explore.

5.1.3 The association of dietary components with reflux

The reported factors which are frequently suggested to lead to the pathogenesis of GOR in general include obesity, being overweight and incorrect dietary habits such as a high fat diet, and spicy food (Dore *et al.*, 2008). GORD patients receive recommendations from their G.P. to adopt lifestyle modifications to lower the frequency and severity of GOR symptoms (Nowak *et al.*, 2005) due to the numerous studies providing associations with refluxogenic foods. G.P's recommend GORD patients to avoid alcohol, avoid spicy food, maintain a low fat diet and reduce weight. Research suggests specific dietary components such as, saturated fats may directly worsen GORD (Dore *et al.*, 2008; Zheng *et al.*, 2007; Nebel *et al.*, 1972) but the research to date is contradictory (Friedenburg *et al.*, 2010; Fox *et al.*, 2007).

Numerous studies report a relationship between high fat diets and GOR (Meyer *et al*, 2001; Holloway *et al.*, 1997; Iwakiri *et al.*, 1996). It was research conducted in 1975 (Nebal et al, 1975) that first illustrated high fat foods are associated with GOR in the general population providing an insight and a starting point into foods causing GOR symptoms. Becker *et al.*, (1989) found that high fat foods increased oesophageal acid exposure in twenty subjects. Later, it was found that oesophageal pH monitoring highlighted high fat foods such as chocolate to be positively associated with GOR (Murphy *et al.*, 1988). However, other clinical studies involving oesophageal pH monitoring provide inconsistent evidence regarding fatty foods and GOR (Saberi-Firoozi

et al., 2007; Fox *et al.,* 2007). Fox *et al.,* (2007) observed that reflux symptoms were significantly higher when subjects consumed a high fat diet than low fat ($p<0.05$), but this was not supported by the presence of intra-oesophageal acid exposure to explain the cause of increased symptoms. This was supported by the findings of Shaprio *et al.,* (2007) who found GORD patients consumed more cholesterol, saturated fatty acids and % of calories from fat and were more likely to experience acidic reflux events (Odds Ratio (OR) ¼ 2.8, 95% Confidence Interval (CI): 1.2–6.5). Furthermore, cholesterol was 3 times as likely to trigger a reflux event compared to no consumption of cholesterol (OR 2.8; CI 95% 1.2 – 6.5). In addition, in 2005 it was observed that increased amounts of total fat, saturated fat, cholesterol, average fat servings and % of energy from dietary fat was prominent in the diet of GORD symptom patients when compared to those without symptoms (El Serag *et al.,* 2005). Controversially, Saberi-Firoozi *et al.,* (2007) found that fried food was inversely associated with GOR symptoms and fast food did not have any association with GOR symptoms (Saberi-Firoozi *et al.,* 2007). Importantly, evidence suggests there are no risks of low fat diets or monounsaturated fatty acids and polyunsaturated fatty acids with episodes or symptoms of GOR (Pehl *et al.,* 1999; Fox *et al.,* 2007; Shapiro *et al.,* 2007). There seems to be a large body of data to support the relationship of dietary saturated fat with reflux symptoms but inconclusive data to demonstrate the promotion of GOR episodes within the gut (Penagini *et al.,* 1998 and Becker *et al.,* 1989; Ruhl and Everhart, 1999; Pehl *et al.,* 1999; Penagini, 2000; Colombo *et al.,* 2002). Many authors could not support the general advice given to patients by their GP's to lower saturated fat from their diet as a therapeutic attempt to decrease GOR (Colombo *et al.,* 2002; Penagini, 2000 and Pehl *et al.,* 1999). The volume of food has been questioned in regards to the pressure of the lower oesophageal sphincter (LOSP). Dore *et al.,* (2008) were unable to identify a relationship between GORD and the reported consumption of large meals (Dore *et al.,* 2008).

Increasing BMI status has been associated with GORD symptoms, significantly in obesity ((El-Serag *et al.,* 2005; Hampel *et al.,* 2005; Dore *et al.,* 2008). El-Serag and colleagues (2005) illustrated that the number of classical GOR symptoms increase with higher BMI status in a dose response manner. Moreover, a high fat diet was associated with GORD, but when the data was adjusted for patient BMI status there was no significant association suggesting that BMI could have been the cause of increasing GORD symptoms rather than dietary fat (El Serag *et al.,* 2005). In contrast, evidence by Shaprio *et al.,* (2007) highlighted that the BMI (BMI mean 27.7 ± 1.0) of GORD patients

didn't illustrate a correlation with GOR symptoms and added that it was the cholesterol, saturated fatty acids and % of calories from fat that directly correlated with GORD symptoms.

5.1.4 Aim of the following chapter

CF patients suffer with up to 90% impaired nutrient absorption, therefore causing weight loss and undernutrition which are both associated with a worse clinical outcome (Kraemer *et al.,* 1978; Corey *et al.,* 1988; Sharma *et al.,* 2001). CF patients have increased nutritional requirements and it is therefore imperative to maintain adequate energy intake. Therefore, dietetic advice recommends that fat should not be restricted despite evidence to support that this leads to reflux. However, some saturated fat can be replaced by monounsaturated and there was no reported association between unsaturated fats and GOR. General GORD advice regarding dietary modifications cannot be adopted by CF patients as their increased dietary requirements are imperative to survival (Thomas and Bishop, 2007). Diet has been shown to be associated with GORD in the general population and GORD patients but there have been no reports to date of diet affecting GOR in the CF population.

Due to the inconclusive evidence regarding the unknown risks of over-weight and obese CF patients the following chapter looks at BMI status in a UK regional CF population. The aim of following chapter was to evaluate the relationship between CFTR genotype (homozygous or heterozygous for delta F508) with BMI and pulmonary function in CF patients to assess whether patients that are homozygous are at risk of low BMI compared to patients heterozygous for delta F508 receiving the same dietary advice. As BMI status has been shown to effect pulmonary function the relationship between genotype and pulmonary function (FEV_1%, FVC% predicted and FEV_1/FVC ratio) was investigated:

Aim 1. To explore a potential association between increasing BMI and pulmonary function in CF.

Aim 2. To investigate a potential association of CFTR mutation (homozygous/heterozygous for delta F508) on the BMI status and pulmonary function of CF patients.

Aim 3. To discover a potential association of BMI status with patient symptoms of reflux (GOR/EOR) and markers of gastric aspiration.

5.2 Results

5.2.1 Study design and sampling

The following chapter analyses data that was reported in chapter 3. Seventy two patients were recruited into the study and their weight and height was measured at recruitment during their routine clinical appointment by a trained nurse. The BMI data was categorised according to the definition of under-weight (UW): <18.5 kg/m^2, normal-weight (NW): 18.5-25.0 kg/m^2, over-weight (OW): 25.1-30.0 kg/m^2 and obese (OB): >30.1 kg/m^2 as defined by the World Health Organisation (WHO.). Lung function (FEV_1 % predicted; FVC% predicted and FEV_1/FVC ratio) and genotype were recorded at the time of data collection. To evaluate the relationship between BMI and pulmonary function with genotype the Kruskal-Wallis statistical test was used followed by the Mann Whitney U test.

5.2.2 Patient BMI categories

There were 15% of patients with a BMI below 18.4 and classed as UW (n=11; range 14.1 to 18.2; median 17.5). A large proportion of patients achieved a normal BMI (NW) between 18.5 and 24.9 at 68% of the study population (n=49; range 18.5 to 24.1; median 21.2). There was 15% of patients with a BMI over 25.0 and classified as overweight (OW) (n=11; range 25.3 to 29.8; median 26.8) and one patient had a BMI of 30.9 and classified as obese (OB). The data were examined to observe any differences in BMI between males and females (Figure 5.1). There were no statistical differences between BMI and gender (the median BMI for males (n=39) was 21.5; range 15.2 – 27.9 and the median for females was 21.0; range 14.1 to 30.9) (P=0.43) of the BMI categories assessed by the Kruskal Wallis test with Gaussian Approximation and Dunns multiple comparison tests shown in Figure 5.2.

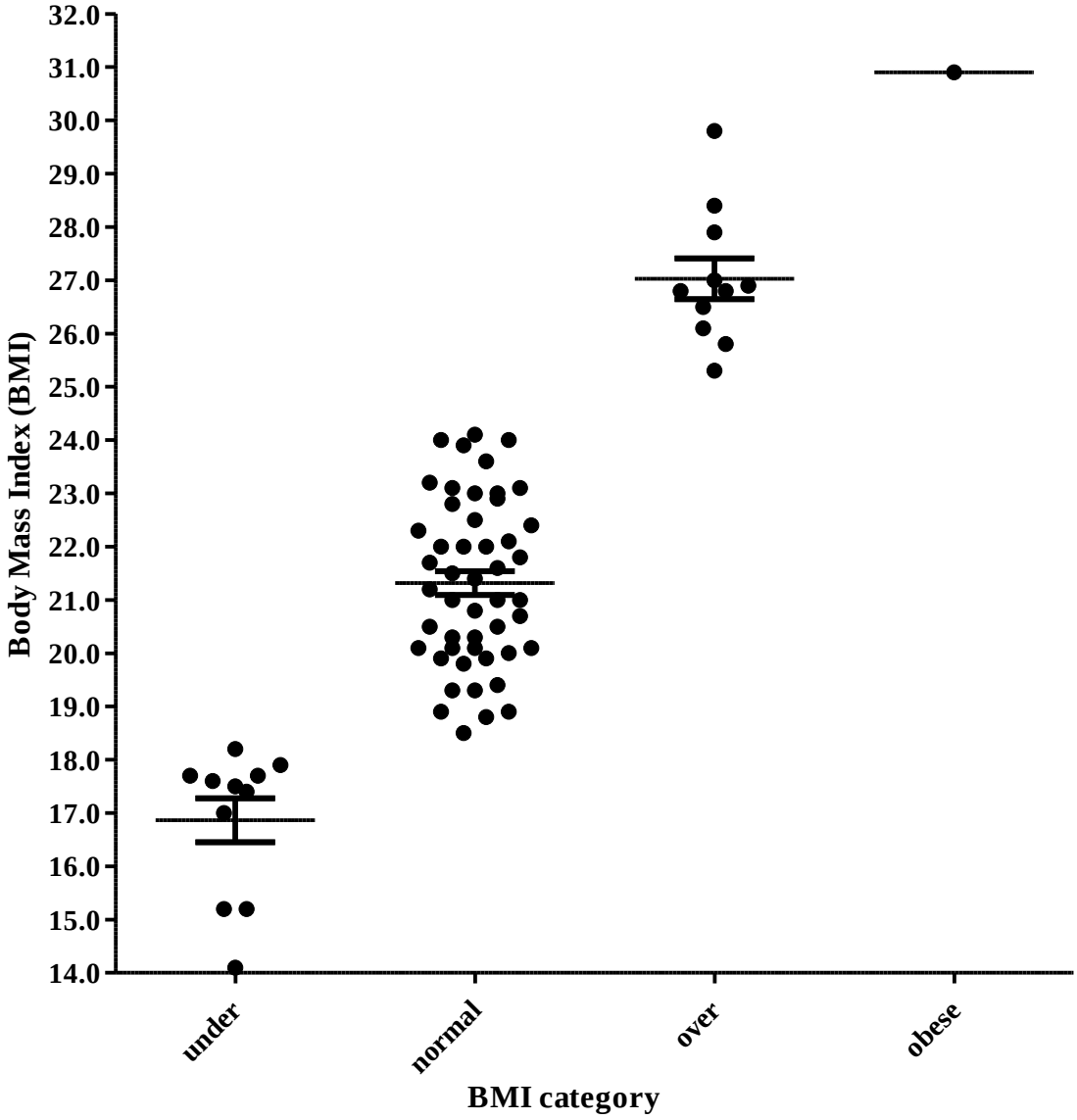


Figure 5.1 The BMI categories of CF patients. Of 72 patients; 11 patients underweight (range 14.1 to 18.2, median 17.5, mean 16.9 SEM 0.4), 49 patients normal weight (range 18.5 to 24.1, median 21.2, mean 21.3 SEM 0.2), 11 patients overweight (range 25.3 to 29.8, median 26.8, mean 27.0 SEM 0.4), 1 patient obese (BMI 30.9).

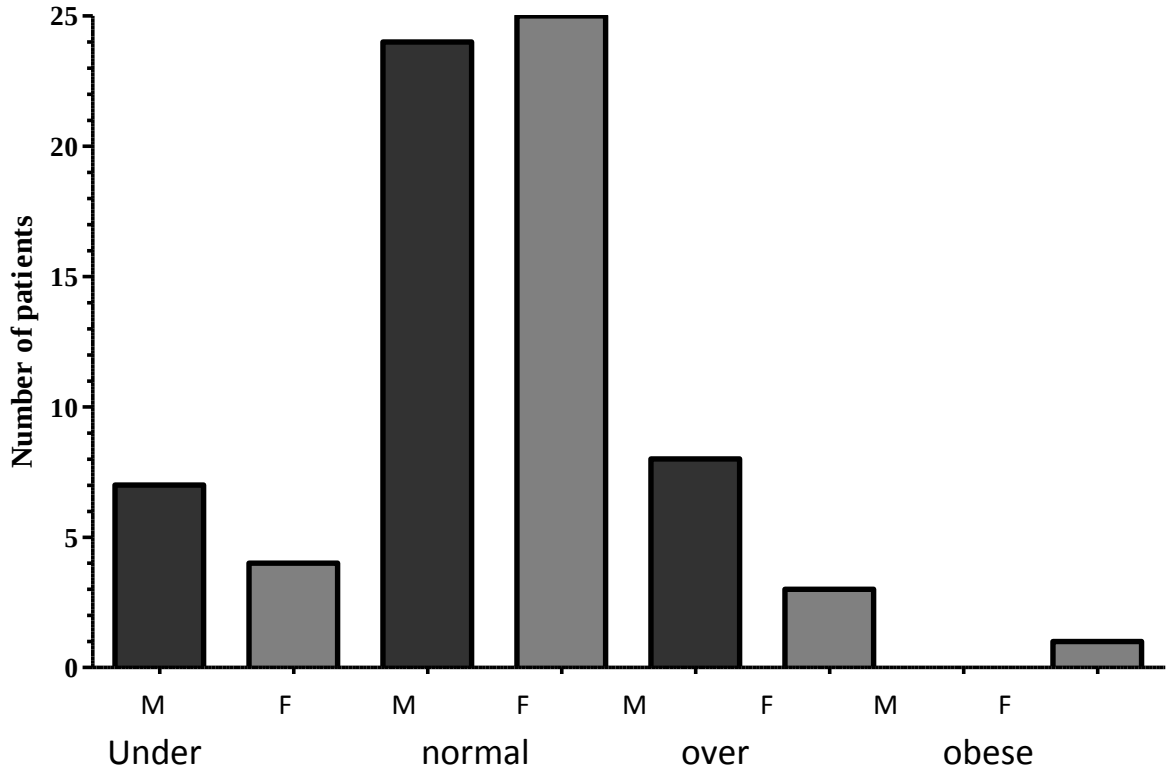


Figure 5.2 The BMI classified groups of CF patients split into gender. Of the underweight patients there were 7 males and 4 females; of the normal BMI patients there were 24 males and 25 females; of the overweight patients there were 8 males and 3 females; the 1 obese patient was female. The median BMI for males (n=39) was 21.5; range 15.2 – 27.9 and the median for females was 21.0; range 14.1 to 30.9. There was no statistical differences of gender and BMI (P=0.423) assessed by the Kruskal Wallis test, Gaussian Approximation and Dunns multiple comparison test.

5.2.3 The association of BMI with CF pulmonary function

The pulmonary function measured by FEV_1%; FVC% predicted and FEV_1/FVC ratio was measured in 63 patients on the day of recruitment by trained pulmonary function staff at the out-patient clinic, RVI, Newcastle according to European Respiratory Society guidelines ((Pellegrino *et al.*, 2005; Laszlo *et al.*, 2006). There were 9 patients that did not have the tests performed and the data was not provided for the study. FEV_1 ranged from 12 to 110% of percentage of predicted values. Increased BMI scores were associated with improved pulmonary function measured by FEV_1 % predicted). The median FEV_1% in the underweight group was 48 (n=11; range 25–56; mean 45.1; SEM 2.9). The median in the normal weight group was 41.5% (n=44, range 12 – 92, mean 45.1, SEM 5.0) and the median score of the overweight group was 69.0% (n=11, range 54-110, mean 75.0, SEM 5.7). The one patient in the obese BMI category had a FEV_1% predicted score of 90.0%. The mean FEV_1% of the over-weight patients was significantly higher than the under-weight group (P=0.02) and normal-weight group (P=0.0003) illustrated in Figure 5.3. The FVC% predicted ranged from 11 to 119 % of the predicted values. Of the 63 patient FVC% predicted scores collected the median of the underweight patients was 59.0% (n=7, range 38 – 84; mean 58.7, SEM 6.6); the median normal weight scores were 59.0% (n=44, range 11 – 100, mean 60.9, SEM 3.2), the median score of the overweight group was 94.0 (n=11, range 68-119, mean 94.3, SEM 4.4) and the one patient in the obese BMI category had a FVC% predicted score of 89.0%. The mean FVC% of the over-weight patients was significantly higher than the under-weight group (P=0.02) and normal-weight group (P=0.0004) illustrated in Figure 5.4. The FEV_1/FVC ratio of the underweight group was 0.60 (n=7, range 0.52-0.87, mean 0.64, SEM 0.05); the normal weight group median was 0.67 (n=44, range 0.35-1.00, mean 0.65, SEM 0.02); and the median of the over-weight group was 0.71 (n=11, range 0.48-0.83, mean 0.68, SEM 0.04). The one patient in the obese category was 0.88. There was no significant differences between the FEV_1/FVC ratio of BMI categories (P=0.38) as shown in Figure 5.5. If the FEV_1% and FVC% are improving then the ratio will not change improvement.

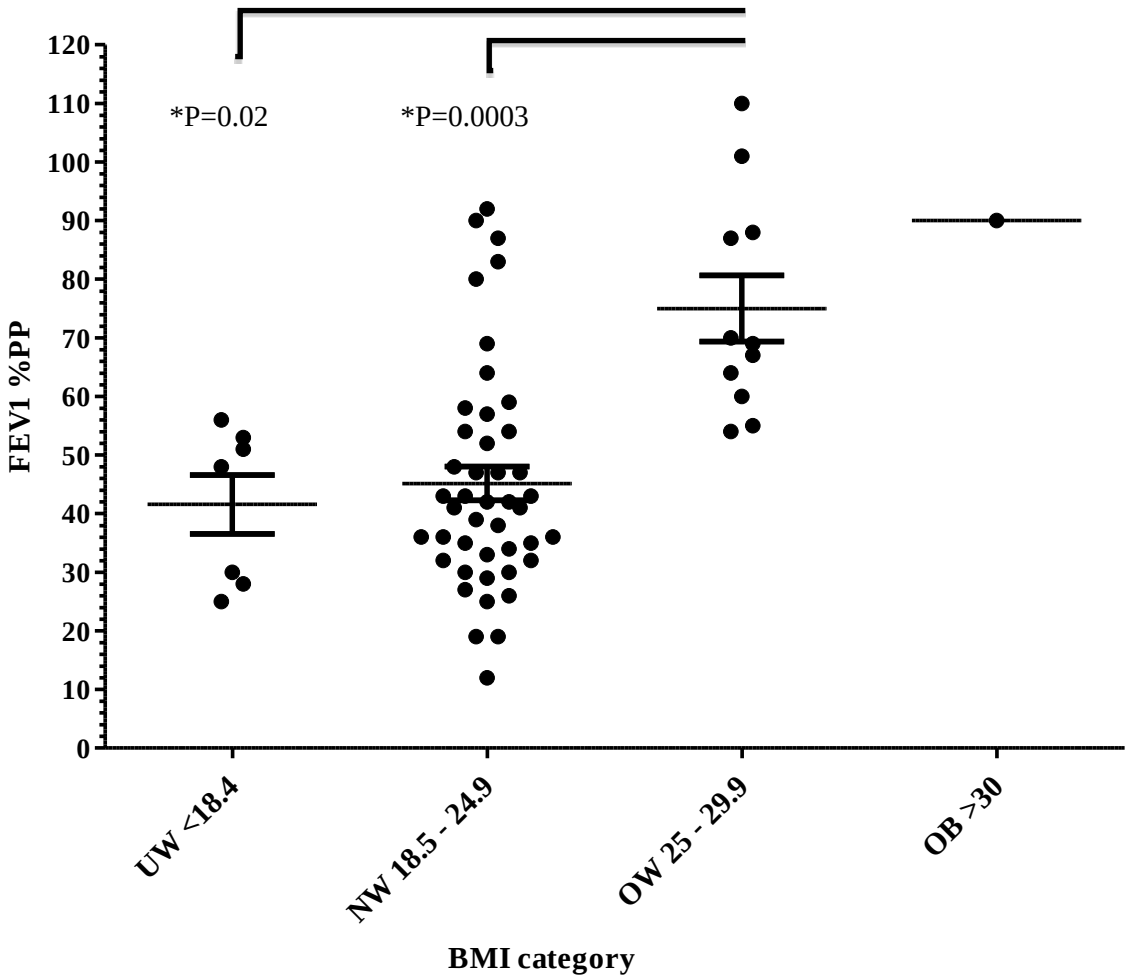


Figure 5.3 The FEV_1% predicted of each CF patient in BMI classified groups. Of the 63 FEV_1% predicted scores collected the median of the underweight patients was 48.0% (n=7, range 25 – 56; mean 41.6, SEM 5.0); median normal weight scores were 41.5% (n=45, range 12 – 92, mean 45.1, SEM 2.9) median score of the overweight group was 69.0 (n=11, range 54-110, mean 75.0, SEM 5.7) and the one patient in the obese BMI category had a FEV_1% predicted score of 90.0%. The mean FEV_1% of the over-weight patients was significantly higher than the under-weight group (P=0.02) and normal-weight group (P=0.0003). Kruskal-Wallis test and Dunns multiple comparisons test.

