Lung Biology Group

Institute for Cell and Molecular Biosciences

Role of Gastro-Oesophageal Reflux in the Development and Severity of Bronchiectasis

A thesis submitted to the School of Medicine, National University of Ireland, Galway, and Newcastle University, in fulfilment of the requirements for a Joint Degree of Doctor of Philosophy

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Summary

Background

Bronchiectasis is a chronic lung disease associated with significant morbidity and mortality, escalating public health costs and profound reductions in health-related quality of life. Comorbidity is a frequent finding in these patients, often with synergistic effects on disease severity and resultant poorer clinical outcomes. The relationship between gastro-oesophageal reflux and bronchiectasis is difficult to elucidate. This thesis aims to explore the contribution and mechanism of gastro-oesophageal reflux disease (GORD), airway reflux and duodeno-gastro-oesophageal microaspiration to the development and severity of lung injury in patients with bronchiectasis using multiple methodologies.

Methods

A qualitative systematic review exploring the association between GORD, airway reflux and pulmonary microaspiration with bronchiectasis in terms of disease prevalence, disease outcomes and potential available treatment strategies was performed. Several large prospective multicentre cohort analyses to derive, validate and compare bronchiectasis-specific disease severity and comorbidity indices were conducted to better define the effect of GORD on bronchiectasis outcomes. A single centre cross-sectional analysis of the associations of the prevalence of hiatal hernias and bronchiectasis severity was also performed. Exploration of the relationship between GORD, proton pump inhibitor use and bronchiectasis outcomes of disease severity, mortality, chronic infection and exacerbations was performed using pan-European multicentre data from the FRIENDS and EMBARC bronchiectasis patient registries. Subsequently, a bicentric parallel prospective observational case-control study assessing the prevalence, mechanism and functional impact of GORD on bronchiectasis patients, utilising a multi-modal diagnostic approach incorporating questionnaires, pH-impedance and biomarkers of duodeno-gastro-oesophageal reflux, was also performed,

comparing findings to age, sex, ethnicity and BMI-matched chronic bronchitis patients and healthy volunteer controls. Finally *in vitro* and *ex-vivo* primary bronchial epithelial cell studies were conducted to investigate the cytotoxic, inflammatory and remodelling effects of physiologically achievable individual and combined bile acids and to determine a potential role for azithromycin in attenuating bile acid-mediated neutrophilic inflammation and remodelling in bronchiectasis with validation of the role of azithromycin in GORD in the EMBARC bronchiectasis patient registry.

Results:

The systematic review highlighted GORD prevalence rates of 11-75% in bronchiectasis depending on methodology used. Derivation, validation and comparison of bronchiectasis-specific disease severity and comorbidity indices showed the BSI and BACI to be superior to other scores with both scores combined having the highest prognostic potential in terms of predicting mortality, exacerbations, hospitalisations and health-related quality of life (QoL). Hiatal hernia presence was associated with worse bronchiectasis disease severity. GORD was associated with a 2.5 fold increase in mortality in the FRIENDS cohort and a 20-30% increased risk of moderate and severe exacerbations requiring hospitalisation but no observed increased mortality risk in the EMBARC cohort; proton pump inhibitor (PPI) use was not associated with an increased hospitalisation rate. In the case control study, bronchiectasis patients were clearly shown to have a dysregulated immune response at baseline compared with chronic bronchitis patients and healthy volunteers, largely driven by neutrophil extracellular trap (NET)-related proteins, immunoglobulins and anti-oxidative stress proteins on proteomic analysis. GORD, airway reflux and pulmonary microaspiration were highly prevalent among bronchiectasis patients (22-91%) and associated with increased bronchiectasis severity manifest by increased exacerbations, reduced functional status, increased chronic infection, increased airways inflammation, and worse quality of life. Bile acids caused direct inflammation and injury in both *in vitro* and *ex-vivo* primary cell culture models with neutrophilic inflammation, epithelial to mesenchymal transition and airways

remodelling. Azithromycin attenuated bile acid-mediated injury in cellular studies with macrolides demonstrating a significant effect in reducing GORD-associated exacerbation frequency and hospitalisations in the EMBARC international database.

Interpretation:

These studies provide novel observational clinical and translational evidence of bronchiectasis disease severity and the associations of GORD, airway reflux and pulmonary microaspiration with increased airways inflammation, epithelial injury, increased disease severity and reduced QoL. We report a novel link between macrolides and the attenuation of GORD-mediated inflammation and exacerbations which may have relevance to other chronic neutrophilic airway conditions. These findings have contributed to recent British and European Clinical Guidelines on Bronchiectasis and have further highlighted future research priorities towards improving our understanding of the disease and quality of care for patients with bronchiectasis.

Declaration

This thesis is submitted jointly to the National University of Ireland, Galway and Newcastle University, UK, in accordance with the requirements for the degree of Doctor of Philosophy (PhD) in the School of Medicine.

I declare that this thesis is a record of my own work and has not been submitted for any other academic award or qualification.

All information sources have been fully acknowledged and referenced appropriately.

Numerous aspects of this work have been presented at national and international respiratory conferences and published in peer-reviewed journals (Appendix).

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Dedication

To my beautiful daughters, Erin Rose and Larissa Grace…

"Nothing is impossible…

the word itself says 'I'm possible!'"

(Audrey Hepburn)

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List of Abbreviations

A1AT: Alpha-1-antitripsin

ABPA: Allergic broncho-pulmonary aspergillosis

ACT: Airway clearance technique

ACG: American College of Gastroenterology

ALI: air-liquid interface

BACI: Bronchiectasis Aetiology and Comorbidity Index

BCI: Bronchiectasis Comorbidity Index

BAL: Bronchoalveolar lavage

BEST: Bronchiectasis Exacerbation and Symptom Tool

BMI: Body Mass Index

BRICS: Bronchiectasis Radiologically Indexed CT Score

BSI: Bronchiectasis Severity Index

BTS: British Thoracic Society

BHQ: Bronchiectasis Health Questionnaire

CAT: COPD Assessment Test

CCI: Charlson Comorbidity Index

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis transmembrane conductance regulator

COPD: Chronic obstructive pulmonary disease

CPA: Clinical Pathology Accreditation

CReSS: Comprehensive Reflux Symptom Score

CTD: Connective tissue diseases

ELISA: enzyme linked immunosorbent assay

EMBARC: European Multicentre Bronchiectasis Audit and Research Collaboration

EMT: epithelial to mesenchymal transition

ENT: Ears, nose and throat

EOR: extra-oesophageal reflux

ERS: European Respiratory Society

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FEV1: Forced expiratory volume in 1 second

FVC: Forced vital capacity

FRIENDS: Facilitating Research Into Existing National DataSets

GERD-Q: Gastro-Esophageal Reflux Disease-Questionnaire

GINA: Global Initiative for Asthma

GOLD: Global initiative for chronic Obstructive Lung Disease

GORD: Gastro-oesophageal reflux disease

GUH: Galway University Hospitals

GSAS: GORD Symptom Assessment Scale

HARQ: Hull Airway Reflux Questionnaire

HRCT: High resolution computed tomography

HRM: High resolution manometry

IB: Idiopathic bronchiectasis

IBD: Inflammatory bowel disease

ICD: International Classification of Diseases

IL: interleukin

LOS: Lower oesophageal sphincter

LPR: Laryngo-pharyngeal reflux

MCID: Minimum clinically important difference

MMP: Matrix metalloproteinase

MRC: Medical Research Council

MWS: Multiple water swallows

NET: neutrophil extracellular trap

NTM: Non-tuberculous mycobacteria

PBEC: Primary bronchial epithelial cell

PCD: Primary Ciliary Dyskinesia

PFTs: Pulmonary function tests

PPI: Proton pump inhibitor

QOL: Quality of life

QOL-B: Quality of life-Bronchiectasis

RA: Rheumatoid arthritis

RDQ: Reflux Disease Questionnaire

RFS: Reflux Finding Score

RSI: Reflux Symptom Index

SERQ: Supra-Esophageal Reflux Questionnaire

SGRQ: St George's Respiratory Questionnaire

SF-36: Short-Form 36-item questionnaire

TB: Tuberculosis

TGF: Transforming growth factor

TEER: trans-epithelial electrical resistance

Chapter 1 - Introduction

Publications relevant to this chapter:

McDonnell MJ, Mokoka MC, O'Toole D, Ward C, De Soyza A, Laffey JG, Rutherford RM. The New Age of Bronchiectasis – state of the art review. In submission 2020.

McDonnell MJ, Ward C, Rutherford RM. Comorbidities and their impact on bronchiectasis. European Respiratory Monograph. Editors: Chalmers JD, Polverino E, Aliberti S. European Respiratory Society 2018; pp 45-61. ISBN 978-1-904097-92-1.

McDonnell MJ, Rutherford RM. Predisposing factors for the development of bronchiectasis. Bronchiectasis: The EMBARC Manual. Editors: Chalmers JD, Polverino E, Aliberti S. Springer link 2016; pp 129-145. ISBN 978-3-319-61452-6.

Hester KLM, McDonnell MJ, De Soyza A. Bronchiectasis: What we don't know, but should. BRN reviews 2016; 2:14-26.

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1.1 Bronchiectasis

1.1.1 The 'New Age' of Bronchiectasis

Bronchiectasis is a chronic debilitating disease characterised by dilated and often chronically infected and inflamed airways leading to a clinical syndrome of persistent cough, purulent sputum production, recurrent chest infections and general malaise.[1, 2] It is increasingly recognised as both a primary disease and as the end result of a number of other pathological processes affecting the lungs, with several common pathophysiological features and "treatable traits" in keeping with other airway diseases.[2, 3] Marked heterogeneity exists in terms of demographic, clinical, radiological, functional, aetiological, comorbid and microbiological features, the combination of which often contributes to a significant disease burden reflected by an increased morbidity and mortality, reduced quality of life (QoL), high utilisation of healthcare resources and resultant socioeconomic costs.[4-8]

Bronchiectasis has historically been a poorly studied disease and, essentially neglected from a therapeutic standpoint with the evidence base for treatment having largely been extrapolated from studies in cystic fibrosis (CF) or based on consensus expert opinion. Guidelines published by the British Thoracic Society (BTS) in 2010 contained primarily Level D evidence provided by anecdotal reports and small case-series.[9] Updated British, Australian, Spanish and European guidelines have since been published but significant knowledge gaps remain with research in this area still lagging behind that of other chronic respiratory diseases.[10-13] There is a continued need to systematically coordinate our research endeavours to enable us to deliver a step wise change in bronchiectasis management from empirical to evidence-based therapies. In recent years, significant progress has been made in developing collaborative networks to capture large patient cohorts including European, American, Asian and Australian patient registries, which are contributing greatly to our understanding of the aetio-pathogenesis of this disorder, helping to define phenotypes of this disease and providing a platform for answering research questions.[14-18] This has already resulted

in a number of advances in bronchiectasis such as the development and validation of tools to characterise disease severity, mortality, exacerbations and symptoms, radiology scores, the impact of aetiologies and comorbidities, and characterisation of the "frequent exacerbator" phenotype.[19-24] Working groups have also been formed to establish unified definitions of bronchiectasis exacerbations and chronic infection to enhance inclusion criteria, interpretation and comparison of observational studies and clinical trials in bronchiectasis.[25] Multidisciplinary and cross-national efforts to characterise epidemiology, microbiology, genetics, immunology, basic science, epithelial biology and new ways to combat inflammation in bronchiectasis are ongoing. In light of the recent Covid-19 pandemic, in which an increase in chronic lung disease and bronchiectasis in particular is expected globally, tackling existing knowledge gaps will hopefully, in years to come, deliver effective, tailored new therapies for this multifactorial disease. This chapter capsulises our current knowledge of bronchiectasis and highlights recent advances in the field.[1]

1.1.2 Epidemiology

There is a common misconception that bronchiectasis is a rare disease of yesteryear, successfully eradicated from our clinical repertoire with the introduction of antibiotics and regular vaccination programmes. International data, however, suggests that prevalence is increasing with estimates of up to 566 per 100,000 population and an incidence that has increased by 40% in the last 10 years.[26] Whether this represents a real increase in disease burden, perhaps linked to an ageing population with increased comorbidities, or is an ascertainment bias due to increased detection in an era when improved sensitivity high resolution computed tomography (HRCT) scans are routinely being performed, is difficult to determine.

Undoubtedly, reported rates of bronchiectasis are likely to be underestimated due to both misdiagnosis (as other common respiratory disorders, e.g. asthma or COPD) and missed diagnosis, with failure to recognise that patients may

[1] This literature review has been updated during the course of thesis preparation and therefore includes work that is discussed in more detail in subsequent chapters.

have dual airway disease in overlap syndromes. Unlike asthma and COPD, bronchiectasis requires an expensive sophisticated imaging test in order to facilitate diagnosis, access to which may be limited in certain healthcare settings and which may result in considerable under-reporting of the disease.

Risk factors for bronchiectasis are intimately associated with the ageing process and these risk factors can have a multiplicative effect. Firstly, the natural history of bronchiectasis is to worsen with time and, the older you live, the more the disease tends to progress and present clinically. Sarcopenia of the swallowing muscles also occurs and the risk of dysphagia and aspiration increases dramatically.[27] Gastro-oesophageal reflux disease (GORD) also increases with age due to a combination of an increasing prevalence of hiatal hernias and oesophageal dysfunction which may contribute to the development of bronchiectasis as a result of airway reflux and subclinical microaspiration over time.[28, 29] When this is combined with immunosenescence, the immune system and potentially the microbiome undergoing its own change with age, the risk of developing lower respiratory tract infections including pneumonia becomes much more prevalent and the development of bronchiectasis much more likely.[30] There is also an ever increasing array of immunosuppressive therapies for treating inflammatory and malignant conditions and these too can predispose to recurrent chest infections and bronchiectasis. Elderly people have more severe disease and atypical presentation with poorer outcomes compared to younger cohorts.[31] Studies of bronchiectasis in the elderly suggest that these patients have more comorbidities, poorer quality of life and a higher mortality, related to overall frailty rather than differences in bronchiectasis aetiology or severity.[32]

1.1.3 Pathophysiology

Our understanding of the pathophysiology of bronchiectasis is limited, in part because of a lack of representative experimental or animal models. The most widely known pathophysiological model of bronchiectasis is Cole's "vicious cycle hypothesis".[33] This proposes that an inflammatory or infective insult, on a background of a possible genetic or acquired susceptibility or defect in host defence, leads to initial damage of the mucociliary apparatus which

doesn’t fully heal. This area is therefore more vulnerable to recurrent infections which damages the airway further and a vicious cycle is thus set in motion. Each step begets the next, resulting in a persistent and progressive process over time. Support for this model is the long prodromal phase in bronchiectasis prior to diagnosis which has been found to be as long as 17 years suggesting that infections get more frequent and severe over time.[34]

Interactions, however, are far more complex than this model allows with the recently coined “vicious vortex” better depicting the underlying pathophysiological interplay driving airway dysfunction, inflammation, infection, and remodelling in bronchiectasis, each pathophysiological step contributing to all others (Figure 1-1).[35] The vortex concept might more appropriately explain why individual treatments in isolation result in only modest benefits on clinical outcomes in bronchiectasis. Breaking a cycle anticipates complete cessation of disease progression, whereas a treatment targeting only one component of a vortex will inevitably have some positive effects but will not necessarily halt progression as inflammation and lung damage may still be sustained by other stimuli, advocating a multimodal therapeutic approach directed at disrupting these interconnecting processes.[35]

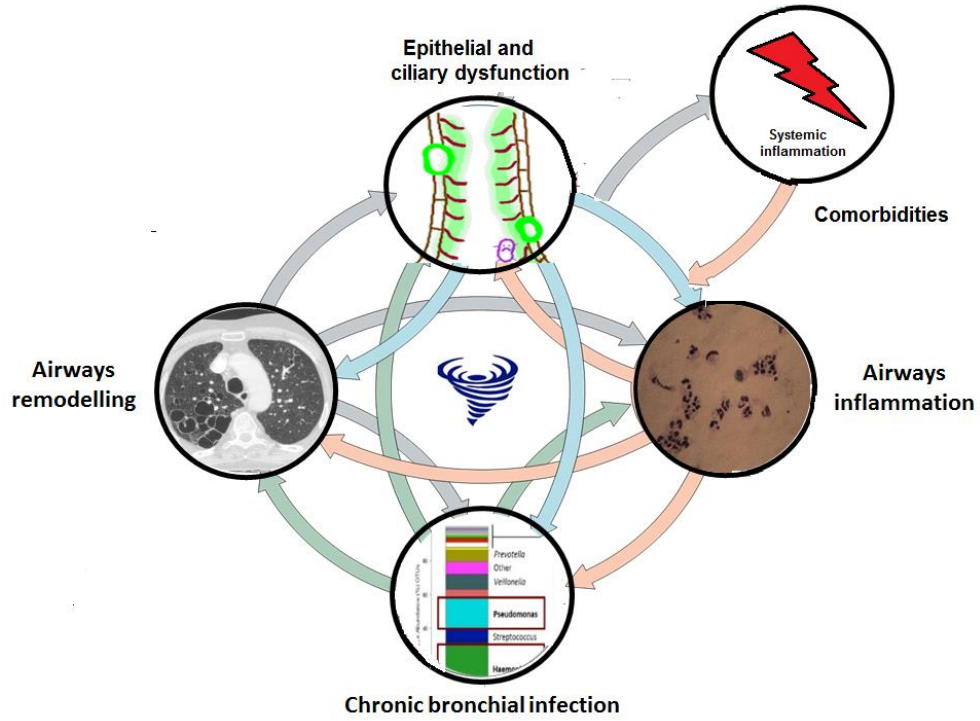

Figure 1-1 Model describing the pathophysiology of bronchiectasis

In the pathophysiology of bronchiectasis, a tetrad of events are considered to occur: (i) airway epithelial and ciliary dysfunction, and mucus hypersecretion, (ii) chronic infections that incite further mucus hypersecretion, (iii) inflammation, resulting in permanent airway injury and dilatation, and (iv) resultant bronchiectatic airways that are poor in airway clearance, perpetuating the continuum of chronic infection, inflammation, and airway remodelling. While this paradigm of chronic airway infection and inflammation is called the Vicious Cycle by Cole, the direction in which these factors occur may be bi- or cross-directional as shown by the double arrows, resulting in a Vicious Vortex.

A dysregulated immune response is thought to drive the pathological process in bronchiectasis. Pro-inflammatory cytokines (e.g. CXCL-8/IL-8), key mediators of neutrophil recruitment and migration and defects of innate and adaptive immunity, have repeatedly been demonstrated in sputum, bronchoalveolar lavage (BAL) and airway biopsies of bronchiectasis patients.[36-38] Higher airway bacterial loads are associated with airway and systemic inflammation and greater risk of exacerbation.[39] Increased plasma fibrinogen has been associated with disease severity, with reference to *P. aeruginosa* chronic infection and health status.[40] Lungs of bronchiectasis patients show active proteolytic damage similar to that observed in COPD with high levels of neutrophil elastase, MMPs and other inflammatory markers.[39] Excessive neutrophilic inflammation is linked to an increased frequency of exacerbations and rapid lung function decline through degradation of airway elastin among other mechanisms.[41-44] Neutrophilic inflammation is thus a key driver of disease progression in bronchiectasis.