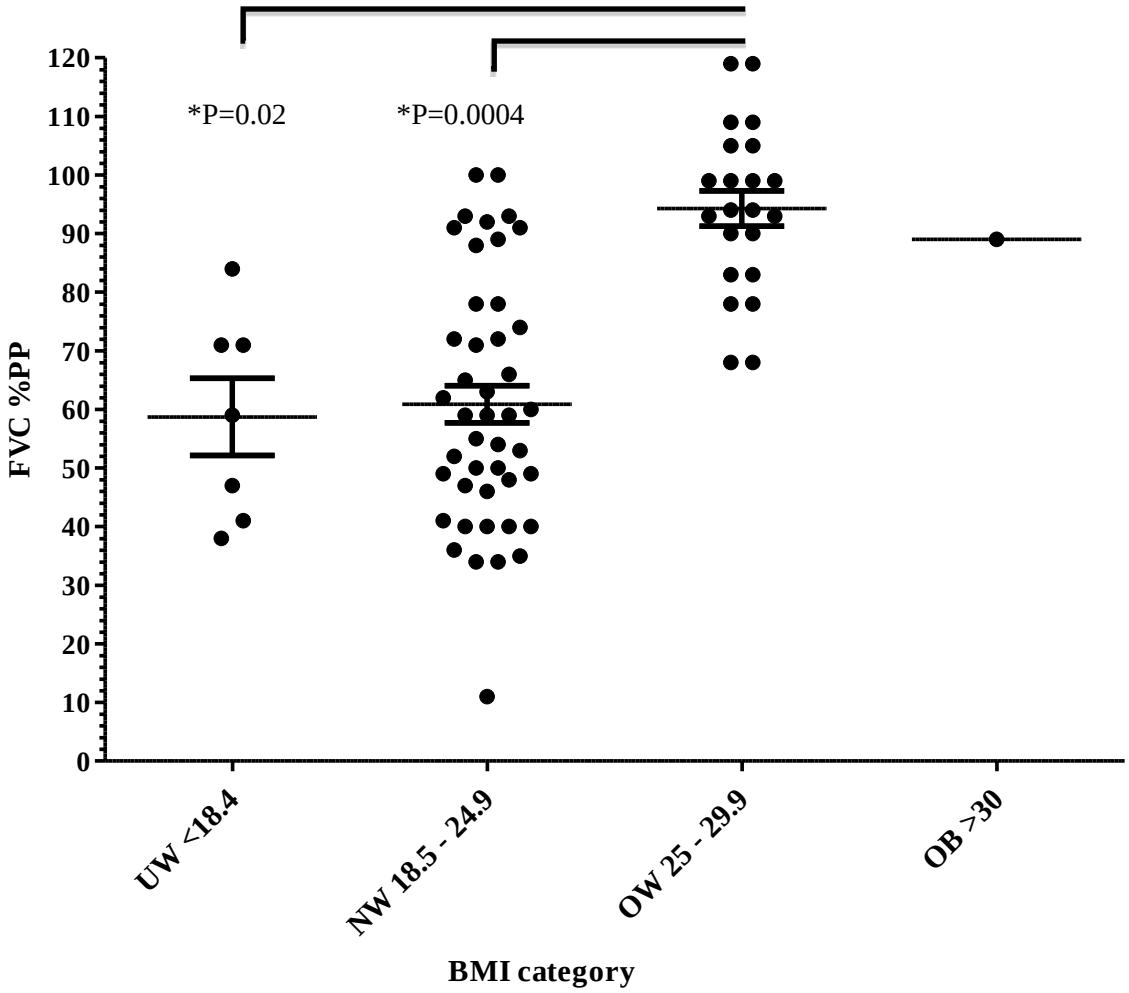


Figure 5.4 The FVC% predicted of each CF patient in BMI classified groups. Of the 63 FVC% predicted scores collected the median of the underweight patients was 59.0% (n=7, range 38 – 84; mean 58.7, SEM 6.6); median normal weight scores were 59.0% (n=45, range 11 – 100, mean 60.9, SEM 3.2) median score of the overweight group was 94.0 (n=11, range 68-119, mean 94.3, SEM 4.4) and the one patient in the obese BMI category had a FVC% predicted score of 89.0%. The mean FVC% of the over-weight patients was significantly higher than the under-weight group (P=0.02) and normal-weight group (P=0.0004). Kruskal-Wallis test and Dunns multiple comparisons test.

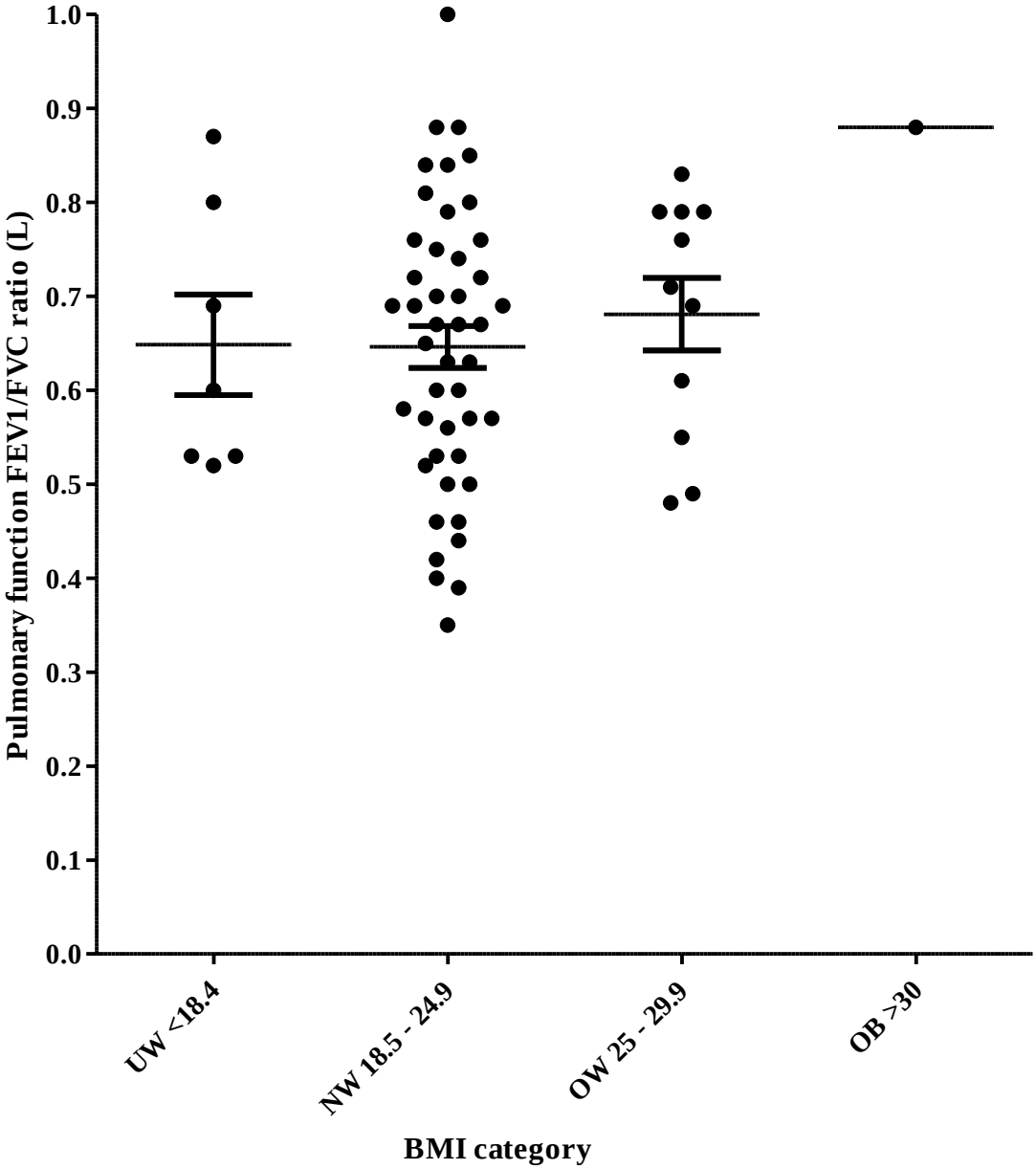


Figure 5.5 The FEV_1/FVC ratio of CF patients n=64 (9 results not available) categorised into BMI groups UW (n=7, median 0.60, range 0.52-0.87, mean 0.64, SEM 0.05); NW (n=45, median 0.67, range 0.35-1.00, mean 0.65, SEM 0.02); OW (n=11, median 0.71, range 0.48-0.83, mean 0.68, SEM 0.04); OB n=1 0.88. Kruskal-Wallis test with Gaussian Approximation Not significantly different; Dunns comparison test (P=0.38).

5.2.4 The association of Delta F508 genotype and BMI category

The relationship between BMI status and the mutation of CFTR was assessed firstly by dividing patients into those homozygous for delta F508 and those heterozygous for delta F508. A comparison was made between the BMI status in each group by a nonparametric Two tailed T-test (Mann-Whitney test with Gaussian approximation). This showed no association between CF genotype status and BMI (P=0.30). The median BMI score of the homozygote patients was 20.5 (range 15.2 to 27.9; mean 21.6; SEM 0.7) and the median BMI of the heterozygotes was 21.8 (range 15.2-30.9; mean 22.2; SEM 0.49) shown in Figure 5.6.

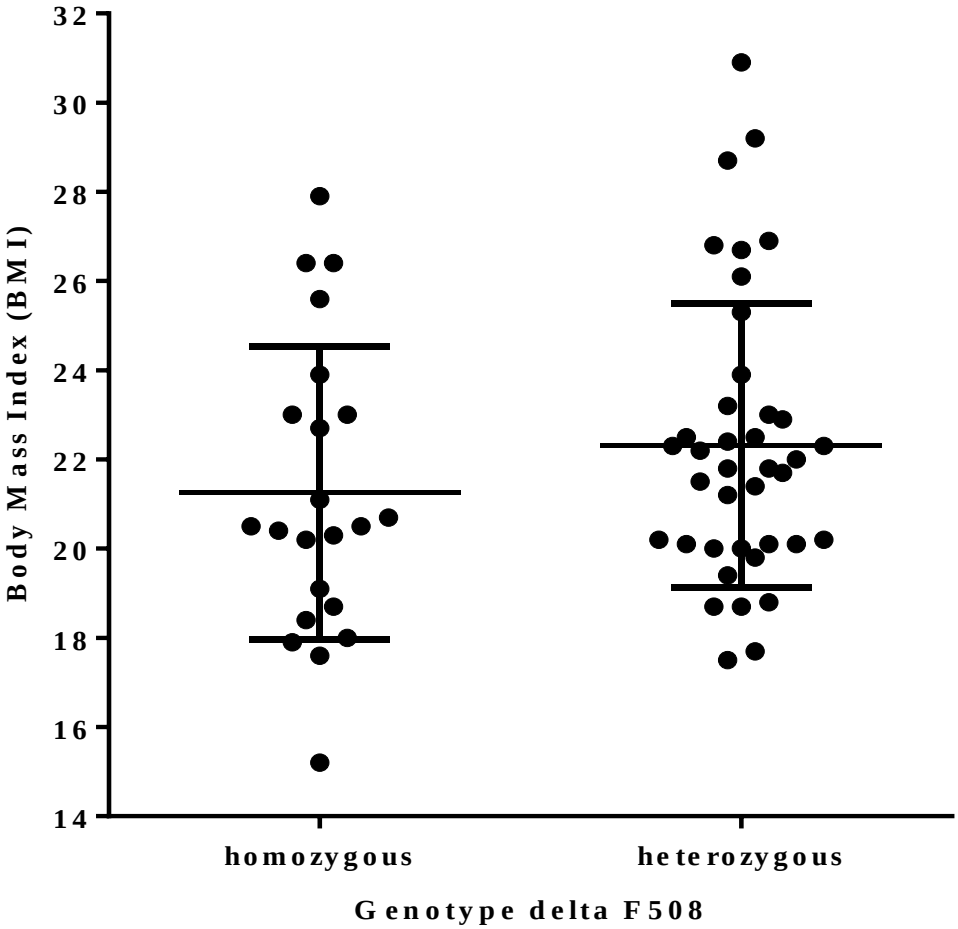


Figure 5.6 The BMI of patients depending on genotype. Of the 22 patients homozygous for Delta F508 the median BMI was 20.5 ranging from 15.2 to 27.9 (mean 21.6, SEM 0.7). The median BMI for those patients heterozygous (n=39) was 21.8 (range 15.2-30.9, mean 22.2, SEM 0.49). Nonparametric Two tailed T-test (Mann-Whitney test with Gaussian approximation) showed no significant difference between the BMI of patient who are homozygous or heterozygous for delta F508 (P=0.30).

5.2.5 The association of Delta F508 genotype and pulmonary function

The genotype data for delta F508 was available for 61 of the total 72 patients recruited onto the study. Twenty two of the 61 patients were homozygous for the delta F508 mutation; the remaining 39 were heterozygous (deltaF508/other). There was no statistical difference observed between delta F508 genotype status and pulmonary function when determined by FEV_1% predicted (P=0.28); FVC% predicted (P=0.18) or FEV_1/FVC ratio (P=0.20). The patients that were homozygous for delta F508 had a median FEV_1% predicted of 43% (range 25-69, mean 44.6, SEM 2.6); median FVC% predicted of 59% (range 34-105, mean 61.9, SEM 4.38) and a median FEV_1/FVC ratio of 0.6L (range 0.5-0.8, mean 0.6, SEM 0.02). This did not statistically differ from the pulmonary function of the heterozygous delta F508 patients. The median FEV_1% predicted was 52% (range 12-110, mean 54.9, SEM 4.23), the median FVC% predicted was 71.0% (range 11-119, mean 70.5, SEM 4.04) and the median FEV_1/FVC ratio was 0.7 L (range 0.4-1.0, mean 0.7, SEM 0.03) in the heterozygotes (Figure 5.7, 5.8 and 5.9). The pulmonary function was higher in the heterozygous group but not significantly.

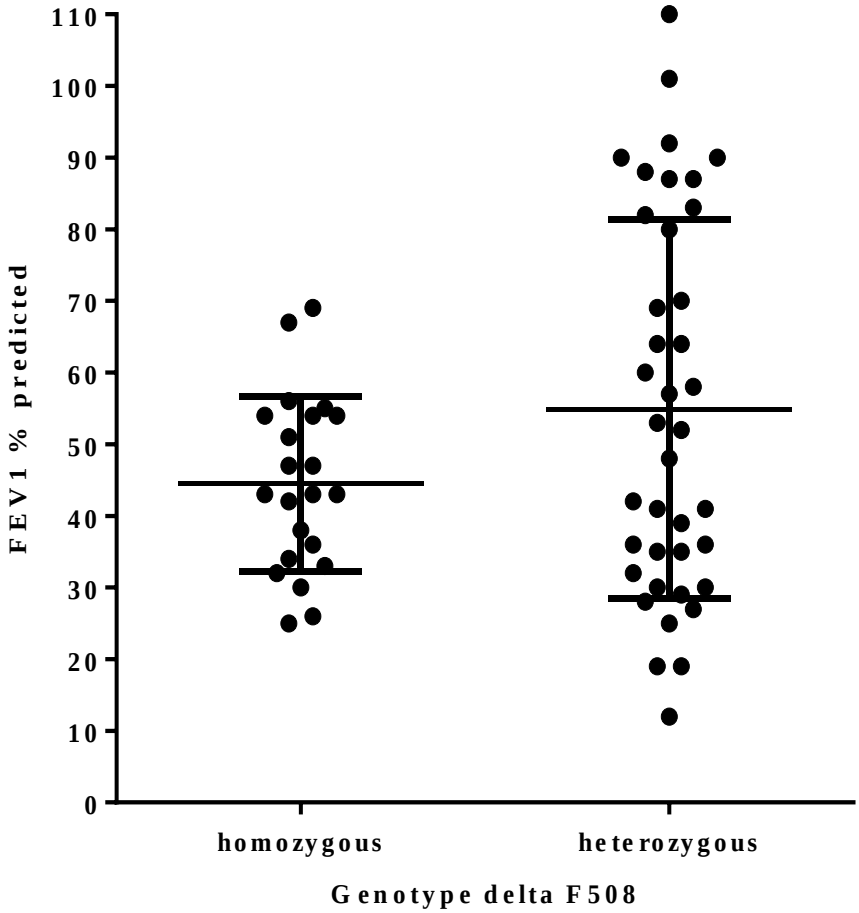


Figure 5.7 The FEV_1% predicted for patients in homo/heterozygous genotypes. There was 22 patients FEV_1% data available for the homozygous for Delta F508 and 39 heterozygous patients with available FEV_1 scores. The median FEV_1% predicted for those patients homozygous for delta F508 was 43% (range 25-69, mean 44.6, SEM 2.6). The median FEV_1% predicted for those patients heterozygous for delta F508 was 52% (range 12-110, mean 54.9, SEM 4.23). Nonparametric Two tailed T-test (Mann-Whitney test with Gaussian approximation) show no significant difference between the FEV_1% predicted of patient who are homozygous or heterozygous for delta F 508 (P=0.28).

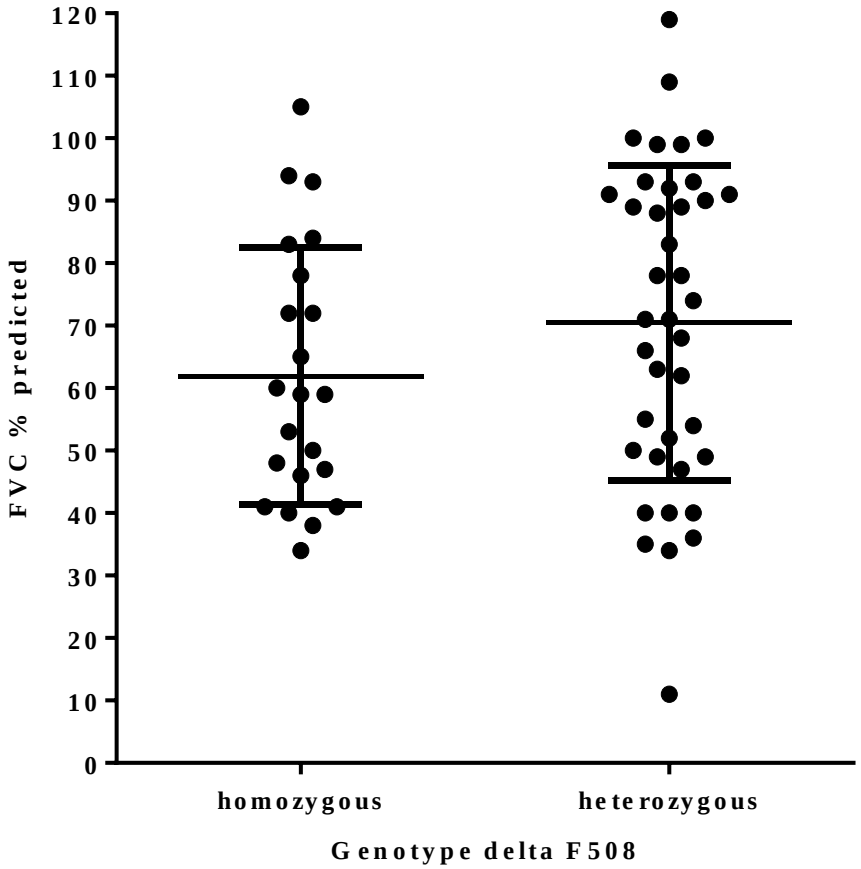


Figure 5.8 The FVC% predicted for patients in homo/heterozygous genotypes There were 22 patients homozygous for Delta F508 and there were 39 heterozygous patients with available FVC% predicted scores. The median FVC% predicted for those patients homozygous for delta F508 was 59% (range 34-105, mean 61.9, SEM 4.38). The median FVC% predicted for those patients heterozygous for delta F508 was 71.0% (range 11-119, mean 70.5, SEM 4.04). Nonparametric Two tailed T-test (Mann-Whitney test with Gaussian approximation) show no significant difference between the FVC% predicted of patient who are homozygous or heterozygous for delta F 508 (P=0.18).

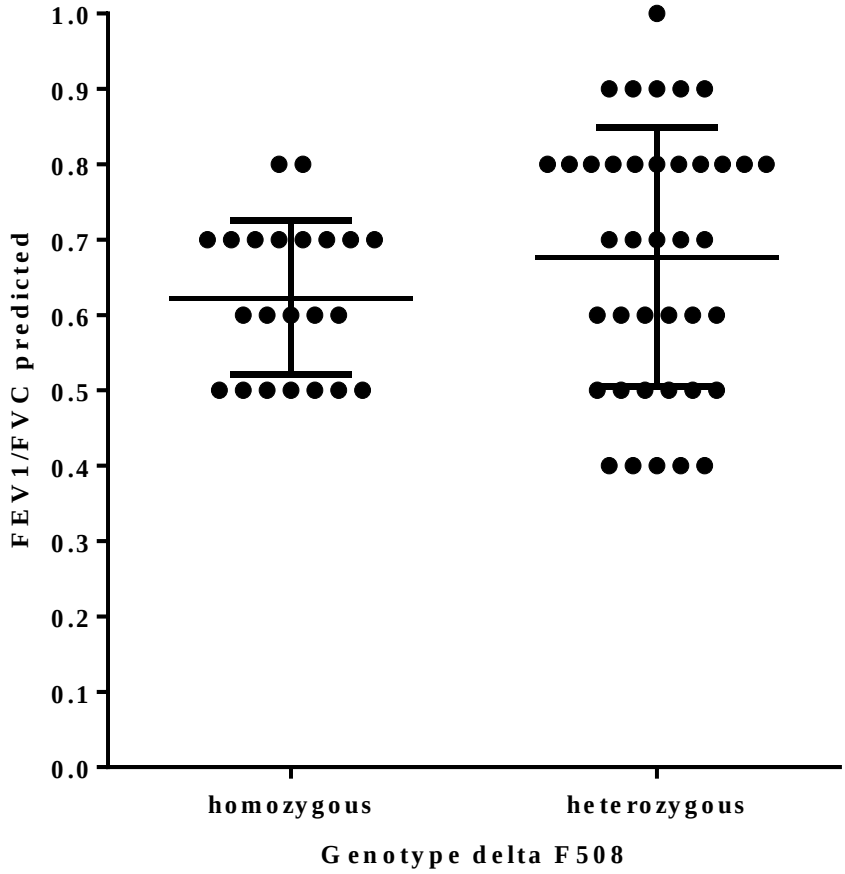


Figure 5.9 The FEV_1/FVC for patients in homo/heterozygous genotypes. There were 22 patients homozygous for Delta F508 and there were 39 heterozygous patients with available FEV_1/FVC ratio scores. The median FEV_1/FVC ratio for those patients homozygous for delta F508 was 0.6 L (range 0.5-0.8, mean 0.6, SEM 0.02). The median for those patients heterozygous for delta F508 was 0.7 L (range 0.4-1.0, mean 0.7, SEM 0.03). Nonparametric Two tailed T-test (Mann-Whitney test with Gaussian approximation) show no significant difference between the FEV_1/FVC scores of patient who are homozygous or heterozygous for delta F 508 (P=0.20).

5.2.6 The association of BMI category with the symptoms of reflux (GOR and EOR)

The effect of BMI status on the symptoms of reflux was assessed in the 72 patients recruited onto the study. The association of BMI with the symptoms of GOR was assessed by comparing GOR symptoms determined by the DeMeester reflux questionnaire in each BMI category. The symptoms of GOR did not statistically correlate with BMI status when assessed by the Kruskal-Wallis test with Gaussian Approximation (P=0.66). The DeMeester scores in each BMI category are shown in Figure 5.10 and the median score (±SEM) of the underweight category was 2 (range 0 to 4, mean 2.1, SEM 0.4), the median score of the normal weight category was 1 (range 0 to 7, mean 1.4, SEM 0.2), the median score of the over-weight category was 1 (range 0 to 5, mean 0.4, SEM 0.4). There was only one patient in the obese category and their DeMeester score was 5.

The effect of BMI status on the presence of EOR symptoms was determined by comparing the RSI questionnaire score of patients in each BMI category. The presence of EOR symptoms were higher in the underweight group at a median of 23 (range 9 to 43, mean 20, SEM 3.1) compared to the normal weight patients that had a median RSI score of 16 (range 0 to 36, mean 16, SEM 1.1) and the overweight patients that had a median RSI score of 11 (range 2 to 18, mean 1.9, SEM 0.4). The one patient in the obese category had a RSI score of 12. The Kruskal-Wallis test with Gaussian Approximation illustrated statistical significance between RSI score to identify EOR symptoms and BMI category (P= 0.038). The median scores of the under-weight and normal-weight group are above the limit of 13 used to identify EOR, and the median score of the over-weight group is below 13 (Figure 5.11). The data was presented in a Scatterplot of BMI status on the y axis and RSI score on the x axis. The Pearson correlation showed a statistical correlation between low BMI leading to higher EOR symptoms (determined by the RSI) (R^2=0.0638/6.4%, n=72, P-Value = 0.034) (Figure 5.12).

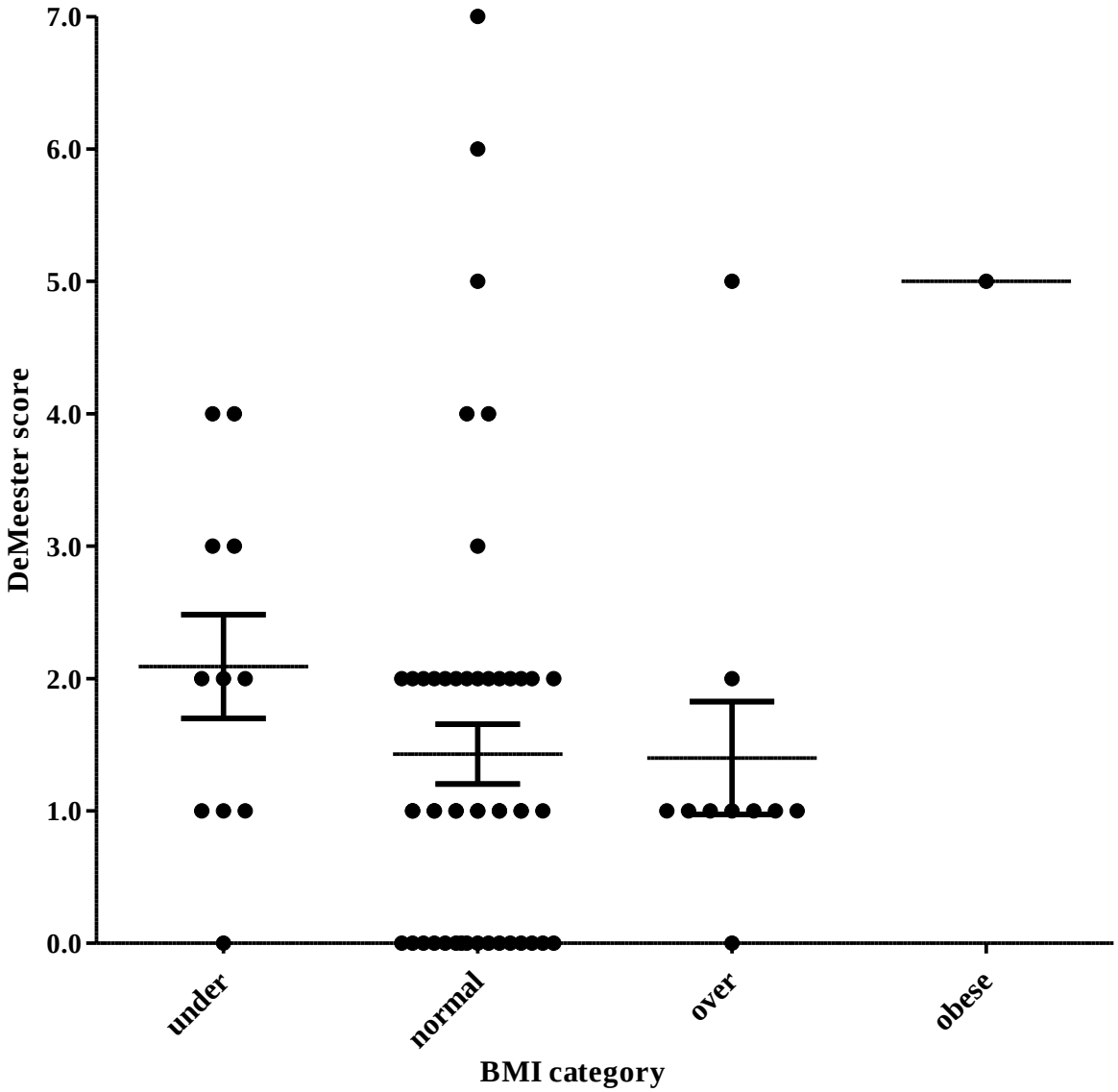


Figure 5.10 The DeMeester score of patients and association with BMI. The median DeMeester score of underweight patients was 2 (range 0 to 4, mean 2.1, SEM 0.4), normal weight patients 1 (range 0 to 7, mean 1.4, SEM 0.2) overweight patients 1 (range 0 to 5, mean 0.4, SEM 0.4) and the obese patients score was 5. There was no statistical significance between DeMeester score to identify GOR symptoms and BMI category (P= 0.13) Kruskal-Wallis test with Gaussian Approximation. The Pearson correlation showed no significance with GOR symptoms determined by DeMeester score and BMI status (P=0.66).

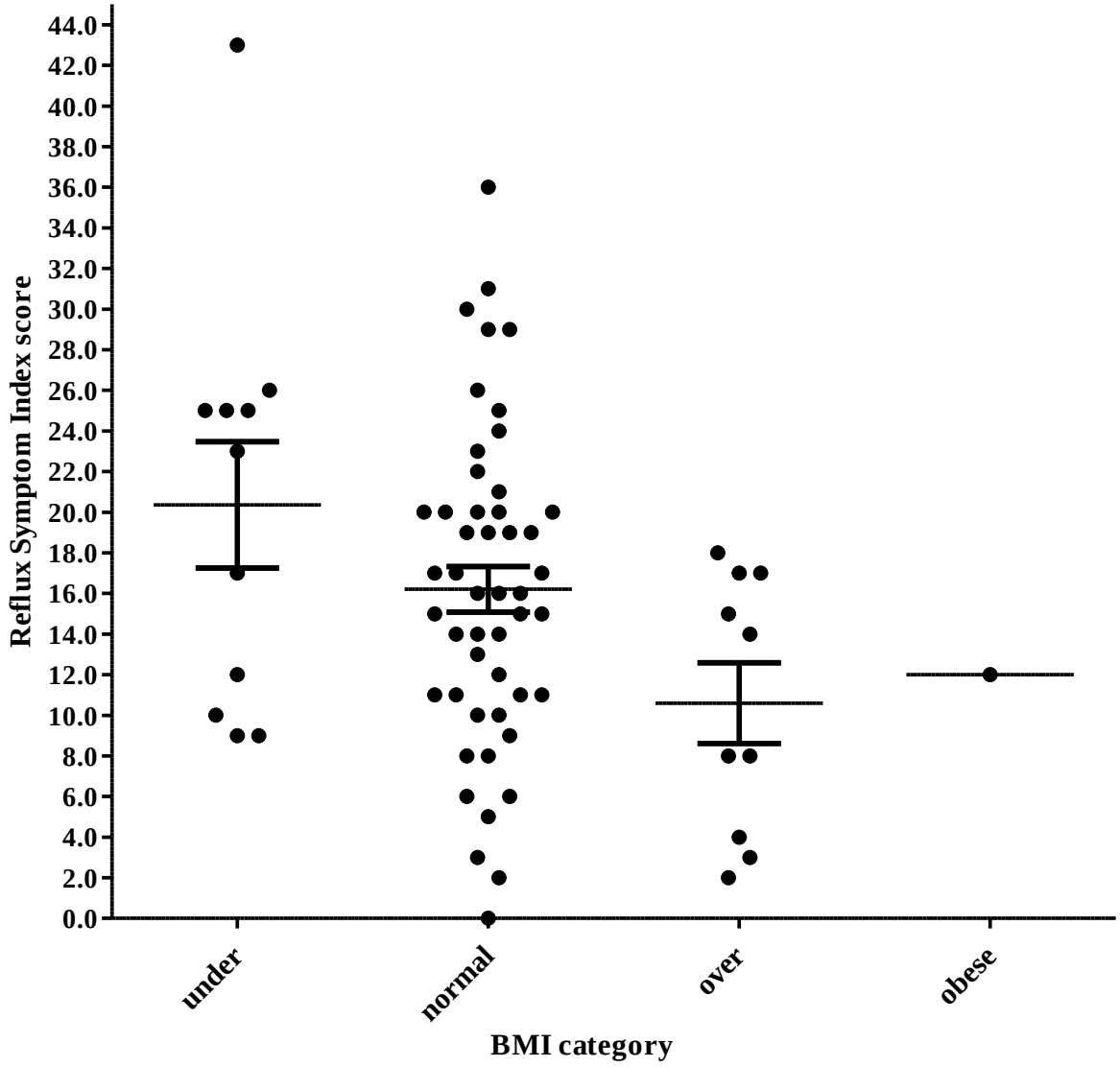


Figure 5.11 The RSI score of patients and association with BMI. The median RSI score of underweight patients was 23 (range 9 to 43, mean 20, SEM 3.1), normal weight patients 16 (range 0 to 36, mean 16, SEM 1.1) overweight patients 11 (range 2 to 18, mean 1.9, SEM 0.4) and the obese patients score was 12. The Kruskal-Wallis test with Gaussian Approximation showed statistical significance between RSI score to identify EOR symptoms and BMI category (P= 0.038).

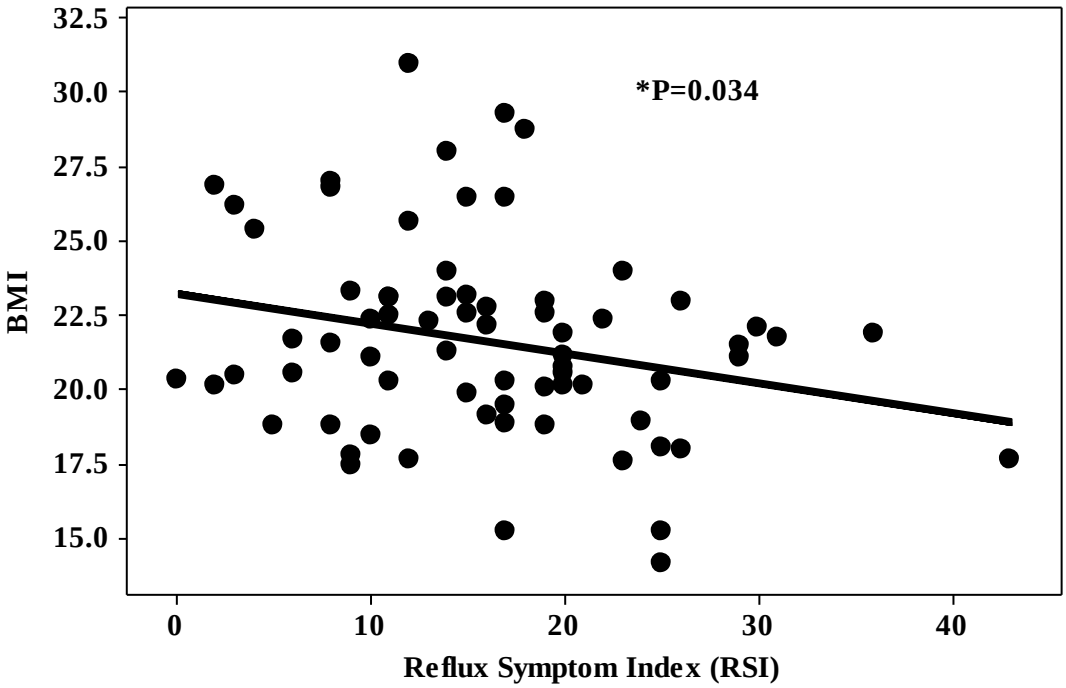


Figure 5.12 Scatterplot of BMI status plotted on the Y axis and RSI score on the X axis. Pearson correlation showed a positive correlation between an increase in BMI and decrease in RSI (n=72; R^2=0.0638/6.4%; P-Value = 0.034).

5.2.7 The association of BMI status and the incidence of gastric aspiration

The detection of pepsin in sputum supernatant samples was performed and analysed as described in chapter 3. The concentration of pepsin of each patient is illustrated in Figure 5.13, grouped by the BMI status category. The median concentration of pepsin was highest in the underweight category at a median of 357ng/ml (n=10, range 0 to 782, mean 319, SEM 74.4). The median level of pepsin in the patients achieving a normal weight BMI status was 193ng/ml (n=48, range 0 to 1151, mean 245, SEM 40.2) which was lower than the underweight category but with a larger range. The median pepsin levels in the over-weight category was 58ng/ml (n=10, range 0 to 611, mean 193, SEM 80.3) and the level of pepsin detected in the sputum supernatant of the one obese patient was 128ng/ml. Figure 5.13 illustrates the mean (±SEM) pepsin concentrations decrease in each BMI category as BMI increases. The Kruskal-Wallis test did not show any statistical differences for pepsin level between BMI categories (P=0.47). There is a non significant decrease of pepsin levels as the BMI of patients increase which supports the RSI score data in Figure 5.11 and 5.12.

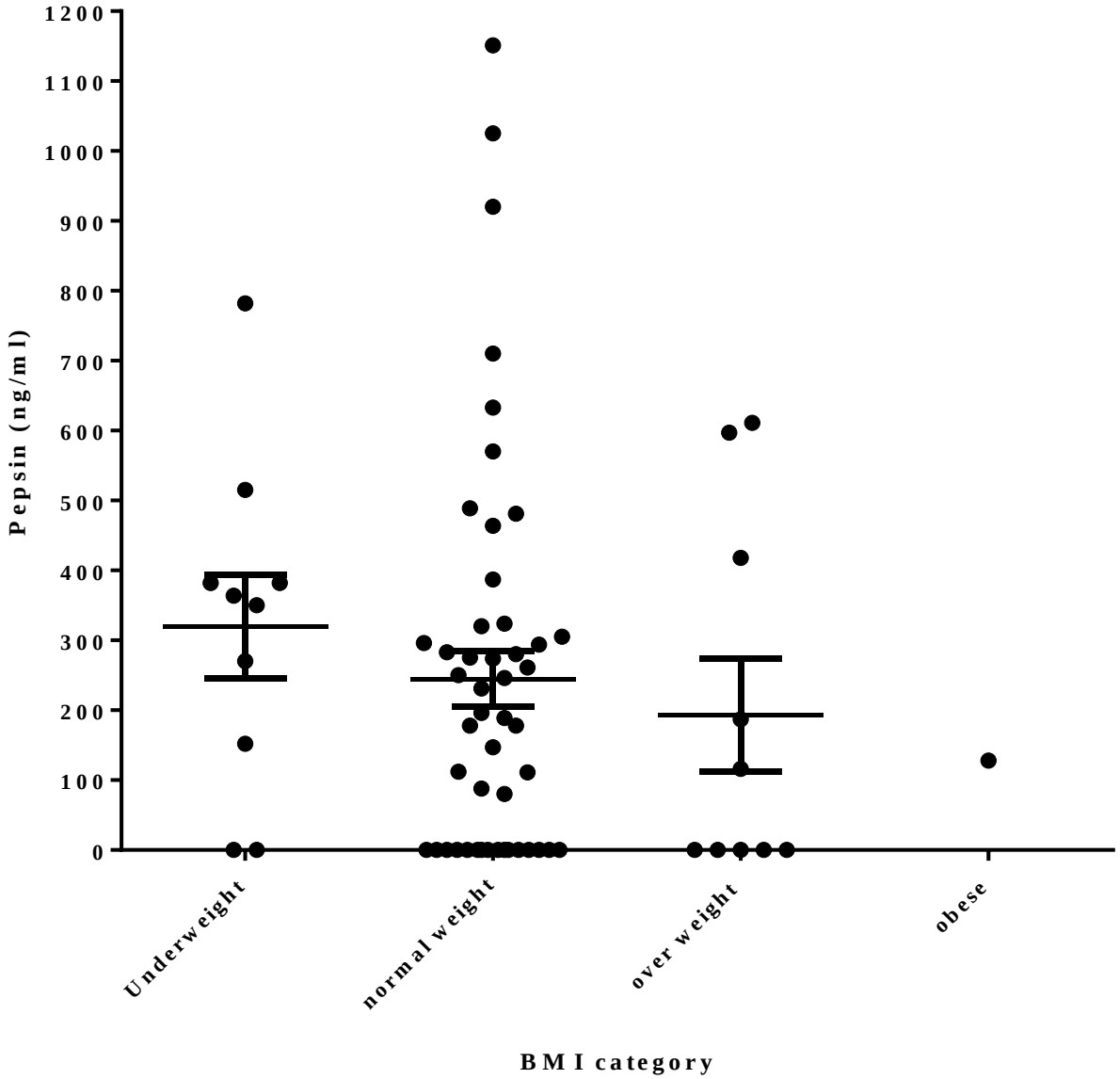


Figure 5.13 The pepsin concentration of CF patients association with BMI category. The median pepsin concentration of underweight patients was 357ng/ml (n=10, range 0 to 782, mean 319, SEM 74.4), normal weight patients 193ng/ml (n=48, range 0 to 1151, mean 245, SEM 40.2) overweight patients 58ng/ml (n=10, range 0 to 611, mean 193, SEM 80.3) and the obese patients pepsin content was 128ng/ml. The Kruskal-Wallis test with Gaussian Approximation showed no statistical significance between gastric aspiration using pepsin identification as a biomarker and BMI category (n=72).

5.3 Discussion

The present study investigated the prevalence of overweight and obesity within a single centre study in the North of England (RVI, Newcastle Upon Tyne, UK) for which 72 CF patients were recruited. Twelve (16.7%) patients were reported to be over-weight/obese (BMI status >25kg/m^2 defined by WHO; 1998) (WHO, 1998); of which 11 patients (15.3%) were classified as overweight and 1 patient (1.4%) was obese. These findings are supported by a number of studies that report the over-weight prevalence to be in a range of 11% to 13.2% and CF obesity to be 0.5% to 3%, reported in Greece, Israel and Canada (Kastner-Cole *et al.*, 2005; Coderre *et al.*, 2012; Kochavi, 2014; Panagopoulou *et al.*, 2014). Our data agrees with observations from a CF clinic in Toronto recorded in the 1990's reporting the average prevalence of BMI >25.0kg/m^2 to be 15.8%. Interestingly, the clinic reported the prevalent to increase by 6.4% 10 years later in 2011, reporting 18.4% to be over-weight and 3.8% to be obese (Stephenson *et al.*, 2013). Additionally, a report of BMI data from the adult CF patients on the register in the United Kingdom (U.K) observed over-weight prevalence to be 9.5% and obese prevalence at 1% recorded 12 years ago (Kastner-Cole *et al.*, 2005), our data may suggest that the prevalence of over-weight BMI status in the U.K has risen by nearly 6% in the past decade. Recently, there has been high prevalence of childhood CF obesity reported in Ireland at 5.9% and Pennsylvania at 8% determined by BMI for age, sex, height and weight. The cut off limits for obesity differed for each study, colleagues in Ireland used the 98th percentile and Pennsylvania used the 95th percentile which may explain the higher prevalence (Stack *et al.*, 2007; Hanna and Weiner, 2014).

Reportedly, CF over-weight and obese BMI status maybe associated with gender. It was identified that there was a significantly higher number of males in the over-weight and obese categories reported in a Canadian CF population (P=0.001) but they did not suggest a mechanism for why this may be. BMI measurement does not take muscle mass into consideration and an increase in BMI may be due to high muscle mass rather than fat mass. The study did not report body fat % (Stephenson *et al.*, 2013). The present study did not find a similar pattern, there was no significant difference observed between gender and BMI status (P=0.43) and the one obese patient was a female. This is supported by a recent study including 226 CF patients (aged 2-18 years) at a CF clinic in Pennsyvania reporting no significant effect of gender on the BMI status of CF patients (Hanna and Weiner, 2014).

Furthermore, Coderre *et al.,* (2012) did not find any significant differences for BMI and gender.

Due to contradictory findings within the literature, the aim of this chapter was to firstly explore the potential association between increasing BMI on pulmonary function in CF patients with a BMI >25.0kg/m^2. The present study observed that median scores of the pulmonary function in the over-weight group increased significantly compared to the ideal weight group and the under-weight group determined by FEV_1% and FVC% predicted. This data was supported by a number of studies that identified a positive association with BMI and pulmonary function even beyond 25.0kg/m^2 (Munck *et al.,* 2007; Coderre *et al.,* 2012; Kochavi, 2014; Panagopoulou *et al.,* 2014). Additionally, FEV_1% was significantly correlated with BMI and percentage of body fat (Panagopoulou *et al.,* 2014) among CF patients from a Greek centre. This suggests that it is the weight gain of body fat rather than muscle may cause the effect, as could be the case by using BMI data alone. However, some researchers have shown that the significant increase in pulmonary function associated with increasing BMI only up to a BMI of 25.0kg/m^2 (Stephenson *et al.,* 2013) and 23.0kg/m^2 (Kastner-Cole *et al.,* 2005) suggesting that there was no benefit to pulmonary function of encouraging over-weight and obese BMI status. Furthermore, it was advised that these CF patients may be required to receive caloric counselling and diet modification to reduce and maintain their BMI to 23.0kg/m^2 or 25.0kg/m^2. It is important to mention the data from these studies show increases in FEV_1% as BMI increases above 25.0kg/$m^{2,}$ although not significant and with no other negative effects on health. A recent study reported that over-weight and obese BMI status was not significantly associated with FEV_1% in CF patients aged 2-18 years, although it is reported that small increases are observed (Hanna and Weiner, 2014).

It has been repeatedly reported that the CF patients with a BMI classified as over-weight or obese are commonly heterozygous for delta F508 (Kastner-Cole *et al.,* 2005; Munck *et al.,* 2007; Stephenson *et al.,* 2013). These patients are said to have a milder genotype and therefore less severe disease outcomes. This may include pancreatic sufficiency and therefore do not have difficulty absorbing dietary fat and it is possible that if they adhere to the high fat diet advised full absorption will occur and result in increased body fat mass. Therefore, our study aimed to explore the potential association of CFTR mutation (homozygous/heterozygous for delta F508) on the BMI status and pulmonary function of CF patients. In the present study genotype data were available for 61 or the 72 total patients, there were 22 patients homozygous for delta F508 and 39 were

heterozygous. The median BMI score for homozygous patients was 20.5 (range 15.2 – 27.9) and for the heterozygous patients 21.8 (range 15.2 – 30.9). There was no association observed between genotype and the BMI outcome. In addition, we could not identify an association between genotype and pulmonary function. The median values for FEV_1%, FVC% and FEV_1/FVC ratio of patients homozygous for delta F508 was lower than the median of the heterozygous CF patients but the differences were not significant. It was reported by Coderre *et al.,* (2012) that a there was a significant increase in heterozygous genotype patients (P=0.001) and less PERT use (P=0.048) in the over-weight/obese BMI categories compared to a BMI of the underweight group of patients. However, the data illustrates that there were 62% of overweight/obese patients using PERT and 54% with a homozygous genotype. Interestingly, Kastner-Cole *et al.,* (2005) recruited only CF patients homozygous for delta F508 and reported a similar prevalence of overweight and obese patients at 10% of the population suggesting that genotype alone cannot be the cause of increased weight gain or increased pulmonary function. A study of CF patients (n=112) aged 40 years to 71 years were explored by Simmonds *et al.,* (2009) highlighted that 30% were homozygous for delta F508 and they had a BMI ranging from 17.4 to 28.7 (median 20.9) suggesting longevity in the CF population cannot be associated with genotype status or BMI status.