Chronic airways infection, most frequently with *Haemophilus influenzae* (14-47%) and *P. aeruginosa* (5-31%) and less frequently with *Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus aureus* and *Enterobacteriaceae*, stimulate and sustain lung inflammation in bronchiectasis.[10, 34, 45-47] Persistent isolation of these organisms in sputum or BAL is associated with an increased frequency of exacerbations, worse quality of life and increased mortality.[48, 49] This is particularly the case with *P. aeruginosa* infection. A systematic review of observational studies identified that *P. aeruginosa* infection is associated with a 3-fold increase in mortality risk, an almost 7-fold increase in risk of hospital admission and an average of 1 additional exacerbation per patient per year.[50] Whilst *P. aeruginosa* colonisation is associated with reduced lung function, a longitudinal study demonstrated *P. aeruginosa* infection across all stages of airflow limitation, highlighting the importance of rigorous sputum surveillance protocols in all bronchiectasis patients even with “mild” airflow limitation.[51]

Mucociliary clearance is impaired by the impact of structural bronchiectasis, airway dehydration, excess mucus volume and viscosity. More than 70% of bronchiectasis patients expectorate sputum daily with highly variable sputum volumes. Treatment aims to prevent mucus stasis and the associated mucus plugging, airflow obstruction and progressive lung damage.[10, 52]

Structural lung disease includes bronchial dilatation, bronchial wall thickening, bronchiolitis and mucus plugging as well as small airways disease and emphysema. More than 50% of patients have airflow obstruction, but restrictive, mixed spirometry and preserved lung function are also frequently observed. Breathlessness is due to the impact of airflow obstruction, impaired gas transfer, exercise deconditioning and the impact of comorbidities.[23, 53, 54] Breathlessness is one of the strongest predictors of mortality.[19, 48] Therapies may aim to treat airflow obstruction (e.g. bronchodilators), to improve exercise capacity (pulmonary rehabilitation), or to remove poorly functioning or diseased lung (e.g. surgery).[10]

Comorbidities are common and significant in bronchiectasis patients, can occur at any stage of the disease process and are often important determinants of outcome, contributing to increased healthcare utilisation, socioeconomic costs and mortality.[23, 32, 55] In a large multicentre observational study of comorbidities in bronchiectasis, a median of four comorbidities per patient were identified with a significantly higher number in males, patients with severe bronchiectasis and non-survivors.[23] 26 of the 81 comorbidities identified were associated with an increased mortality in bronchiectasis, compared to 15 identified in the derivation of the COPD comorbidity test.[23, 56] Systemic inflammation, partly caused by the ageing process, is closely linked to an increased likelihood of developing chronic multiple conditions with lower survival rates associated with the “inflamed comorbids” in COPD.[40, 57, 58] Comorbidities have been shown to predict mortality risk with a higher accuracy than markers of bronchiectasis severity, emphasising the importance of incorporating comorbidities into multidimensional phenotyping of patients with bronchiectasis.[59]

1.1.4 Aetiology

Determining the aetiology of bronchiectasis can be highly challenging. Numerous diseases can lead to bronchiectasis and the specific aetiology may influence clinical manifestations and outcomes. A useful framework in which to characterise aetiology is depicted in Figure 1-2. According to a recent systematic review on the aetiology of bronchiectasis in adults, in approximately 45% of patients, it is seemingly a primary airway disease and in the remainder, a complication of a number of other highly heterogeneous disorders (Table 1-1).[60] History taking is extremely important in determining the most likely aetiology. Finding conditions that have a known association with bronchiectasis does not necessarily mean that they are causal. In a patient with an existing airway disease, it can be difficult to determine if the diagnosis is primary bronchiectasis, or asthma/COPD complicated by bronchiectasis? Similarly, in systemic disorders such as rheumatoid arthritis (RA), is the bronchiectasis part of the disease process that may even precede the joint disease or is it due to pulmonary infection in an immunosuppressed host? Collaborative research efforts utilising data from international datasets have demonstrated wide heterogeneity in the proportion of different aetiologies identified between centres, in part, reflecting possible variations in testing practice or in the definitions of aetiology used.[61, 62] An aetiological algorithm has subsequently been developed to standardise aetiological assessment across centres and enhance our ability to compare results of different studies in different healthcare settings.[34] There is growing awareness of the need to identify modifiable risk factors and to engage these patients in holistic disease management programs. Recent ERS guidelines provide a minimum bundle of aetiological investigations to standardise aetiological assessment across Europe.[10] Tailoring treatment is particularly likely to benefit patients with immunodeficiency states, allergic bronchopulmonary aspergillosis (ABPA), non-tuberculous mycobacterial infection (NTM), recurrent aspiration and patients with very focal bronchiectasis who may benefit from lung resection.[63]

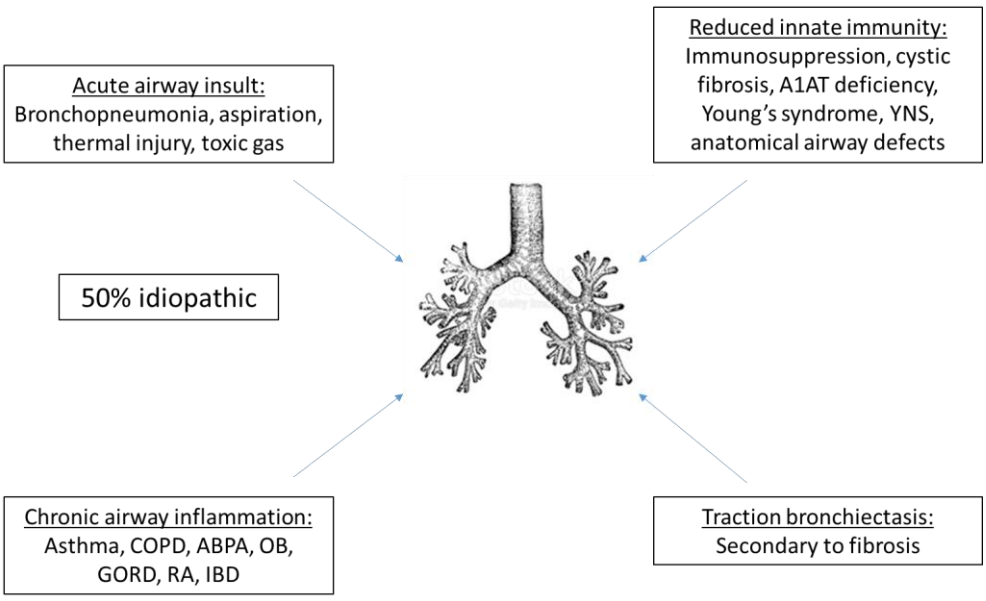

Figure 1-2 Aetiological framework for bronchiectasis

Abbreviations: COPD: chronic obstructive pulmonary disease; ABPA: allergic bronchopulmonary aspergillosis; OB: obliterative bronchiolitis; GORD: gastro-oesophageal reflux disease; RA: rheumatoid arthritis; IBD: inflammatory bowel disease, A1AT: alpha-1 anti-trypsin deficiency; YNS: yellow nail syndrome.

Table 1-1 Breakdown of bronchiectasis aetiologies according to recent systematic review

Risk factors	Total number	% of total
Idiopathic bronchiectasis	3, 857	44.8
Post-infective bronchiectasis	2, 574	29.9
Immunodeficiency	429	5.0
Chronic obstructive pulmonary disease	333	3.9
Connective tissue disease	328	3.8
Allergic bronchopulmonary aspergillosis	223	2.6
Ciliary dysfunction	218	2.5
Asthma	120	1.4
Inflammatory bowel disease	66	0.8
Obstructive	67	0.8
Aspiration/gastro-oesophageal reflux	64	0.7
Congenital malformation	33	0.4
α_1-Antitrypsin deficiency	36	0.4
Diffuse panbronchiolitis	27	0.3
Young's syndrome	26	0.3
Pink's disease	20	0.2
Yellow nail syndrome	11	0.1
Bronchiolitis obliterans	3	<0.1
Others*	221	2.6

** Other aetiologies include sinobronchial syndrome (n = 27), amyloid (n = 1), smoke inhalation (n = 1), eosinophilic bronchiolitis (n = 1), bronchiolitis obliterans (n = 3), vasculitis (n = 5), interstitial lung disease (n = 63), cystic fibrosis or cystic fibrosis transmembrane conductance regulator related bronchiectasis (n = 20), systematic disease (n = 47) and other unreported (n = 42).*

1.1.5 Radiology

Radiological findings in bronchiectasis are associated closely with aetiology, symptoms, exacerbation frequency and mortality.[59] Classification can occur in a number of ways: according to anatomical phenotype if cylindrical, varicose or cystic; if localised or diffuse; or according to lobar distribution. The most common pattern is lower lobe bronchiectasis, which is characteristic of idiopathic bronchiectasis but may also be associated with COPD, infection or aspiration. Bronchiectasis of the middle lobes is classically associated with NTM infection or primary ciliary dyskinesia. Upper lobe bronchiectasis is suggestive of cystic fibrosis (CF); hence, all patients presenting with upper lobe predominant disease should be screened for CF. Central bronchiectasis is less common and is typically a manifestation of ABPA or tracheobronchomegaly (Mounier-Kuhn syndrome).

Plain chest radiograph is less sensitive than CT for diagnosing bronchiectasis but findings include ring opacities due to cross-sectional view of dilated bronchi with thickened walls, tram tracks with longitudinal view of abnormal airways, and dense tubular structures representing mucoid impaction (finger-in-glove sign) (Figure 1-3 (a)).[64] Classic HRCT criteria for diagnosing bronchiectasis (Figure 1-3(b) and (c)) are described below.[63]

- Bronchial wall dilatation – where the internal diameter of a bronchus is greater than the diameter of the accompanying pulmonary artery ("signet ring" sign) when the dilated airway is seen in cross section end on). This pattern is usually seen in the upper and lower lobes.
- Bronchial wall thickening- represented by parallel (tram track) lines
- Failure of bronchial tapering - usually seen in the middle lobe and lingula when the bronchi are travelling horizontally.
- Visible bronchi within 1 cm of the pleura
- Crowding of bronchi with lobar volume loss
- Thickening and plugging of small airways resulting in "tree-in-bud" appearance of bronchiolitis

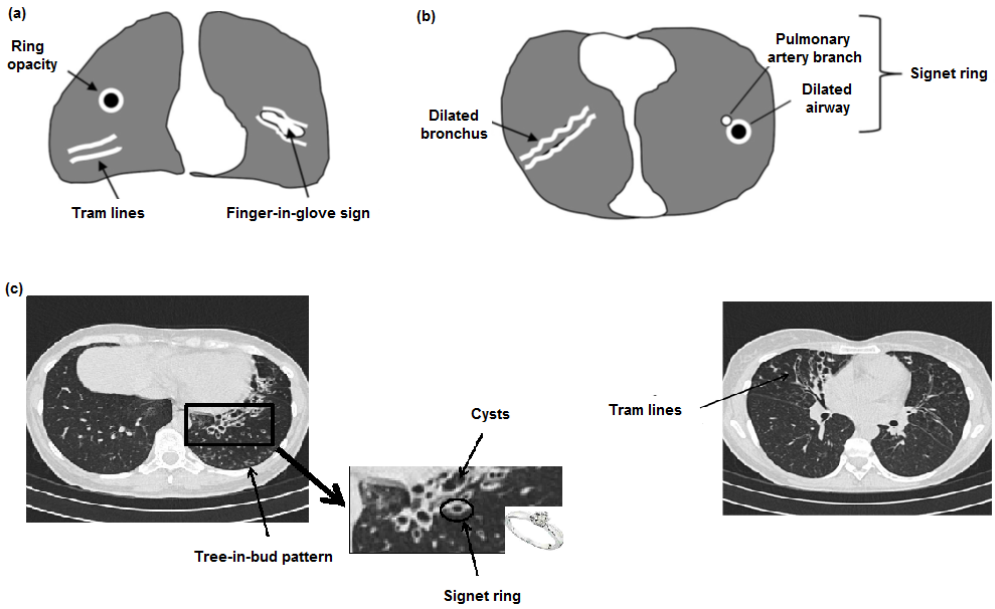

Figure 1-3 Pictorial representation and high resolution computed tomography of chest imaging features of bronchiectasis

(a) Plain chest radiograph showing dilated and thick-walled bronchi visualised in cross-section (ring-opacities) or longitudinally (tram lines). (b) and (c) Axial HRCT pictorial representation and images demonstrating typical radiographic findings of bronchiectasis with (i) a dilated, thick-walled airway larger than the companion pulmonary artery branch viewed in cross-section resulting in a signet ring shadow (enhanced picture), (ii) dilated, thick-walled bronchi viewed longitudinally that fails to taper in the periphery of the lung resulting in tram lines of bronchial thickening, (iii) thickening and plugging of the small airways visualised at the periphery resulting in a tree-in-bud pattern, and (iv) bronchial cysts associated with more severe bronchiectasis disease.

Bronchiectasis shows considerable radiological heterogeneity. Extensive bronchiectasis in terms of the number of lobes involved or the presence of cystic bronchiectasis has been shown to be independently associated with severe exacerbations (hazard ratio [HR] 1.48; 95% confidence interval [CI]: 1.02−2.15), but was not an independent predictor of mortality.[19] The finding was in agreement with Loebinger *et al.* who showed, in 91 patients, that the extent of bronchiectasis, severity of dilatation, bronchial wall thickness, mucus plugging, mosaicism and emphysema were all associated with mortality on univariate analysis; however, none were independently associated with mortality on multivariate analysis.[49]

Radiological scoring systems in bronchiectasis have consisted of the Bhalla, modified Bhalla, and Reiff scores.[65-67] More recently, the Bronchiectasis Radiologically Indexed CT Score (BRICS), was developed and validated in idiopathic and post-infectious bronchiectasis using bronchial dilatation and number of bronchopulmonary segments with emphysema on HRCT.[22] This score was significantly associated with lung function, sputum purulence, and hospital admissions for bronchiectasis exacerbations.[22] Neutrophil elastase was also noted to be significantly higher in patients with radiological emphysema, suggesting that enhanced neutrophil elastase predominance may result in, or perhaps intensify, pathological states such as emphysema.[22, 68]

There are some limitations to interpreting pathological airway dilatation radiologically. Studies acknowledge that a clinical scenario highly suggestive of bronchiectasis may not correlate with a 'positive' HRCT, which raises questions about the links between clinical symptoms and the pathophysiological processes involved.[49, 69] Whether this suggests early bronchiectasis and radiological changes lag behind clinical features has yet to be determined. Several case-series have also demonstrated that approximately 15% of radiologically diagnosed patients had their bronchiectasis diagnosis refuted on re-read of their scans suggesting ambiguity in radiological interpretation.[29, 49, 70] Difficulty also remains in understanding which disease process is the primary disease driver in patients with co-existing lung diseases, for example, determining COPD-driven bronchiectasis versus idiopathic bronchiectasis in an ex-or current smoker.

1.1.6 Disease severity and prognosis

Multidimensional scoring systems have improved our understanding of disease heterogeneity and helped to prognosticate clinical outcome in several chronic lung diseases. Two such scales have been devised in bronchiectasis: the bronchiectasis severity index (BSI) (Table 1-2), and the FACED score with its extended version, the E-FACED score (Table 1-3)[19, 20, 71] The BSI was developed to determine disease severity by identifying independent risk factors for mortality, exacerbations, hospitalisations, and QoL. Scores range from a minimum of 0 to a maximum of 25. Based on disease severity,

it is possible to describe bronchiectasis phenotypes that relate to clinically relevant outcomes.[19]

Table 1-2 Bronchiectasis Severity Index

Bronchiectasis Severity Index							
Criteria							
	0	**1**	**2**	**3**	**4**	**5**	**6**
Age (years)	< 50		50-69		70–79		+ 80
BMI kg/m2	> 18.5		< 18.5				
FEV_1 % pred	≥ 80%	50-79 %	30-49 %	<30 %			
Colonisation/ chronic infection	No	Yes		PA			
MRC dyspnea score	1 - 3		4	5			
Radiological extent	< 3 lobes		≥ 3 lobes / cystic				
Exacerbations in past year	0 - 2		3				
Hospitalisation in prev. 2 years	No					Yes	

BSI risk: mild (0-4 points), moderate (5-8 points), and severe (>8 points). FEV1: forced expiratory volume in one second; MRC = Medical Research Council dyspnoea score; PA = Pseudomonas aeruginosa chronic infection.

The FACED score was designed to predict mortality. Patients with higher scores (5–7 points) have a significantly higher risk of mortality compared to those with lower scores.[20] The addition of exacerbations extended the score to a maximum of 9 points and significantly increased its capacity to predict future yearly exacerbations while maintaining the score's simplicity and prognostic capacity for mortality.[71]

Table 1-3 FACED score and extended E-FACED score

FACED			
	Points		
	0	**1**	**2**
P. aeruginosa colonisation/ chronic infection	No	Yes	
mMRC dyspnea scale	0 - 2	3 - 4	
FEV_1 % predicted	≥ 50 %		< 50 %
Age (years)	< 70		≥ 70
Number of lobes affected	1 - 2	> 2	
E-FACED - adds exacerbation history			
At least one exacerbation in the previous year	No		Yes

FACED and E-FACED score: Acronym for F (FEV1 % pred), A (Age), C (Colonisation status), E (radiological Extension) and D (Dyspnoea scale). FACED: mild (0-2 points), moderate (3-4 points) and severe (5-7 points). E-FACED: mild (0-3 points), moderate (4-6 points) and severe (7-9 points). FEV1 = forced expiratory volume in one second; mMRC: modified Medical Research Council dyspnoea score.

Since the publication of the BSI and FACED scoring systems, the predictive ability of both tools has been evaluated in five separate studies.[48, 72-75] The BSI and FACED scores have a similar capacity to predict mortality but there are important differences. The BSI accurately reflects disease severity and disease impact such as exacerbation frequency, hospital admissions, quality of life, exercise capacity and symptoms such as cough, whereas the FACED score only predicts mortality, with E-FACED extending its ability to predict exacerbations and hospitalisations (Table 1.4). The FACED score is heavily weighted by age, which means that in patients < 70 years, all other risk factors must be present to be deemed high risk. This can limit its purpose, for instance, in a younger patient awaiting transplant, in whom disease is

universally considered to be severe, these patients may be classified as mild or moderate by the FACED score. Arguably, the FACED score may be slightly better at predicting long-term mortality (Table 1-4).[73]

Table 1-4 Comparative area under the receiver operator characteristic curve (AUC) values for bronchiectasis severity scores

Study	Score	5-year Mortality	Exacerbations	Hospitalisation
McDonnell[23] (n=1612)	**BSI**	0.73 - 0.93	-	0.71 - 0.97*
	FACED	0.68 - 0.87		0.56 - 0.79
Ellis[73] (n=74)	**BSI**	0.79 (15 yr: 0.69)		
	FACED	0.80 (15 yr: 0.82*)		
Menendez (n=319)	**BSI**	0.79	-	-
	FACED	0.81		
Rosales (n=182)	**BSI**	-	0.81*	0.89*
	FACED		0.73	0.81
	E-FACED		0.76*	0.82

For optimal results with any scoring system, each instrument must be used solely for its intended purpose. Bronchiectasis-specific tools that can classify patients according to disease severity, predicting mortality, hospitalisation and exacerbation frequency may allow caregivers to inform patients and increase their awareness in relation to admission rates, mortality and exacerbations. In addition, such a tool may help treatment allocation and help avoid over and under-treatment as well as provide researchers and clinicians with the opportunity to uniformly and consistently monitor disease and set-up trials that target the population the investigated drug aims for.

1.1.7 Symptom and QoL scores

Measuring symptoms and quality of life in bronchiectasis has proved challenging with research often limited by extrapolation of questionnaires and treatments from other diseases. Quality of life (QoL) is a key endpoint often

used to determine disease severity in bronchiectasis. A wide range of tools have been applied, ranging from generic tools such as the Medical Outcomes Study 36-Short Form Health Survey (SF-36); organ specific tools such as the St. George's Respiratory Questionnaire (SGRQ), the Leicester Cough Questionnaire (LCQ) and the COPD Assessment Tool (CAT); and the disease-specific QOL-Bronchiectasis (QOL-B) and more recently developed Bronchiectasis Health Questionnaire (BHQ).[76-80] A large meta-analysis of the associations between QoL and clinical measures revealed that most QoL measures showed the strongest correlation with subjectively reported symptoms; dyspnea and fatigue.[81] Correlation with objective measures was moderate for exercise capacity, with lung function, radiological extent and exacerbation rate showing only weak correlations.[81] Good correlations between SGRQ and FEV_1, and between LCQ and bacterial colonisation and radiological extent have been demonstrated in some studies.[82] The only study including QoL measures in their investigation into factors associated with mortality in bronchiectasis identified the SGRQ activity domain as an independent predictor of survival.[49]

Symptom scores are often considered the "missing ingredient" of assessing a patient's perspective of disease severity.[83] Bronchiectasis symptoms are highly individual with a recent qualitative assessment identifying symptom burden, symptom variation, personal measurement, quality of life and symptom control as patient priorities.[83] The Bronchiectasis Exacerbation and Symptom Tool (BEST) is a novel symptom diary that measures day-to-day changes in symptoms to facilitate early detection of the onset, peak and duration of exacerbations.[21] This has concurrent validity with current health questionnaires, and is responsive at onset and recovery from exacerbation. Of relevance to future clinical trials in bronchiectasis, the BEST tool demonstrated a 44% rate of unreported exacerbations.[21] A failure to capture the expected number of exacerbations during study timescales has rendered many bronchiectasis clinical trials underpowered. Inclusion of symptom diaries in future trials would increase our ability to detect reported and unreported events which, in COPD, are both associated with the same medium-term health consequences, and therefore could have major

consequences for bronchiectasis patients at an individual and population-based level.[21, 84] Symptom scores and QoL are important determinants of outcome in bronchiectasis, more accurately reflect disease burden from a patient's perspective, and are rightfully being incorporated into clinical trials, which will hopefully provide invaluable information on how therapies affect patients' symptoms in a far more detailed way than is currently captured.[21, 85]

1.1.8 Comorbidity and multimorbidity in bronchiectasis

Chronic diseases rarely occur in isolation and frequently co-exist with other chronic diseases as comorbidities or multimorbidities, potentially adding to disease burden or accelerating decline in a uni- or bi-directional manner. "Comorbidity" is defined as 'any distinct additional clinical entity that has existed or that may occur during the clinical course of a patient with the index disease under study'.[86] Debate about the accuracy of the term centres on which condition is the primary or index disease, the manner in which these multiple conditions interact in terms of cause and effect, the role of shared risk factors and common underlying mechanisms.[87] In contrast, "multimorbidity" is described as 'any co-occurrence of medical conditions within a person'.[88] Patients with multimorbidity are becoming the norm rather than the exception, and managing multimorbidity requires an evolution away from the single disease focus that has dominated medicine for centuries.[89]

The recognition of the potentially pathogenic role of comorbidities in bronchiectasis is still at an embryonic stage. Comorbidities can provoke acute exacerbations, interfere with acute and chronic pharmacotherapy and rehabilitation, and potentially contribute to chronic disease progression. For example, patients with co-existent angina or peripheral vascular disease may not be able to undergo pulmonary rehabilitation, or marked bronchorrhoea in a COPD patient with secondary bronchiectasis admitted with acidotic hypercapnic respiratory failure, may render the patient intolerant of acute non-invasive ventilation, which may have effects on long-term survival. Patients with multimorbidity are more likely to have prolonged length of

hospital stays for severe exacerbations and a higher inpatient mortality risk; both GORD and heart failure have been shown to be independent predictors of future hospitalisation over 1 year follow up.[18] A significant proportion of deaths and healthcare costs associated with bronchiectasis are attributable to comorbid conditions. Studies have shown that in approximately 30-40% of patients with bronchiectasis, the primary cause of death is attributed to non-respiratory disease.[49] In the original BSI validation study, 16 deaths among 62 patients (26%) were due to myocardial infarction, heart failure, and stroke.[22] Similarly in the Galway cohort of the Bronchiectasis Aetiology Comorbidity Index (BACI) derivation study, 12 of 44 (27%) were attributable to cardiovascular disease and 6 (14%) due to malignancy.[23]

In patients with bronchiectasis complicating known airway diseases such as asthma, COPD and ABPA, the mechanism of deterioration is clear, feeding into the vicious vortex hypothesis of recurrent infections and progressive airways damage leading to poorer control of the underlying disease. Bronchiectasis associated with rheumatoid arthritis has been shown to carry a higher risk of bronchiectasis progression and increased mortality.[90] Airway inflammation is often post-ceded by the development of bronchiectasis either due to or as a result of progression of the primary inflammation or intercurrent infection. Once bronchiectasis develops, however, the immunosuppressive effects of the disease and its treatment, often leads to marked acceleration of airway disease and frequent exacerbations.[91]

The association of bronchiectasis with accelerated vascular and other seemingly non-related diseases, however, is more complex. Systemic spill-over of airway and lung parenchymal inflammation, on a potential background of a congenital or acquired heightened susceptibility to exaggerated inflammatory responses, is suspected to be a key link in the mechanistic pathway relating bronchiectasis with its comorbidities.[57] It is hypothesised that the source of systemic inflammation in bronchiectasis may occur when a sufficient trigger is encountered and that individuals with this predisposition may then develop bronchiectasis as part of a systemic

inflammatory syndrome (Figure 1-4).[57] The association between biomarkers of systemic inflammation, and outcomes in bronchiectasis, including comorbidities, has not been well documented to date. Aside from inflammation, however, there are some specific anatomical, mechanical and pathophysiological links with certain comorbidities, many of which interact with each other as well as with bronchiectasis and may form part of a wider network of disease. Addressing this knowledge gap may allow us to identify pathway-specific treatment targets that could be beneficial in the treatment of multi-diseased bronchiectasis patients.