Review of the literature has highlighted the need for further study into the health outcomes of CF patients who are over-weight or obese (Munck *et al.,* 2007; Hanna and Weiner, 2014). Hanna and Weiner (2014) stated that the high fat and high calorie diet has been shown to result in adverse consequences for some patients with CF. It was highlighted that there are no adult or paediatric guidelines on nutrition for CF patients who are overweight or obese. Proposed guidelines for this increasing patient group are needed to advise the correct nutritional requirements (Kochavi *et al.,* 2014; Hanna and Weiner, 2014). Stephenson *et al.,* (2013) advised that these CF patients may be required to receive caloric counselling and diet modification to reduce and maintain their BMI to ideal weight recommended at 23.0kg/m^2 (Kastner-Cole *et al.,* 2005) and 25.0kg/m^2 (Stephenson *et al.,* 2013). Stephenson *et al.,* (2013) suggested that CF patients with milder CF disease including CF patients that are older, pancreatic sufficient and heterozygous for delta F508 are at risk of obesity and suggested a modified diet may be required (Stephenson *et al.,* 2013). However, contradictory advice was proposed by Kastner-Cole *et al.,* (2005) who suggested calorie restriction was not needed for paediatric CF patients in the over-weight and obese BMI groups due to the positive effects on pulmonary function. The present

study does not support the requirement for dietary modification due to the significant benefits of BMI on pulmonary function which were not associated with genotype.

The literature has provided a strong argument for the advantages of over-weight and obese BMI (BMI >25.0kg/m^2) in a wide variety of CF patient studies from around the world. It was observed that there was a lower prevalence of CF related diabetes in this patient group compared to the under-weight group (Munck *et al.*, 2007; Stephenson *et al.*, 2013; Panagopoulou *et al.*, 2014). In addition, there were less chronic disease observed in patients with increased BMI beyond 25.0kg/m^2 such as liver disease (Panagopoulou *et al.*, 2014) and cirrhosis (Munck *et al.*, 2007). As it was repeatedly identified that pulmonary function was higher in over-weight and obese patients whether significant or not it is a reason to explain why there were no patients on the lung transplant waiting list or mortality rates reported by Munck *et al.,* (2007) compared to a reported 77 patients waiting for transplant and 45 patients deceased with a BMI <24.9kg/m^2 in a French CF cohort (n=4533). This data could also be due to the observations found by numerous studies describing less *Pseudomonas aeruginosa* colonisation of the sputum in the airways of patients with a BMI >25.0kg/m^2 (Munck *et al.*, 2007; Stephenson *et al.*, 2013; Panagopoulou *et al.,* 2014). This would be the opposite in the general population. The reason that higher BMI serves to be protective for the CF population is yet to be elucidated. It could be possible that an increased BMI may lead to better health due to increased absorption of nutrients. The cells of the body require fat to function correctly and the high fat diet and adequate absorption may lead to a cell environment close to normal.

There were negative effects associated with an over-weight and obese BMI status within the CF population. Two studies observed increased triglyceride concentration in the blood samples of over-weight and obese patients compared to underweight and ideal weight patients (Coderre *et al.*, 2012; Stephenson *et al.*, 2013). Blood triglyceride concentrations were shown to be significantly increased in the overweight group (mean 1.17mmol/L (±0.71)) and the obese group (mean 1.74mmol/L (±1.41) compared to the underweight (mean 0.89mmol/L (±0.49)) and the ideal weight group (mean 0.94mmol/L (±0.47)) (P=0.001). However, the fasting blood lipid profiles were collected from the closest recording within 2 years of the most recent clinic visit at which BMI and FEV_1% were recorded which may lead to a weakness in the methodology if BMI status changed since the triglyceride content was measured (Stephenson *et al.*, 2013). The studies also reported increased total cholesterol levels from fasting blood samples. Coderre *et al.*, (2012) noted that although the levels were significantly higher (P=<0.05) than patients

with a BMI <24.9kg/m2 they were in the normal range with the highest median of 4.2mmol/L (±0.8) in the over-weight group (Coderre *et al.*, 2012). However, Stephenson *et al.,* (2013) reported total cholesterol levels above 5.2mmol/L at 9% in the ideal weight group, 19% in the over-weight group and 39% in the obese group (Stephenson *et al.*, 2013). Interestingly, Coderre *et al.,* (2012) illustrated the same finding with low density cholesterol (LDL), significant progressive increases were observed with increasing BMI categories whereas high density cholesterol (HDL) was similar in all groups. This is interesting because it gives insight into the risks associated with the high fat and calorie diet for patients with a BMI above the ideal cut off at 25.0kg/m^2. LDL cholesterol carries more cholesterol with the potential to oxidise into particles that may attach to plaque in the arterial wall leading to plaque build-up and thus increasing the chance of atherosclerosis. HDL hold positive benefits because they escort cholesterol to the liver for metabolism and excretion and hold protective properties against heart disease by decreasing plaque build-up in the arteries (Brown, 2013).

This knowledge makes it difficult to ignore the relationship observed between the increased risk factors for GOR in the over-weight and obese CF population. The reported factors which are frequently suggested to lead to the pathogenesis of GORD in the literature include obesity, being overweight (El-Serag *et al.,* 2005; Hampel *et al.,* 2005) and incorrect dietary habits such as a high fat diet (Iwakiri *et al.,* 1996; Holloway *et al.,* 1997; Meyer *et al.,* 2001; Dore *et al.,* 2008). Moreover, it was observed in two separate studies that CF patients with a BMI above 25.0kg/m^2 are associated with higher cholesterol levels which have been shown to increase the risk of GOR in previous studies. Important findings by El Serag *et al.,* (2005) and Shaprio *et al.,* (2007) found positive associations between cholesterol from the diet and GORD in the general population. A considerable amount of literature has been published highlighting the high prevalence of GOR in the CF population (Gustafsson *et al.,*1991; Navarro *et al.,* 2001; Blondeau *et al.,* 2008; Pauwels *et al.,* 2013). These findings provide an important opportunity to advance the understanding into risks of over-weight and obesity with GOR in the CF population as it has not been studied previously. Therefore, the study aimed to explore the potential association of BMI status with patient symptoms of reflux (GOR/EOR) and gastric aspiration.

Our study did not show any significant association with GOR symptoms and BMI status across all BMI groups, although the one patient in the obese BMI category scored 5 (maximum 7) on the DeMeester score and experienced heartburn that was predictable on

the position of straining and reported interference with daily activities. There were higher scores seen in the normal (DeMeester score 7) and over-weight groups (DeMeester score 5) to illustrate that BMI was not the cause. Interestingly, we showed that EOR symptoms were significantly correlated with a low BMI suggesting under-weight BMI carries a higher risk of EOR than over-weight and obesity. We illustrated that the symptoms of EOR reduced as the BMI status increased. There was a non-significant decrease in RSI questionnaire score as the BMI increased, this is supported by a non-significant decrease in pepsin levels as the BMI increased. Although GOR and EOR has not been studied in relation to the increasing prevalence of over-weight and obesity in CF there has been reports of GOR in CF patients with failure to thrive and malnutrition (Malfroot and Dab, 1991; Dray *et al.,* 2005). Interestingly, Malfroot and Dab (1991) observed that refluxing children shared characteristics of recurrent coughing, vomiting, respiratory infections and failing to thrive and these were not so common in the group of infants without reflux.

The lessons learned from these findings are that the prevalence of over-weight and obesity is increasing in the UK CF population. This is associated with significantly increased pulmonary function and is not dependant on genotype or gender. Opposite to the effects seen in the general population, in fact the presence of EOR symptoms in CF was positively associated with low BMI status. In the general population the risks detrimental to health are increasingly associated. Such as GORD, other diseases including heart disease and cancer and decreased pulmonary function (Friedenberg *et al.,* 2008; Festi *et al.,* 2009). These are due to changes in the anatomy and physiology of the body, it has been reported that central adiposity fat maybe a sign. The overweight and obesity levels in CF are not as high as the general population. For example a median BMI of 45kg/m^2 has been reported to be associated with GORD (Dixon *et al.,* 1999) and this is not the level observed in CF. Thus, the observed protective aspect of high BMI overweight and obesity in CF may be down to the overall health of the patient. It could suggest that they have experienced less malnutrition in their lifetime which physically strains the body. Vitamin status could play a role, a well-nourished body may see less negative effects on health. It has been demonstrated that the membrane lipid composition of the cell has a role to play in the phenotype of the CFTR demonstrated in the ilium, pancreas and airway of a CFTR mouse. These results illustrate the importance of lipid membrane of cells in CF and lipid alterations of fat from the diet may affect the phenotype of the CF patient as seen in the results herein. (Freedman *et al.,* 1999).

The limitations of this study begin with small sample size of 72 CF patients compared to previous studies reporting prevalence in the UK, France and Canada with a sample size from n=651 to n=1489. A larger sample size of the total number of CF patients at the adult CF clinic (n=235) would have allowed stronger conclusions to be made. This study was a single centre study in the North East of England (RVI, Newcastle Upon Tyne, UK) so regional differences of CF patient care, dietary advice and variety of diet may affect the results. Secondly, the study used BMI as a tool to assess over-weight and obesity in the patient cohort. The BMI was calculated by dividing the weight (kg) by height (m^2) and classified using the BMI scores set by the WHO. This could have been strengthened by collecting anthropometric body measurements and bioelectrical impedance data to assess body fat mass so that weight increases caused by muscle mass could be accounted for. Central adiposity could be explored and compared to the health risks associated with this in the general population. The study did not include such measurements into the initial ethical application at the beginning of the study. Furthermore, our data set for genotype (n=61) and pulmonary function (n=64) was incomplete. The study holds many strengths and the most important being the novel findings of a protective effect of over-weight and obesity to gastric aspiration and EOR symptoms in the CF population. Further, all the data provided was collected at the time of recruitment therefore symptoms and gastric aspiration are directly linked to pulmonary function and BMI status.

Our data provides a positive overview of over-weight and obesity in the CF population. For adults, the CF Foundation recommends maintenance of normal weight-for-height because this was associated with better FEV_1 and survival (Stallings *et al.*, 2008) but our data suggests that the benefit to health increases as the BMI status increases.

We suggest that there is a need for further study into the effect of a high fat diet in patients with a BMI over 25.0kg/m^2 as there is a possibility of high triglyceride and cholesterol levels that were not measured in our study. The dietary fat consumed in the diet may be the focus for further study.

To conclude the body weight of the CF population is increasing due to improvements in clinical care and today we observe BMI scores reaching OW and OB categories. The potential health risks of OW and OB in the CF population are not completely understood but appears to be at odds with the non-CF population. The benefits of increased pulmonary function scores regardless of gender and genotype were observed. This may be linked to less EOR symptoms among this patient group. Further research is needed before a revision of dietary advice is proposed to CF patients with a BMI above 25.0kg/m^2.

6.0 General discussion

The survival rates are increasing for the CF population and many CF patients are reaching the age of 40 and 50 years in present times (Thomson and Harris, 2008; Salvatore *et al.*, 2012; Simmonds, *et al.,* 2013). The CF community observe age related diseases in many patients reaching adulthood, for example CF related diabetes, liver disease and GORD (Bourke and Burns, 2011). GOR is the main focus of the present thesis due to the high prevalence, the potential for related health problems in adult CF patients and the lack of research available. The focus of CF research is changing and it is becoming increasingly important to ensure the years gained are of the best quality as possible (Simmonds *et al.*, 2009). The prevalence of GOR in the CF population has been a long standing problem for a high percentage of patients (Chen *et al.,* 2010; Armstrong, 2005). Living with GOR has been demonstrated to decrease the quality of life and in worse cases is associated with mortality (Ronkainen *et al.,* 2013; Holt *et al.,* 2013). Aside from the GOR symptoms affecting the quality of life, the concerning factor regarding GOR in CF is the potential damage it can cause to the airway. GOR may lead to aspiration of the gastric contents and it has been demonstrated that it is not acid that is the potent contents but pepsin and bile acids (Gustafsson *et al.*,1991; Navarro *et al.,* 2001; Blondeau *et al.,* 2008; Pauwels *et al.*, 2013). Increasing exposure to gastric aspiration has been associated with lung damage, decreased pulmonary function and previously observed in lung transplant patients (Blondeau *et al.*, 2008; Nally *et al.*, 2011; Aseeri *et al*, 2012; Pauwels *et al.*, 2012), but there is limited data in CF. Other health implications, associated with gastric aspiration and relevant in CF include EOR symptoms like coughing, wheezing, shortness of breath and persistent clearing of the throat (Dettmar *et al.*, 2011).

The present thesis first emphasises the prevalence of classical GOR symptoms in the studied CF population to be 42% which is twice that reported in the general population (Dent *et al.,* 2005), the prevalence of EOR symptoms were higher affecting 63% of the study population. These findings highlight the high number of CF patients that are affected by symptoms, therefore outlining the importance of exploring the topic. Secondly, the study indicated the high degree of patients that are not responsive to acid suppression medication (PPI or H_2RA) to treat such symptoms. It was presented that 70% of patients with classical GOR symptoms and 73% of patients experiencing EOR symptoms were receiving acid suppression medication. i.e. Symptoms persisted despite the use of medication. This has also been illustrated in the literature over the previous years and the

prevalence remains high despite medication (Zerbib *et al.,* 2013). It is agreed that acid suppression medication such as PPI and H_2RA relieves the symptoms of heartburn for patients but many remain to be non-responsive and continue to experience symptoms (Sabati *et al.,* 2010). These patients may be likely to receive anti-reflux surgery (Kahrilas, *et al.,* 2013). Medicated acid suppression has not been shown to be useful for EOR symptoms. A potential reason for this resides in the fact that it is not the acid in the refluxate that is harmful to the aero-digestive tract, nasal cavity and airways but it is the potent contents such as pepsin and bile acids causing the epithelial damage (Jolly, *et al.,* 2004; Bulmer, *et al.,* 2010).

Since it has been suggested that the aspiration of gastric contents negatively affects pulmonary function (Gustafsson *et al.,*1991; Navarro *et al.,* 2001; Blondeau *et al.,* 2008; Pauwels *et al.,* 2013) the present study investigated reflux biomarkers within the expectorated sputum from the CF airway and correlated the results of this with pulmonary function test results. It was observed that the aspiration of gastric pepsin was present in 70% of the study population. The concentration of bile acid in the 22 patient samples analysed was not above the maximum serum concentration of 10μmol/L. The possible correlations between the presence of bile acids and other health outcomes inclusing pulmonary function could not be assessed at a median bile salt concentration of 0.22μmol/L as it is not known where the origin of these bile acids have resulted from, it could be present in the airway due to serum levels or gastric contents (Parikh *et al.,* 2013). Contrary to the literature, the present study did not demonstrate a relationship between the presence of pepsin or bile acids in sputum from CF airways with pulmonary function. The sputum sample contained a high neutrophil content and the IL-8 concentration was high illustrating inflammation within the CF airway. There was no association between gastric aspiration and inflammation with pulmonary function. The present study did not mirror the negative effects of gastric aspiration as seen in the literature and this could be because of the different methods used to analyse reflux biomarkers.

The CFTR potentiator named as 'Ivacaftor' aims to normalise the flow of chloride ions through the gating channel of the CFTR in the G551D mutation (Kotha and Clancy, 2013). The CFTR modulator has been shown to restore the function of the CFTR in clinical trials by demonstrating the reduction in sweat chloride concentrations, and in some cases the sweat chloride concentrations were normalised (<30.0mmol/L). In CF defective CFTR protein is expressed in the epithelium of the gastrointestinal tract leading to thick

and sticky mucus blocking the pancreatic ducts which minimises the release of pancreatic enzymes. It has been reported that 85-90% of CF patients have pancreatic insufficiency resulting in the patient having difficulty gaining weight and malabsorption of fat soluble vitamins A,D,E and K (Cohen *et al.,* 2005) which leads to poor weight gain the patient becoming malnourished. The Ivacaftor treatment has demonstrated an increase in weight demonstrating the CFTR correction reversing the effects of poor weight gain. As the CFTR is expressed in the airways the accumulation of mucus results in poor pulmonary function, and the effect of malnutrition also decreases pulmonary function (Accurso *et al.,* 2010; Ramsey *et al.,* 2011; Barry *et al.,* 2014). The present study supported the observed findings of the literature in the 12 patients studied with the G551D mutation. The sweat chloride concentration significantly decreased after 6 weeks of treatment, with 6 patients demonstrating normalised sweat chloride concentrations at the 6 month follow-up. There were significant improvements in patient weight after 6 month of treatment and a significantly improved pulmonary function after 6 weeks of treatment.

As the CFTR mutation results in multiple complications in exocrine tissues there is now a strong interest in the possible clinical benefits of Ivacaftor that have not yet been investigated. The CFTR proteins are expressed in the gastrointestinal tract and this may have a role to play in the high prevalence of GOR. Given the high prevalence of GOR and EOR in the CF community this was explored for the first time within the CF population in the present study. Longitudinal observations over a 12 month follow-up period showed that the number of patients presenting abnormal GOR, EOR and airway reflux questionnaire scores reduced. In addition, the severity of GOR, EOR and airway reflux symptoms decreased during the treatment of Ivacaftor. This is a novel and interesting finding, not previously shown to my knowledge. The results are limited however could be confounded and are in a small number of patients. Confounders could include other factors such as patients achieving a healthier weight and increased pulmonary function.

It has been reported that the BMI status of the CF population is increasing with the highest overweight prevalence to be 18% in Canada and the obese prevalence to be 6% in Ireland (Stephenson *et al.,* 2013; Stack *et al.,* 2007). This has not been extensively researched however, the present thesis explored the prevalence of overweight and obese BMI status within the total study population. In the present study there were 16.7% of patients with a BMI above the ideal 25.0kg/m^2. This was associated with significantly increased lung function. The symptoms of EOR were demonstrated to be significantly reduced in the over-weight and obese BMI group, this was supported by the decreased

prevalence of gastric aspiration within these groups but the effect was not significant. Over-weight and obesity did not show a relationship to F508del/F508del genotype status or to gender. It is difficult to reach any firm conclusions as there is a possibility of confounding and conflicting variables such as those with obesity may have milder clinical disease, or be at an earlier stage in the disease process with less severe lung infections and associated catabolic state.

It is important to mention that the present study did not provide comparable data obtained from a healthy control group. This is a limitation of the study, however, the use of validated questionnaires allowed for the analysis of questionnaire scores to be deemed abnormal if higher than the validated cut off score previously evaluated in healthy individuals. Also, the study of reflux biomarkers and inflammation obtained from the collection of sputum was a key part of the study to assess the effect of gastric aspiration into the airway. The collection of expectorated sputum collection from healthy volunteers would not be possible. To assess declining pulmonary function the spirometry values were compared to the spirometric reference equations collected from healthy non-smokers with no symptoms of asthma and respiratory problems, this is recommended by the European Respiratory Society. The reference equations are compared to the patient spirometry value according to gender, sex and height (Pellegrino *et al.*, 2005), the value from the healthy population is set a 100% and the value from the CF patient is calculated as a percentage of what a healthy person would achieve of the same anthropometric characteristics. The inclusion of a healthy control group in further studies would strengthen the data obtained from the questionnaires and the assessment of reflux symptoms with pulmonary function would be a possible correlation. Although healthy controls would not provide a sputum sample, it would be possible to obtain a BAL sample to obtain data regarding gastric aspiration and inflammation within the airway.

6.1 Conclusion and future direction

The study has observed a high prevalence of GOR and EOR symptoms in the CF population that is not alleviated by acid suppression medication. The presence of gastric aspiration was not correlated to pulmonary function impairment but this cannot be out ruled due to the demonstrated a high number of neutrophils in the airway fluid and high inflammatory cytokine presence. CFTR modulators were shown to decrease the number of symptomatic patients and symptoms severity of reflux for most patients. The increase of BMI to over-weight and obese categories was associated with less EOR symptoms and gastric aspiration. These findings may be due to better overall health but may suggest that EOR symptoms are related with weight and pulmonary function of CF patients.

As the results of this study do not support the suggested harmful effects of the gastric aspiration collected from one sample of the airway fluid on the airways in terms of lowering pulmonary function and increasing inflammation there is room for further research. On our first observation taken by analysing one expectorated sputum sample during the outpatient appointment there was little opportunity to assume a reflux event occurred and therefore may not identify pepsin or bile acids during one sample at one time point therefore suggesting a longitudinal study of CF patients with GOR/EOR symptoms and using non GOR/EOR CF patients as a comparison group maybe an ideal way to observe the effects. Multiple samples throughout the day and one during symptoms would eliminate the chances of missing the detection of the reflux biomarkers.

For the first time, our study researched the presence of reflux in a novel group of CF patients receiving Ivacaftor treatment to correct the CFTR class III mutation. In this patient group the effect of correcting the CFTR function brought about the observed health benefits including decreased sweat chloride, weight gain and improved pulmonary function with the novel finding of reduced symptoms of reflux. The longitudinal study of other CFTR correcting treatments including Lumacaftor for the homozygous delta F508 patients would be beneficial to observe the effects it has on symptoms of reflux. The association of CFTR expression in the gastrointestinal epithelia and in the high prevalence of gastric reflux in the CF population can be studied using patients receiving CFTR correcting medication.

The arising concern of overweight and obesity in the CF population requires further study in a larger population. Although the prevalence is increasing the use of single study data includes a small number of patients and further multicentre studies are suggested to

address the outcome and provide answers to the questions raised during single centre studies regarding the benefits over the risks.

7.0 Bibliography

Accurso, F.J., Van Goor, F., Zha, J., Stone, A.J., Dong, Q., Ordonez, C.L., Rowe, S.M., Clancy, J.P., Konstan, M.W., Hoch, H.E., Heltshe, S.L., Ramsey, B.W., Campbell, P.W. and Ashlock, M.A. (2014) 'Sweat chloride as a biomarker of CFTR activity: Proof of concept and ivacaftor clinical trial data', *Journal of Cystic Fibrosis*, 13(2), pp. 139-147.

Accurso FJ, Rowe SM, Clancy JP, Boyle MP, Dunitz JM, Durie PR, Sagel SD, Hornick DB, Konstan MW, Donaldson SH, Moss RB, Pilewski JM, Rubenstein RC, Uluer AZ, Aitken ML, Freedman SD, Rose LM, Mayer-Hamblett N, Dong Q, Zha J, Stone AJ, Olson ER, Ordoñez CL, Campbell PW, Ashlock MA, Ramsey BW. (2010) 'Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation' New England Journal of Medicine 18;363(21):1991-2003

Anthony, H., Collins, C.E., Davidson, G., Mews, C., Robinson, P., Shepherd, R. and Stapleton, D. (1999) 'Pancreatic enzyme replacement therapy in cystic fibrosis: Australian guidelines', *Journal of Paediatrics and Child Health*, 35(2), pp. 125-129.

Armstrong, D. (2005) 'Gastroesophageal reflux disease', *Current Opinion in Pharmacology*, 5(6), pp. 589-595.

Armstrong, D. and Sifrim, D. (2010) 'New Pharmacologic Approaches in Gastroesophageal Reflux Disease', *Gastroenterology Clinics of North America*, 39(3), pp. 393-418.

Austin, G. L., M. T. Thiny, et al. (2006). "A very low-carbohydrate diet improves gastroesophageal reflux and its symptoms." Digestive Diseases and Sciences 51(8): 1307-1312.

Aseeri, A., Brodlie, M., Lordan, J., Corris, P., Pearson, J., Ward, C. and Manning, N. (2012) 'Bile Acids Are Present in the Lower Airways of People with Cystic Fibrosis', *American Journal of Respiratory and Critical Care Medicine*, 185(4), pp. 463-463.

Bardhan, K.D., Strugala, V. and Dettmar, P.W. (2012) 'Reflux Revisited: Advancing the Role of Pepsin', *International Journal of Otolaryngology*, 2012.

Avidan, B., Sonnenberg, A., Schnell, T.G. and Sontag, S.J. (2001) 'Temporal associations between coughing or wheezing and acid reflux in asthmatics', *Gut*, 49(6), pp. 767-772.

Banner, K.H., De Jonge, H., Elborn, S., Growcott, E., Gulbins, E., Konstan, M., Moss, R., Poll, C., Randell, S.H., Rossi, A.G., Thomas, L. and Waltz, D. (2009) 'Highlights of a workshop to discuss targeting inflammation in cystic fibrosis', *Journal of Cystic Fibrosis*, 8(1), pp. 1-8.

Bardhan, K.D., Strugala, V. and Dettmar, P.W. (2012) 'Reflux Revisited: Advancing the Role of Pepsin', *International Journal of Otolaryngology*, 2012.

Barth, A.L. and Pitt, T.L. (1996) 'The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic Pseudomonas aeruginosa', *Journal of Medical Microbiology*, 45(2), pp. 110-119.

Barry, P.J., Plant, B.J., Nair, A., Bicknell, S., Simmonds, N.J., Bell, N.J., Shafi, N.T., Daniels, T., Shelmerdine, S., Felton, I., Gunaratnam, C., Jones, A.M. and Horsley, A.R. (2014) 'EFfects of ivacaftor in cystic fibrosis patients carrying the g551d mutation with severe lung disease', *CHEST Journal*.

Belafsky, P.C., Postma, G.N. and Koufman, J.A. (2002) 'Validity and Reliability of the Reflux Symptom Index (RSI)', *Journal of Voice*, 16(2), pp. 274-277.
Boeckxstaens, G.E. (2005) 'The lower oesophageal sphincter', *Neurogastroenterology & Motility*, 17, pp. 13-21.

Becker DJ, Sinclair J, Castell DO, et al. A comparison of high and low fat meals on postprandial esophageal acid exposure. Am J Gastroenterol 1989;84:782–6.
Bell, S.C., De Boeck, K. and Amaral, M.D. (2014) 'New pharmacological approaches for cystic fibrosis: Promises, progress, pitfalls', *Pharmacology & Therapeutics*, (0).

Bendig, D.W., Seilheimer, D.K., Wagner, M.L., Ferry, G.D. and Harrison, G.M. (1982) 'Complications of gastroesophageal reflux in patients with cystic fibrosis', *The Journal of Pediatrics*, 100(4), pp. 536-540.

Bergin, D.A., Hurley, K., Mehta, A., Cox, S., D, R., O'Neill, S.J., Reeves, E.P. and McElvaney, N.G. (2013) 'Airway inflammatory markers in individuals with cystic fibrosis and non-cystic fibrosis bronchiectasis', *Journal of Inflammation Research*, 6, pp. 1-11.

Bhatt, J.M. (2013) 'Treatment of pulmonary exacerbations in cystic fibrosis', *European Respiratory Review*, 22(129), pp. 205-216.

Blondeau, K., Dupont, L.J., Mertens, V., Verleden, G., Malfroot, A., Vandenplas, Y., Hauser, B. and Sifrim, D. (2008a) 'Gastro-oesophageal reflux and aspiration of gastric contents in adult patients with cystic fibrosis', *Gut*, 57(8), pp. 1049-1055.

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

Blondeau, K., Pauwels, A., Dupont, L.J., Mertens, V., Proesmans, M., Orel, R., Brecelj, J., Lopez-Alonso, M., Moya, M.J., Malfroot, A., De Wachter, E., Vandenplas, Y., Hauser, B. and Sifrim, D. (2010) 'Characteristics of Gastroesophageal Reflux and Potential Risk of Gastric Content Aspiration in Children With Cystic Fibrosis.', *Journal of Pediatric Gastroenterology and Nutrition*, Feb;50(2), pp. 116-6.

Boeckxstaens, G.E. (2005) 'The lower oesophageal sphincter', *Neurogastroenterology & Motility*, 17, pp. 13-21.

Boesch, R.P. and Acton, J.D. (2007) 'Outcomes of fundoplication in children with cystic fibrosis', *Journal of Pediatric Surgery*, 42(8), pp. 1341-1344.

Bohman, J.K., Kor, D.J., Kashyap, R., Gajic, O., Festic, E., He, Z. and Lee, A.S. (2013) 'AIrway pepsin levels in otherwise healthy surgical patients receiving general anesthesia with endotracheal intubation', *Chest*, 143(5), pp. 1407-1413.

Bonfield, T.L., Panuska, J.R., Konstan, M.W., Hilliard, K.A., Hilliard, J.B., Ghnaim, H. and Berger, M. (1995) 'Inflammatory cytokines in cystic fibrosis lungs', *American Journal of Respiratory and Critical Care Medicine*, 152(6), pp. 2111-2118.

Bulmer, D.M., Ali, M.S., Brownlee, I.A., Dettmar, P.W. and Pearson, J.P. (2010) 'Laryngeal mucosa: Its susceptibility to damage by acid and pepsin', *The Laryngoscope*, 120(4), pp. 777-782.

Boyle, M.P. and De Boeck, K. (2013) 'A new era in the treatment of cystic fibrosis: correction of the underlying CFTR defect', *The Lancet Respiratory Medicine*, 1(2), pp. 158-163.

Button BM, Roberts S, Kotsimbos TC, et al. Gastroesophageal reflux (symptomatic and silent): a potentially significant problem in patients with cystic fibrosis before and after lung transplantation. J Heart Lung Transplant 2005;24:1522–9.

Bourke, S.J. and Burns, G.P. (2011) *Lecture Notes: Respiratory Medicine*. 8th Edition edn. United Kingdom: Wiley-Blackwell.

Boyle, M.P., Bell, S.C., Konstan, M.W., McColley, S.A., Rowe, S.M., Rietschel, E., Huang, X., Waltz, D., Patel, N.R. and Rodman, D. (2014) 'A CFTR corrector (lumacaftor) and a CFTR potentiator (ivacaftor) for treatment of patients with cystic fibrosis who have a phe508del CFTR mutation: a phase 2 randomised controlled trial', *The Lancet Respiratory Medicine*, 2(7), pp. 527-538.

Bredenoord, A.J. (2013) 'Management of Belching, Hiccups, and Aerophagia', *Clinical Gastroenterology and Hepatology*, 11(1), pp. 6-12.

Brodzicki J, Trawinska-Bartnicka M, Korzon M. Frequency, consequences and pharmacological treatment of gastroesophageal reflux in children with cystic fibrosis. Med Sci Monit 2002;8:CR529–37.

Brown, J. (2013) *Nutrition Now*. Cengage Learning.

Cohen, J.R., Schall, J.I., Ittenbach, R.F., Zemel, B.S. and Stallings, V.A. (2005) 'Fecal Elastase: Pancreatic Status Verification and Influence on Nutritional Status in Children with Cystic Fibrosis', *Journal of Pediatric Gastroenterology and Nutrition*, 40(4), pp. 438-444 10.1097/01.MPG.0000158222.23181.1C.

Chang, A.B., Cox, N.C., Purcell, J., Marchant, J.M., Lewindon, P.J., Cleghorn, G.J., Ee, L.C., Withers, G.D., Patrick, M.K. and Faoagali, J. (2005) 'Airway cellularity, lipid laden macrophages and microbiology of gastric juice and airways in children with reflux oesophagitis', *Respiratory Research*, 6(1), pp. 1-5.

Chen, S.-h., Wang, J.-w. and Li, Y.-m. (2010) 'Is alcohol consumption associated with gastroesophageal reflux disease?', *Journal of Zhejiang University SCIENCE B*, 11(6), pp. 423-8.

Chiba, N., Thomson, A., Barkun, A., Armstrong, D., van Zanten, S.V., White, R., Escobedo, S. and Sinclair, P. (2003) 'The ROME II definition of dyspepsia does not exclude patients with gastroesophageal reflux disease (GERD) in primary care', *Gastroenterology*, 124(4, Supplement 1), pp. A223-A224.

NHS Commissioning Board. Clinical Commissioning Policy: Ivacaftor for cystic fibrosis (G551D gene)Reference : NHSCB/A01/P/a.

Coderre, L., C. Fadainia, L. Belson, V. Belisle, S. Ziai, G. Maillhot, Y. Berthiaume and R. Rabasa-Lhoret (2012). "LDL-cholesterol and insulin are independently associated with body mass index in adult cystic fibrosis patients." Journal of Cystic Fibrosis 11(5): 393-397.

Cohen, J.R., Schall, J.I., Ittenbach, R.F., Zemel, B.S. and Stallings, V.A. (2005) 'Fecal Elastase: Pancreatic Status Verification and Influence on Nutritional Status in Children with Cystic Fibrosis', *Journal of Pediatric Gastroenterology and Nutrition*, 40(4), pp. 438-444 10.1097/01.MPG.0000158222.23181.1C.

Committee on Safety of Medicines., C.o.S.o. (1995) 'Report of the pancreatic enzymes working party; London: Committee on Safety of Medicines'.

Colombo, J.L. and Hallberg, T.K. (1987) 'Recurrent aspiration in children: Lipid-laden alveolar macrophage quantitation', *Pediatric Pulmonology*, 3(2), pp. 86-89.

Colombo, P., M. Mangano, et al. (2002). "Effect of calories and fat on postprandial gastro-oesophageal reflux." Scandinavian Journal of Gastroenterology 37(1): 3-5.

Colombo, C., Faelli, N., Tirelli, T.S., Fortunato, F., Biffi, A., Claut, L., Cariani, L., Daccò, V., Prato, R. and Conese, M. (2011) 'Analysis of inflammatory and immune response biomarkers in sputum and exhaled breath condensate by a multi-parametric biochip array in cystic fibrosis.'.

Couturier O, Bodet-Milin C, Querellou S, et al. Gastric scintigraphy with a liquid–solid radiolabelled meal: performances of solid and liquid parameters. Nucl Med Commun 2004;25:1143–50.

Corey, M., F. J. McLaughlin, M. Williams and H. Levison (1988). "A comparison of survival, growth, and pulmonary function in patients with cystic fibrosis in Boston and Toronto." Journal of Clinical Epidemiology 41(6): 583-591.
Crozier, D. N. (1974). "Cystic fibrosis: a not-so-fatal disease." Pediatric Clinic North America Nov;21(4): 935-950.

Cucchiara S, Santamaria F, Andreotti MR, Minella R, Ercolini P, Oggero V and G., d.R. (1991) 'Mechanisms of gastro-oesophageal reflux in cystic fibrosis.', *Archives of Diseases in Children*, May;66(5), pp. 617-22.

Cystic Fibrosis Foundation 2011
G.L.Crossfield, A. Krishnan, J. Lordan, S. Bourke, A. Anderson, P.W. Dettmar, I.A. Brownlee, C. Ward, J.P. Pearson (2013) 'WS3.1 Gastric aspiration into the CF lung – relationship with reflux symptoms and lung function' [ABSTRACT] *Journal of Cystic Fibrosis* 12*,1*:*S5*

Davies, J., Sheridan, H., Bell, N., Cunningham, S., Davis, S.D., Elborn, J.S., Milla, C.E., Starner, T.D., Weiner, D.J., Lee, P.-S. and Ratjen, F. (2013) 'Assessment of clinical response to ivacaftor with lung clearance index in cystic fibrosis patients with a G551D-CFTR mutation and preserved spirometry: a randomised controlled trial', *The Lancet Respiratory Medicine*, 1(8), pp. 630-638.

Davies, J.C., Cunningham, S., Alton, E.W.F.W. and Innes, J.A. (2008) 'Lung clearance index in CF: a sensitive marker of lung disease severity', *Thorax*, 63(2), pp. 96-97.

De Boeck, K., Kent, L., Davies, J., Derichs, N., Amaral, M., Rowe, S.M., Middleton, P., De Jonge, H., Bronsveld, I., Wilschanski, M., Melotti, P., Danner-Boucher, I., Boerner, S., Fajac, I., Southern, K., De Nooijer, R.A., Bot, A., De Rijke, Y., De Wachter, E., Leal, T.,

Vermeulen, F., Hug, M.J., Rault, G., Nguyen-Khoa, T., Barreto, C., Proesmans, M. and Sermet-Gaudelus, I. (2013) 'CFTR biomarkers: Time for promotion to surrogate end-point?', *European Respiratory Journal*, 41(1), pp. 203-216.

de Bentzmann, S., Plotkowski, C. and Puchelle, E. (1996) 'Receptors in the Pseudomonas Aeruginosa Adherence to Injured and Repairing Airway Epithelium', *American Journal of Respiratory and Critical Care Medicine*, 154(4_pt_2), pp. S155-S162.

Dennersten, U., Lannefors, L., Höglund, P., Hellberg, K., Johansson, H., Lagerkvist, A.-L., Ortfelt, M., Sahlberg, M. and Eriksson, L. (2009) 'Lung function in the aging Swedish cystic fibrosis population', *Respiratory Medicine*, 103(7), pp. 1076-1082.

DeMeester, T.R., Lafontaine, E., Joelsson, B.E., Skinner, D.B., Ryan, J.W., OSullivan, G.C., Brunsden, B.S. and Johnson, L.F. (1981) 'Relationship of a hiatal hernia to the function of the body of the esophagus and the gastresophageal junction.', *Journal of Thoracic Cardiovascular Surgery*, 82, pp. 547–558.

DeMeester, T.R., Wang, C.I., Wernly, J.A., Pellegrini, C.A., Little, A.G., Klementschitsch, P., Bermudez, G., Johnson, L., F. and Skinner, D.B. (1980) 'Technique, indications, and clinical use of 24 hour esophageal pH monitoring.', *Journal of Thorac Cardiovascular Surgery* 79, pp. 656–670.

Dent, J., Dodds, W.J., Friedman, R.H., Sekiguchi, T., Hogan, W.J., Arndorfer, R.C. and Petrie, D.J. (1980) 'Mechanism of gastroesophageal reflux in recumbent asymptomatic human subjects', *The Journal of Clinical Investigation*, 65(2), pp. 256-267.

Dent, J., El-Serag, H.B., Wallander, M.A. and Johansson, S. (2005) 'Epidemiology of gastro-oesophageal reflux disease: a systematic review', *Gut*, 54(5), pp. 710-717.

Dixon, J., Strugala, V., Welfare, M.R., Dettmar, P.W., Allen, A. and Pearson, J.P. (2001) 'Esophageal mucin: an adherent mucus gel barrier is absent in the normal esophagus but present in columnar-lined Barrett's esophagus', *Am J Gastroenterol*, 96(9), pp. 2575-2583.

Dettmar, P.W., Castell, D.O., Heading, R.C., Ell, S., Fagan, M.J., McGlashan, J.A., Morice, A.H. and Watson, M. (2011) 'Review article: reflux and its consequences – the laryngeal, pulmonary and oesophageal manifestations', *Alimentary Pharmacology & Therapeutics*, 33, pp. 1-71.

Dodge, J.A., Lewis, P.A., Stanton, M. and Wilsher, J. (2007) 'Cystic fibrosis mortality and survival in the UK: 1947-2003', *European Respiratory Journal*, 29(3), pp. 522-526.

Dore, M. P., E. Maragkoudakis, et al. (2008). "Diet, lifestyle and gender in gastro-esophageal reflux disease." Digestive Diseases & Sciences 53(8): 2027-2032.

Dixon, J. and O'Brien, P. (1999) 'Gastroesophageal Reflux in Obesity: The Effect of Lap-Band Placement', *Obesity Surgery*, 9(6), pp. 527-531.

di Sant'Agnese, P.A. and Davis, P.B. (1979) 'Cystic fibrosis in adults: 75 cases and a review of 232 cases in the literature', *The American Journal of Medicine*, 66(1), pp. 121-132.

Dore, M.P., Maragkoudakis, E., Fraley, K., Pedroni, A., Tadeu, V., Realdi, G., Graham, D.Y., Delitala, G. and Malaty, H.M. (2008) 'Diet, lifestyle and gender in gastro-esophageal reflux disease', *Digestive Diseases & Sciences*, 53(8), pp. 2027-32.

Dray, X., Hubert, D., Munck, A., Moreau, J. and Marteau, P. (2005) 'Manifestations digestives de la mucoviscidose de l'adulte', *Gastroentérologie Clinique et Biologique*, 29(12), pp. 1279-1285.

Efthimiadis, A.S., Hamid, Q., Kelly, M.M., Linden, M., Louis, R., Pizzichini, M.M.M., Pizzichini, E., Ronchi, C., Van Overveld, F. and Djukanović, R. (2002) 'Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation', *European Respiratory Journal*, 20(37 suppl), pp. 19s-23s.

Eckford, P.D.W., Li, C., Ramjeesingh, M. and Bear, C.E. (2012) 'Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Potentiator VX-770 (Ivacaftor) Opens the Defective Channel Gate of Mutant CFTR in a Phosphorylation-dependent but ATP-independent Manner', *Journal of Biological Chemistry*, 287(44), pp. 36639-36649.

Eckford, Paul D.W., Ramjeesingh, M., Molinski, S., Pasyk, S., Dekkers, J.F., Li, C., Ahmadi, S., Ip, W., Chung, Timothy E., Du, K., Yeger, H., Beekman, J., Gonska, T. and Bear, Christine E. (2014) 'VX-809 and Related Corrector Compounds Exhibit Secondary Activity Stabilizing Active F508del-CFTR after Its Partial Rescue to the Cell Surface', *Chemistry & Biology*, 21(5), pp. 666-678.

Escobar, C.H., Perdomo, G.M., Gimeno, B.R., Máiz, C.L. and Suárez, C.L. (1996) 'Gastroesophageal reflux, pulmonary and gastric function in patients with cystic fibrosis. Results of a randomized trial', *Acta Gastroenterol Latinoam*, 26(3), pp. 183-6.

Ervine, E., McMaster, C., McCallion, W. and Shields, M.D. (2009) 'Pepsin measured in induced sputum—a test for pulmonary aspiration in children?', *Journal of Pediatric Surgery*, 44(10), pp. 1938-1941.

El-Serag, H. B., J. A. Satia, et al. (2005). "Dietary intake and the risk of gastro oesophageal reflux disease: a cross sectional study in volunteers." Gut 54(1): 11-17.

Fathi H, Moon T, Donaldson J, Jackson W, Sedman P, Morice AH. Cough in adult cystic fibrosis: diagnosis and response to fundoplication. Cough. 2009 Jan 18;5:1.

Farré, R. (2013) 'Pathophysiology of gastro-esophageal reflux disease: a role for mucosa integrity?', *Neurogastroenterology & Motility*, 25(10), pp. 783-799.

Fanen, P., Wohlhuter-Haddad, A. and Hinzpeter, A. (2014) 'Genetics of cystic fibrosis: CFTR mutation classifications toward genotype-based CF therapies', *The International Journal of Biochemistry & Cell Biology*, 52(0), pp. 94-102.

Farrell, P.M., Rosenstein, B.J., White, T.B., Accurso, F.J., Castellani, C., Cutting, G.R., Durie, P.R., LeGrys, V.A., Massie, J., Parad, R.B., Rock, M.J. and Campbell Iii, P.W. (2008) 'Guidelines for Diagnosis of Cystic Fibrosis in Newborns through Older Adults: Cystic Fibrosis Foundation Consensus Report', *The Journal of Pediatrics*, 153(2), pp. S4-S14.

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

Fischer, N., Hentschel, J., Markert, U.R., Keller, P.M., Pletz, M.W. and Mainz, J.G. (2014) 'Non-invasive assessment of upper and lower airway infection and inflammation in CF patients', *Pediatric Pulmonology*, pp. n/a-n/a.

Flume, P., Wainwright, C., Tullis, E., Rodriguez, S., Davies, J. and Wagener, J. (2013) '58 Pulmonary exacerbations in CF patients with the G551D-CFTR mutation treated with ivacaftor', *Journal of Cystic Fibrosis*, 12, Supplement 1(0), p. S63.

Flume P A, Liou T G, Borowitz D S, Li H, Yen K, Ordoñez K L, Geller D E. (2012) 'Ivacaftor in Subjects With Cystic Fibrosis Who Are Homozygous for the F508del-CFTR Mutation' Chest 142, 3: 718-724

Fox, M., C. Barr, et al. (2007). "The Effects of Dietary Fat and Calorie Density on Esophageal Acid Exposure and Reflux Symptoms." Clinical Gastroenterology and Hepatology 5(4): 439-444.e431.

Friedenberg, F.K., Xanthopoulos, M., Foster, G.D. and Richter, J.E. (2008) 'The Association Between Gastroesophageal Reflux Disease and Obesity', *Am J Gastroenterol*, 103(8), pp. 2111-2122.

Freedman, S.D., Katz, M.H., Parker, E.M., Laposata, M., Urman, M.Y. and Alvarez, J.G. (1999) 'A membrane lipid imbalance plays a role in the phenotypic expression of cystic

fibrosis in cftr−/− mice', *Proceedings of the National Academy of Sciences*, 96(24), pp. 13995-14000.

Feigelson, J. and Sauvegrain, J. (1975) 'Letter: Gastro-esophageal reflux in mucoviscidosis', *Nouv Presse Med.*, Nov 8; 4 (38), pp. 2729-30.

Gaskin, K., D. Gurwitz, P. Durie, M. Corey, H. Levison and G. Forstner (1982). "Improved respiratory prognosis in patients with cystic fibrosis with normal fat absorption." The Journal of Pediatrics 100(6): 857-862.

Gillen, D. and McColl, K. (2011) 'Symptoms and signs of upper gastrointestinal disease', *Medicine*, 39(2), pp. 67-71.

Gerson LB, Boparai V, Ullah N, Triadafilopoulos G. Oesophageal and gastric pH profiles in patients with gastroesophageal reflux disease and Barrett's oesophagus treated with proton pump inhibitors. Aliment Pharmacol Ther 2004; 20: 637–43.

Gerson, L. B., K. Shetler, et al. (2005). "Control of intra-oesophageal and intra-gastric pH with proton pump inhibitors in patients with Barrett's oesophagus." Digestive and Liver Disease **37**(9): 651-658.

Gibson, L.E. and Cooke, R.E. (1959) 'A TEST FOR CONCENTRATION OF ELECTROLYTES IN SWEAT IN CYSTIC FIBROSIS OF THE PANCREAS UTILIZING PILOCARPINE BY IONTOPHORESIS', *Pediatrics*, 23(3), pp. 545-549.

Gustafsson, P.M., Fransson, S.G., Kjellman, N.I. and Tibbling, L. (1991) 'Gastro-oesophageal reflux and severity of pulmonary disease in cystic fibrosis', *Scand J Gastroenterol*, 26(5), pp. 449 - 56.

Grabowski, M., Kasran, A., Seys, S., Pauwels, A., Medrala, W., Dupont, L., Panaszek, B. and Bullens, D. (2011) 'Pepsin and bile acids in induced sputum of chronic cough patients', *Respiratory Medicine*, 105(8), pp. 1257-1261.

Hallberg, K., Fandriks, L. and Strandvik, B. (2004) 'Duodenogastric Bile Reflux Is Common in Cystic Fibrosis.', *Journal of Pediatric Gastroenterology & Nutrition.* , 38(3), pp. 312-316.

Hampel, H., Abraham, N.S. and El-Serag, H.B. (2005) 'Meta-Analysis: Obesity and the Risk for Gastroesophageal Reflux Disease and Its Complications', *Annals of Internal Medicine*, 143(3), pp. 199-211.

Hassal. E (1993) 'Barrett's esophagus: new definitions and approaches in children.', *Journal of Paediatric Gastroenterology and Nutrition*, 15:, pp. 345–64.