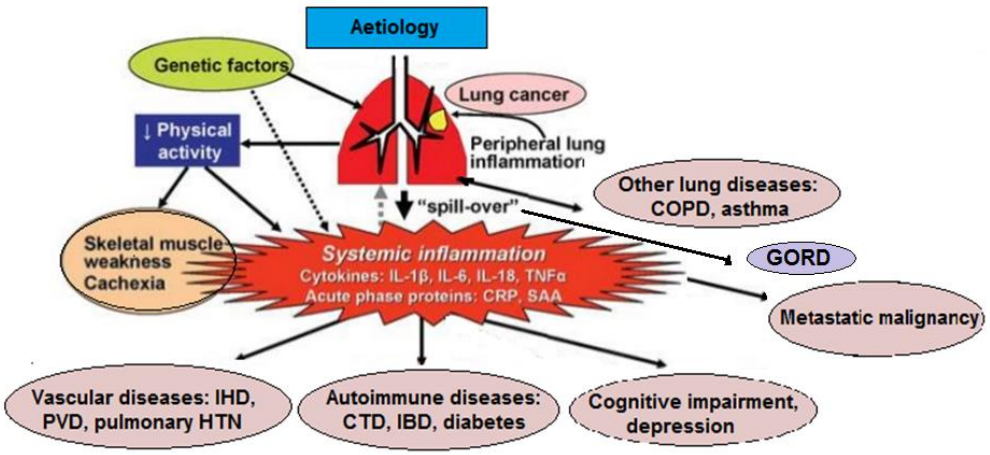

Figure 1-4 Systemic effects and comorbidities of bronchiectasis

Peripheral lung inflammation may cause a "spill-over" of cytokines increasing acute-phase proteins, potentially initiating or worsening comorbid conditions. CRP: C-reactive protein; SAA: serum amyloid; IHD: ischaemic heart disease; PVD: peripheral vascular disease; PH: pulmonary hypertension; CTD: connective tissue disease.

1.1.9 Clinical phenotypes

Bronchiectasis phenotypes are currently emerging. Grouping patients on the basis of common clinical characteristics as they relate to clinically meaningful outcomes in COPD and asthma has been shown to influence clinical decision making; for example, identifying patients that may benefit from closer monitoring due to a higher risk of exacerbations or mortality, or identifying specific interventions based on response to therapy.[4, 59, 92] Acute

exacerbations are critical events in bronchiectasis. The "frequent exacerbator" phenotype, defined as patients with three or more exacerbations per year, is associated with worse health status, increased hospitalisations and increased mortality.[24] Similar to COPD, a history of frequent exacerbations was the strongest predictor of future exacerbations over time.[93] Other independent predictors of frequent exacerbations were the presence of *H. influenzae* and *P. aeruginosa*, reduced FEV1, radiologic severity, and the presence of coexisting COPD.[24] This group may be the optimal target population for future clinical trials and may be the most appropriate candidates for currently available treatments, including long-term use of macrolides and inhaled antibiotics.[4, 94]

Other phenotypic groups from multidimensional clustering have also been proposed, such as those patients with dry bronchiectasis versus those with daily sputum production, patients with early versus late onset idiopathic bronchiectasis, and disease in the young versus disease in the elderly.[95-97] Whilst these analyses have a certain degree of value in informing future research, they can be criticised for not extending current knowledge.[98] Only the *P. aeruginosa* infection frequently exacerbating phenotype was consistent across all four analyses and indeed, is the most robust phenotype identified to date in bronchiectasis.[59]

Defining phenotypes is a major research priority in bronchiectasis.[94] However, although clinical phenotypes can be useful, they can also mask significant complexity. For instance, patients may exacerbate for multiple reasons including neutrophilic or eosinophilic inflammation, immunodeficiency, comorbidity, genetic susceptibility or microbial dysbiosis, among others.[99, 100] Insight into the underlying biology is therefore key to determining how to treat a phenotype.[101] The aim of disease stratification studies is to define endotypes - subtypes of a condition defined by distinct functional and pathobiological mechanisms.[35] As a highly heterogeneous disease, bronchiectasis may have the most to benefit from careful definition of phenotypes and endotypes. The goal is to apply emerging technologies of proteomics, metabolomics, and genomics to well-

characterised groups of patients with bronchiectasis to better understand exact causes and identify targets for future therapies.[4] Adopting a "treatable traits" strategy based on the recognition of clinical phenotypes and endotypes, may enhance personalised and targeted precision medicine approaches resulting in improved better clinical outcomes.[99, 101, 102] Progress in this area is currently underway via EMBARC-BRIDGE, BRONCH-UK and other international projects, with key emerging data on the microbiome and proteome in bronchiectasis likely to herald future promise.

1.1.10 Microbiology and the microbiome

Microbiology in bronchiectasis is becoming increasingly complex. Traditional culture methods are largely being replaced with the lung microbiome, a technology that uses next-generation sequencing to produce a DNA profile of the diverse bacterial communities present in the lung.[103] Detection of uncultivable microorganisms has challenged our understanding of the pathogenesis, progression and management of bronchiectasis. These technologies reveal colonisation with organisms previously not recognised by culture-based studies including *Veilonella sp., Prevotella sp.* and *Neisseria spp*.[45, 104-106] Loss of microbiome diversity, with dominance of one or a few species, is associated with worse lung function, increased exacerbations and higher inflammatory markers.[45, 105] An inverse relationship between the abundance of *P. aeruginosa* and that of *H. influenzae* within the bronchiectasis lung bacterial community, suggests a progression in microbial states.[45]

The role of viruses, fungi, NTM and air pollution is poorly studied in bronchiectasis. Exacerbations in bronchiectasis are frequently managed with antibiotics; however, viral infections, particularly in light of the recent Covid-19 pandemic, may also be significant in triggering bronchiectasis exacerbations. Respiratory viruses are commonly detected in patients with stable bronchiectasis including during asymptomatic viral periods with multiple viruses often present concurrently.[107] Previous work in the UK suggests a role for viruses where bacterial density and diversity remains stable during exacerbations.[105] Work from the US, Canada, and New Zealand

reported viral infection, specifically influenza B and adenovirus to be associated with post-infection bronchiectasis.[108, 109] China have previously demonstrated coronavirus, rhinovirus, and influenza A and B detection during exacerbations with concomitant increases in both airway and systemic inflammation.[110] These data however, do not elucidate whether viruses are a cause or consequence of exacerbations. A recently published study based on data from the Canadian Respiratory Virus Detection Surveillance program showed that viruses (influenza A and B, respiratory syncytial virus, parainfluenza, adenovirus, human metapneumovirus, rhinovirus and coronavirus) over 2010-2013 accounted for 67% of emergency department (ED) visits and 74% of hospitalisations for respiratory tract infections (RTI), 53% of visits and 48% of hospitalisations for COPD, and only 13% of visits and 10% of hospitalisations for asthma, suggesting that community respiratory viral epidemics are major drivers of ED visits and hospitalisations with RTIs and COPD.[111] Recent work in bronchiectasis has focused on the role of Epstein-Barr virus (EBV) which has been implicated in other chronic lung diseases and malignancies such as COPD, IPF and lymphoproliferative disease post-lung transplant.[112-114] Data suggests that bronchiectasis patients with detectable EBV DNA have a higher inflammatory load, a shorter time to exacerbation and a faster lung function decline than those without, suggesting that EBV may contribute to bronchiectasis progression.[115] There appears to be a potential protective effect of macrolides against viral load due to suppression of inflammation, strengthening of epithelial defense, reduction of airway hypersecretion and acceleration of lymphocyte apoptosis.[116, 117] Moreover, macrolides have reportedly inhibited the growth of EBV-transformed B lymphocytes, providing further hints on their protective effects of EBV in bronchiectasis.[118] In the above study, patients with more prominent airway inflammation were less likely to have EBV detection, probably because of concurrent macrolide therapy. [115]

The long-term effects of the Covid-19 pandemic are yet to be determined but it is anticipated that a significant proportion of patients with severe infection will go on to develop post-infectious chronic bronchiectasis or lung fibrosis.

How these diseases will progress remains to be seen. Viral exacerbations in bronchiectasis and COPD inevitably drive disease progression, lead to FEV1 decline and increase risk of mortality directly implicating any acute respiratory virus infection in disease pathogenesis.[119] However, the mechanisms underlying virus-induced exacerbations remain relatively poorly elucidated and current anti-viral therapies are limited. The majority of acute treatments are supportive and antimicrobial prescribing is often empirical in the absence of diagnostic microbiological samples. In the age of personalised medicine a more targeted therapeutic approach is mandated and further research should focus on developing pathogen-specific therapy for viral induced exacerbations.

A great paucity of data exists on the role of fungi in the airways of bronchiectasis patients. Dedicated fungal cultures are rarely performed routinely with most published reports based on an incidental detection. *Candida spp*. (34-48%) and *Aspergillus spp.* (7-24%) have been reported to contribute the highest proportion of fungi isolated by traditional culture methods from the bronchiectasis airway.[120-123] Within the *Aspergillus* genus, *A. fumigatus* is the most common coloniser with other filamentous fungi such as *Penicillium, Scedosporium* and *Fusarium* less frequently seen.[124] A recent mycobiome sequencing study reported that *Candida, Penicillium* and *Saccharomyces* are commonly identified in both healthy controls and patients with bronchiectasis, while *Aspergillus, Alternaria, Botrytis, Clavispora* and *Cryptococcus* were associated only with bronchiectasis.[125] The authors described geographical differences in the bronchiectasis-associated genera, with *Aspergillus* particularly relevant in samples derived from Asia and the remaining associated with European samples.[124, 125] Moreover, a significant positive correlation between the abundance of *A. terreus* and exacerbations was noted, with abundance of *Aspergillus, Penicillium* and *Cryptococcus* associated with increased disease severity.[125]

NTM is frequently associated with bronchiectasis. *Mycobacterium avium complex* (MAC) is generally the most common form affecting bronchiectasis

patients although geographic and gender variation exists.[126-129] NTM are notoriously difficult to culture, especially in the presence of other colonising organisms.[130] There is mounting evidence to suggest that patients with NTM-related bronchiectasis have a distinct immunologic phenotype that results in an imbalance of cytokines leading to inability of the host to resist mycobacterial infection.[131, 132] The cause and effect relationship between NTM and bronchiectasis is difficult to elucidate and is likely bi-directional. In some cases, it can precede bronchiectasis, most likely related to a Lady Windermere type presentation due to cough suppression resulting in a milder phenotype with lower exacerbations and better pulmonary function.[70, 133, 134] It can also occur secondary to bronchiectasis with airway distortion predisposing to a super-added infection, likely correlating with studies suggesting poorer outcomes and more aggressive disease. Studies of prevalence in bronchiectasis are conflicting with variations from 2-10% in Europe, even in centres routinely using bronchial lavage which is reported to be twice as sensitive as sputum for isolation of NTM, with 19-30% reported in the US.[15, 70, 127, 129, 135-137]

Critically, chronic antibiotic use has been associated with prolonged colonisation by fungi and an increased incidence of NTM.[120, 137] Two separate UK studies have demonstrated that co-existence of chronic pulmonary aspergillosis and NTM is common and predicts mortality in bronchiectasis.[138, 139] Emerging next generation sequencing approaches with targeted amplicon sequencing and whole-genome shotgun metagenomics will hopefully allow comprehensive detection of bacterial, viral and fungal populations simultaneously, while also potentially providing data for the carriage of virulence genes and genes associated with antimicrobial resistance, hopefully facilitating targeted therapeutic approaches in the future.

1.1.11 Inflammation and the proteome

Several studies of bronchiectasis airways have investigated extracellular protein abundance in bronchiectasis sputum, BAL and bronchial epithelial cell secretions. Although these studies have contributed to our understanding

of bronchiectasis, investigations of single cell types or *in vitro* cultures have limited relevance to in situ bronchiectasis lung pathogenesis. Tissue damage in bronchiectasis is mediated mostly by extracellular proteases, but other cellular proteins may also contribute to damage through their effect on cell activities and/or release into sputum fluid by means of active secretion or cell death.[140] Untargeted exploratory proteomic studies of bronchiectasis sputum or BAL using highly sensitive shotgun technologies are currently being investigated to characterise the global activity and protein composition of cells in bronchiectasis sputum and BAL. This will provide insight into potential mechanisms of lung disease and identify potential candidate biomarkers associated with poor outcomes in this population. By employing these methods in clinical samples, we may be able to identify novel cellular proteins and activities likely to be clinically relevant as mechanisms for tissue damage and disease progression in bronchiectasis.

Of the few proteomic studies performed to date, a study in Dundee identified 3 vectors driving heterogeneity in inflammation suggesting that bronchiectasis is composed of multiple inflammatory endotypes which may represent different "treatable traits".[141] At the extremes of these vectors were eosinophilic and epithelial dominant (IL-5, IL-13 and Gro-α in sputum), systemic (GMCF, IL-6, VEGF, IL-10, IL1β in serum) and airway neutrophilic inflammatory (neutrophil extracellular traps, resistin, TNFα, CXCL-8, IL-10, MMP9 and elastase) endotypes.[141] Disease severity was worse in patients in the neutrophilic group reflected by increased BSI scores, higher sputum volumes and decreased lung function. Notably, frequently exacerbating patients were identified in all groups. Treatment factors in terms of inhaled corticosteroids and macrolides showed little difference between groups, although inhaled antibiotics were more frequently used in the neutrophilic group.[141]

Several other studies have compared overlapping clinical features of bronchiectasis with other disease entities versus bronchiectasis alone to determine potential endotypic differences that might support a "treatable traits" approach. Comparing bronchiectasis with COPD demonstrated two

clusters that partially separated COPD patients with bronchiectasis from those without.[142] COPD patients with bronchiectasis were co-clustered with other bronchiectasis patients. Regardless of COPD status, however, bronchiectasis patients were predominantly associated with a proteome profile over-represented with the "neutrophil degranulation" pathway and a proteobacteria dominant microbiome profile. In contrast, COPD patients without bronchiectasis displayed a higher expression of IGK, PIGR, AZGP1 and TCN1 and a higher microbiome diversity.[142] An Australian group also studied proteomic sputum differences between bronchiectasis, PCD and healthy controls. Approximately 200 proteins exhibited significant differences between the three cell types with pathway analysis showing activation of lung injury and tumour pathways. Similar to above, results suggested that although disease-specific proteome patterns were observed, both disease entities shared features of overlap, in this case, increased mucin hyperconcentration, and elevated neutrophil collagenase and eosinophil peroxidase.[143]

A further study from Dundee looking at proteomic responses to antibiotic therapy in bronchiectasis identified three endotypes of treatment response: type 1 associated with marked changes in the airway proteome associated with pathogen clearance and significant improvements in symptoms and FEV1; type 2 associated with minimal proteomic change; and type 3 associated with a mixed response and protein profiles suggestive of non-neutrophil driven exacerbations.[144] These could potentially be further explored to personalise treatment approaches in the future. Further work in this area will expand our current understanding of the mechanisms driving bronchiectasis lung disease and identify sputum and BAL cellular proteins with potential for use as indicators of disease severity, prognosis, stratification determinants for treatment prescription and potential therapeutic targets.

1.1.12 Treatment of bronchiectasis

The mainstays of treatment for bronchiectasis can be divided into general measures with patient education and vaccination advice, physiotherapy

consisting of airway clearance techniques, mucoactive therapy and pulmonary rehabilitation, pharmacological treatment for acute exacerbations and prophylactic antibiotic strategies, and surgery for localised disease or transplant. It is extremely important to treat the underlying cause - for example with antibody deficiency syndromes, where a change in management will likely lead to improved outcomes. A multidisciplinary treatment approach is key to optimise management of these patients.

1.1.12.1 Non-pharmacological treatment

All patients should receive disease-specific education and advice regarding general health measures such as smoking cessation advice if indicated, vaccination advice, psychological support, and nutritional support, particularly for those with low body mass index.[145] While there is limited evidence for influenza vaccine in bronchiectasis patients, indirect evidence suggests annual influenza vaccinations reduce morbidity, mortality and health care costs in "at risk" groups.[146, 147] For pneumococcal vaccination, limited evidence supports the use of the 23-valent pneumococcal vaccine in reducing acute infective exacerbations (number needed to treat (NNT) benefit of 6, 95% CI 4-32 over 2 years).[147-149] In the coming year, it is likely that a Covid-19 vaccine will be produced but several challenges remain before it will be widely available, let alone demonstrating efficacy in bronchiectasis. Besides the complexity of virological, pharmacological and immunological challenges associated with any vaccine development and uptake, we first need to establish accurate estimates of disease burden; increase public confidence in vaccine safety and effectiveness; and challenge the poor uptake of existing vaccines given negative public perceptions of disease severity. High-quality experimental and non-experimental studies using current state-of-the-art microbiological methods and validated, standardised case definitions are needed across the depth and breadth of the vaccine development pathway.[147]

Current international guidelines advocate patient education and personalised self-management plans although the limited evidence available does not suggest it improves QoL or frequency of exacerbations.[150, 151] Patient

education and self-management plans have been highlighted as top patient research priorities that, until recently, have been somewhat lacking in bronchiectasis compared to other chronic lung diseases.[94] Qualitative studies show that patients feel a lack of information is a significant barrier to self-management and describe the importance of information in improving patients' confidence and in developing the skills to live with and manage their condition.[152, 153] A digital bronchiectasis-specific information resource based upon in-depth qualitative exploration and understanding of the needs and experiences of patients and their families has recently been developed and trialled in a single-centre pilot study whereby 93% of users found it helpful.[154, 155] There is often a high treatment burden in bronchiectasis and promoting treatment adherence is important. Factors predicting adherence to treatment in bronchiectasis include patients' beliefs about treatments, perceived treatment burden and number of prescribed treatments.[156] Treatment adherence affects important health outcomes and should be considered in bronchiectasis management.[157] Work to develop a behaviour change intervention to promote adherence to treatment in bronchiectasis has begun.[158]

There is limited literature available on the management of comorbidities in bronchiectasis. Whilst screening for high-risk comorbidities that may contribute to worse outcomes in bronchiectasis is important, management of these comorbidities generally conforms to standard practice guidelines. Given the potential complications of treatment and the interactions between comorbid diseases and bronchiectasis, an integrated multidisciplinary care approach to the management of patients with bronchiectasis is essential to optimise patient outcomes.

1.1.12.2 Physiotherapy

(a) Airway clearance techniques

There are a number of airway clearance techniques (ACTs) available including postural drainage, manual techniques (percussion or clapping), breathing strategies (active cycle of breathing or autogenic drainage (AD)),

positive expiratory pressure (PEP) devices, oscillatory PEP devices and high-frequency chest wall percussion. Such techniques are simple, inexpensive, well tolerated, and can be performed independently or with the aid of a physiotherapist. Although observational studies have demonstrated significant improvements in 24-h sputum volume, symptom scores, exercise capacity and QoL with regular use of ACT, few trials have evaluated the optimal technique with recent Cochrane reviews rating evidence as low quality.[159-161] Despite all published guidelines advocating the use of airway clearance techniques as a standard therapeutic treatment in bronchiectasis, data from the US bronchiectasis registry suggests that non-pharmacological measures are only prescribed in 56% of patients, perhaps due to the lack of RCTs to support the use of ACTs.[4, 15] More substantial evidence is emerging to confirm the benefits of ACTs. Two RCTs have been performed in recent years promoting the use of ELTGOL (L'Expiration Lente Totale Glotte Ouverte en décubitus Latéral) which translates to slow expiration with the glottis opened in the lateral posture. The ELTGOL technique has been shown to increase sputum clearance, reduce symptom-based exacerbations and improve QoL after 12 months and is comparable with AD and PEP techniques.[162-164]

(b) Pulmonary rehabilitation and exercise training

Pulmonary rehabilitation and/or exercise training are recommended in all current bronchiectasis guidelines. There is a clear physiological rationale that muscle weakness and physical inactivity may play a role in disease progression as well as impacting QoL, exacerbation frequency and ability to mobilise sputum.[165] In a systematic review of 4 trials, short-term improvements in exercise capacity and QoL were achieved with both supervised pulmonary rehabilitation and exercise training programs, but these effects were not maintained at 6 months.[166] Of potential importance, one trial of supervised exercise training plus a review of ACT (versus standard therapy incorporating encouragement of regular exercise) demonstrated a reduced frequency of exacerbations and prolonged time to exacerbations in the treatment group.[167] A recent real-life, propensity-matched control

study showed that patients with bronchiectasis had similar completion rates and improvements in exercise and health outcomes as those with COPD where the evidence base is well-established, with COPD patients experiencing greater improvements in fatigue only.[168] However, as with any pulmonary rehabilitation study, it is difficult to ascertain whether observed benefits could perhaps result from other components of the rehabilitation package such as education or ACT. While it seems likely that the exercise training is responsible for improved exercise capacity, the basis of the improved QoL is less intuitive, since effective airway clearance may improve both dyspnoea and QoL in patients with bronchiectasis.[159, 169]

There are numerous studies assessing the impact of pulmonary rehabilitation after an acute exacerbation of COPD, demonstrating an improved exercise capacity and reduced risk of future exacerbations, but only one recent pilot study of post-exacerbation rehabilitation in bronchiectasis.[170, 171] Given the small patient numbers, no significant differences in exercise capacity, time to next exacerbation, lung function or QoL were noted.[172] An analysis of probability based on the patients enrolled suggested that more than 1000 participants would be needed to have an > 80% probability of observing a statistically significant difference between pulmonary rehabilitation post-exacerbation versus standard care, with differences likely being too small to be deemed clinically relevant.[172] As with any pulmonary rehabilitation, significant barriers to referral, patient uptake and completion exist. Future research should focus on investigating how various components of the programme affect outcomes, how the organisation of such programmes within specific healthcare systems determines their effects, and how best to refine programmes for patients with bronchiectasis overlap conditions.