Harmon, J., Johnson, L. and Maydonovitch, C. (1981) 'Effects of acid and bile salts on the rabbit esophageal mucosa', *Digestive Diseases and Sciences*, 26(1), pp. 65-72.

Harrison, M.J., Murphy, D.M. and Plant, B.J. (2013) 'Ivacaftor in a G551D Homozygote with Cystic Fibrosis', *New England Journal of Medicine*, 369(13), pp. 1280-1282.

Hayat, J.O., Kang, J.-Y., Dettmar, P.W., Moore, A.T., Hicklin, L.C., Yazaki, E. and Sifrim, D. (2012) 'Su1056 Do Patients With Hoarseness and Endoscopic Signs of LPR Have Abnormal Esophago-Pharyngeal Reflux? a Study Using Simultaneous Impedance-Phmetry, Oro-Pharyngeal pH Monitoring (Restech) and Pepsin Measurements in Saliva', *Gastroenterology*, 142(5, Supplement 1), pp. S-411-S-412.

Hayes, D., Kirkby, S., S. McCoy, K., Mansour, H.M., Khosravi, M., Strawbridge, H. and Tobias, J.D. (2013) 'Reduction of lipid-laden macrophage index after laparoscopic Nissen fundoplication in cystic fibrosis patients after lung transplantation', *Clinical Transplantation*, 27(1), pp. 121-125.

Hanna, R.M. and Weiner, D.J. (2014) 'Overweight and obesity in patients with cystic fibrosis: A center-based analysis', *Pediatric Pulmonology*, pp. n/a-n/a.

Hebestreit, H., Sauer-Heilborn, A., Fischer, R., Käding, M. and Mainz, J.G. (2013) 'Effects of ivacaftor on severely ill patients with cystic fibrosis carrying a G551D mutation', *Journal of Cystic Fibrosis*, 12(6), pp. 599-603.

Heijerman, H. (1992) 'New modalities in the treatment of exocrine pancreatic insufficiency in cystic fibrosis.', *Netherlands Journal of Medicine*, Oct;41(3-4), pp. 105-9.

Hodson, M., A. Bush and D. Geddes (2007). Cystic Fibrosis, Third Edition, Taylor & Francis.

Holloway, R. H. (2000). "Treatment of gastro-oesophageal reflux disease through control of transient lower oesophageal sphincter relaxations - feasibility, practicality and possibilities." Digestive and Liver Disease 32(3): 183-187.

Holloway RH, Lyrenas E, Ireland A, Dent J. Effect of intraduodenal fat on lower oesophageal sphincter function and gastro-oesophageal reflux. *Gut* 1997; 40: 449-453

Holt, E.W., Yimam, K.K. and Liberman, M.S. (2013) 'Esophageal adenocarcinoma in a 40-year-old man with cystic fibrosis: coincidence or not?', *Ochsner J.*, Summer;13(2), pp. 252-5.

Hofmann, A.F. (1999) 'THe continuing importance of bile acids in liver and intestinal disease', *Archives of Internal Medicine*, 159(22), pp. 2647-2658.

Hopkins, P.M., Kermeen, F., Duhig, E., Fletcher, L., Gradwell, J., Whitfield, L., Godinez, C., Musk, M., Chambers, D., Gotley, D. and McNeil, K. (2010) 'Oil red O stain of alveolar macrophages is an effective screening test for gastroesophageal reflux disease in lung transplant recipients', *The Journal of Heart and Lung Transplantation*, 29(8), pp. 859-864.

Irwin, R.S., Pratter, M.R., Holland, P.S., Corwin, R.W. and Hughes, J.P. (1984) 'POstnasal drip causes cough and is associated with reversible upper airway obstruction', *Chest*, 85(3), pp. 346-352.

Iwakiri K, Kobayashi M, Kotoyori M, Yamada H, Sugiura T, Nakagawa Y. Relationship between postprandial esophageal acid exposure and meal volume and fat content. *Dig Dis Sci* 1996; 41: 926-930

Jaspersen, D., Labenz, J., Willich, S.N., Kulig, M., Nocon, M., Leodolter, A., Lind, T., Meyer-Sabellek, W., Vieth, M., Stolte, M. and Malfertheiner, P. (2006) 'Long-term clinical course of extra-oesophageal manifestations in patients with gastro-oesophageal reflux disease: A prospective follow-up analysis based on the ProGERD study', *Digestive and Liver Disease*, 38(4), pp. 233-238.

Jelalian, E., L. J. Stark, L. Reynolds and R. Seifer (1998). "Nutrition intervention for weight gain in cystic fibrosis: A meta analysis." The Journal of Pediatrics 132(3): 486-492.
Jolly, A.J., Wild, C.P. and Hardie, L.J. (2004) 'Acid and bile salts induce DNA damage in human oesophageal cell lines', *Mutagenesis*, 19(4), pp. 319-324.

Johnston N, Bulmer D, Gill GA, et al. Cell biology of laryngeal epithelial defences in health and disease: further studies. Ann Otol Rhinol Laryngol 2003; 112: 481–91.
Kageyama T. Pepsinogens, progastricsin and prochymosins: structure, function, evolution and development. Cell Mol Life Sci 2002; 59: 288–306.

Kahrilas, P.J., Boeckxstaens, G. and Smout, A.J.P.M. (2013) 'Management of the patient with incomplete response to PPI therapy', *Best Practice & Research Clinical Gastroenterology*, 27(3), pp. 401-414.

Kastner-Cole, D., C. N. A. Palmer, S. A. Ogston, A. Mehta and S. Mukhopadhyay (2005). "Overweight and Obesity in ΔF508 Homozygous Cystic Fibrosis." The Journal of Pediatrics 147(3): 402-404.

Kennedy, J.H. (1962) 'Silent gastroesophageal reflux: an important but little known cause of pulmonary complications. ', *Diseases of the Chest* 42, pp. 42–5.

Klauser, A.G., Schindlbeck, N.E. and Müller-Lissner, S.A. (1990) 'Symptoms in gastro-oesophageal reflux disease', *The Lancet*, 335(8683), pp. 205-208.

Kitz, R., Boehels, H.J., Rosewich, M. and Rose, M.A. (2012) 'Lipid-Laden Alveolar Macrophages and pH Monitoring in Gastroesophageal Reflux-Related Respiratory Symptoms', *Pulmonary Medicine*, 2012.

Kivilaakso, E., Fromm, D. and Silen, W. (1980) 'Effect of bile salts and related compounds on isolated esophageal mucosa.', *Surgery*, mar;87(3), pp. 280-5.

Köksal, D., Özkan, B., Þimþek, C., Köksal, A.Þ., Aðaçkýran, Y. and Þaþmaz, N. (2005) 'Lipid-Laden Alveolar Macrophage Index in Sputum is Not Useful in the Differential Diagnosis of Pulmonary Symptoms Secondary to Gastroesophageal Reflux', *Archives of Medical Research*, 36(5), pp. 485-489.

Konstan, M.W., Morgan, W.J., Butler, S.M., Pasta, D.J., Craib, M.L., Silva, S.J., Stokes, D.C., Wohl, M.E.B., Wagener, J.S., Regelmann, W.E. and Johnson, C.A. (2007) 'Risk Factors For Rate of Decline in Forced Expiratory Volume in One Second in Children and Adolescents with Cystic Fibrosis', *The Journal of Pediatrics*, 151(2), pp. 134-139.e1.

Kochavi, B. (2014) '211 Overweight and obesity in the cystic fibrosis Israeli national center', *Journal of Cystic Fibrosis*, 13, Supplement 2(0), p. S100.

Kotha K and Clancy J P. (2013) ‘Ivacaftor treatment of cystic fibrosis patients with the G551D mutation: a review of the evidence’ Therapeutic Advances in Respiratory Disease 7: 288 2013

Kulig, M., Nocon, M., Vieth, M., Leodolter, A., Jaspersen, D., Labenz, J., Meyer-Sabellek, W., Stolte, M., Lind, T., Malfertheiner, P. and Willich, S.N. (2004) 'Risk factors of gastroesophageal reflux disease: methodology and first epidemiological results of the ProGERD study', *Journal of Clinical Epidemiology*, 57(6), pp. 580-589.

Kraemer, R., A. RÜDeberg, B. Hadorn and E. Rossi (1978). "RELATIVE UNDERWEIGHT IN CYSTIC FIBROSIS AND ITS PROGNOSTIC VALUE." Acta Pædiatrica 67(1): 33-37.

Laszlo, G. (2006) 'Standardisation of lung function testing: helpful guidance from the ATS/ERS Task Force', *Thorax*, 61, pp. 744-746

Ledson, M.J., Tran, J. and Walshaw, M.J. (1998) 'Prevalence and mechanisms of gastro-oesophageal reflux in adult cystic fibrosis patients.', *Journal of the Royal Society of Medicine*, 91, pp. 7-9.

Laohasiriwong, S., Johnston, N. and Woodson, B.T. (2013) 'Extra-esophageal Reflux, NOSE score, and sleep quality in an adult clinic population', *The Laryngoscope*, 123(12), pp. 3233-3238.

Lerín, M., Prados, C., Martínez, M.T., Maíz, L., Girón, R., Solé, A., Cabanillas, J.J. and Álvarez-Sala, R. (2014) 'Cystic fibrosis in adult age', *Revista Clínica Española (English Edition)*, 214(6), pp. 289-295.

Littlewood, J.M. (1992) 'Gastrointestinal complications in cystic fibrosis', *Journal of the Royal Society of Medicine*, 85(19), pp. 13-19.

Mandal, P., Morice, A.H., Chalmers, J.D. and Hill, A.T. (2013) 'Symptoms of airway reflux predict exacerbations and quality of life in bronchiectasis', *Respiratory Medicine*, 107(7), pp. 1008-1013.

Malfroot, A. and Dab, I. (1991) 'New insights on gastro-oesophageal reflux in cystic fibrosis by longitudinal follow up.', *Archives of Disease in Childhood*, 66, pp. 1339-1345.

McNally, P., Ervine, E., Shields, M.D., Dimitrov, B.D., El Nazir, B., Taggart, C.C., Greene, C.M., McElvaney, N.G. and Greally, P. (2011) 'High concentrations of pepsin in bronchoalveolar lavage fluid from children with cystic fibrosis are associated with high interleukin-8 concentrations', *Thorax*, 66(2), pp. 140-143.

Meyer JH, Lembo A, Elashoff JD, Fass R, Mayer EA. Duodenal fat intensifies the perception of heartburn. *Gut* 2001; 49: 624-628

Meining, A. and Classen, M. (2000) 'The role of diet and lifestyle measures in the pathogenesis and treatment of gastroesophageal reflux disease', *The American Journal of Gastroenterology*, 95(10), pp. 2692-2697.

Mittal, R.K., Holloway, R.H., Penagini, R., Ashley Blackshaw, L. and Dent, J. (1995) 'Transient lower esophageal sphincter relaxation', *Gastroenterology*, 109(2), pp. 601-610.
Mousa, H. and Woodley, F. (2012) 'Gastroesophageal Reflux in Cystic Fibrosis: Current Understandings of Mechanisms and Management', *Current Gastroenterology Reports*, 14(3), pp. 226-235.

Moayyedi, P. and Talley, N.J. (2006) 'Gastro-oesophageal reflux disease', *The Lancet*, 367(9528), pp. 2086-2100.

Moazzez, R., Bartlett, D. and Anggiansah, A. (2004) 'Dental erosion, gastro-oesophageal reflux disease and saliva: how are they related?', *Journal of Dentistry*, 32(6), pp. 489-494.

Morice, A.H., Faruqi, S., Wright, C.E., Thompson, R. and Bland, J.M. (2011) 'Cough Hypersensitivity Syndrome: A Distinct Clinical Entity', *Lung*, 189(1), pp. 73-79.

Morton, A.M. (2009) 'Symposium 6: Young people, artificial nutrition and transitional care The nutritional challenges of the young adult with cystic fibrosis: transition', *Proceedings of the Nutrition Society*, 68(04), pp. 430-440.

Mousa, H. and Woodley, F. (2012) 'Gastroesophageal Reflux in Cystic Fibrosis: Current Understandings of Mechanisms and Management', *Current Gastroenterology Reports*, 14(3), pp. 226-235.

Murphy DW, Castell DO. Chocolate and heartburn: evidence of increased esophageal acid exposure after chocolate ingestion. Am J Gastroenterol. 1988;83:633–636.

Munck, A., G. Bellis, S. Ravilly and F. Huet (2007). "221* Do overweight and obesity improve clinical outcome in the French cystic fibrosis population?" Journal of Cystic Fibrosis 6, Supplement 1(0): S54.

Nandurkar, S., G. R. Locke, et al. (2004). "Relationship between body mass index, diet, exercise and gastro-oesophageal reflux symptoms in a community." Alimentary Pharmacology & Therapeutics **20**(5): 497-505.

Navarro, J., Rainisio, M., Harms, H.K., Hodson, M.E., Koch, C., Mastella, G., Strandvik, B., McKenzie, S.G. and on behalf of the investigators of the European Epidemiologic Registry of Cystic, F. (2001) 'Factors associated with poor pulmonary function: cross-sectional analysis of data from the ERCF', *European Respiratory Journal*, 18(2), pp. 298-305.

Nessbaum, E., Maggi, J.C., Mathis, R. and Galant, S.P. (1987) 'Association of lipid-laden alveolar macrophages and gastroesophageal reflux in children', *Journal of Peadiatrics*, Feb; 110(2), pp. 190-4.

Nebel OT, Fornes MF, Castell DO. Symptomatic gastroesophageal reflux: incidence and precipitating factors. Am J Dig Dis. 1976;21:953–956.

Nowak M, Büttner P, Raasch B, Daniell K, McCutchan C, Harrison S (2005) Lifestyle changes as a treatment of gastroesophageal reflux disease: a survey of general practitioners in North Queensland, Australia. Ther Clin Risk Manag.1(3):219-24.

Orel, R. and Markovic, S. (2003) 'Bile in the Esophagus: A Factor in the Pathogenesis of Reflux Esophagitis in Children', *Journal of Pediatric Gastroenterology and Nutrition*, 36(2), pp. 266-273.

Orenstein, D.M., Rosenstein, B.J. and Stern, R.C. (2000) *Cystic Fibrosis: Medical Care*. Philadelphia, USA.: Lippincott Williams & Wilkins.
O'Sullivan, B. P. and S. D. Freedman (2009). "Cystic fibrosis." The Lancet 373(9678): 1891-1904.

Palm, K., Sawicki, G. and Rosen, R. (2012) 'The impact of reflux burden on Pseudomonas positivity in children with Cystic Fibrosis', *Pediatric Pulmonology*, 47(6), pp. 582-587.

Panagopoulou, P., M. Fotoulaki, A. Nikolaou and S. Nousia-Arvanitakis (2014). "Prevalence of malnutrition and obesity among cystic fibrosis patients." Pediatrics International 56(1): 89-94.

Parameswaran, K., Anvari, M., Efthimiadis, A., Kamada, D., Hargreave, F.E. and Allen, C.J. (2000) 'Lipid-laden macrophages in induced sputum are a marker of oropharyngeal reflux and possible gastric aspiration', *European Respiratory Journal*, 16(6), pp. 1119-1122.

Parikh, S., Brownlee, I.A., Robertson, A.G., Manning, N.T., Johnson, G.E., Brodlie, M., Corris, P.A., Ward, C. and Pearson, J.P. (2013) 'Are the enzymatic methods currently being used to measure bronchoalveolar lavage bile salt levels fit for purpose?', *The Journal of Heart and Lung Transplantation*, 32(4), pp. 418-423.

Pauwels, A., Verleden, S., Farre, R., Vanaudenaerde, B.M., Van Raemdonck, D., Verleden, G., Sifrim, D. and Dupont, L.J. (2013) 'The effect of gastric juice on interleukin-8 production by cystic fibrosis primary bronchial epithelial cells', *Journal of Cystic Fibrosis*, 12(6), pp. 700-705.

Pauwels, A., Blondeau, K., Mertens, V., Farré, R., Dupont, L. and Sifrim, D. (2011) 'A Distinct Mechanism for Reflux in Patients With Cystic Fibrosis: A Study Using High Resolution Manometry-Impedance', *Gastroenterology*, 140(5, Supplement 1), pp. S-43.

Pauwels, A., Blondeau, K., Dupont, L.J. and Sifrim, D. (2012) 'Mechanisms of Increased Gastroesophageal Reflux in Patients With Cystic Fibrosis', *Am J Gastroenterol*, 107(9), pp. 1346-1353.

Pier, G.B., Grout, M., Zaidi, T.S., Olsen, J.C., Johnson, L.G., Yankaskas, J.R. and Goldberg, J.B. (1996) 'Role of mutant CFTR in hypersusceptibility of cystic fibrosis patients to lung infections.', *Science.*, 271, pp. 64-67.

Pearson, J.P and Parikh, S (2011) 'Reviewarticle:refluxanditsconsequences—the laryngeal, pulmonaryandoesophagealmanifestations.', *Alimentary Pharmacology and Therapeutics* 33, pp. 2-7.

Pearson, J P & Brownlee, I A, Stomach – Structure and function, Encyclopedia of human nutrition, Volume 4, 2005, pp. 180-190, Academic Press.

Polenakovik, H.M. and Sanville, B. (2013) 'The use of ivacaftor in an adult with severe lung disease due to cystic fibrosis (ΔF508/G551D)', *Journal of Cystic Fibrosis*, 12(5), pp. 530-531.

Pehl, C., A. Waizenhoefer, et al. (1999). "Effect of low and high fat meals on lower esophageal sphincter motility and gastroesophageal reflux in healthy subjects." The American Journal of Gastroenterology 94(5): 1192-1196.

Pellegrino, R., Viegi, G., Brusasco, V., Crapo, R.O., Burgos, F., Casaburi, R., Coates, A., van der Grinten, C.P.M., Gustafsson, P., Hankinson, J., Jensen, R., Johnson, D.C., MacIntyre, N., McKay, R., Miller, M.R., Navajas, D., Pedersen, O.F. and Wanger, J. (2005) 'Interpretative strategies for lung function tests', *European Respiratory Journal*, 26(5), pp. 948-968.

Penagini, R., Mangano, M. and Bianchi, P.A. (1998) 'Effect of increasing the fat content but not the energy load of a meal on gastro-oesophageal reflux and lower oesophageal sphincter motor function', *Gut*, 42(3), pp. 330-333.

Penagini R (2000) 'Fat and gastro-oesophageal reflux disease.', *European Journal of Gastroenterology and Hepatology*, Dec;12(12), pp. 1343-5.

Phitidis, M.E., Murthy, M., Hunt, N., Jones, G.H., Fox, J., Ledson, M., Greenwood, J. and Walshaw, M. (2014) '168 Cystic fibrosis: an ageing condition', *Journal of Cystic Fibrosis*, 13, Supplement 2(0), p. S89.

Quintana-Gallego, E., Delgado-Pecellín, I. and Calero Acuña, C. (2014) 'CFTR Protein Repair Therapy in Cystic Fibrosis', *Archivos de Bronconeumología (English Edition)*, 50(4), pp. 146-150.

Quon, B.S. and Aitken, M.L. (2012) 'Cystic Fibrosis: What to Expect now in the Early Adult Years', *Paediatric Respiratory Reviews*, 13(4), pp. 206-214.

Ramsey, B. W., J. Davies, et al. (2011). "A CFTR Potentiator in Patients with Cystic Fibrosis and the G551D Mutation." New England Journal of Medicine **365**(18): 1663-1672.

Robertson, A.G.N., Krishnan, A., Ward, C., Pearson, J.P., Small, T., Corris, P.A., Dark, J.H., Karat, D., Shenfine, J. and Griffin, S.M. (2012) 'Anti-reflux surgery in lung transplant recipients: outcomes and effects on quality of life', *European Respiratory Journal*, 39(3), pp. 691-697.

Robinson, N.B. and DiMango, E. (2014) 'Prevalence of Gastroesophageal Reflux in Cystic Fibrosis and Implications for Lung Disease', *Annals of the American Thoracic Society*, 11(6), pp. 964-968.

Robinson, P.J., Smith, A.L. and Sly, P. (1990) 'Duodenal pH in cystic fibrosis and its relationship to fat malabsorption.', *Digestive Diseases & Sciences*, Oct;35(10), pp. 1299-304.

Ronkainen, J. and Agréus, L. (2013) 'Epidemiology of reflux symptoms and GORD', *Best Practice & Research Clinical Gastroenterology*, 27(3), pp. 325-337.

Rosaida MS, Goh KL. (2004) Gastro-oesophageal reflux disease, reflux oesophagitis and non-erosive reflux disease in a multiracial Asian population: a prospective, endoscopy based study. *Eur J Gastroenterol Hepatol.* 16: 495-501

Ruhl, C. E. and J. E. Everhart (1999). "Overweight, but Not High Dietary Fat Intake, Increases Risk of Gastroesophageal Reflux Disease Hospitalization: The NHANES I Epidemiologic Followup Study." Annals of Epidemiology 9(7): 424-435.

Ruigómez, A., García Rodríguez, L.A., Wallander, M.A., Johansson, S., Graffner, H. and Dent, J. (2004) 'Natural history of gastro-oesophageal reflux disease diagnosed in general practice', *Alimentary Pharmacology & Therapeutics*, 20(7), pp. 751-760.

Ruigomez, A., Wallander, M.-A., Lundborg, P., Johansson, S. and Garcia-Rodriguez, L. (2008) 'T1981 Gastroesophageal Reflux Disease in Children and Adolescents in Primary Care: Incidence, Prevalence and Treatment Patterns', *Gastroenterology*, 134(4, Supplement 1), pp. A-593.

Reznikov, L.R., Abou Alaiwa, M.H., Dohrn, C.L., Gansemer, N.D., Diekema, D.J., Stoltz, D.A. and Welsh, M.J.(2014) 'Antibacterial properties of the CFTR potentiator ivacaftor', *Journal of Cystic Fibrosis*, (0).

Sabati, A.A., Kempainen, R.R., Milla, C.E., Ireland, M., Schwarzenberg, S.J., Dunitz, J.M. and Khan, K.M. (2010) 'Characteristics of gastroesophageal reflux in adults with cystic fibrosis', *Journal of Cystic Fibrosis*, 9(5), pp. 365-370.

Saberi-Firoozi Mehdi, Yazdanbakhsh Mohammad A, Heidari Seyed-Taghi, Khademolhosseini Farnaz, Mehrabani Davood (2007) Correlation of gastroesophageal reflux disease with positive family history and headache in Shiraz city, Southern Iran. Saudi J Gastroenterol.13; 4, 176-179

Sagel, S.D., Chmiel, J.F. and Konstan, M.W. (2007) 'Sputum Biomarkers of Inflammation in Cystic Fibrosis Lung Disease', *Proceedings of the American Thoracic Society*, 4(4), pp. 406-417.

Saiman, L. and Prince, A. (1993) 'Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells', *The Journal of Clinical Investigation*, 92(4), pp. 1875-1880.

Salvatore, D., Buzzetti, R., Baldo, E., Furnari, M.L., Lucidi, V., Manunza, D., Marinelli, I., Messore, B., Neri, A.S., Raia, V. and Mastella, G. (2012) 'An overview of international literature from cystic fibrosis registries. Part 4: Update 2011', *Journal of Cystic Fibrosis*, 11(6), pp. 480-493.

Savarino, E., Carbone, R., Marabotto, E., Furnari, M., Sconfienza, L., Ghio, M., Zentilin, P. and Savarino, V. (2013) 'Gastro-oesophageal reflux and gastric aspiration in idiopathic pulmonary fibrosis patients', *European Respiratory Journal*, 42(5), pp. 1322-1331.

Scott, R.B., O'Loughlin, E.V. and Gall, D.G. (1985) 'Gastroesophageal reflux in patients with cystic fibrosis', *The Journal of Pediatrics*, 106(2), pp. 223-227.

Sagel, S.D., Chmiel, J.F. and Konstan, M.W. (2007) 'Sputum Biomarkers of Inflammation in Cystic Fibrosis Lung Disease', *Proceedings of the American Thoracic Society*, 4(4), pp. 406-417.

Sharma, R., Florea, V.G., Bolger, A.P., Doehner, W., Florea, N.D., Coats, A.J.S., Hodson, M.E., Anker, S.D. and Henein, M.Y. (2001) 'Wasting as an independent predictor of mortality in patients with cystic fibrosis', *Thorax*, 56(10), pp. 746-750.

Shapiro, M., C. Green, et al. (2007). "Assessment of dietary nutrients that influence perception of intra-oesophageal acid reflux events in patients with gastro-oesophageal reflux disease." Alimentary Pharmacology & Therapeutics 25(1): 93-101.

Shanks, K.K., Guang, W., Kim, K.C. and Lillehoj, E.P. (2010) 'Interleukin-8 Production by Human Airway Epithelial Cells in Response to Pseudomonas aeruginosa Clinical Isolates Expressing Type a or Type b Flagellins', *Clinical and Vaccine Immunology*, 17(8), pp. 1196-1202.

Sheikh, S.I., Ryan-Wenger, N.A. and McCoy, K.S. (2013) 'Outcomes of surgical management of severe GERD in patients with cystic fibrosis', *Pediatric Pulmonology*, 48(6), pp. 556-562.

Sifrim, D. and Holloway, R. (2001) 'Transient lower esophageal sphincter relaxations: how many or how harmful[quest]', *Am J Gastroenterol*, 96(9), pp. 2529-2532.

Simmonds, N. J. (2013). "Ageing in Cystic Fibrosis and Long-term Survival." Paediatric Respiratory Reviews 14, Supplement 1(0): 6-9.
Simmonds, N.J., Cullinan, P. and Hodson, M.E. (2009) 'Growing old with cystic fibrosis – The characteristics of long-term survivors of cystic fibrosis', *Respiratory Medicine*, 103(4), pp. 629-635.

Simon, M. I. S. S., M. Drehmer and S. S. Menna-Barreto (2009). "Association between nutritional status and dietary intake in patients with cystic fibrosis." Associação entre o estado nutricional e a ingestão dietética em pacientes com fibrose cística 35(10): 966-972.

Sinaasappel, M., M. Stern, J. Littlewood, S. Wolfe, G. Steinkamp, H. G. M. Heijerman, E. Robberecht and G. Döring (2002). "Nutrition in patients with cystic fibrosis: a European Consensus." Journal of Cystic Fibrosis 1(2): 51-75.
Smyth, A. and Elborn, J.S. (2008) 'Exacerbations in cystic fibrosis: 3 · Management', *Thorax*, 63(2), pp. 180-184.

Smyth, A.R., Bell, S.C., Bojcin, S., Bryon, M., Duff, A., Flume, P., Kashirskaya, N., Munck, A., Ratjen, F., Schwarzenberg, S.J., Sermet-Gaudelus, I., Southern, K.W., Taccetti, G., Ullrich, G. and Wolfe, S. (2014) 'European Cystic Fibrosis Society Standards of Care: Best Practice guidelines', *Journal of Cystic Fibrosis*, 13, Supplement 1(0), pp. S23-S42.

Stack, M., M. Mahony and C. Boyle (2007). "220 Obesity in childhood cystic fibrosis." Journal of Cystic Fibrosis 6, Supplement 1(0): S54.

Stallings, V. A., L. J. Stark, K. A. Robinson, A. P. Feranchak and H. Quinton (2008). "Evidence-Based Practice Recommendations for Nutrition-Related Management of Children and Adults with Cystic Fibrosis and Pancreatic Insufficiency: Results of a Systematic Review." Journal of the American Dietetic Association 108(5): 832-839.

Stephenson, A. L., L. A. Mannik, S. Walsh, M. Brotherwood, R. Robert, P. B. Darling, R. Rosane Nisenbaum, J. Moerman and S. Stanojevic (2013). "Longitudinal trends in nutritional status and the relation between lungfunction and BMI in cystic fibrosis: a population-based cohort study." Am J Clin Nutr 97: 872-827.

Starosta, V., Kitz, R., Hartl, D., Marcos, V., Reinhardt, D. and Griese, M. (2007) 'BRonchoalveolar pepsin, bile acids, oxidation, and inflammation in children with gastroesophageal reflux disease*', *CHEST Journal*, 132(5), pp. 1557-1564.

Steinkamp, G. and Wiedemann, B. (2002) 'Relationship between nutritional status and lung function in cystic fibrosis: cross sectional and longitudinal analyses from the German CF quality assurance (CFQA) project', *Thorax*, 57(7), pp. 596-601.

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

Strugala, V., McGlashan, J.A., M.G, W., Morice, A.H., Granier, B. and Dettmar, P.W. (2007) 'Detection of pepsin using a non-invasive lateral flow test for the diagnosis of extra-oesophageal reflux - results of a pilot study', *Gut*, Suppl III, p. A212.

Tasker, A., Dettmar, P.W., Panetti, M., Koufman, J.A., Birchall, J.P. and Pearson, J.P. (2002) 'Reflux of gastric juice and glue ear in children', *The Lancet*, 359(9305), p. 493.

Thomson, A. and A. Harris (2008). Cystic Fibrosis The Facts, Oxford University Press, 2008.

Thomas B. and Bishop J, (2007) Manual of Dietetic Practice, 4th Edition. Oxford, Blackwell Publishing LTD

Tsui, L.C., Buchwald, M., Barker, D., Braman, J.C., Knowlton, R., Schumm, J.W., Eiberg, H., Mohr, J., Kennedy, D. and Plavsic, N. (1985) 'Cystic fibrosis locus defined by a genetically linked polymorphic DNA marker', *Science.*, 230(4729), pp. 1054-7.

van der Doef, H.P.J., Arets, H.G.M., Froeling, S.P., Westers, P. and Houwen, R.H.J. (2009) 'Gastric Acid Inhibition for Fat Malabsorption or Gastroesophageal Reflux Disease in Cystic Fibrosis: Longitudinal Effect on Bacterial Colonization and Pulmonary Function', *The Journal of Pediatrics*, 155(5), pp. 629-633.

Van Goor F, Hadida S, Grootenhuis P D J, Burton B, Cao D, Neuberger T, Turnbull A, Singh A, Joubran J, Hazlewood A, Zhou J, McCartney J, Arumugam V, Decker C, Yang J, Young C, Olsonb E R, Winec J J, Frizzelld R A, Ashlocke M, Negulescua P. (2009) 'Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770.' PNAS 106 , 44: 18825–18830

Van Goor F, Yu H, Burton B, Hoffman B J. (2013) 'Effect of ivacaftor on CFTR forms with missense mutations associated with defects in protein processing or function.' Journal of Cystic Fibrosis IN PRESS

van Pinxteren B, Numans M, Lau J, de Wit NJ, Hungin APS, Bonis PAL: Short-term treatment of gastroesophageal reflux disease. A systematic review and meta-analysis of the effect of acid-suppressant drugs in empirical treatment and in endoscopy-negative patients. J Gen Intern Med 2003, 18:755-763.

Vakil, N. (2010). "Disease definition, clinical manifestations, epidemiology and natural history of GERD." Best Practice & Research Clinical Gastroenterology 24(6): 759-764.

Vemulapalli, R. (2008). "Diet and lifestyle modifications in the management of gastroesophageal reflux disease." Nutrition in Clinical Practice 23(3): 293-298.
Veeze, H.J. (1995) 'Diagnosis of cystic fibrosis', *The Netherlands Journal of Medicine*, 46(6), pp. 271-274.

Vinocur, C.D., Marmon, L., Schidlow, D.V. and Weintraub, W.H. (1985) 'Gastroesophageal reflux in the infant with cystic fibrosis', *The American Journal of Surgery*, 149(1), pp. 182-186.

Wang, Y., Goulart, R.A. and Pantanowitz, L. (2010) 'Oil Red O Staining in Cytopathology', *Diagnostic Cytopathology*, 39(4), pp. 272-3.

Wang JH, Luo JY, Dong L, Gong J, Tong M. Epidemiology of gastroesophageal reflux disease: a general population-based study in Xi'an of Northwest China. *World J Gastroenterol* 2004; 10: 1647-1651

Waugh, A., Grant, A.W., Chambers, G., Ross, J.S. and Wilson, K.J.W. (2006) *Ross and Wilson anatomy and physiology in health and illness*. Churchill Livingstone.

Weusten, B.L., Akkermans, L.M., vanBerge-Henegouwen, G.P. and Smout, A.J. (1995) 'Dynamic characteristic of gastro-oesophageal reflux in ambulatory patients with gastro-oesophageal reflux disease and normal control subjects.', *Scand J Gastroenterol.*, Aug;30(8), pp. 731-7.

Webster-Gandy, J., A. Madden and M. Holdsworth (2012). Oxford Handbook of Nutrition and Dietetics, OUP Oxford.

White, H., A. M. Morton, D. G. Peckham and S. P. Conway (2004). "Dietary intakes in adult patients with cystic fibrosis–do they achieve guidelines?" Journal of Cystic Fibrosis 3(1): 1-7.

WHO. (1998). "International Obesity Task Force. Obesity: preventing and managing the global epidemic. Report of the WHO consultation on obesity, Geneva, 3-5th June 1998." Geneva: WHO, 1998.

Wilson, D. C. and P. B. Pencharz (1998). "Nutrition and cystic fibrosis." Nutrition 14(10): 792-795.

Woodley, F., Machado, R., Hayes, D., Jr., Lorenzo, C., Kaul, A., Skaggs, B., McCoy, K., Patel, A. and Mousa, H. (2014) 'Children with Cystic Fibrosis Have Prolonged Chemical Clearance of Acid Reflux Compared to Symptomatic Children Without Cystic Fibrosis', *Digestive Diseases and Sciences*, 59(3), pp. 623-630.

Yankaskas, J. R., B. C. Marshall, B. Sufian, R. H. Simon and D. Rodman (2004). "Cystic fibrosis adult care*: Consensus conference report." CHEST Journal 125(1_suppl): 1S-39S.

W, Chiaw P K and Bear C E. (2011) 'Probing Conformational Rescue Induced by a Chemical Corrector of F508del-Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Mutant.' Journal of Biological Chemistry 286, 28: 24714-24725

Yu H, Burton B, Huang C J, Worley J, Cao D, Johnson J P, Urrutia A, Joubran J, Seepersaud, Sussky S K, Hoffman B J, Van Goor F. (2012) 'Ivacaftor potentiation of multiple CFTR channels with gating mutations.' Journal of Cystic Fibrosis 11: 237–245

Zerbib, F., Bruley Des Varannes, S., Roman, S., Pouderoux, P., Artigue, F., Chaput, U., Mion, F., Caillol, F., Verin, E., Bommelaer, G., DucrottÉ, P., Galmiche, J.P. and Sifrim, D. (2005) 'Normal values and day-to-day variability of 24-h ambulatory oesophageal impedance-pH monitoring in a Belgian–French cohort of healthy subjects', *Alimentary Pharmacology & Therapeutics*, 22(10), pp. 1011-1021.

Zerbib, F., Sifrim, D., Tutuian, R., Attwood, S. and Lundell, L. (2013) 'Modern medical and surgical management of difficult-to-treat GORD', *Jornal of United European Gastroenterol*, Feb;1(1), pp. 21-31.

Zheng, Z., H. Nordenstedt, et al. (2007). "Lifestyle Factors and Risk for Symptomatic Gastroesophageal Reflux in Monozygotic Twins." Gastroenterology **132**(1): 87-95.

8.0 Appendix

8.1 Appendix 1 Ethical approval and NHS R&D approval

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

LRF/JW

The Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Tel: 0191 233 6161
Fax: 0191 213 1968
www.newcastle-hospitals.nhs.uk

18th May 2010

Professor S Griffin
Professor of Gastrointestinal Surgery
Northern Oesophagogastric Unit
Royal Victoria Infirmary

Dear Professor Griffin

Trust R&D Project:	**5183**
Title of Project:	**The use of impedance pH measurements to determine the effect of gastro-oesophageal reflux in patients with cystic fibrosis and idiopathic pulmonary fibrosis**
Principal Investigator	**Professor Michael Griffin**
Number of patients:	**60**
Funder (proposed):	**Own Account**
Sponsor (proposed):	**Newcastle upon Tyne Hospitals NHS Foundation Trust**
REC number:	**10/H0908/8**

Having carried out the necessary risk and site assessment for the above research project, Newcastle upon Tyne Hospitals NHS Foundation Trust grants NHS R&D approval for this research to take place at this Trust dependent upon:

(i) you, as Principal Investigator, agreeing to comply with the Department of Health's Research Governance Framework for Health and Social Care, and understanding their responsibilities and duties (a copy of responsibilities prepared by the Trust R&D Office is enclosed)

(ii) you, as Principal Investigator, ensuring compliance of the project with all other legislation and guidelines including Caldicott Guardian approvals and compliance with the Data Protection Act 1998, Health and Safety at Work Act 1974, any requirements of the MHRA (*eg* CTA, EudraCT registration), and any other relevant UK/European guidelines or legislation (*eg* reporting of suspected adverse incidents).

(iii) where applicable, you, as Principal Investigator, should also adhere to the GMC supplementary guidance *Good practice in research* and *Consent to research* which sets out the good practice principles that doctors are expected to understand and follow if they are involved in research – see http://www.gmc-uk.org/guidance/ethical_guidance/5991.asp

Sponsorship

The Newcastle upon Tyne Hospitals NHS Foundation Trust will act as Sponsor for this project, under the Department of Health's guidelines for research in health and social care.

NHS

National Research Ethics Service

County Durham & Tees Valley 2 Research Ethics Committee
The Tatchell Centre
University Hospital of North Tees
Piperknowle Road
Stockton-on-Tees
TS19 8PE

Telephone: 01642 624164
Facsimile: 01642 624164

22 February 2010

Professor S.Michael Griffin
Professor of Gastrointestinal Surgery
Northern Oesophagogastric Unit
Royal Victoria Infirmary
Queen Victoria Road
NE1 4LP

Dear Professor Griffin

Study Title:	**The use of impedance pH measurements to determine the effect of gastro-oesophageal reflux in patients with cystic fibrosis and idiopathic pulmonary fibrosis**
REC reference number:	**10/H0908/8**
Protocol number:	**1**

Thank you for your letter of 18 February 2010, 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 Vice Chair.

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.

Ethical review of research 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).

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

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

8.2 Appendix 2 Patient information sheet and consent form

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Study Information for patients with Cystic Fibrosis;

Providing sputum for bile and pepsin analysis

You are being asked to provide sputum for a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

It is thought that patients with cystic fibrosis (CF) experience reflux. This is when the stomach contents travel up the gullet and enter the airways causing lung damage. This reflux may or may not cause symptoms; however the long term consequences of stomach contents within the lung is the deterioration of lung function, which can have a considerable effect on quality of life. We would like to use sputum coughed up from the lung to test for stomach chemicals called bile and pepsin. This will provide important evidence in understanding the role of reflux in cystic fibrosis.

Why have I been chosen?

We are requesting all patients with cystic fibrosis attending their routine clinic appointment to provide a sputum sample for the study.

Do I have to provide a sample?

Providing a sample is entirely voluntary. If you do decide to give a sample you will be asked to sign a consent form.

What will happen to me if provide a sample?

Once a sample has been provided at the clinic you will simply continue with your clinic schedule as planned.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. No identifiable data will be used in the study, and all data will be stored on a secure, encrypted database which is password locked. Any samples or information that leaves the hospital will have any information that identifies you removed. All samples will remain under the direct control of Professor Jeffrey Pearson and Dr Chris Ward at the University of Newcastle, in a secure facility.

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Patient Consent Form

STUDY TITLE: The use of sputum samples for the analysis of bile salts and pepsin to determine the effect of gastro-oesophageal reflux in patients with cystic fibrosis

Lead Investigator: Amaran Krishnan
Supervisors: Prof. S.M. Griffin, Prof. J. Pearson, Mr. Jon Shenfine, Dr. Chris Ward, Dr. Alistair Gascoigne, Dr. Stephen Bourke

1. I confirm that I have read and understand the information sheet
   I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I understand that all data will be handled with the strictest of confidentiality. ☐
5. I understand that samples taken from me will be stored and maybe used for future related studies. ☐
6. I agree to for my sputum sample to be used in the above study ☐

Name of Patient	Signature	Date
Name of Person taking consent	Signature	Date

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

8.3 Appendix 3 Questionnaire(s)

1 The DeMeester reflux questionnaire

DeMeester Reflux Questionnaire

1) In the last 2 weeks have you suffered from heartburn (i.e. a burning sensation in the chest)?

grade 0, no symptoms	grade 1, occasional episodes	grade 2, reason for medical visit	grade 3, interference with daily activities

2) In the last 2 weeks have you suffered from regurgitation (acid or stomach contents coming up into your throat, mouth or lungs)?

grade 0, no regurgitation	grade 1, occasional episodes	grade 2, predictable on position of straining	grade 3, episodes of pulmonary aspiration, nocturnal cough or recurrent pneumonia

3) In the last 2 weeks have you suffered from dysphagia (difficulty swallowing or food getting stuck)?

grade 0, no dysphagia	grade 1, occasional episodes	grade 2, require liquid-to-clear diet	grade 3, episodes of esophageal obstruction

2. The Reflux Symptom Index (RSI)

The Reflux Symptom Index (RSI)						
Within the last month, how did the following problems affect you?	0 = No Problem					5 = Severe Problem
Circle the appropriate response.						
1.Hoarseness or a problem with your voice	0	1	2	3	4	5
2. Clearing your throat	0	1	2	3	4	5
3. Excess throat mucus or postnasal drip	0	1	2	3	4	5
4. Difficulty swallowing food, liquids, or pills	0	1	2	3	4	5
5. Coughing after you ate or after lying down	0	1	2	3	4	5
6. Breathing difficulties or choking episodes	0	1	2	3	4	5
7. Troublesome or annoying cough	0	1	2	3	4	5
8. Sensations of something sticking in your throat or a lump in your throat	0	1	2	3	4	5
9. Heartburn, chest pain, indigestion, or stomach acid coming up	0	1	2	3	4	5
	TOTAL RSI SCORE					

3. The Hull Airway Reflux Questionnaire (HARQ)

Within the last month, how did the following problems affect you? 0 = no problem 5= severe/frequent problem						
	0	1	2	3	4	5
Hoarseness or problem with your voice						
Clearing your throat						
The feeling of something dripping down the back of your nose or throat						
Retching or vomiting when you cough						
cough or shortness of breath on first lying down or bending over						
chest tightness or wheeze when coughing						
heartburn, indigestion, stomach acid coming up (or medication =5)						
A tickle in your throat, or a lump in your throat						
cough or shortness of breath with eating (during or soon after)						
cough or shortness of breath when you get out of bed in the morning						
cough or shortness of breath brought on by singing or speaking (telephone)						
coughing more when awake rather than asleep						
a strange taste in your mouth						

8.4 Appendix 4 Letter for Ivacaftor patients

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

DEPARTMENT OF RESPIRATORY MEDICINE

Dr S J Bourke MD FRCPI FRCP DCH
Dr A D Gascoigne MBBS FRCP BSc
Dr Simon Doe MRCP
Karen Heslop: Nurse Consultant(Tel: 0191 2820151)
Alan Anderson: Nurse Specialist (Tel: 0191 2820151)
Marie Caraher: Dietician (Tel: 0191 282 5930)
Sarah Lenaghan: Physiotherapist (Tel 282 1873)

Royal Victoria Infirmary
Queen Victoria Road
Newcastle upon Tyne
NE1 4LP

Tel: 0191 233 6161
www.newcastle-hospitals.nhs.uk

Direct line: 0191 2824776
Fax: 0191 2820112

NEWCASTLE ADULT CYSTIC FIBROSIS CENTRE

Date as postmark

Dear

The Research Team are interested in studying some aspects of Ivacaftor (Kalydeco) treatment, including its effect on acid reflux symptoms.
It is now about 9 months since you started taking Ivacaftor (Kalydeco) and it would be very helpful to the research if you would complete the following questionnaires to help us find out if the medication is affecting your symptoms.
You will find the following questionnaires in this letter:

1. Reflux questionnaire 1
2. Reflux symptom questionnaire 2
3. Reflux and cough questionnaire

The questionnaires repeat the same information but they are completely different and all should be completed. If you have any problems or need any assistance when filling out the questionnaires please contact Gemma by ringing 07801959520 or email g.l.crossfield@ncl.ac.uk.
Please find a paid stamped addressed envelope for return of the completed questionnaires to Dr Bourke at the CF Centre. Please complete these and return them at your earliest convenience.

Thank you
Gemma Crossfield

Dr SJ Bourke
Consultant Physician

8.5 Appendix 5 Abstracts

Gastric aspiration into the CF lung; relationship with reflux symptoms and lung function

G.L. Crossfield[1], A. Krishnan[2], J. Lordan[3], S. Bourke[4], A. Anderson[4], P.W. Dettmar[5], I.A. Brownlee[6], C. Ward[7], J.P. Pearson[1]

[1]Institute of Cell and Molecular Bioscience, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, United Kingdom, [2]Northern Oesophago-Gastric Unit, Royal Victoria Infirmary, Newcastle Upon Tyne, United Kingdom, [3]Institute of Transplantation, The Freeman Hospital, Newcastle Upon Tyne, United Kingdom, [4]Royal Victoria Infirmary, Newcastle Upon Tyne, United Kingdom, [5]Technostics Ltd, Daisy Building, 2nd Floor, Castle Hill Hospital, Cottingham, East Yorkshire, United Kingdom, [6]Food and Human Nutrition Department, Nanyang Polytechnic, Newcastle University, Singapore, [7]Institute of Cellular Medicine, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, United Kingdom

Objectives: Gastro-oesophageal reflux (GOR) is the retrograde movement of gastric contents up the oesophagus. This results in both classical and atypical symptoms. GOR is common in CF affecting 35-81% of patients and it may be associated with deteriorating lung function. We investigated microaspiration into the CF lung, characterised symptoms of reflux and compared these parameters with lung function.

Methods: FEV_1 % predicted was recorded in 37 CF patients (19 male), mean age 29 (range 17-60) years. GOR symptom severity was assessed using the DeMeester Reflux score (0-9; < 1 normal) and extra oesophageal reflux (EOR) using the Reflux Symptom Index (RSI) score (0-45; < 13 normal). Pepsin (ELISA) and total bile acids (TBA) (mass spectrometry) were measured in the sputum of 29 and 24 CF patients respectively.

Results: Pepsin was identified in 17 of 29 (59%) (median 111ng/ml) and TBA in 23 of 24 (96%) (median 0.18µMol/L) patients. RSI scores showed atypical symptoms in 87% of patients, whereas, classical symptoms (DeMeester scores) were identified in 43% of patients. 88% pepsin-positive and 87% TBA- positive patients suffered EOR symptoms, however 53% pepsin-positive and 61% TBA-positive patients experienced GOR symptoms. FEV_1 ranged from 12% to 101% of predicted (median 43%) but was not correlated with biomarkers or symptoms of reflux.