(c) Mucoactive therapies

The ERS guidelines summarise the findings of three Cochrane systematic reviews that comprehensively examined the current evidence for mucoactive therapies (inhaled mannitol, isotonic saline, hypertonic saline, and carbocysteine among others), demonstrating limited effectiveness in

increased airways clearance and sputum yield and suggesting a weak recommendation to trial inhaled rather than oral mucoactive therapies in patients with significant sputum expectoration and poor QoL where standard airway clearance techniques have failed.[10, 173-175] Several studies suggest a role for inhaled hyperosmolar agents with hypertonic saline having been shown to reduce exacerbations, reduce sputum volume and purulence and improve QoL in small RCTs to date, although the precise mechanism of action is still somewhat controversial.[176, 177] Whether mucociliary clearance is enhanced by hydrating effects associated with an osmostic drawing of water into the airways or whether the saline directly stimulates coughing is unknown. Inhaled mannitol in bronchiectasis has been assessed in five trials, only two of which were greater than 3 months in duration, with the most recently published systematic review suggesting that, although inhaled mannitol benefits mucociliary clearance, reduces sputum load and reduces exacerbations, evidence over long periods is still lacking.[178-180] Screening is required before initiating mannitol and hypertonic saline due to the potential for bronchospasm. Carbocysteine and N-acetylcysteine are mucolytic agents that target the mucin disulphide bond and have been used in bronchiectasis for many years. The most recent trial published in this area is an open-label Chinese RCT assessing the effects of 600 mg of twice daily oral N-acetylcysteine for 12 months versus as needed therapy in 121 patients. This demonstrated a reduced exacerbation frequency (1.31 *vs.* 1.98 exacerbations per patient-year; risk ratio, 0.41; 95% CI 0.17– 0.66; p=0.0011), reduced 24-h sputum volume and improved QoL with long-term use of N-acetylcysteine, suggesting that oral mucoactive therapies may have a role for some patients with bronchiectasis.[181] There are two active clinical trials exploring the efficacy of commonly used mucoactives (HTS and carbocisteine) in bronchiectasis, the UK CLEAR trial and the Australian/New Zealand trial.[165] The results of these trials are likely to have an important impact on future practice, not least because of the differential cost associated with these mucoactive therapies. Data from the European registry suggests that up to 20% patients currently use HTS (7%) or carbocisteine in their treatment regime despite multiple guidelines indicating insufficient evidence to recommend their use. If ineffective, this

suggests that up to 20% of patients are using ineffective treatments associated with a high treatment and psychosocial burden; and if they are effective, then up to 80% of patients are receiving suboptimal care. [165] Until these definitive trials are completed, a pragmatic approach is to consider stopping the use of mucoactive therapies in patients if there is no benefit after a 4-week trial. Mucoactive therapies should be prescribed in conjunction with ACT and therefore the mechanism of action of mucoactive drugs and their timing with ACT should be taken into consideration.[165] Of note, guidelines strongly advise against the use of inhaled DNase in bronchiectasis despite its efficacy in the CF population, due to its association with increased exacerbation rates, increased use of antibiotics and increased hospitalisation rates in idiopathic bronchiectasis.[10, 11, 182]

1.1.12.3 Antibiotic therapy

Antibiotic therapy forms the cornerstone of bronchiectasis treatment in treating acute exacerbations and as prophylaxis to prevent further exacerbations.[63] To date, there are very few RCTs evaluating the efficacy of antibiotic treatment in infective exacerbations. Current guidelines recommend prompt antibiotic treatment for all patients presenting with an exacerbation. Oral antibiotic therapy should be used first-line for 10-14 days in the absence of any direct data comparing longer and shorter courses of antibiotics. Antibiotic choice should be guided by local or national guidelines based on antimicrobial susceptibility and resistance patterns and the severity of the exacerbation. Intravenous antibiotics may be needed if there has been no response to oral antimicrobials, systemic deterioration or if pathogenic organisms sensitive only to intravenous agents are cultured. Self-management of exacerbations is strongly encouraged with home supply of antibiotics where appropriate to facilitate prompt treatment. Sending a sputum sample at the start of an exacerbation is helpful to guide choice of antibiotics in the event of inadequate response to initial therapy. Sputum specimens for microbiological culture should routinely be collected at different time points to facilitate targeted antibiotic therapy in the development of chronic infection. Guidelines recommended a combined approach to

eradication/suppression of *P. aeruginosa* suggesting three strategies, all of which included at least 3 months of inhaled antibiotic use.[10] BTS guidelines also suggest eradication for first isolation or regrowth in the context of intermittently positive cultures of methicillin-resistant *Staphylococcus aureus* (MRSA) associated with clinical deterioration.[11] Further research studies assessing the optimal duration of antibiotics in acute exacerbations and comparative efficacy studies for treatment of acute exacerbations and eradication regimes are recommended.

The rationale for prescribing long-term antibiotics (oral or inhaled) in clinically stable bronchiectasis is to reduce the bacterial burden in the airways, reduce infection and inflammation and thus reduce daily symptoms and exacerbation frequency.[183] Antibiotic intervention has been shown to lower bacterial burden and reduce most markers of inflammation in stable and exacerbating bronchiectasis patients.[39] The evidence for this approach, however, is limited. Current guidelines recommend that patients suffering from three or more exacerbations per year, should be considered for long-term antibiotics, with recent evidence supporting macrolides as the first-line treatment of choice of prevention in all bronchiectasis frequent exacerbators.[10, 11, 184, 185]

(a) Macrolides

Given the role of inflammation in the pathogenesis of bronchiectasis, there is a focus on strategies that attenuate the inflammatory pathway. Macrolides have been shown to exert immunomodulatory effects on innate and adaptive immune responses in a biphasic manner, initially by promoting host defence by stimulating immune and epithelial cells and later by reducing tissue injury by interactions with structural cells, leukocytes, and modulation of transcription factors to promote inflammation resolution.[186, 187] In the past 20 years, several observational studies and RCTs (Table 1.5), systematic reviews and a 2018 Cochrane review investigating long-term macrolide use in bronchiectasis have been performed with the majority of weighting coming from the EMBRACE, BLESS and BAT trials, all demonstrating a statistically

significant reduction in exacerbations.[188-197] These have subsequently informed international guidelines and consequently long-term macrolides are recommended in frequent exacerbators without *P. aeruginosa* infection.[10, 11] Two recently published systematic reviews with detailed sub-group analyses have helped to expand our existing evidence base.[185, 198] The first conducted an adjusted indirect treatment comparison between macrolides to evaluate their efficacy and safety, demonstrating that azithromycin outperforms both erythromycin and roxithromycin in reducing exacerbation frequency, but was associated with a higher adverse effect profile.[198] The second performed an individual patient data meta-analysis suggesting that long-term macrolide therapy is indeed highly effective in reducing exacerbations (adjusted incidence rate ratio [IRR]=0.49; 95% CI, 0.36–0.66; $p < 0.0001$) and should in fact be considered in all frequent exacerbators including those who are infected with *P. aeruginosa*, the latter statement representing a change in guidelines moving forwards.[185] Baseline exacerbation frequency, lung function, symptoms or quality of life did not impact upon efficacy.[185]

While the evidence clearly supports the use of macrolides for exacerbation prevention in bronchiectasis, there are significant challenges.[184] The optimal dose and dosing regimens for use in bronchiectasis have not been identified. We have remarkably little information about what happens to patients with bronchiectasis treated with macrolides beyond the first 12 months. Gastrointestinal and other adverse effects associated with chronic macrolide use are relatively common including QT interval prolongation, ototoxicity, and bacterial resistance. Although observed much less frequently in the smaller bronchiectasis studies compared with COPD, these potential adverse effects still need to be considered in clinical practice. Reassuringly, however, recent large-scale studies have shown no significant increase in cardiac arrhythmias with macrolides with the resultant lower all-cause mortality in older adults thought to be attributable to its effects on systemic inflammation and comorbidities.[199] Antibiotic resistance emerges rapidly in the respiratory flora following macrolide treatment and changes in the microbiome have been observed, but the clinical significance of these

changes is as yet unknown.[200] [201, 202] Two post-hoc sub-analyses of 86 participants included in the original BLESS study have examined the potential effects of long-term macrolide treatment on respiratory microbiota.[201, 202] Rogers *et al.* demonstrated a significant reduction in exacerbation frequency in patients with *P. aeruginosa*-dominated infection compared to those without, with microbiome evidence suggesting that erythromycin did not significantly change microbiota composition in patients with *P. aeruginosa*-dominant infection, but resulted in displacement of *H. influenzae* by more macrolide-tolerant pathogens in those without.[201] A subsequent analysis showed that changes in the oropharygeal microbiota composition from long-term erythromycin treatment were modest and limited to a discrete group of taxa. Erythromycin treatment did not result in a significant increase in resistance gene reservoir carriage but the abundance of erm(B) and mef(A/E) gene copies within carriers who had received erythromycin did increase significantly, highlighting the potential for this microbial system to act as a reservoir for resistance.[202] Exclusion of NTM infection is also recommended prior to and during treatment with macrolides because of the risk of inducing macrolide resistance which is an issue of particular importance in populations with high NTM prevalence such as the USA.[203]

Table 1.5 Summary of clinical studies of macrolides in bronchiectasis

Study	No. patients	Target population	Study design	Study drug and dose	Duration of study	Location of study	Findings
Koh *et al.*, 1997[204]	25	Children	Randomised, double-blind, placebo-controlled trial	Roxithromycin (8mg/kg)	12 weeks	South Korea	Reduced airway reactivity to metacholine Improved sputum features No change in lung function
Tsang *et al.*, 1999[205]	21	Adults	Randomised, double-blind, placebo-controlled trial	Erythromycin (1000mg/day)	8 weeks	Hong Kong	Increased FEV1 and FVC Reduced 24-h sputum volume No change in sputum inflammatory markers
Davies and Wilson, 2004[206]	39	Adults	Non-randomised, prospective observational study	Azithromycin (dose variation)	16 weeks	UK	Reduced symptoms Reduced sputum volume Reduced exacerbation frequency Reduced requirement for intravenous antibiotics Increased DLCO
Ming and Zhang, 2005 (Respirology abstract)	42	Adults	Non-randomised observational study	Macrolide and theophylline (not specified)	26 weeks	Asia	Reduced symptoms Improved disease control Reduced costs

Table 1.5 (continued) Summary of clinical studies of macrolides in bronchiectasis

Study	No. patients	Target population	Study design	Study drug and dose	Duration of study	Location of study	Findings
Cymbala *et al.*, 2005[207]	11	Adults	Randomised, open-label, crossover study	Azithromycin (1000 mg/day)	26 weeks	USA	Reduced exacerbation frequency Reduced sputum volume Improved QoL
Yalcin *et al.*, 2006[208]	34	Children	Randomised trial, not placebo-controlled	Clarithromycin (15 mg/kg, once daily)	12 weeks	Turkey	Reduced symptoms Reduced sputum volume Reduced sputum inflammatory markers No change in lung function
Anwar *et al.*, 2008[209]	56	Adults	Non-randomised, retrospective observational study	Azithromycin (250 mg, three times weekly)	26 weeks	UK	Reduced exacerbation frequency Increased FEV1 Reduced sputum volume
Coeman *et al.*, 2011[210]	61	Adults	Non-randomised, retrospective observational study	Azithromycin or clarithromycin (dose variation)	3-8 weeks	Belgium	Improved symptom scores
Sersier and Martin, 2011[211]	24	Adults	Non-randomised, prospective observational study	Erythromycin (250 mg/day)	52 weeks	Australia	Reduced exacerbation frequency Reduced annual days of antibiotic use

Table 1.5 (continued) Summary of clinical studies of macrolides in bronchiectasis

Study	No. patients	Target population	Study design	Study drug and dose	Duration of study	Location of study	Findings
Juthong and Eiamsaard, 2011[212] (ERS abstract)	20	Adults	Randomised, double-blind, placebo-controlled trial	Roxithromycin (300 mg, once daily)	8 weeks	Thailand	Improved symptoms Improved QoL (SGRQ)
Asintam *et al*., 2012[213] (ERS abstract)	30	Adults with post-TB bronchiecasis	Randomised, double-blind, placebo-controlled trial	Roxithromycin (300 mg, once daily)	12 weeks	Thailand	No change in sputum volume, QoL or lung function
Liu *et al*, 2012[214] (Article published in Chinese)	50	Adults	Randomised, double-blind, placebo-controlled trial	Roxithromycin dispersible (0.15 g, once daily)	26 weeks	China	Improved dyspnoea scores Improved QoL Improved radiology scores
Wong *et al.* (EMBRACE), 2012[188]	141	Adults	Randomised, double-blind, placebo-controlled trial	Azithromycin (500 mg, three times weekly)	26 weeks	New Zealand (multicentre)	Reduced exacerbation frequency No change in lung function or QoL

Table 1.5 (continued) Summary of clinical studies of macrolides in bronchiectasis

Study	No. patients	Target population	Study design	Study drug and dose	Duration of study	Location of study	Findings
Altenberg *et al*. (BAT), 2013[190]	83	Adults	Randomised, double-blind, placebo-controlled trial	Azithromycin (250 mg, once daily)	52 weeks	Australia (multicentre)	Reduced exacerbation frequency Improved FEV1 Increased treatment side effects Increased macrolide-resistance rate
Valery *et al*., 2013[215] (children)	89	Children	Randomised, double-blind, placebo-controlled trial	Azithromycin (30 mg/kg to max.600 mg, once weekly)	91 weeks	Australia and New Zealand (multicentre)	Reduced exacerbation frequency Higher carriage of azithromycin-resistant bacteria
Masekala *et al*., 2013 (children)	31	Children with HIV-associated bronchiectasis	Randomised, double-blind, placebo-controlled trial	Erythromycin (150 mg <15 kg; and 250 mg ≥15 kg)	52 weeks	South Africa	No change in exacerbation frequency, lung function or inflammatory markers
Sersier *et al*. (BLESS), 2013[189]	117	Adults	Randomised, double-blind, placebo-controlled trial	Erythromycin ethylsuccinate (400 mg, once daily)	48 weeks	Australia	Reduced exacerbation frequency overall, per patient year and in patients with chronic *Pseudomonas* infection Reduced 24-h sputum volume Attenuated FEV1 decline Increased macrolide-resistant organisms

Table 1.5 (continued) Summary of clinical studies of macrolides in bronchiectasis

Study	No. patients	Target population	Study design	Study drug and dose	Duration of study	Location of study	Findings
Diego *et al*., 2013[216]	36	Adults	Randomised, double-blind, placebo-controlled trial	Azithromycin (250 mg, three times weekly)	12 weeks	Spain	Reduced exacerbation frequency Reduced sputum volume Improved dyspnoea scores Improved QoL (SGRQ) No change in oxidative stress levels, inflammatory markers or lung function
Sadigov *et al*., 2013[217] (ATS abstract)	65	Adults	Randomised, double-blind, placebo-controlled trial	Azithromycin (500 mg, three times weekly)	26 weeks	Azerbaijan	Reduced exacerbation frequency Increased time to first exacerbation Reduced sputum inflammatory markers Increased FEV1
Liu *et al*., 2014[218]	52	Adults	Open-label randomised controlled trial	Roxithromycin (150 mg, once daily)	26 weeks	China	Reduced exacerbation frequency Reduced sputum inflammatory markers Reduced bronchial wall thickness on imaging
Lourdesamy and Muthukumaru, 2014[219]	68	Adults	Randomised, double-blind, placebo-controlled trial	Azithromycin (1000mg, once weekly)	12 weeks (plus 12 weeks off active treatment)	Malaysia	Reduced 24-h sputum volume Attenuated FEV1 decline Improved health status

(b) Inhaled antibiotics

Inhaled antibiotics have the proposed benefit of targeted drug delivery, limitation of systemic drug absorption, reduction of side effects and therefore, the possibility of safely prolonging treatment times.[220] A recently published meta-analysis that included the RESPIRE and ORBIT trials, published after the 2017 ERS guidelines, showed that inhaled antibiotics significantly reduced the frequency of all exacerbations (rate ratio 0.81; 95% CI 0.67–0.97; p=0.020) and severe exacerbations (rate ratio 0.43; 95% CI 0.24–0.78; p=0.0050).[221] Time to first exacerbation was significantly prolonged (hazard ratio 0.83; 95% CI 0.69– 0.99; p=0.028), and the proportion of patients with at least one exacerbation decreased (risk ratio 0.85; 95% CI 0.74–0.97; p=0.015).[221] The RESPIRE (ciprofloxacin dry powder inhalation) studies found benefit in terms of exacerbation frequency with reduction of 39% in RESPIRE 1 14-day on/off arm, but no clear demonstrable benefit in the other three arms of the trial.[222] Similarly, inhaled liposomal ciprofloxacin met its primary endpoint in ORBIT 4 with a 28 day on/off regime but not in ORBIT 3.[223] Meta-analyses suggest that inhaled antibiotics are effective, but prompts us to consider reasons why individually, many trials fail to meet their primary endpoints, with patient selection and lack of statistical power most likely contributory.[224] Standardisation of design, outcome measures, and analysis would help to improve future trials.[35, 225, 226] There is now a consensus that exacerbation frequency is the most clinically relevant endpoint, and that a trial duration of 12 months allows sufficient time for an adequate number of events to occur while also removing seasonal influences and other potential confounders.[226] The prior reticence for interventional trials in bronchiectasis arose in part, from the perceived disease heterogeneity. This is in stark contrast to the enthusiasm for clinical trials in asthma which has similarly diverse phenotypes and a similar need for stratified medicine approaches. The common belief amongst clinicians is that inhaled antibiotics are effective in reducing exacerbation frequency, and these agents are widely used despite not being licenced for this purpose. In a recent report from the US Bronchiectasis Research Registry, inhaled antibiotics were used in 10%

of patients.[15] In addition, international guidelines endorse the use of inhaled antibiotics, particularly for patients with chronic *P. aeruginosa* infection and in selected patients with frequent exacerbations.[10-13] We still have to identify the "best responder" phenotype in bronchiectasis. To date, there has been a significant focus on those with chronic *P. aeruginosa* infections, a sub-group with high healthcare costs due to admissions but potentially more severe and possibly less responsive disease. There is a rationale for having both different therapeutic regimens and outcome measures in different severities in bronchiectasis. Further studies should focus on identifying the optimal patient population to benefit from these treatments in bronchiectasis. In the absence of randomised clinical trials, observational "real-life" data can provide important information about comparative effectiveness and safety as well as generating hypotheses that can be explored and investigated in future trials.

1.1.12.4 Surgery and lung transplant

Before the introduction of antibiotic therapy, the most effective management for bronchiectasis was surgery. Aggressive medical therapy is recommended before surgery is contemplated. Surgical indications include life-threatening haemoptysis or localised disease causing significant morbidity unresponsive to medical therapy. To date there have been no RCTs of surgical versus non-surgical management of bronchiectasis such that it is not possible to provide an unbiased estimate of comparative treatment.[63] Lung transplantation can be a useful intervention in very advanced disease. A double-lung transplant is the method of choice as overwhelming sepsis would likely ensue from retention of either native lung. Experiences from large centres have shown 5-year survival rates between 55-73% with a reported 10-year survival of 48% in a large UK centre.[227-229] Lung transplantation continues to be a useful therapeutic option in selected patients, with good survival and lung function outcomes.

1.1.12.5 Other therapies

Anti-inflammatory agents may play a role in attenuating airway inflammation in bronchiectasis. Although data with statins and inhaled corticosteroids has been somewhat controversial, with the European guidelines advocating that further sufficiently powered RCTs are needed, inhaled corticosteroids continue to be used in 39-55% of bronchiectasis patients, likely driven by disease overlap with asthma and COPD.[15, 94] A recent study of 618,303 patients with a diagnosis of bronchiectasis in the Medicare database followed a subset of 90,089 patients receiving long-term prophylaxis to compare the relative benefit of inhaled corticosteroids (83, 589) versus oral macrolides (6,500) between 2006 and 2014. The results showed a striking advantage of macrolide treatment over ICS in terms of exacerbation reduction and prevention of severe exacerbations with ICS leading to a 39% higher risk of hospitalisation and a 56% likelihood of developing further acute exacerbations in adjusted models.[230] Interestingly, there was no difference in mortality, contrary to predictions given the established link between exacerbations and mortality.[230] Inhaled corticosteroids have been shown to increase the risk of NTM, adversely affect neutrophilic inflammation, increase bacterial load and increase the risk of pneumonia across the spectrum of obstructive lung disease, though the risk is notably higher with fluticasone compared to budenoside preparations.[231-234] In COPD, where the use of ICS in combination with bronchodilators is established, the field is moving towards targeted therapy with the release of guidelines to step-down treatment in patients with normal eosinophil counts who are less likely to respond to treatment.[235] In bronchiectasis, inflammation is predominantly neutrophilic rather than eosinophilic but eosinophilic subtypes are emerging and anti-eosinophil therapy may play a role in selected responders in the future.[184, 236] Statins have also been addressed in two small RCTs to improve clinical outcomes in bronchiectasis, owing to their anti-inflammatory effects and their role on systemic inflammation.[237, 238] Concomitant with the reduction in cough and improved QoL were improvements in various indices of neutrophilic inflammation, consistent with the known pleotropic effects of statins.[239] As in the case with inhaled

antibiotics and inhaled corticosteroids, future studies may identify specific groups of patients who may benefit from statins safely.

On the upside, there is hope with INS1007, Brensocatib, a novel, oral, selective, reversible inhibitor of dipeptidyl peptidase 1 (DPP1) which inhibits neutrophil serine proteases (neutrophil elastase, proteinase 3 and cathepsin), with the WILLOW trial showing statistically significant improvements in its primary outcome, time to first exacerbation, and secondary outcomes, reduced frequency of exacerbation (36% and 25% reduction) and reduced concentrations of neutrophil elastase for both 10mg and 25mg dosage strengths respectively over 24 weeks. Perhaps even more importantly, the US Food and Drug Administration (FDA) granted breakthrough therapy designation for Brensocatib for reducing exacerbations in adults with bronchiectasis, hopefully serving to expedite drug development on the basis of preliminary clinical evidence indicating that the drug may demonstrate substantial improvement over available therapy in this patient population.

1.1.13 Conclusion

Bronchiectasis is a rapidly developing field. While the research investment into the management of bronchiectasis has previously been poor, we are currently in an exciting time of focus, with a pipeline of drugs that offer promise in bronchiectasis. There is, however, still a need for more basic science research as underlying pathological processes are still poorly understood and targets for therapy are likely to have been missed. Although disease phenotyping and endotyping in bronchiectasis is still in its infancy, analyses performed to date suggest enormous heterogeneity in disease severity and presentation as well as potential to identify populations with greater likelihood of treatment response and varying prognoses. By recognising the clinical and biological complexity of bronchiectasis, we may be able to pave the way toward a more precise, safe and effective therapy in these patients aligned with causal mechanistic disease pathways, as demonstrated with the success of Brensocatib. Bronchiectasis management is expected to undergo considerable change over the next few years as we look forward to the results of further ongoing clinical trials including novel anti-

inflammatory agents and comparator antibiotic trials, as well as long-term follow-up studies from real-life clinical data networks and registries that will hopefully offer new insights as to the real benefits of these promising therapies. Collaborative work and harnessing the enthusiasm and engagement of a patient population that has been frustrated by delays in diagnosis and treatment, will be crucial to achieving these goals.

1.2 Rationale for investigation of GORD in bronchiectasis

Bronchiectasis is a challenging disease associated with considerable phenotypic and biological complexity and heterogeneity. Extra-pulmonary comorbidities, which often have pathophysiological links, can have a profound impact on morbidity and mortality and are often important determinants of poorer clinical outcomes, contributing to increased healthcare utilisation and socioeconomic cost.[240, 241] The recognition of the potentially pathogenic role of comorbidities in bronchiectasis is still at an embryonic stage. GORD has emerged as a common comorbidity in 26-75% of bronchiectasis patients utilising questionnaires or 24-hour pH monitoring with co-existing disease associated with worse bronchiectasis severity manifest by increased symptoms, increased exacerbations and hospitalisations, worse radiological parameters, reduced quality of life (QoL) and a more than doubling of 5-year mortality.[28, 242] In a real-world setting, GORD is usually diagnosed using a combination of symptoms or response to empiric anti-secretory therapy with objective measurements less frequently measured despite pH-impedance having become the gold-standard assessment.[243] Airway reflux is often asymptomatic and may only be detected by 24h pH-impedance. The main factors that determine the significance of GORD and airway reflux include the frequency, duration and extent of reflux episodes as well as the volume, composition and destination of the refluxate.[242] Of studies performed prior to this thesis, many were somewhat limited in that they were small single centre studies that used varying definitions of reflux with a lack of gold-standard diagnostic investigations or accepted biomarkers of microaspiration, potentially underestimating the detrimental effects of GORD in these patients. The detection of pepsin and bile acids, markers of gastric and duodenal reflux respectively, have been shown in other chronic lung conditions to exacerbate airway inflammation and chronic infection. The extent and effects of airway reflux and pulmonary microaspiration and potential mechanisms contributing to GORD have yet to be determined in this patient population. Reflux may be more prevalent in bronchiectasis for a number of reasons including an increased prevalence of hiatal hernias, oesophageal motility dysfunction,

respiratory medications altering sphincter tone and changes in respiratory mechanics, with increased cough and lung hyperinflation potentially compromising the diaphragm–oesophageal interface.[59] The hypothesis is that recurrent GORD can irritate upper airways, causing bronchial hyper-responsiveness and an exaggerated inflammatory response and potentially an increased susceptibility to lung infections such as aspiration pneumonia, consequently leading to bronchiectasis. It may be possible that frequent exacerbations drive further GORD and that they act in a bi-directional manner. GORD and microaspiration have been associated with an increased risk of oesophageal, laryngopharyngeal and lung cancer in the general population, and may be responsible for the increased prevalence and mortality associated with these malignancies in bronchiectasis.[244, 245] *In vitro* studies have shown that bile acid challenge of airway epithelial cells leads to epithelial to mesenchymal transition.[246, 247] The index of suspicion for GORD in bronchiectasis should remain high, particularly in patients with frequent exacerbations, rapidly deteriorating airway radiology or associated bronchiolitis, or where conventional bronchiectasis management has failed as this may have important therapeutic and prognostic implications.

There are a range of medical and surgical options available for the treatment of GORD and while extensive studies have not yet been taken in this patient population, it may be amenable to treatment. Macrolides have been demonstrated to be highly effective in reducing exacerbation frequency in patients with bronchiectasis, which may in part, be due to its prokinetic properties. Given the current move towards attenuating the anti-bacterial and pro-kinetic effects of macrolides, it is important to fully elucidate the mechanism of action of macrolides in this patient population. Although there is a clear association between GORD and bronchiectasis, a causal connection has not yet been established. Understanding this relationship at every level from molecules to population epidemiology is key to enable future intervention and prevention of this deleterious comorbidity in bronchiectasis. By recognising the phenotypic properties of GORD in bronchiectasis and aligning these findings with causal mechanistic disease pathways, we may be able to improve therapy and outcomes in these patients.

1.3 Aims and Hypotheses

1.3.1 Overall objective

Given the burden of bronchiectasis, the potential synergistic effect of GORD in bronchiectasis, and its associations with significant morbidity and mortality, escalating public health costs and profound reductions in QoL, further studies in this area are greatly needed. This thesis aims to explore the contribution and mechanism of GORD, airway reflux and microaspiration to the development and severity of lung injury in patients with bronchiectasis using multiple methodologies, further expanding the existing evidence-base to inform guidelines that target bronchiectasis severity and progression.

1.3.2 Specific objectives

First, a systematic review will be performed to explore the association between GORD, airway reflux and pulmonary microaspiration with bronchiectasis in terms of disease prevalence, disease outcomes and potential available treatment strategies. Second, the derivation, validation and comparison of bronchiectasis disease severity and comorbidity indices using large prospective multicentre cohort analyses will be conducted to better define the effect of GORD on bronchiectasis outcomes. Third, an exploration of the associations of the prevalence of hiatal hernias and bronchiectasis severity will be determined. Fourth, to build on the existing evidence base, exploration of the associations between GORD and bronchiectasis outcomes of disease severity, mortality, chronic infection and exacerbations will be performed using pan-European multicentre data from the FRIENDS and EMBARC bronchiectasis patient registries. Fifth, a bi-centric parallel prospective observational case-control study to assess the prevalence, mechanism and functional impact of GORD, airway reflux and microaspration on bronchiectasis patients, utilising a multi-modal diagnostic approach incorporating questionnaires, pH-impedance and biomarkers of duodeno-gastro-oesophageal reflux, will be performed, comparing findings to age, sex, ethnicity and BMI-matched chronic bronchitis patients and healthy volunteer controls. Finally, *in vitro* and *ex-vivo* primary bronchial epithelial cell studies will be performed to investigate the cytotoxic,

inflammatory and remodelling effects of physiologically achievable individual and combined bile acids and to determine a potential role for azithromycin in attenuating bile acid-mediated neutrophilic inflammation and remodelling in bronchiectasis. It is envisaged that these studies will provide novel observational clinical and translational data relating to disease severity and the associations between gastro-oesophageal reflux and bronchiectasis.

1.3.3 Study hypotheses

This PhD is based on the hypothesis that GORD, airway reflux and microaspiration of duodeno-gastro-oesophageal contents into the lung causes bronchial epithelial cell damage, stimulation of cytokine production and an airway inflammatory and remodelling response that contributes to airway damage and disease severity in bronchiectasis (Figure 1-5 and Figure 1-6).

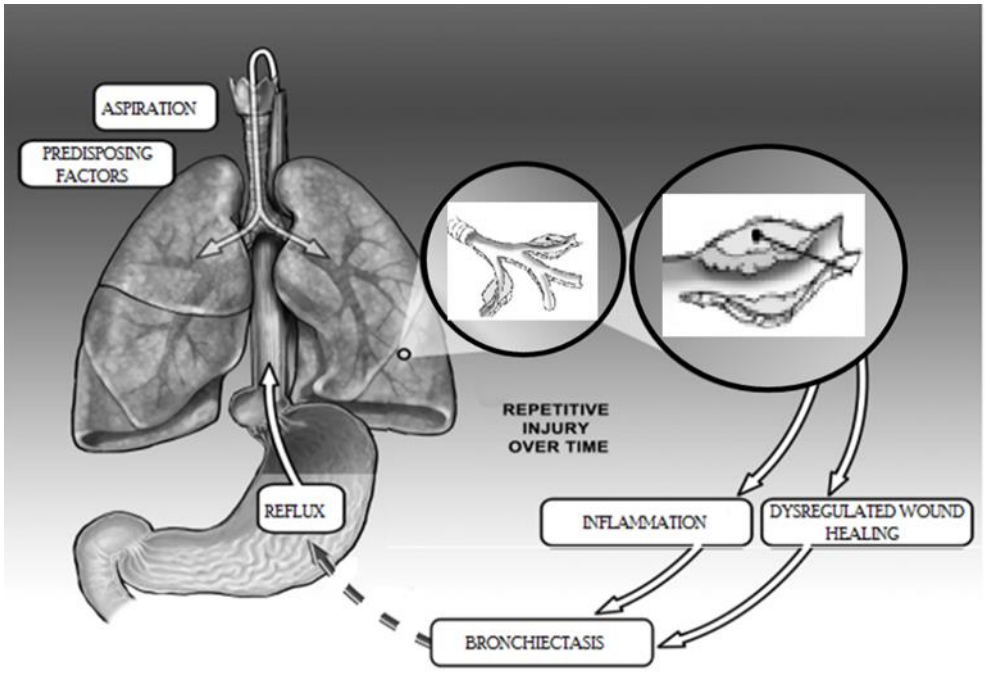

Figure 1-5 Proposed pathophysiology of the airways-reflux paradigm

Repetitive injury from components of gastro-oesophageal reflux disease, airway reflux and microaspiration in bronchiectasis over time likely contributes to lung injury that drives the vicious vortex of increased inflammation, dysregulated wound healing, impaired ciliogenesis (surplus mucus collection in widened airways (arrow)) and chronic infection, resulting in frequent exacerbations, worse bronchiectasis severity and disease progression.

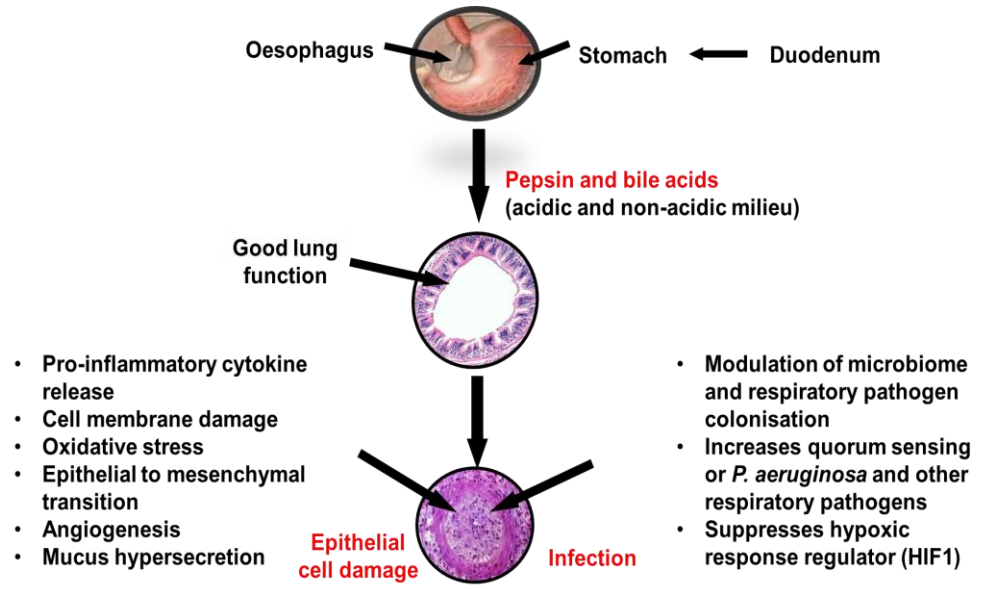

Figure 1-6 Proposed cellular pathophysiology contributing to airways injury and remodelling

Summary of the hypothetical routes through which gastro-oesophageal reflux disease, airway reflux and microaspiration could potentially drive mucosal inflammatory responses within the airways.

Direct or indirect epithelial cell injury from components of liquid or gaseous acid or non-acid reflux or injurious properties of gastric acid, bile, pepsin and duodenal contents from duodeno-gastro-oesophageal microaspiration will likely lead to an increased inflammatory response with an increased drive in neutrophil recruitment resulting in further inflammation-induced damage. This will cause airway epithelial cells to release a range of local inflammatory mediators resulting in oxidative stress, cell membrane damage, apoptosis, dysregulated wound healing and airways remodelling with epithelial to mesenchymal transition, angiogenesis and mucus hypersecretion from goblet cells. This subsequently leads to a loss in mucociliary clearance and further exposure of mucosa to damaging or noxious stimuli contributing to the development of chronic infection due to bacterial persistence via modulation of the lung microbiome and respiratory pathogen biofilm formation, contributing to increased infection and bronchiectasis disease severity and chronicity.

Chapter 2 - A Qualitative Synthesis of Gastro-Oesophageal Reflux in Bronchiectasis: Current Understanding and Future Risk

Publications relevant to this chapter:

McDonnell MJ, O'Toole D, Ward C, Pearson JP, Lordan JL, De Soyza A, Loebinger M, Chalmers JD, Laffey JG, Rutherford RM. A qualitative synthesis of gastro-oesophageal reflux in bronchiectasis: Current understanding and future risk. *Respir Med.* 2018;141:132-143.

McDonnell MJ, Hunt EB, Ward C, Pearson JP, O'Toole D, Laffey JG, Murphy DM, Rutherford RM. Current therapies for gastro-oesophageal reflux in the setting of chronic lung disease: state of the art review. *Eur Respir J Open Res* 2020.

2.1 Introduction

Bronchiectasis is an umbrella term for patients with a chronic inflammatory lung disease characterised radiologically, by the permanent dilation of bronchi, and clinically, by persistent cough, sputum production, and recurrent respiratory tract infections.[1] Data across multiple healthcare systems suggest that the prevalence of bronchiectasis is increasing.[248-250] The common pathophysiological pathway of bronchiectasis consists of Cole's "vicious cycle" hypothesis of infection, inflammation and airway structural changes.[33] The interesting feature is that the initial herald event may be a once-off phenomenon such as aspiration, an inhaled foreign body or pneumonia, but once initiated, the vicious cycle is often self-perpetuating. The clinical profile of bronchiectasis is frequently punctuated by acute exacerbations, which are associated with accelerated lung function decline and deterioration in quality of life (QoL).[3] Bronchiectasis patients are also frequently afflicted by comorbidities, often associated with severe disease, poor clinical outcomes, and an increased mortality.[23]

Gastro-oesophageal reflux (GOR) is a normal physiological event in healthy individuals, referring to the involuntary passage of gastric contents into the oesophagus.[251, 252] Gastro-oesophageal reflux disease (GORD) comprises symptoms or end-organ complications resulting from the reflux of gastric contents into the oesophagus, or beyond into the oral cavity, larynx or lung – a continuum termed extra-oesophageal reflux (EOR).[253] Reflux disease affects 9-27% of Europeans, and may be associated with either oesophageal or extra-oesophageal syndromes.[253, 254] Reflux may be acidic, weakly acidic or non-acidic (alkaline), and may be liquid, gaseous or mixed.[255] The main factors that determine the significance of GORD include the frequency, duration and extent of episodes as well as the volume, composition and destination of the refluxed contents.

As both bronchiectasis and GORD are highly prevalent conditions, the possibility of an interaction has long been recognised. GORD has been attributed as an aetiological factor in several aetiological studies of

bronchiectasis but is more commonly perceived as a comorbidity that may exacerbate the underlying lung disease. Given the potential for bronchiectasis and GORD to aggravate each other in a bi-directional manner, it is important to better understand the relationship and possible consequences of the two conditions co-existing. This area has generated significant interest despite the relative paucity of good-outcome data because the potential landscape for the treatment of GORD, both medically and surgically, is significant.

In this thesis, GORD refers to all symptoms or end-organ complications resulting from the reflux of gastric contents into the oesophagus, or beyond, into the oral cavity, larynx or lung by any mechanism including typical GORD, airway reflux and microaspiration. This review explores the underlying pathophysiology of GORD, its nomenclature and clinical presentation, risk factors, commonly applied diagnostic tools, and a detailed synthesis of original articles evaluating the prevalence of GORD, its influence on disease severity and current management strategies within the context of bronchiectasis.

2.1.1 Pathophysiology of GORD

Gastro-oesophageal reflux (GOR) is a normal physiological occurrence. In health, reflux is prevented through the combined action of the components of the anti-reflux barrier: the lower oesophageal sphincter (LOS), the crural diaphragm and the anatomical flap valve.[256] GORD usually occurs in the event of failure of one or more of the anatomical or physiological protective mechanisms of the anti-reflux barrier, such that the aggressive forces (injurious properties of gastric acid, bile, pepsin and duodenal contents) outweigh the defensive forces (anti-reflux barrier and oesophageal clearance), potentially leading to histological damage in the oesophagus and extra-oesophageal organs, including the exposed respiratory epithelium.[251, 257] GORD typically occurs during periods of gastro-oesophageal junction incompetence that may be functional (due to an increased number of transient LOS relaxations or the presence of a hiatal hernia) or mechanical (due to reduced LOS tone, oesophageal body dysfunction, delayed proximal gastric emptying, or increased intragastric pressure); with age, gender, smoking,

obesity, spicy foods, alcohol consumption, positional and physiological changes in respiratory mechanics and medications all potential contributing factors.[251, 256, 258] It is also important to consider that GORD may result from progressive incompetence of the anti-reflux barrier due to the failure of multiple anti-reflux mechanisms rather than one single process, with the frequency and duration of reflux events increasing progressively with each protective mechanism that becomes compromised (Figure 2-1).