Conclusion: Gastric and biliary reflux into the lungs are very common in CF patients. EOR symptoms may be more closely associated with CF than GOR. Microaspiration of gastric content into the lung was not correlated with FEV_1 and occurred across the spectrum of disease severity.

Keywords: Gastro-oesophageal reflux, cystic fibrosis, FEV_1

Overweight and obesity challenges in the Cystic Fibrosis population – a need for revision in nutritional advice? By G. L. Crossfield [1], A. Krishnan [2], S. Bourke [3], A. Anderson [3], A. Gurney [3], P. W. Dettmar [4], I. Brownlee 5, C. Ward [6] and J. P. Pearson [1], *[1]Institute of Cell and Molecular Bioscience, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, NE2 4HH; [2]Northern Oesophago-Gastric Unit, Royal Victoria Infirmary, Newcastle Upon Tyne, NE1 4LP; [3]Royal Victoria Infirmary, Newcastle Upon Tyne, NE1 4LP; [4]Technostics Ltd, Daisy Building, 2nd Floor, Castle Hill Hospital, Cottingham, East Yorkshire, HU16 5JQ; [5]Food and Human Nutrition Department, Nanyang Polytechnic, Newcastle University, Singapore; [6] Institute of Cellular Medicine, Faculty of Medical Sciences, Newcastle University, Newcastle Upon Tyne, NE2 4HH.*

Cystic fibrosis (CF) is the most common inherited autosomal recessive disease in Caucasian populations leading to pulmonary and digestive complications. CF is often associated with malnutrition due to the increased energy demands of chronic lung infection and reduced nutritional intake associated with anorexia and fat malabsorption. A high fat and high calorie diet is therefore advised. The importance of nutritional care has been evident for >3 decades as an increase in Body Mass Index (BMI) has been associated with an increase in pulmonary function (FEV_1) and improved survival. In recent times reported overweight and obese BMI scores are increasing and the potential health risks are not understood in our ever aging CF population[(1)]. Authors suggested that there were lower numbers of homozygous ΔF508 genotypes with a BMI >25. It has been proposed that dietary advice may need to be modified for CF patients with a BMI >25 or those expressing a heterozygous ΔF508 genotype[(2)]. The aim of the study was to evaluate the relationship between CFTR genotype with BMI and pulmonary function in CF patients.

Between April 2011 and April 2013 sixty four CF patients (37M/27F, median age 29 (17-60) years) were recruited from their routine appointments at the adult CF clinic and were measured for BMI (km/m^2). The patients were grouped according to the following BMI categories: underweight (UW): <18.5 km/m^2, normal weight (NW): 18.5-25.0 km/m^2, overweight (OW): 25.1-30.0 km/m^2 and obese (OB): >30.1 km/m^2. Lung function (FEV_1 % predicted) and genotype were recorded at the time of data collection. To evaluate the relationship between BMI or pulmonary function with genotype the Kruskal-Wallis statistical test was used followed by the Mann Whitney U test.

Of the total 64 patients 9% were UW, 72% achieved a NW, 17% were OW, and 1 patient was OB. Increased BMI scores were associated with improved pulmonary function (FEV_1 % predicted, mean (range): UW:41 (25–53); NW:45 (12–92); OW:75 (54–110); OB:90 ($P < 0.05$). Twenty of the 64 CF patients were homozygous for the ΔF508 mutation; the remaining 44 were heterozygous (ΔF508/other). No association was observed between CF genotype status and BMI or pulmonary function.

The body weight of the CF population is increasing due to improvements in clinical care and today we observe BMI scores reaching OW and OB categories. The potential health risks of OW and OB in the CF population are not completely understood but the benefits of increased pulmonary function scores regardless of genotype are evident. Long term follow up studies are needed before a revision of dietary advice is proposed to CF patients.

1. Kastner-Cole D, Palmer CNA, Ogston SA et al. (2005) J Pediatrics **147,** 402-404.
2. Stephenson AL, Mannik LA, Walsh S et al. (2013) Am J Clin Nutr **97**, 872-7.

Pepsin detection despite the use of acid suppressant medication in patients with airway reflux related chronic cough

Gemma L Crossfield, Warren Jackson, Jennifer Burke, Andrew D Woodcock, Vicki Strugala, Chris Ward, Jeffrey P Pearson, Peter W Dettmar, Alyn H Morice

Background

Chronic cough (CC) is an increasing problem that is not easy to treat with medication. Associated symptoms include hoarse voice, dysphonia, persistent tickling and irritation of the throat or chest. These lead to poor sleeping and eating patterns, loss of vocal independence and social isolation all resulting in an impaired quality of life. Airway reflux is a common cause of unexplained chronic cough and proton pump inhibitor (PPI) medication is commonly prescribed as initial therapy. The following study assessed pepsin identification in CC patients as a marker of airway reflux on PPI.

Methods

Symptomatic expectorated saliva samples were obtained from 16 patients (6 male/10 female, 50 years (37–76), Body Mass Index (BMI) 30 (24-44) median (range)) attending clinical appointment with symptoms of chronic cough. Pepsin was identified using the Peptest™ an in vitro diagnostic medical device specific for human pepsin A (RD Biomed Ltd, UK). All patients completed the Hull Airways Reflux Questionnaire (HARQ) to determine airway reflux related cough (range 0-70; <13 normal). Patient demographics and medication data was provided on sample collection.

Results

Fourteen (88%) of the CC patients were positive for pepsin in saliva samples (median 83ng/ml; range 25–250), providing non-invasive verification of presence of reflux in this CC population. Thirteen pepsin positive patients were symptomatic of airway reflux related cough according to abnormal HARQ score (median 40; range 25–59) and all were taking PPI (20-60mg/d range collected from referral letter and patient questionnaire). The median BMI of the pepsin positive patients was 30 (range 25–44).

Conclusion

Pepsin was present in 88% of suspected airway reflux related chronic cough patients therefore corroborating the diagnosis of reflux. Airway reflux is associated with unexplained chronic cough in patients receiving PPI highlighting that symptoms and reflux are still present despite acid suppression. Overweight and obese BMI status is a common feature of airway reflux related chronic cough patients. A reconsideration of the empiric use of acid suppression use maybe warranted for unexplained chronic cough.

Key words: Chronic cough, pepsin, airway reflux, acid suppressants

8.6 Appendix 6 ECFS travel grant

ECFS 2013 Grant Notification

Dear Miss Gemma Crossfield,

On behalf of the European Cystic Fibrosis Society, we would like to thank you for your application and abstract entitled "***Gastric aspiration into the CF lung: relationship with reflux symptoms and lung function***" submitted to the 2013 Conference.
After careful consideration by the Scientific Committee, your application for a ***Travel Grant*** has been successful and we wish to extend our congratulations.

In order to register for the conference, please complete the free registration form which you will find at the following link and send it per fax to: +49 30 24 603 269.

Free Registration Form

Should you receive funding from another source, we would be grateful if you declined the monetary compensation in order for the funds to be awarded to another applicant.

You will be contacted by the ECFS office in the near future with further information on the free ECFS Membership and practical details related to the receipt of the monetary award.

We look forward to welcoming you in Lisbon.
Once again, our congratulations.

Yours sincerely,

The ECFS 2013 Secretariat
36th European Cystic Fibrosis Conference

Please visit http://www.ecfs.eu/lisbon2013 for further details and updates on the conference.

8.7 Appendix 7 Patient demographics

Patient no	Sex (m/f)	Age	Height (m)	Weight (kg)	BMI
1	F	23	1.73	65.00	21.70
2	M	22	1.85	61.70	18.00
3	F	29	1.60	57.20	22.30
4	M	29	1.63	61.20	23.00
5	M	40	1.77	82.80	26.40
6	M	20	1.77	58.50	18.70
7	F	24	1.52	55.30	23.90
8	M	35	1.83	73.02	21.80
9	F	19	1.47	63.00	29.20
10	F	60	1.67	56.24	20.10
11	M	32	1.70	62.00	21.50
12	F	21	1.50	46.10	20.50
13	F	23	1.73	62.80	21.00
14	F	28	1.60	58.50	22.90
15	M	40	1.77	82.80	26.40
16	F	24	1.52	53.40	23.00
17	M	26	1.75	68.03	22.20
18	M	19	1.67	57.20	20.50
19	F	59	1.67	54.00	19.40
20	M	21	1.85	52.00	15.20
21	M	17	1.72	60.30	20.40
22	F	34	1.67	58.96	21.10
23	F	22	1.50	57.70	25.60
24	F	41	1.65	60.78	22.30
25	F	31	1.52	52.00	22.50
26	F	29	1.60	56.30	22.00
27	M	44	1.67	56.20	20.20
28	F	25	1.57	59.42	23.90
29	M	22	1.85	63.00	18.40
30	M	32	1.70	51.00	17.60
31	M	24	1.75	58.00	18.90
32	M	20	1.72	64.00	21.60
33	F	19	1.48	46.00	21.00
34	M	21	1.78	73.00	23.00
35	M	21	1.73	80.00	26.70
36	M	34	1.78	63.50	20.00
37	F	43	1.60	45.00	17.60
38	F	39	1.65	78.00	28.70
39	F	17	1.55	48.50	20.20
40	M	29	1.73	57.20	19.10
41	M	24	1.73	60.50	20.20
42	M	23	1.70	54.00	18.70

43	M	44	1.83	76.00	22.70
44	F	30	1.70	66.20	22.90
45	F	32	1.56	50.20	20.70
46	M	19	1.70	54.00	18.70
47	M	20	1.69	68.00	23.80
48	F	32	1.53	47.00	20.10
49	M	20	1.15	48.40	21.20
50	M	22	1.72	79.40	26.80
51	F	21	1.54	41.40	17.50
52	F	21	1.66	61.50	22.40
53	F	17	1.50	47.00	18.80
54	F	38	1.59	54.00	21.40
55	F	20	1.46	65.80	30.90
56	M	23	1.71	57.80	19.80
57	M	25	1.72	77.00	26.10
58	M	28	1.70	73.80	25.30
59	M	24	1.73	67.40	22.50
60	M	28	1.75	82.80	26.90
61	M	36	1.81	70.40	21.80
62	F	25	1.54	55.00	20.30
63	M	31	1.80	87.00	27.90
64	M	24	1.80	55.8	17.70
65	M	43	1.77	69.30	22.10
66	M	20	1.77	64.40	23.20
67	F	25	1.54	55.00	23.10
68	F	42	1.77	63.20	20.10
69	M	16	1.65	47.50	17.40
70	F	28	1.55	34.10	14.10
71	F	24	1.55	43.10	17.90
72	M	26	1.92	56.40	15.20
NA	F	38	1.52	47.62	20.60
NA	F	39	1.53	36.74	15.70

8.8 Appendix 8 Questionnaire scores

Patient no	DeMeester score	Reflux Symptom Index (RSI)	Hull Airway Reflux Cough Questionnaire (HARQ)
1	6	31	
2	2	25	
3	4	22	
4	2	11	
5	1	15	
6	2	19	
7	2	14	
8	7	36	
9	5	17	
10	4	21	
11	1	8	
12	1	20	
13	2	29	
14	2	26	
15	1	17	
16	2	14	
17	1	13	
18	0	6	
19	2	17	
20	2	25	
21	0	3	
22	2	20	
23	1	12	
24	5	10	
25	0	19	
26	1	30	
27	1	17	
28	0	23	
29	3	10	
30	3	12	
31	1	24	
32	0	6	
33	0	10	
34	2	11	
35	1	8	
36	2	19	
37	4	43	
38	1	18	

39	2	25	
40	0	16	
41	1	11	
42	0	8	
43	0	16	
44	0	19	
45	0	20	
46	2	5	
47	0	8	
48	3	20	
49	1	14	12
50	1	2	3
51	1	23	31
52	0	11	7
53	1	17	19
54	2	29	33
55	5	12	12
56	2	15	15
57	0	3	3
58	1	4	4
59	0	15	27
60	1	8	8
61	1	20	
62	1	0	0
63	2	14	25
64	2	9	9
65	0	16	36
66	1	9	
67	0	15	26
68	0	2	1
69	0	9	15
70	1	25	44
71	4	26	42
72	1	17	32

8.10 Appendix 10 Medication

Patient no	Medication
1	40mg BD
2	30mg BD
3	40mg BD
4	30mg BD
5	30mg BD
6	20mg BD
7	Ranitidine 300mg OD
8	20mg BD
9	20mg BD
10	30mg OD
11	15mg OD
12	omeprazole 20mg od
13	40mg BD
14	omeprazole 40mg bd
15	lansoprazole 30mg bd
16	omeprazole 20mg bd
17	lansoprazole 30mg od
18	omeprazole 20mg bd
19	lansoprazole 30mg od
20	
21	omeprazole 10mg bd
22	no
23	no
24	omeprazole 20mg bd
25	omeprazole 20mg bd
26	no
27	Lansoprazole 30mg od
28	no
29	no
30	no
31	
32	omeprazole 20mg bd
33	lansoprazole 30mg od
34	lansoprazole 30mg prn
35	omeprazole 20mg bd
36	omeprazole 20mg
37	no
38	omeprazole X2 daily
39	ranitidine 150mg OD
40	lansoprazole 30mg od

41	no
42	omeprazole 20mg bd
43	omeprazole 20mg bd
44	omeprazole 20mg BD
45	lansoprazole 30mg od
46	lansoprazole 30mg bd
47	no
48	no
49	omeprazole 20mg
50	ranitidine 150mg
51	lansoprazole 30mg bd
52	omeprazole 20mg
53	gaviscon prn/omeprazole 20mg
54	lansoprazole 30mg bd
55	omeprazole 20mg
56	no
57	gaviscon prn/omeprazole 20mg
58	no
59	lansoprazole 30mg
60	no
61	lansoprazole 30mg
62	no
63	no
64	no
65	no
66	lansoprazole
67	lansoprazole 15mg
68	no
69	lansoprazole 20mg
70	no
71	omeprazole 20mg/2
72	lansoprazole 30mg/2

8.11 Appendix 11 Pepsin identification and quantification

Patient no	Pepsin ng/ml
1	0
2	152
3	88
4	0
5	0
6	324
7	196
8	112
9	0
10	80
11	111
12	296
13	320
14	0
15	187
16	0
17	0
18	305
19	0
20	0
21	0
22	0
23	0
24	275
25	0
26	0
27	189
28	147
29	364
30	611
31	
32	831
33	178
34	0
35	
36	800
37	382
38	0
39	0
40	570
41	633
42	489

43	920
44	1025
45	274
46	0
47	231
48	294
49	261
50	0
51	870
52	1151
53	283
54	178
55	128
56	710
57	116
58	418
59	481
60	611
61	387
62	
63	597
64	515
65	0
66	0
67	246
68	464
69	782
70	382
71	270
72	350

8.12 Appendix 12 Bile acid identification and quantification

Patient no	TBA µMol/L	G-DHC	G-THC	T-DHC	T-THC	Total	Free LITHOCHOLATE
1	3.168	0.64	1.6	0.08	0.88	3.2	0.32
2	0.2348	0	0.08	0	0.08	0.16	0.16
3	0.11	0	0	0	0.08	0.08	0
4	0.144	0	0.08	0	0.08	0.16	0.16
5	0.0428	0	0	0	0	0	0.08
6	0.1288	0.08	0	0	0.08	0.16	0.48
7	0.374	0.24	0.08	0	0	0.32	0.24
8	0.3216	0.16	0.16	0	0.08	0.4	0.24
9	0.1268	0	0	0.08	0	0.08	0.24
10	0.2744	0.16	0	0.08	0.08	0.32	0.48
11	0.192	0	0	0	0	0	0.4
12	0.2336	0.08	0	0.08	0.08	0.24	0.3824
13	0	0	0	0	0	0	0.94
14	2.42	1.44	0.48	0.32	0.24	2.48	0.9424
15	0.0896	0	0	0	0	0	0.2176
16	0.1208	0	0.08	0	0	0.08	0.228
17	0.0856	0	0	0	0.08	0.08	2.0984
18	0.2216	0.08	0.08	0	0	0.16	0.7184
19	0.3056	0.16	0	0.16	0	0.32	0.532
20	0.792	0.16	0.08	0.56	0.08	0.88	0.448
21	0.2216	0.08	0.08	0	0	0.16	0.2064
22	0.4216	0.24	0.08	0.08	0	0.4	0.4128
23	0.1832	0.08	0.08	0	0.08	0.24	0.7704
24	1.0488	0.8	0.16	0.08	0	1.04	0.584

8.13 Appendix 13 Pulmonary function

Patient no	FEV1	FEV1 pred %	FVC	FVC pred %	ratio L
1	1.05	30	1.4	34	0.75
2	2.55	51	4.95	84	0.52
3	1.35	42	2.35	63	0.57
4	1.6	43	3.1	72	0.52
5	2.25	54	4.65	93	0.48
6	2.1	48	4.56	88	0.46
7	0.35	12	0.35	11	1.00
8	1.55	36	2.05	40	0.76
9	2.2	87	2.65	90	0.83
10	0.91	36	1.52	50	0.60
11	1.2	30	2.4	52	0.50
12	0.95	36	1.5	47	0.63
13	2.2	47	3.3	59	0.67
14	1.35	41	2.35	62	0.57
15	2.3	55	4.7	94	0.49
16	0.7	25	1.2	36	0.58
17	3.4	80	4.7	93	0.72
18	1.05	25	2	41	0.53
19	0.75	29	1.5	49	0.50
20	2.2	48	3.65	71	0.60
21	2.1	47	4	72	0.53
22	0.9	26	1.3	34	0.69
23	1.8	67	3.3	105	0.55
24	1.75	57	2.5	66	0.70
25	1.45	52	1.8	54	0.81
26	2.5	83	3.15	91	0.79
27	0.8	27	1.9	55	0.42
28	1.25	43	1.7	53	0.74
29	2.2	47	3.3	59	0.67
30	1.35	32	1.95	40	0.69
31	2.55	59	3.05	59	0.84
32					
33					
34	1.93	42	3.36	60	0.57
35	4.15	101	5.24	109	0.79
36	1.4	35	3.6	71	0.39
37					
38	1.9	60	2.4	68	0.79
39	1	38	1.25	41	0.80
40	1.45	33	2.59	50	0.56
41	0.74	19	2.1	49	0.35
42	3	69	4.65	91	0.65

43	1.7	43	2.45	48	0.69
44					
45	0.95	34	1.5	46	0.63
46	2.06	54	2.95	65	0.70
47	3.35	82	3.97	83	0.84
48	1.15	41	1.3	40	0.88
49	2.05	92	2.4	92	0.85
50	4.51	110	5.73	119	0.79
51	0.85	28	1.6	47	0.53
52	3	90	3.95	100	0.76
53	1.94	87	2.2	89	0.88
54	0.95	35	2.38	78	0.40
55	2.3	90	2.6	89	0.88
56	0.8	19	1.75	35	0.46
57	3.7	88	4.9	99	0.76
58	2.85	70	4.7	99	0.61
59	2.75	64	3.8	74	0.72
60	2.75	64	4	78	0.69
61	1.4	32	2.1	40	0.67
62	1.75	54	2.9	78	0.60
63	3.05	69	4.3	83	0.71
64	3.25	53	3.75	71	0.87
65					
66	2.1	58	3.96	93	0.53
67					
68	1.4	39	3.2	100	0.44
69					
70					
71	0.9	30	1.3	38	0.69
72	2.15	56	2.7	59	0.80

8.14 Appendix 14 Genetics

Patient no	genetic mutation
1	(?508/-)
2	(?508/?508)
3	(?508/N1303K)
4	(?508/?508)
5	(?508/?508)
6	(?508/NMD)
7	(?508/2184delA)
8	(?508)
9	(?508/9551D)
10	(?508/D1152H)
11	(?508)
12	?F508/ ?F508
13	
14	?F508/ N1303K
15	?F508/ ?F508
16	?F508/ 2184DELA A
17	1145INSTC/ 3659DEL C
18	?F508/ ?F508
19	?F508/ 01152H
20	
21	?F508/ ?F508
22	?F508/ ?F508
23	?F508/ ?F508
24	?F508/ P67l
25	?F508/ E60X
26	?F508/ 1154
27	?F508/ ?
28	?F508/ ?F508
29	?F508/ ?F508
30	?F508/ ?F508
31	
32	
33	
34	?F508/ ?F508
35	?F508/G551D
36	?F508/ ?1507
37	
38	?F508/c.3140-26A.G
39	?F508/ ?F508
40	?F508/ ?F508
41	?F508/ R560T
42	?F508/A1507

43	?F508/ ?F508
44	
45	?F508/ ?F508
46	?F508/ ?F508
47	?F508/R56OT
48	?F508/E6OX
49	?F508/G551D
50	?F508/G551D
51	?F508/G551D
52	?F508/G551D
53	?F508/G551D
54	?F508/G551D
55	?F508/G551D
56	?F508/G551D
57	?F508/G551D
58	?F508/G551D
59	?F508/G551D
60	?F508/G551D
61	?F508/?
62	?F508/?F508
63	?F508/?F508
64	?F508/621+1G>T
65	
66	?F508/R117H-5T
67	
68	^ F508/P67L
69	
70	
71	^ F508/^ F508
72	^ F508/^ F508

8.15 Appendix 15 Inflammatory analysis

patient no	IL8 ng/ml	IL6 pg/ml
1	12	0
2	4.5	0
3	9	0
4	14	0
5	4.5	78
6	17	0
7	7.5	0
8	8	0
9	8	0
10	6	266
11	9	70
12	3.1	
13	10	32
14	1	0
15		
16	27	105
17		
18		
19		
20	24	0
21	2	0
22	5	0
23		
24	6	0
25	14	0
26	19	105
27	8	0
28	14	166
29	6	0
30	10	0
31		
32	17	0
33	16	0
34	14	0
35		
36	4	0
37	12	0
38	2	0

39	19	0
40	3	0
41	5	244
42	19	58
43	23	0
44	8	0
45	5	0
46	9	0
47	2	0
48	11	0
49	7	290
50	10	249
51	4	
52	9	0
53	15	94
54	2	0
55	16	0
56	11	87
57	3	0
58	4	0
59	11	0
60	9	107
61	35	0
62		
63	12	41
64	30	0
65	8	0
66	17	704
67	8	0
68	14	0
69	5	0
70	7	0
71	3	0
72	4	0

Patient no	Neutrophils %	Eosinophils %	Macrophages %	Lymphocytes %
1	100	0	0	0
2	100	0	0	0
3	99	0	0	1
4	100	0	0	0
5	100	0	0	0
6	99.4	0	0	0.6
7	100	0	0	0
8	99.4	0	0	0.6
9	99.2	0.8	0	0
10	100	0	0	0
11	100	0	0	0
12	99.6	0.4	0	0
12	100	0	0	0
14	97.2	0	1.6	1.2
16	100	0	0	0
17	100	0	0	0
18	100	0	0	0
19	99.4	0	0	0.6
20	99.8	0	0	0.2
22	100	0	0	0
23	100	0	0	0
24	100	0	0	0
25	100	0	0	0
26	99.4	0.6	0	0
27	100	0	0	0
28	99.8	0	0	0.2
29	100	0	0	0
34	100	0	0	0
35	100	0	0	0
36	100	0	0	0
37	100	0	0	0
49	100	0	0	0
51	100	0	0	0
53	99	0	1	0
56	100	0	0	0
60	100	0	0	0