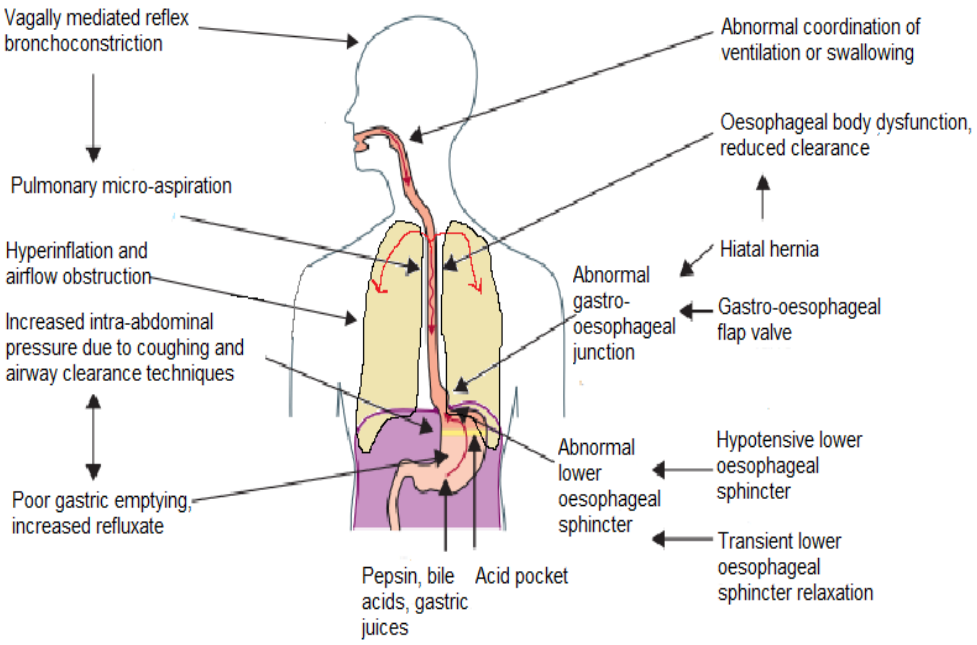

Figure 2-1 Potential pathophysiological mechanisms contributing to gastro-oesophageal reflux disease in chronic lung disease

2.1.2. Clinical presentation and nomenclature

GORD may manifest as typical reflux symptoms such as heartburn, acid regurgitation, odynophagia, dysphagia, chest pain, epigastric pain or sleep disturbances.[253, 256, 259] These clinical features together with oesophageal complications, including reflux oesophagitis, Barrett's oesophagus, and adenocarcinoma, are collectively referred to as oesophageal syndromes.[253] It is therefore necessary to establish a temporal relationship between GORD symptoms with food, posture, and stress.[242] Symptoms from EOR are frequently not inquired about by physicians or offered by patients and can include peri-prandial or persistent cough (laryngeal

irritation), dysphonia, globus, laryngitis, sinusitis, metallic taste, dental caries and halitosis.[260] The prevalence of EOR is difficult to determine; extra-oesophageal symptoms can occur concurrently with typical GORD symptoms or in isolation.[259] It is estimated that approximately one third of patients with GORD have concurrent extra-oesophageal symptoms; however, establishing that an individual patient's extra-oesophageal symptoms are caused by reflux is extremely difficult.[254]

The classical definition of GORD refers to liquid acid reflux defined by 24h pH-monitoring with most epidemiological studies of GORD referring to this phenomenon. In recent years, however, the concept of airway reflux, which consists of neither acid nor liquid reflux but rather a gaseous mist containing mainly non-acid components, has become widely established, with 24h pH-impedance studies now the gold standard investigation of choice for the same. pH impedance quantifies the type, number, phase, duration and proximal extent of each reflux episode.[255, 261, 262] Airway reflux may be entirely asymptomatic, and gaseous or mixed reflux may be as pathogenic to the oesophagus, oropharynx and upper and lower respiratory tract, as liquid acid reflux.[255, 259, 263-266] It is also increasingly recognised that the refluxate may also be from the duodenum and contain bile acids which again can be very pro-inflammatory and have been associated with changes in the gut microbiome.[267]

The nomenclature remains confusing with GOR considered to be a normal physiological process and GORD a disease caused by pathological GOR. Does GOR refer only to the involuntary passage of liquid acid contents or does it encompass all potential gastric contents? Should airway reflux be considered under the same umbrella term of GORD if it contributes to a disease process comprising symptoms or end-organ complications? Airway reflux is more likely to contribute to extra-oesophageal reflux disease (EORD) caused by EOR which can consist of liquid acid reflux (typical GORD) or gaseous non-acid reflux (airway reflux). In this review, therefore, GORD is the overarching term that refers to symptoms or end-organ complications resulting from the reflux of gastric contents into the

oesophagus, or beyond, into the oral cavity, larynx or lung by any mechanism including typical GORD, airway reflux and microaspiration.

An outline of oesophageal and extra-oesophageal clinical presentations of GORD is presented in Figure 2-2. Either may be present in patients with bronchiectasis.

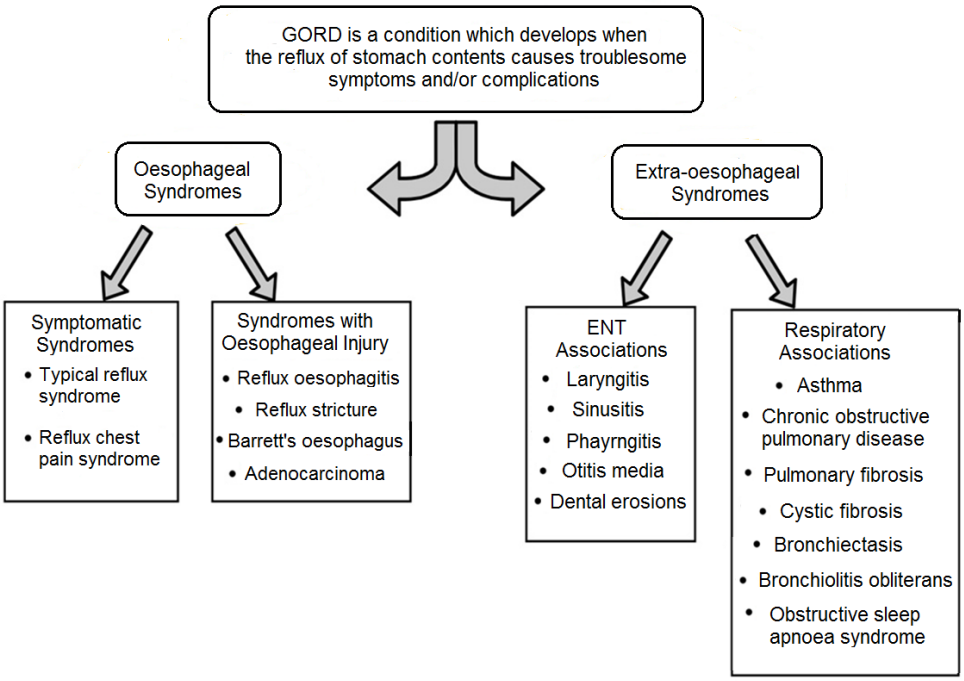

Figure 2-2 Representation of the working definition of gastro-oesophageal reflux disease encompassing oesophageal and extra-oesophageal end-organ effects and complications.

The most common approach to the diagnosis of GORD is through an accurate medical history, enquiring about typical symptoms and their relationship to food, posture, and stress.[259] However, some of the extra-oesophageal symptoms of GORD may be similar to those of bronchiectasis. Therefore, it is necessary to enquire as to the timing of GORD symptoms and their association with awakening from sleep, or the presence of respiratory symptoms or coughing after meals.[242] Symptom evaluation alone may be insufficient for a diagnosis of GORD due to limited sensitivity and specificity.[268] Symptom assessment through validated questionnaires,

which ideally incorporate both oesophageal and extra-oesophageal symptoms so as not to limit their applicability in the setting of silent reflux, may be needed.[269] In the presence of typical reflux symptoms, an empirical trial of acid suppression therapy is often undertaken, with resolution of symptoms considered clinically indicative of GORD.[259] In those with persisting symptoms despite therapy, objective tools such as an oesophago-gastro-duodenoscopy may be used to identify secondary complications of mucosal injury and oesophagitis.[270]

In patients without typical symptoms or where asymptomatic reflux is suspected, alternative options for diagnosing GORD include ambulatory 24-hour oesophageal pH monitoring, with or without multichannel intraluminal impedance testing – the current "gold standard" for diagnosing GORD.[255, 259, 263-266] pH-monitoring is generally performed after cessation of acid suppression drugs for a minimum period of five days to allow tracking of overall oesophageal acid exposure and investigate whether or not a temporal relationship is present between symptoms and reflux events.[256] Oesophageal manometry testing is generally performed prior to insertion of the pH-impedance probe to ensure correct positioning for electrode placement and to rule out severe oesophageal motility disorders.[271] Dual-channel pH monitoring measures proximal and distal oesophageal pH, providing data on the frequency and duration of reflux episodes and the proximal spread of the refluxed material over a complete circadian cycle.[264, 265]. A variation on this is telemetry capsule pH monitoring, which offers increased patient tolerability and the option to extend the monitoring period to 48 or 96 hours, but which does not allow for a combined impedance assessment.[272] Combining pH monitoring with multichannel intraluminal impedance allows the additional identification of acid versus weakly acid or non-acid reflux, and measurement of gaseous versus liquid or mixed reflux, recording GORD at all pH levels and enabling confirmation in patients whose diagnoses may have been missed using pH-testing alone.[255] This technique quantifies the type, number, composition, duration and extent of each reflux episode, giving an exact assessment of the proximal extent of refluxed material and a detailed characterisation of each reflux episode.[255, 261, 262]

2.1.4. Diagnosis of pulmonary microaspiration

Pulmonary microaspiration of duodeno-gastric contents into the lungs, hypothesised to drive the progression of an exaggerated bronchial inflammatory response, can be detected through various methods.[257, 273] This hypothesis is very difficult to test, due to both the difficulties in assessing the presence of reflux clinically and diagnostically, and the potential confounding effects of anti-inflammatory and prokinetic therapies used in the treatment of bronchiectasis.[273] Although dual chamber pH and impedance monitoring both detect proximal reflux, the extent of reflux within the hypopharynx and airway is not measured. The detection of pepsin and bile salts, as markers of gastric and duodenal reflux, respectively, in saliva, sputum, tracheal aspirates or bronchoalveolar lavage (BAL) fluid have been proposed as surrogate markers of reflux aspiration.[268, 269] Pepsin has been detected in lung transplant recipients with GORD confirmed on oesophageal pH monitoring or impedance monitoring, and more recently in sputum and exhaled breath condensate (EBC) in individuals with bronchiectasis, suggesting that these biological markers are reliable in assessing the effect of pulmonary microaspiration in lung disease severity.[274, 275]

2.1.5. Treatment of GORD

Current therapy for GORD focuses on modifying risk factors, inhibiting the production of gastric acid and enhancing oesophageal and gastric motility.[259] Lifestyle modifications to minimise GORD include weight loss, avoidance of late-night meals, avoidance of food and drink that might relax the LOS, stress reduction and altered posture, including adapting a semi-recumbent posture when sleeping.[259, 276] Medical approaches include the use of antacids, histamine antagonists and proton pump inhibitor (PPI) therapy, as determined by the severity of GORD.[256, 259, 277, 278] The beneficial effect of antacids, with or without alginic acid, is related to the neutralisation of acid, which provides temporary symptomatic relief. PPIs and histamine antagonists inhibit gastric acid secretion. PPIs are thought to be more effective and promote faster healing than histamine antagonists. However, recent population-based studies have suggested that long-term PPI

use may be associated with a variety of adverse events including osteoporosis-related hip and spine fractures, community-acquired and nosocomial pneumonia, vitamin B12 deficiency, various enteric and non-enteric infections, and many others.[279] Studies involving oesophageal multichannel intraluminal impedance have revealed a potential role of weakly acidic or non-acid reflux in patients with persistent symptoms despite treatment with a PPI.[280] PPI therapy may result in a paradoxical increase in the number and frequency of weakly acid and non-acid reflux events rather than eradicating the problem, and therefore do not provide a long-term solution for GORD.[280] The mechanism by which weakly acidic reflux causes GORD-related symptoms remains poorly understood but could be related to one or both of oesophageal distension by increased reflux volume or oesophageal hypersensitivity to weakly acidic reflux.[280]

In cases of oesophageal dysmotility, prokinetic therapy can be trialled to speed up gastric emptying by increasing peristaltic muscle contractions of the lower oesophagus and strengthening the LOS, limiting exposure of acid to the oesophagus. More commonly used drugs are domperidone and metoclopramide, but the prokinetic effects of erythromycin and azithromycin should not be ignored. Macrolides work by increasing gastric emptying, increasing proximal stomach tone and lowering LOS pressure via a cholinergic pathway mediated by motilin receptors.[281] Given their success in the treatment of bronchiectasis exacerbations, further work into the effect of GORD and bronchiectasis exacerbations is needed.

There is currently a growing interest in non-PPI-related therapeutic strategies for GORD with a resurgence in endoscopic treatment and anti-reflux surgeries, which at present are reserved for patients with persistence of the underlying mechanism causing GORD.[282] A Nissen fundoplication is the most commonly performed surgical procedure for GORD, consisting of a complete 360° wrap to create an anti-reflux valve at the fundus of the stomach that inhibits the regurgitation of gastric contents into the oesophagus. Endoscopic treatments, such as the LINX or Stretta therapy, may not offer the same degree of relief provided by surgery, but might represent viable

alternatives for patients seeking relief from lifelong dependence on pharmacological therapy, its cost, associated side effects, and long-term adverse outcomes.[259] A systematic review and qualitative synthesis of original articles evaluating the prevalence of GORD, its influence on disease severity and current management strategies within the context of bronchiectasis was therefore undertaken.

2.2 Methodology

2.2.1 Literature search

We searched electronic databases including Pubmed, Medline (Ovid), EMBASE, Scopus and the Cochrane Central Register of Controlled Trials (CENTRAL) for all reports published from inception until May 2017 using the following search string: reflux[majr] OR gastro-oesophageal reflux[Majr] OR GORD[tiab] OR gastroesophageal reflux[majr] OR GERD[tiab] OR duodenogastric reflux[Majr] OR laryngopharyngeal reflux[Majr] AND bronchiectasis[majr] OR NCFB[tiab] OR NCFBr[tiab]. To ensure a complete review of available studies, manual review of conference proceedings and review of references from selected papers was also performed.

2.2.2 Eligibility criteria

Limits were not applied to the search strategy to enable a comprehensive search to be performed. However, the following article types were excluded from our qualitative synthesis: reviews, editorials, case reports, case series and non-English publications. Full-text articles were independently reviewed by two investigators with disagreements regarding eligibility resolved by consensus. Data on study type and design, population characteristics, diagnosis of GORD (method of assessment and result) and outcome variables were extracted. A positive association was defined as worsening of any bronchiectasis outcome associated with the presence of GORD. As bronchiectasis outcomes differed between studies (e.g. reduced pulmonary function, increased radiological severity, etc), we report bronchiectasis outcomes as defined in the study manuscripts. Due to known heterogeneity in the methods of GORD detection and the number of different bronchiectasis

outcomes, a decision was made a priori not to perform a meta-analysis or generate quantitative summary estimates. Instead a qualitative summary in tabular format was planned, with studies divided into those assessing the prevalence of GORD in bronchiectasis, the role of *Helicobacter pylori* in bronchiectasis, and treatment options for GORD in bronchiectasis.

2.3 Results

2.3.1 Study selection

Of 968 total articles identified, 24 studies (n=4,605) fulfilled the eligibility criteria (Figure 2-3), including 7 prospective case control studies (n=662), 13 prospective cohort studies (n=3,375) and 4 retrospective cohort studies (n=568). No randomised controlled trials (RCTs) have been performed to date. 15 studies assessed the prevalence of GORD in bronchiectasis (n=3,679) with 2 of these incorporating the prevalence of pulmonary microaspiration in bronchiectasis (n=57); 7 assessed the role of *H. pylori* (n=662), and 2 assessed treatment options for GORD in bronchiectasis (n=264). Effects of GORD on markers of bronchiectasis disease severity were noted in 12 studies (n=3,876). 2 studies focussed on the effects of chest physiotherapy on GORD in bronchiectasis (n=62). 4 studies were paediatric-based (n=330); the remaining 20 (n=4, 275) were adult-based.

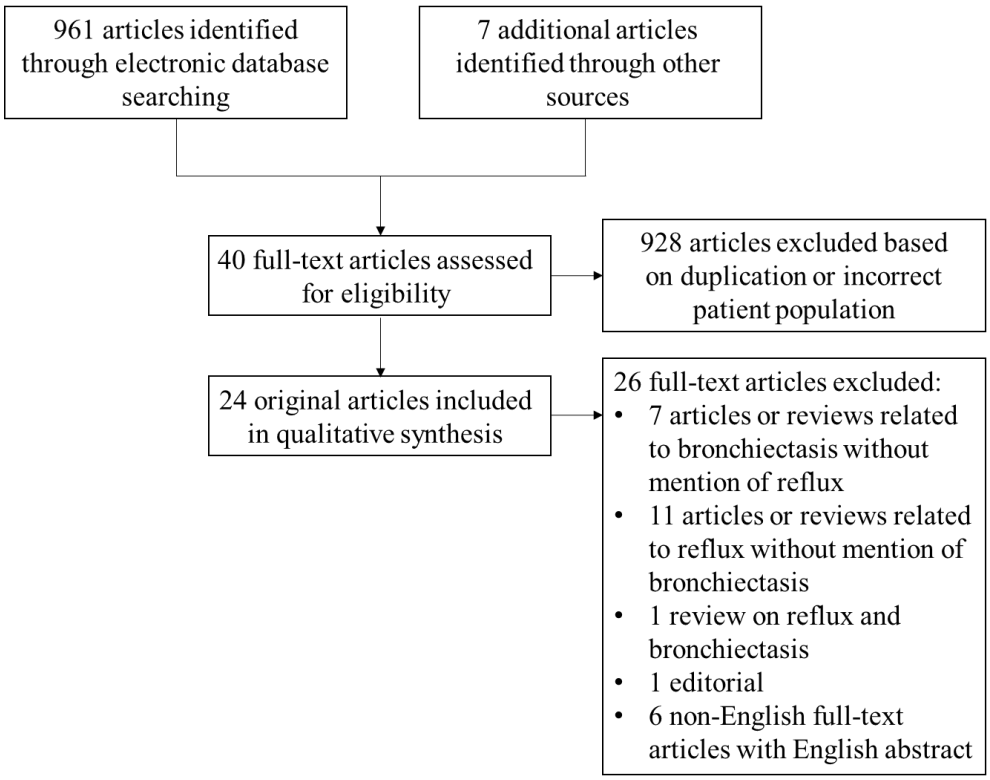

Figure 2-3 Synthesis of electronic database literature search

2.3.2 Prevalence of GORD in bronchiectasis

The prevalence of GORD in individuals with bronchiectasis has been explored in a number of studies to date.[15, 23, 29, 274, 275, 283-292] A range of diagnostic tools have been used, including symptom assessment, questionnaires and objective measurements, outlined in Table 2-1. Based on self-reported symptoms and questionnaires, the prevalence of GORD ranges from 34% to 74%.[15, 23, 29, 274, 291, 292] Although a detailed clinical history of symptom presentation is recommended, this method of diagnosis is reliant upon the provocation of symptoms by reflux events, which in the event of asymptomatic or clinically silent reflux is not a reliable indicator. By comparison, according to oesophageal pH monitoring, prevalence ranges from 11% to 75% were noted.[274, 275, 283-286, 288, 290] Such a wide spread may be related to several factors, such as selective investigation which may miss patients with silent reflux, the application of different GORD criteria, and whether tests were undertaken on or off anti-reflux medication. Mixed patterns of reflux are evident, with distal reflux only, proximal reflux

only, and a mix of both demonstrated. In those with bronchiectasis, a prevalence greater than that seen in COPD and more than twice that of healthy controls has been reported for proximal and distal reflux.[274] GORD can affect patients with mild, moderate and severe bronchiectasis and appears to be particularly prevalent among bronchiectasis patients with co-existing NTM disease.[15, 286] The confirmed presence of asymptomatic reflux in 42% to 73% of bronchiectasis patients emphasises the importance of objective confirmation of GORD in certain individuals.[274, 286]

Table 2-1 Summary of prevalence studies of gastro-oesophageal reflux disease and associations with disease severity in bronchiectasis

Original paper	Patient group	Investigations	Population and study design	Outcome
Ahmed *et al.*, 1995[283]	Stable bronchiectasis	Single probe 24-hour oesophageal pH monitoring Tracheal monitoring	19 Adults Cohort Prospective	Prevalence of reflux = 42% using DeMeester score on pH monitoring Positive correlation with symptoms of nocturnal reflux and distal reflux on pH-monitoring No microaspiration of tracheal contents demonstrated on tracheal monitoring
Chen *et al.*, 1998[284]	Stable bronchiectasis during physiotherapy	Single probe 24-hour oesophageal pH-monitoring	32 Adults (bronchiectasis n=23, chronic bronchitis, n=9) Cohort Prospective	Prevalence of reflux = 41% using DeMeester score on pH monitoring Chest physiotherapy including postural drainage, percussion and forced expiration techniques in different positions did not induce or increase the incidence of reflux events in bronchiectasis or chronic bronchitis patients with or without confirmed GORD
Sweet *et al.*, 2006[285]	End-stage bronchiectasis	Symptomatic evaluation (non-validated questionnaire) Manometry Dual-probe 24-hour oesophageal pH monitoring	4 Adults Cohort Prospective	Prevalence of distal reflux = 75% using DeMeester score on pH monitoring; prevalence of proximal reflux = 50% Poor concordance between symptoms and pH diagnosis of GORD Increased oesophageal length noted in bronchiectasis patients

Table 2-1 (continued) Summary of prevalence studies of gastro-oesophageal reflux disease and associations with disease severity in bronchiectasis

Original paper	Patient group	Investigations	Population and study design	Outcome
Koh *et al.*, 2007[286]	Non-tuberculous mycobacteria (NTM) bronchiectasis	Symptomatic evaluation (no questionnaire) Single probe 24-hour oesophageal pH monitoring	56 Adults Cohort Prospective	Prevalence of reflux = 26% using DeMeester score on pH monitoring; clinically silent reflux = 73% (no reflux on symptom assessment in 27%) Presence of GORD associated with: • Increased positivity for AFB on sputum smear • Increased lobar extent of bronchiectasis and bronchiolitis
Banjar *et al.*, 2007[287]	Stable bronchiectasis	Barium swallow, milk scan or combination of both where clinically indicated	151 Children Cohort Prospective	Prevalence of GORD = 32% using: • Barium swallow alone 67% • Milk scan alone 21% • Combination of both radiological procedures 12% 45% of those diagnosed with GORD required Nissen fundoplication
Fortunato *et al.*, 2008[288]	End-stage bronchiectasis	Dual probe 24-hour oesophageal pH-monitoring preceded by conventional oesophageal manometry	7 Adults Cohort Prospective	Prevalence of reflux = 50% using DeMeester score on pH monitoring Bronchiectasis patients had highest prevalence of GORD and highest mean DeMeester scores compared to all other lung diseases Abnormal manometry noted in 71% with LOS hypotonia in 57% and UOS hypotonia in 14%
Zaid *et al.*, 2010[289]	Stable bronchiectasis	Barium swallow and/or single probe 24-hour oesophageal pH monitoring where clinically indicated	92 Children Cohort Retrospective	Prevalence of GORD = 11%

Table 2-1 (continued) Summary of prevalence studies of gastro-oesophageal reflux disease and associations with disease severity in bronchiectasis

Original paper	Patient group	Investigations	Population and study design	Outcome
Lee *et al.*, 2012[290]	Stable bronchiectasis during physiotherapy	Dual-probe 24-hour oesophageal pH-monitoring	30 Adults Cohort Prospective	Prevalence of reflux = 40% using DeMeester score on pH monitoring 57% of all bronchiectasis patients experienced GORD during at least one physiotherapy task but irrespective of GORD diagnosis, there were fewer distal reflux episodes compared to background reflux time during all physiotherapy interventions of PEP, 6MWT and upper limb movements. No significant difference in reflux with physiotherapy type
Mandal *et al.*, 2013[291]	Stable bronchiectasis	Hull Airway Reflux Questionnaire (HARQ)	163 Adults Cohort Prospective	Prevalence of reflux = 74% Presence of GORD associated with: • Increased cough severity and reduced HrQoL • Increased sputum inflammatory markers • Reduced FEV1% predicted • Increased radiological severity • Increased chronic infection and polymicrobial growth • Increased exacerbations
Lee *et al.*, 2014[274]	Mild (n=15) and moderate (n=12) Bronchiectasis	Carlsson-Dent reflux symptom questionnaire Dual probe 24-hour oesophageal pH monitoring	27 Adults Cohort Prospective	Prevalence of reflux = 40% using questionnaires Clinically silent reflux = 42% using dual-probe pH monitoring No association with sputum pepsin or markers of severity

Table 2-1 (continued) Summary of prevalence studies of gastro-oesophageal reflux disease and associations with disease severity in bronchiectasis

Original paper	Patient group	Investigations	Population and study design	Outcome
McDonnell *et al.*, 2015[29]	Stable bronchiectasis	Evaluation on high resolution computed tomography (HRCT) by independent expert radiologist	81 Adults Cohort Prospective	Prevalence of symptomatic reflux on PPI = 41% Prevalence of confirmed hiatal hernia = 36% Presence of a hiatal hernia associated with: • Increased prevalence of GORD • Increased no. of lobes affected • Increased prevalence of cystic bronchiectasis • Decreased parenchymal attenuation • Reduced FEV1% • Increased disease severity (using BSI and FACED)
McDonnell *et al.*, 2015[292]	Stable bronchiectasis	Symptomatic evaluation and medication review	212 Adults Cohort Retrospective	Prevalence of reflux = 34% Presence of GORD associated with: • Increased cough, sputum production and wheeze • Increased exacerbations and hospitalisations • Increased lobar extent and cystic bronchiectasis • Reduced FEV1% • Increased *P. aeruginosa* infection and polymicrobial culture growth • Increased disease severity (BSI) OR 2.2 (95% CI 1.1-6.7) • Increased mortality OR 2.5 (95% CI 1.1-7.8)

Table 2-1 (continued) Summary of prevalence studies of gastro-oesophageal reflux disease and associations with disease severity in bronchiectasis

Original paper	Patient group	Investigations	Population and study design	Outcome
Lee *et al.*, 2015[275]	Stable bronchiectasis (n=10), COPD (n=10), healthy controls (n=10)	Dual probe 24-hour oesophageal pH-monitoring Exhaled breath condensate pepsin	30 Adults Case control Prospective	Prevalence of reflux = 40% using definition of positive distal and proximal reflux on oesophageal pH monitoring Prevalence of reflux = 70% using sputum pepsin and 60% using exhaled breath condensate (EBC) pepsin Presence of EBC pepsin associated with: • Moderate correlation of sputum pepsin • No correlation with total DeMeester score, distal reflux index or proximal reflux index on oesophageal pH monitoring • No association with lung function
McDonnell *et al.*, 2016[23]	Stable bronchiectasis	Symptomatic evaluation and medication review	986 Adults Cohort Prospective	Prevalence of reflux = 34% Presence of GORD associated with: • Increased mortality (GORD non-survivors 48%, GORD survivors 32%; p=0.001)
Aksamit *et al.*, 2017[15]	Stable bronchiectasis (US registry data)	Symptomatic evaluation	1789 Adults Cohort Prospective	Prevalence of reflux = 47% Presence of GORD associated with: • NTM disease in bronchiectasis (GORD NTM 51%, GORD non-NTM 40%; p<0.01)

2.3.2 Presence of pulmonary microaspiration in bronchiectasis

Surrogate indicators of pulmonary microaspiration of gastric contents have also been examined in bronchiectasis. Pepsin in sputum samples has been detected in 26% to 70% of individuals with mild to moderate bronchiectasis.[274, 275] Although no significant association between oesophageal pH monitoring indices or lung disease severity and pepsin concentrations in sputum has been demonstrated to date, this has been observed previously in individuals with other types of lung disease.[293-295] This may be explained by isolated reflux events that could be aspirated being insufficiently frequent to contribute to the criteria defining GORD. A pilot study of exhaled breath condensate (EBC) in ten individuals with bronchiectasis found pepsin in 60%, irrespective of a diagnosis of GORD on oesophageal pH monitoring.[275] The EBC pH was significantly lower in bronchiectasis patients compared to controls and was strongly correlated with higher concentrations of EBC pepsin.[275] Low EBC pH may be related to several factors including airway inflammation, oxidative stress, bacterial colonisation or severe GORD in bronchiectasis. Low EBC pH has been related to severe GORD symptoms in COPD, suggesting that EBC pH may reflect acid reflux rather than tracheobronchial inflammation.[242]

2.3.4 Influence of GORD on bronchiectasis severity

The relationship between the severity of bronchiectasis and GORD remains somewhat controversial. Although some studies performed to date have observed significant relationships between GORD and markers of bronchiectasis disease severity (Table 2-1), the majority of these have been based on a subjective evaluation of GORD determined by symptom evaluation, questionnaires and medication review. Three large prospective observational cohort studies suggest that GORD (particularly in the presence of a hiatal hernia) is associated with increased symptoms, increased exacerbations and hospitalisations, increased radiological severity, increased colonisation rates, reduced lung function and reduced HrQoL in bronchiectasis patients.[29, 291, 292] An increase in mortality has been described in two studies, a single centre study of 212 patients and a

multicentre study of 986 patients.[23, 292] Koh *et al.* were the first and only group using 24h oesophageal pH monitoring to report an association with increased radiological disease extent in their cohort of bronchiectasis patients with co-existing NTM.[286] The increased prevalence of GORD in bronchiectasis and NTM has also been observed among patients in the US bronchiectasis registry.[15] Ahmed *et al.* described a correlation between symptoms of nocturnal reflux and distal reflux on pH-monitoring, which suggests that GORD may influence nocturnal respiratory status in some patients.[283] Two case-control studies of GORD in bronchiectasis failed to observe any association with reduced lung function or other markers of disease severity. However, due to the difficulties in recruiting, these studies were significantly underpowered to detect such effects, and a single dimension of time may be insufficient to accurately reflect the relationship between GORD and bronchiectasis.[274, 275]

2.3.5 Role of Helicobacter pylori in bronchiectasis

H. pylori is a pathogenic organism linked with a number of gastric (gastritis, peptic ulcer, gastric, colorectal and pancreatic malignancy) and non-gastric (ischaemic heart disease, cerebrovascular disease, diabetes mellitus, vitamin B12 deficiency, and idiopathic thrombocytopenic purpura) disorders.[296] Interestingly, a potential role has also been described for lung diseases including bronchiectasis (Table 2-2), COPD and lung cancer.[297-305] Different mechanisms of action have been proposed, ranging from the induction of a low grade inflammatory state to the occurrence of molecular mimicry mechanisms.[296, 306] There are no known common factors implicated in the susceptibility to both bronchiectasis and *H. pylori* infection, but it has been hypothesised that aspiration or inhalation of *H. pylori* or its endotoxins into the respiratory tract from upper respiratory territories or the gastric reservoir, particularly in bronchiectasis patients with symptomatic GORD, could be an underlying mechanism of the pathogenic role of *H. pylori* in bronchiectasis.[298] Given the lack of bronchial findings in the majority of studies, it is unlikely that *H. pylori* plays a direct role in the pathogenesis of bronchiectasis; however, we cannot exclude an indirect role of the products of *H. pylori* in the pathogenesis of bronchiectasis. Further work is also needed

to corroborate previous findings that suggest *H. pylori* may be responsible for increased disease severity, manifest by reduced lung function and increased radiological severity.[298, 303]

Table 2-2 Summary of clinical studies assessing the role of *Helicobacter pylori* in bronchiectasis

Original paper	Patient group	Investigations	No. of patients	Outcome
Tsang *et al.*, 1998[297]	Stable bronchiectasis (n=100), healthy controls (n=94)	*H. pylori* serology	194 Adults Case control Prospective	High seroprevalence of *H. pylori* among bronchiectasis patients *vs.* controls = 76% *vs*. 54%; p=0.001 Positive correlation with increased sputum volume and age No association with lung function or causes of bronchiectasis
Tsang *et al.*, 1999[298]	Stable bronchiectasis (n=100), healthy controls (n=94)	Symptomatic evaluation (A patient questionnaire to identify bowel disease) Anti- *H. Pylori* Cag A serology	194 Adults Case control Prospective	Symptomatic prevalence of reflux = 32% Positive anti- *H. Pylori* Cag A serology in of bronchiectasis patients *vs*. controls = 24% *vs*. 12%; p=0.03 No association with anti- *H. Pylori* Cag A and lung function, sputum volume, respiratory symptoms or upper gastrointestinal symptoms Positive correlation of patients reporting acid reflux or upper abdominal distension with reduced FEV1% and FVC%
Yalcin *et al.*, 2002[299]	Idiopathic bronchiectasis	Bronchoalveolar lavage (BAL) fluid PCR	30 Children Cohort Prospective	PCR for *H. Pylori* negative in all BAL samples

Table 2-2 (continued) Summary of clinical studies assessing the role of *Helicobacter pylori* in bronchiectasis

Original paper	Patient group	Investigations	No. of patients	Outcome
Ilvan *et al.*, 2004[300]	Male patients with bronchiectasis (n=31), healthy male controls (n=56)	*H. pylori* serology Bronchial brush and biopsy for urease activity, culture and histopathological examination	87 Adults Case control Prospective	Seroprevalence of *H. pylori* noted in 58% bronchiectasis patients *vs.* 68% healthy controls *H. pylori* was not isolated from protected brush or mucosal biopsy samples in any patient and urease test was negative in all patients No associations were observed between *H. pylori* seropositivity and sputum volume, lung function or radiological extent
Angrill *et al.*, 2006[301]	Stable bronchiectasis (n=46), controls undergoing bronchoscopic exploration in search of a primary malignancy (n=8)	*H. pylori* serology Immunostaining of bronchial biopsy for anti- *H. pylori* antibody	54 Adults Case control Prospective	Seroprevalence of *H. pylori* among bronchiectasis patients = 46% No evidence of *H. pylori* was obtained in the bronchial samples of bronchiectasis patients or controls
Gulhan *et al.*, 2007[302]	Stable bronchiectasis (n=26), controls without pulmonary disease, (n=20)	*H. pylori* serology Bronchoalveolar lavage (BAL) fluid PCR and ELISA PCR in surgically removed tissues	46 Adults Case control Prospective	Seroprevalence of *H. pylori* noted in 92% bronchiectasis patients *vs*. 80% controls PCR for *H. pylori* negative in all BAL samples from patients and controls and in surgically resected tissue in bronchiectasis patients

Table 2-2 (continued) Summary of clinical studies assessing the role of *Helicobacter pylori* in bronchiectasis

Original paper	Patient group	Investigations	No. of patients	Outcome
Aydin Teke *et al.*, 2016[303]	Stable bronchiectasis (n=41), healthy controls (n=16)	Bronchoalveolar lavage (BAL) fluid PCR and culture Gastric juice PCR and culture Urea breath test	57 Children Case control Prospective	PCR for *H. pylori* in BAL positive in 22% bronchiectasis patients *vs.* 19% controls ($p>0.05$) PCR for *H. pylori* in gastric juice positive in 39% bronchiectasis patients *vs.* 44% controls ($p>0.05$) Urea breath test positive in 27% bronchiectasis patients *vs.* 19% controls ($p>0.05$) No associations was observed between BAL *H. pylori* positivity and lung function Positive association between BAL *H. pylori* positivity and increased CT score was observed ($p<0.05$)

2.3.6 Treatment of GORD in bronchiectasis

Two retrospective studies assessing potential treatment strategies for GORD have been performed in the bronchiectasis population (Table 2-3). In a recent study of 257 bronchiectasis patients with GORD, a comparison of 27 patients treated with long-term PPIs compared to 230 without PPI treatment, was performed. No significant differences were observed between groups in terms of lung function 6 months after PPI therapy. However, there was a significant improvement in lung function in patients with high BMI in the PPI treatment group, thought to result from obesity causing increased oesophageal acid exposure time compared to the non-obese population.[307] There have been no RCTs of anti-reflux therapy in this patient population, and the effects of pharmacological management on other markers of disease severity, the co-occurrence of respiratory and GORD symptoms, and the use of respiratory medications remain to be clarified.

A retrospective review of the clinical outcomes of seven patients with GORD-related deteriorated bronchiectasis showed that active anti-reflux treatment with Stretta radiofrequency (SRF) and/or laparoscopic fundoplication was beneficial to patient symptoms and outcome.[308] Patients were followed up for a period of one to five years. Typical GORD symptoms, respiratory symptoms, medication consumption and general health status were assessed during follow-up. GORD symptoms disappeared in five people and were significantly improved in the remaining two.[308] Complete remission of both respiratory symptoms and bronchiectasis exacerbations was reported in two patients. Four had significantly improved respiratory symptoms to mild or moderate degrees as well as reduced or zero bronchiectasis exacerbations, enabling them to resume normal physical and social functions.[308] Surgical management, with a Nissen Fundoplication, has been successfully applied to bronchiectasis patients awaiting transplantation, with reductions in symptoms of GORD as well as of lung disease.[309, 310] Anti-reflux surgery is not widely used in bronchiectasis but should be considered when medical management fails, especially when GORD remains severe in individuals with bronchiectasis at risk of respiratory deterioration.

Table 2-3 Summary of clinical studies assessing treatment options for GORD in patients with bronchiectasis

Original paper	Patient group	Treatment intervention	No. of patients	Outcome
Hu 2014 *et al.*, [308]	Deteriorating bronchiectasis in presence of GORD	Stretta radiofrequency (SRF) and/or laparoscopic fundoplication	7 Adults Cohort Retrospective	Laparoscopic fundoplication (n=2) Laparoscopic fundoplication with repair of hiatal hernia (n=2) Stretta radiofrequency (n=2) Combined laparoscopic fundoplication and Stretta radiofrequency (n=1) Significant reduction in reflux and respiratory symptoms and exacerbations/hospitalisations noted on follow-up with negation of therapy for GORD in n=4 patients.
Ahn 2016 *et al.*, [307]	Bronchiectasis and GORD with (n=27) or without (n=250) long-term PPI therapy	PPI therapy	257 Adults Cohort Retrospective	No significant differences were observed between groups in terms of lung function 6 months after PPI therapy. A significant improvement in lung function was noted in patients with high BMI in the PPI treatment group that was significantly related to the severity of obesity.

2.4 Discussion

GORD is a common comorbidity in bronchiectasis with a prevalence ranging from 26%-75%. Associations between the presence of GORD and increased bronchiectasis severity were observed in several large prospective cohort studies manifest by increased symptoms, exacerbations, hospitalisations, radiological extent, chronic infection, and mortality, with reduced pulmonary function and quality of life. However, this effect was not replicated in several case control studies, most likely due to the small sample sizes and reduced power to detect such effects. All the above clinical studies of GORD in bronchiectasis are somewhat limited in that they lack a comprehensive multimodal assessment of GORD that can ascertain the mechanism of disease in this patient population which may increase the vulnerability to GORD in patients with bronchiectasis.

Two of the possible mechanisms by which GORD may impact on the severity of bronchiectasis are vagally mediated reflex bronchoconstriction and pulmonary microaspiration. Vagally mediated reflex bronchoconstriction originates from the shared autonomic innervation between the tracheobronchial tree and the oesophagus. The presence of oesophageal acid in the distal oesophagus activates a GORD-induced vagal reflex arc which stimulates airway irritation, and triggers the release of potent mediators associated with coughing and bronchoconstriction.[311] During microaspiration, refluxed gastric material extends proximally to the oesophagus and then enters the hypopharynx, directly triggering a laryngeal or tracheal response, which may manifest as coughing, wheezing, or a sensation of dyspnoea, and with the potential to enter the lungs and trigger a direct intra-pulmonary inflammatory response.[273] This process of inflammation involves a complex cascade of cellular, molecular and systemic events aimed at benefitting the clearance of noxious agents from the mucosal surface. In most pathophysiological cases, the inflammatory response appears to be in excess of the normal state, and is believed to play a role in disease progression.[273] This mechanism has been extensively studied in CF and IPF patients, whereby higher documented levels of BAL pepsin and bile acids

were found compared with healthy controls, confirming active reflux and microaspiration, not suppressed by PPI therapy.[294, 312] Challenge of primary bronchial epithelial cells cultured from CF lungs with physiologically achievable levels of primary and secondary bile acids led to increased release of the key pro-neutrophilic mediators IL-8 and IL-17.[313] These findings suggest that duodeno-gastro-oesophageal reflux and subsequent microaspiration may contribute to the neutrophilic inflammation that is a hallmark of suppurative lung diseases.[44]

Other possible explanations for pulmonary aspiration secondary to GORD may be related to swallowing dysfunction in bronchiectasis. Precise coordination between swallowing and respiration is necessary, with the swallowing reflex an important defence against airway infection and aspiration.[242] Compared to healthy controls, the swallowing reflex may be impaired in patients with bronchiectasis, with a lack of coordination of the pharyngeal musculature and disruption of the breathing–swallowing coordination which, if altered, may increase the risk of aspiration in patients with bronchiectasis and contribute to exacerbations. Swallowing dysfunction may result from a range of pathologies, including neurological impairment, vocal cord injury, surgery, and radiation, and is often overlooked as an aetiological cause of bronchiectasis.

It has long been postulated that the development of an abdomino-thoracic pressure gradient in patients with chronic respiratory disease may drive reflux and gastric aspiration.[273] Pulmonary hyperinflation contributes to flattening of the hemi-diaphragms and diaphragmatic dysfunction, which not only reduces the diaphragmatic crural support augmenting lower oesophageal pressures, but also changes the angle of the oesophagus, making it easier for reflux to occur. In primary lung disease, the intra-thoracic pressure is negative in relation to the abdominal cavity and varies during the respiratory cycle. As such, pre-existing LOS incompetence may be worsened by factors producing an increased trans-diaphragmatic pressure gradient, e.g. increased negative intra-thoracic pressure during inspiration and bouts of coughing, or with progressive bronchoconstriction of the airways.[29] Heightened anxiety is

also known to aggravate GORD symptoms by increasing acid production.[314] As increased anxiety is common in bronchiectasis, this may be an additional contributory factor to GORD.[23, 315] Respiratory medications, including beta agonists, anticholinergics and corticosteroids, may alter oesophageal function by reducing LOS pressure or oesophageal motility.[269, 316] However, this association could also be a reflection of the severity of lung disease rather than the specific physiological effects of these medications on oesophageal function; further exploration of the cause and effect relationship between respiratory medications and GORD in bronchiectasis is needed.

Although oesophageal motility studies have not yet been extensively applied in bronchiectasis, one study using conventional manometry showed abnormal results in 71% of bronchiectasis patients, manifest by LOS hypotonia in 57% of patients and upper oesophageal sphincter hypotonia in 14%.[288] This would be expected to be relatively low in a healthy patient population. An increased prevalence of hiatal hernias has also been reported in patients with bronchiectasis.[29] A hiatal hernia occurs when part of the stomach protrudes into the thoracic cavity through the oesophageal hiatus of the diaphragm due to disruption of the anti-reflux barrier. If the anatomical flap valve disrupts, the LOS moves above the crural diaphragm, causing it to lose its synergistic configuration, hence both the LOS and diaphragm sphincters become appreciably weaker, compromising oesophageal acid clearance and facilitating the development of reflux.[256, 317] Pandolfino *et al.* clearly demonstrated that a hiatal hernia was associated with an increased distensibility of the gastro-oesophageal junction, increasing the risk of liquid reflux and contributing to the increased acid exposure observed in patients with hiatal hernias.[318] The presence of a post-prandial acid pocket, a layer of unbuffered acidic gastric juice that sits on top of a meal, may be located more proximally or above the crural diaphragm in the presence of a large hiatal hernia, resulting in an increased number of acid reflux episodes during transient LOS relaxations.[319] Treatment with alginate-antacids and azithromycin have been shown to abolish or reduce the pocket, reducing acid oesophageal acid exposure. Azithromycin has been shown to reduce

exacerbations in bronchiectasis and it is therefore tempting to speculate that perhaps some of its therapeutic benefit may relate to its ability to treat reflux.[320, 321]

A deficient mucus barrier function may play a role in facilitating lung injury associated with gastric aspiration.[273] Mucus hypersecretion is a common endpoint in many respiratory diseases, often arising as a result of increased release of mucin granules by epithelial goblet cells or IL-8 driven increased gland-based secretion.[273] In patients with impaired mucociliary clearance, the underlying tissues may become more susceptible to damage with an accumulation of pathogenic microbial organisms from elsewhere in the respiratory or gastro-intestinal tracts. Inflammatory responses may also be driven in part by the presence of specific receptors within the epithelium. Preliminary data suggests that some receptors may be triggered by the presence of gastric juice, pepsin and bile by receptor-mediated endocytosis.[322, 323] Uptake of pepsin in non-acid reflux was shown to cause mitochondrial damage by becoming reactivated inside the cell, changing the expression of several genes implicated in stress and toxicity. Irreversible inhibitors of peptic activity hold promise as a new therapy for reflux.[323]

The potential for horizontal transmission of microorganisms between the gut-lung axis may indicate that the upper gastrointestinal tract could act as a potential reservoir of microorganisms.[324] Chronic colonisation with *P. aeruginosa* has been shown in several studies to be an independent predictor of mortality and lung function decline in bronchiectasis.[19, 49, 325] Horizontal transmission may be suggested by the demonstrated association between reflux and *P. aeruginosa* positivity in CF.[326] After adjusting for age and FEV1, total reflux burden was found to be associated with *P. aeruginosa* positivity, suggesting that an increased reflux burden may predispose patients to *P. aeruginosa* infection and worse lung function.[326, 327] More recently, similar bacterial profiles of CF sputum and gastric juice samples were demonstrated, which were distinct from non-CF gastric juice, perhaps providing novel evidence of an aerodigestive microbiome in

CF.[327] However, it is difficult to establish whether cross-infection relates to swallowing of sputum leading to seeding of the gastrointestinal microbiome or if reflux and aspiration into the lungs may be causative. The microbiome in bronchiectasis is frequently dominated by enteric Gram-negative organisms suggesting that the lower airway is constantly being "replenished" by the upper airway, giving plausibility to there being a link in health and disease between the gut, the upper airway and the lung.[105, 324]

Recent research investigating the impact of bile on the behaviour of *P. aeruginosa* and other CF-associated respiratory pathogens showed that bile increases biofilm formation and quorum sensing in *P. aeruginosa*, driving the switch from acute to persistent infection.[328] Bile also modulates biofilm formation in a range of other CF-associated respiratory pathogens, including *Burkholderia cepacia* and *Staphylococcus aureus*, suggesting, perhaps, that GORD-derived bile could be a host determinant contributing to chronic respiratory infection in chronic suppurative lung diseases.[328, 329]

A mix of demographic factors may also increase the risk of GORD in bronchiectasis. Older age and female sex are well-described risk factors for GORD, and given the median age and female predominance of bronchiectasis patients, an increased co-existence of the two is perhaps not surprising.[23] A larger body mass index is also a risk for GORD, increasing as BMI increases.[330] A greater BMI has been demonstrated in bronchiectasis patients with GORD, and may impact on the contour of the diaphragm, increasing the elastic work of breathing.[29] When combined with respiratory-related risk factors, this may increase the contribution of a higher BMI to GORD in bronchiectasis. Comorbidities, such as ischaemic heart disease, have also been associated with a heightened risk of GORD and have been shown to occur frequently and contribute to mortality in patients with bronchiectasis.[23] Whether these are independent variables, common consequences of age, diet and obesity, or part of an integrated pathway of systemic inflammation, remains to be established.

2.5 Conclusion

In conclusion, GORD is a common comorbidity in patients with bronchiectasis and has a variety of clinical presentations. The index of clinical suspicion should remain high across the spectrum of disease severity in bronchiectasis, and objective measures should be used for diagnostic confirmation where possible due to high detection rates of asymptomatic or clinically silent reflux. The presence of GORD is associated with increased disease severity and mortality in patients with bronchiectasis. Therefore, identifying GORD in bronchiectasis patients may have important therapeutic and prognostic implications, although clinical trial evidence that treatment targeted at GORD can improve outcomes in bronchiectasis is currently lacking.

Chapter 3 – Methodology

Publications relevant to this chapter:

McDonnell MJ, Rutherford RM. Other Predisposing Factors for Bronchiectasis. *In:* Chalmers J, Polverino E, Aliberti S. Bronchiectasis: The EMBARC Manual. Springer International Publishing, 2018; pp. 129-145.

Araújo D, Shteinberg M, Aliberti S, Goeminne PC, Hill AT, Fardon T, Obradovic D, Dimakou K, Polverino E, De Soyza A, McDonnell MJ, Chalmers JD. Standardised classification of the aetiology of bronchiectasis using an objective algorithm. *Eur Respir J.* 2017; 14; 50 (6).

Chalmers JD, Goeminne P, Aliberti S, McDonnell MJ, Lonni S, Davidson J, Poppelwell L, Salih W, Pesci A, Dupont LJ, Fardon TC, De Soyza A, Hill AT. The Bronchiectasis Severity Index: An International Derivation and Validation Study. *Am J Respir Crit Care Med.* 2014; 189(5): 576-585.

*Anwar GA, *McDonnell MJ, Worthy SA, Bourke SC, Afolabi G, Lordan J, Corris PA, De Soyza A, Middleton P, Ward C, Rutherford RM (joint first authors). Phenotyping adults with non-cystic fibrosis bronchiectasis: a prospective observational cohort study. *Respir Med.* 2013; 107(7):1001-1007.

3.1 Introduction

The methodologies described in this chapter relate to the diagnostic work-up of bronchiectasis patients in Galway University Hospitals (GUH) and are universal to all studies performed in this thesis. Detailed additional information pertaining to clinical investigations and laboratory techniques for Chapters 7-9 are also included. Related studies enhancing our methodological approach to key research questions are discussed in the relevant sections. Variations for individual studies will be described in the corresponding chapters.

3.2 Study design and bronchiectasis work-up

3.2.1 Patient population

The studies in this thesis focus on patients with a confirmed clinical and radiological diagnosis of bronchiectasis, defined according to the British Thoracic Society (BTS) 2010 guidelines as a chronic inflammatory lung disease characterised clinically by persistent cough, daily sputum production, shortness of breath and recurrent respiratory tract infections, and radiologically, by the permanent dilation of bronchi.[9] Consecutive patients aged ≥18 years with a diagnosis of bronchiectasis confirmed by a specialist pulmonary physician were enrolled into retrospective (Chapter 6) and prospective (Chapters 4, 5 and 7) cohort and case-control (Chapters 8, 9 and 10) studies respectively.

Patients with cystic fibrosis (CF), traction bronchiectasis due to pulmonary fibrosis or active non-tuberculous mycobacterial (NTM) disease were excluded from all studies due to the different mechanisms contributing to the development and progression of bronchiectasis in these patients. CF represents a distinct patient population defined by the presence of recognised genotypes and dysfunction of the CF transmembrane conductance regulator (CFTR) protein that leads to progressive and permanent disease in these predominantly younger patients.[331] Current consensus suggests that the term “non-CF” bronchiectasis is somewhat misleading as it is often applied

to populations who have not been formally assessed for CF, hence we cannot define our population by the absence of a condition that has not been tested for.[332] Traction bronchiectasis often occurs as a consequence of parenchymal lung disease, resulting from interstitial fibrosis pulling the airway wider, rather than direct damage to the bronchial wall. Similarly, there is mounting evidence to suggest that patients with NTM-related bronchiectasis have a distinct immunological phenotype that results in an imbalance of cytokines, leading to an inability of the host to resist mycobacterial infection.[333, 334] Coupled with the wide geographical and genetic variations in both the prevalence and causative organisms of NTM infection, and the notorious difficulties in culturing NTM in the presence of other organisms, these patients were excluded.

3.2.2 Data collection

In all patients, a comprehensive history was recorded including: age at symptom onset and diagnosis; total duration of symptoms; sex; body mass index (BMI); smoking status and pack year history; aetiological screening for history of previous acute respiratory illnesses or associated diseases or complications and a temporal relationship with onset of bronchiectasis symptoms; baseline bronchiectasis symptoms including clinical data relating to sputum production, appearance, volume and previous colonisation; modified Medical Research Council (mMRC) dyspnoea scale; number of exacerbations; number of severe exacerbations requiring hospitalisation over the previous 24 months; medical comorbidities according to the Charlson Comorbidity Index (CCI); and treatment modalities.[335-338]

Sputum colour was assessed using a sputum colour chart validated in bronchiectasis (Figure 3-1).[335] This differentiates sputum according to three categories: mucoid, mucopurulent and purulent, with colour changes from grey to yellow to green correlating with increased levels of bacterial colonisation and neutrophilic inflammation.[335, 339, 340]

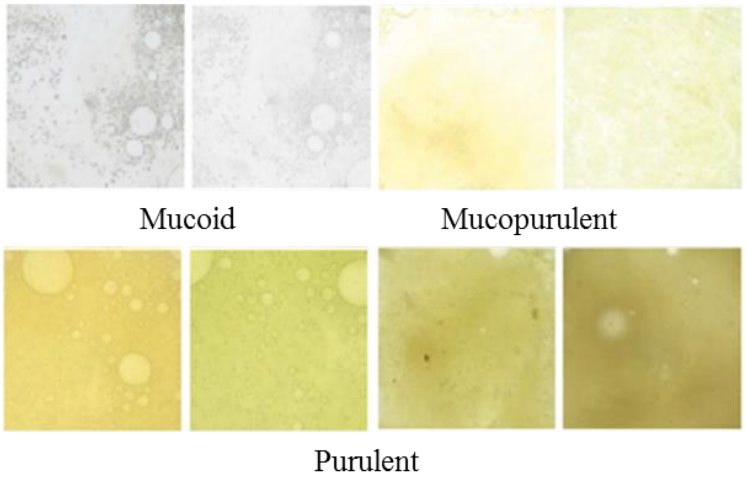

Figure 3-1 Sputum colour chart for bronchiectasis

Individuals' perceived effect of breathlessness on daily activities was assessed using the mMRC dyspnoea scale.[336, 337] This is a simple and valid method of categorising patients with bronchiectasis in terms of their respiratory disability using clinical dyspnoea grades of 0 to 4 according to Table 3-1 below.

Table 3-1 Modified Medical Research Council dyspnoea scale

mMRC dyspnoea scale	
0	Breathlessness only with strenuous exercise
1	Short of breath when hurrying on the level or up a slight hill
2	Slower than most people of the same age on a level surface or Have to stop when walking at my own pace on the level
3	Stop for breath walking 100 metres or After walking a few minutes at my own pace on the level
4	Too breathless to leave the house

An exacerbation was defined according to BTS guidelines as the requirement for antibiotics in the presence of one or more symptoms of increasing cough, increasing sputum volume, worsening sputum purulence, worsening dyspnoea, increased fatigue/malaise, fever, and haemoptysis.[9] Severe exacerbations were defined as unscheduled hospitalisations or emergency

department visits for exacerbations or complications as recorded from patient histories and verified using administrative databases.[9]

The CCI is the most widely utilised method of categorising patients based on the International Classification of Diseases (ICD) diagnosis codes found in administrative data (Table 3-2).[338] Each comorbidity category has an associated weight (from 1 to 6) based on the adjusted risk of mortality or resource use, and the sum of all the weights results in a single comorbidity score for a patient. A score of zero indicates that no comorbidities were found. The higher the score, the more likely the predicted outcome will result in mortality or higher resource use.

Table 3-2 Charlson Comorbidity Index

Condition	Points	Notes
Myocardial infarction	1	
Congestive heart failure	1	
Peripheral vascular disease	1	Includes gangrene, acute arterial insufficiency and thoracic or abdominal or aortic aneurysm
Cerebrovascular disease	1	CVA with no or minor residual effects and patients who have had transient ischaemic attacks
Hemiplegia	2	If hemiplegia, do not count CVA separately. Includes hemiplegia or paraplegia from any cause
Chronic pulmonary disease	1	Includes any chronic lung disease where patients have ongoing symptoms despite treatment
Diabetes	1	Includes all patients with diabetes treated with insulin or medications do not diet alone or gestational diabetes
Diabetes with end-organ damage	2	Includes nephropathy, neuropath and retinopathy secondary to diabetes
Renal disease	2	Includes moderate or severe renal disease, patients on dialysis, post-transplant or those with uraemia
Mild liver disease	2	Includes chronic hepatitis (B or C) or cirrhosis without portal hypertension
Severe liver disease	3	Moderate disease consists of cirrhosis with portal hypertension but without bleeding. Severe disease consists of patients with ascites, chronic jaundice, portal hypertension, history of variceal bleeding or post-transplant.
Gastric or peptic ulcer	1	Treated for
Malignancy	2	Solid organ, lymphoma or leukaemia. Non-metastatic cancer only.
Metastatic solid tumour	6	If metastatic, do not count malignancy separately.
Cognitive impairment	1	
Connective tissue disease	1	
HIV or AIDS	6	

3.2.3 Aetiological assessment

A comprehensive diagnostic bronchiectasis aetiological work-up was performed in all patients according to BTS guidelines and previous studies by our group, including full blood count, serum immunoglobulins (Ig) G, IgA, IgM, total IgE, specific IgE and/or precipitins for *Aspergillus fumigatus*, serum electrophoresis, specific antibody levels (against tetanus toxoid, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b - repeated 6-8 weeks after vaccination to assess response if initially low), and additional investigations in select cases where a specific underlying aetiology was suspected.[9, 34]

Patients with recurrent sinusitis, chronic otitis media and/or infertility in whom a clinical suspicion of primary ciliary dyskinesia (PCD) was considered, were referred for further testing with nasal mucociliary clearance measured by the saccharin test and/or nasal nitric oxide at the National Specialist Centre for investigation of PCD.[341-344] Alpha-1-antitripsin (A1AT) deficiency was evaluated in the presence of emphysema affecting lower lobes on high resolution computed tomography (HRCT) scan and/or significant family history.[345-347] Autoimmunity testing including anti-nuclear antibodies, extractable nuclear antigens, anti-neutrophil cytoplasmic antibodies, rheumatoid factor and anti–citrullinated protein antibody were requested if a rheumatological disease was clinically suspected. Sweat test and CFTR genetic testing were requested in any patient with signs or symptoms suggestive for CF such as symptoms since childhood, upper lobe disease, persistent *Staphylococcus aureus* or *Pseudomonas aeruginosa* growth, features of malabsorption and/or history of infertility.[348, 349]

Evaluation of CT images and aetiological screening bloods enabled differentiation of patients into definitive diagnoses of congenital abnormalities, post-obstructive bronchiectasis, diffuse panbronchiolitis, primary or secondary immunodeficiency, and A1AT deficiency, CF, PCD or Young's syndrome in cases where additional tests were performed. In the presence of a diagnosis of both bronchiectasis and connective tissue disease (CTD), including rheumatoid arthritis (RA), Sjögren's syndrome, systemic

sclerosis, psoriatic arthritis, ankylosing spondylosis or mixed CTD, a diagnosis of CTD-associated bronchiectasis was made. Bronchiectasis associated with inflammatory bowel disease (IBD) was diagnosed if patients had ulcerative colitis or Crohn's disease, a definite symptom onset of bronchiectasis following a confirmed diagnosis of IBD, and no other suggested aetiology for bronchiectasis. If findings were compatible with one of these aetiologies, a definitive diagnosis was achieved. Yellow nail syndrome was diagnosed when examination showed yellow discoloration of dystrophic nails together with bronchiectasis and sinusitis, whether or not patients had other features of the syndrome.[350, 351] Young's syndrome was diagnosed when there was a history of bronchiectasis, sinusitis and azoospermia in males and negative CF testing.[352, 353]

In cases where all the above tests were negative, other possible diagnoses, associations or complications including allergic bronchopulmonary aspergillosis (ABPA), post-NTM infection, post-tuberculosis (TB), chronic obstructive pulmonary disease (COPD), asthma, gastro-oesophageal reflux disease (GORD), overt aspiration, and secondary immunodeficiency were considered. If one of these diseases was the only possible aetiology present and if there were no atypical features, we considered this a probable diagnosis. A serum IgE of $\geq$ 1000 IU/ml, positive Aspergillus precipitins and/or skin prick, a blood eosinophilia of $\geq$ 0.4 and compatible radiology was required to fulfil the diagnosis of ABPA.[354-356] Post-NTM infection was diagnosed in patients with previous positive cultures for NTM and/or nodular changes on HRCT scan, associated with progressive decline in lung function or significant weight loss and recurrent exacerbations unresponsive to standard antibiotics.[334] Post-TB bronchiectasis was diagnosed in patients with clearly documented prior TB and compatible radiology. COPD-associated bronchiectasis was classified in the presence of significant smoking history and airflow obstruction according to Global initiative for chronic Obstructive Lung Disease (GOLD) criteria.[357] Bronchiectasis associated with asthma was diagnosed in patients without post-infective bronchiectasis and with normal or negative results of blood investigations, according to Global Initiative for Asthma (GINA) guidelines.[358] Causation

secondary to COPD or asthma was attributed when bronchiectasis symptoms were reported to develop ≥ 5 years after the initial primary diagnosis. GORD-associated bronchiectasis was considered in patients with self-reported symptoms of heartburn and regurgitation or those with improvement in symptoms after trial of therapy as per the American College of Gastroenterology (ACG) recommendations.[259] Overt aspiration was considered causative in patients with objective confirmation of aspiration on barium swallow studies. Pink's disease was diagnosed based on a history of mercury poisoning in childhood. Mouhnier-Kuhn's syndrome was diagnosed based on the presence of abnormally large air passages on CT and/or bronchoscopy, with dilation of the trachea and main bronchi during inspiration, and constriction and collapse during expiration and coughing.[359] In adults, the diagnostic criteria are diameters of the trachea of >30 mm; right main bronchus > 20 mm; and left main bronchus > 18 mm.[360]

If no association or complication could be attributed, a history of prior severe respiratory infections was investigated. Post-infective bronchiectasis was diagnosed in patients reporting a history of symptom onset within 10 years of a severe respiratory tract infection, such as pneumonia, whooping cough or complicated measles infection. Where a patient reported a history of severe respiratory infections, but with a prolonged symptom free period, a post-infective diagnosis was not attributed. If all screening tests were negative and no association with any other disease was found, a diagnosis of idiopathic bronchiectasis (IB) was made by exclusion of any known cause.

Collaborative international research efforts utilising data from the FRIENDS (Facilitating Research In to Existing National DataSets) and EMBARC (European Multicentre Bronchiectasis Audit and Research Collaboration) bronchiectasis patient registries have generated numerous discussions regarding the standardisation of bronchiectasis aetiology to ensure consistency across multiple patient cohorts. Studies demonstrate wide heterogeneity in the proportion of different aetiologies identified between centres, in part, reflecting possible variations in testing practice or in the

definitions of aetiology used. Clinicians frequently diagnose idiopathic bronchiectasis in the presence of disease associated with bronchiectasis. This extensive phenotyping process has been described previously and has now been incorporated into an aetiological algorithm that should enhance our ability to compare results of different studies across numerous centres (Figure 3-2).[62]

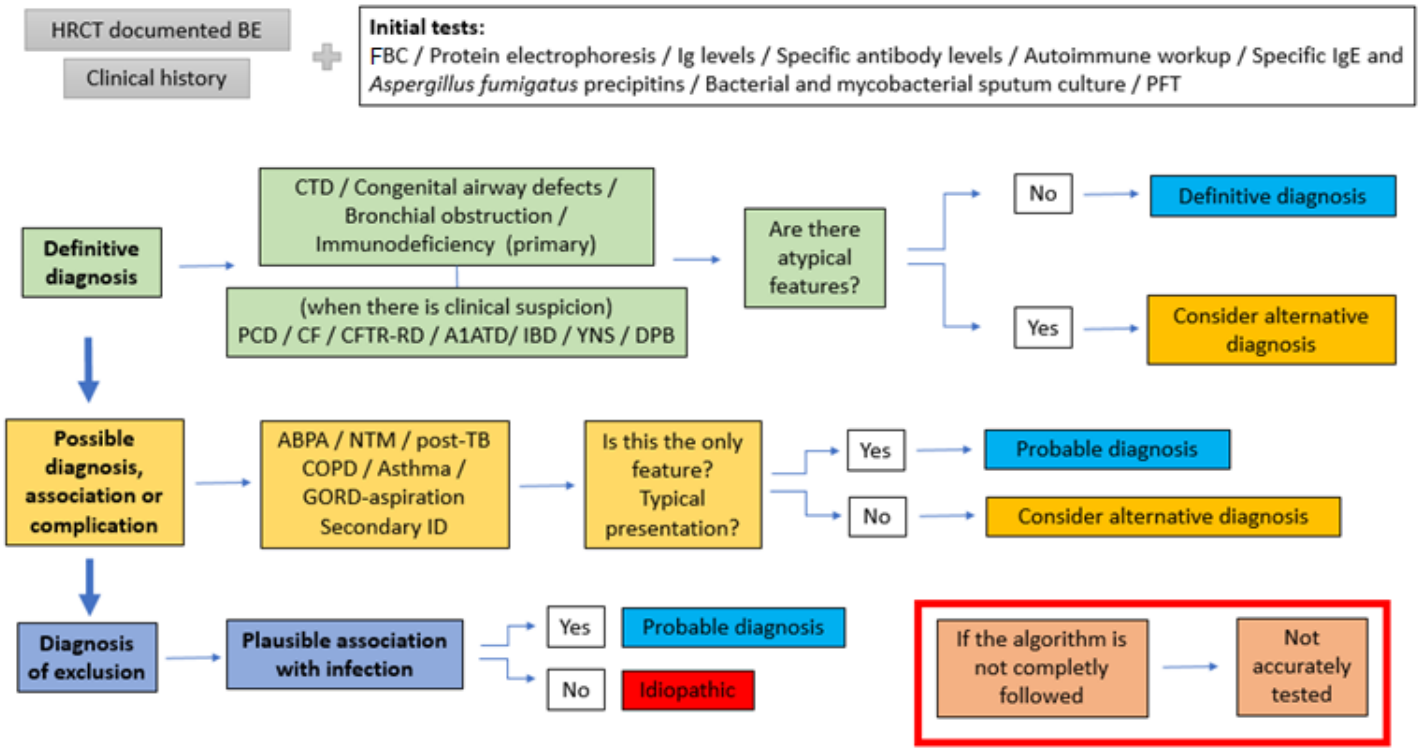

Figure 3-2 Aetiological algorithm of bronchiectasis

FBC: full blood count; Ig: immunoglobulin; PFT: pulmonary function test; CTD: connective tissue disease; PCD: primary ciliary dyskinesia; CF: cystic fibrosis; CFTR-RD: cystic fibrosis transmembrane receptor-related disease; A1ATD:alpha-1-antitrypsin deficiency; IBD: inflammatory bowel disease; YNS: yellow nail syndrome; DPB: diffuse pan-bronchiolitis; ABPA: allergic bronchopulmonary aspergillosis; NTM: non-tuberculous mycobacteria; TB: tuberculosis; COPD: chronic obstructive pulmonary disease; GORD: gastro-oesophageal reflux disease; ID: immune deficiency.

3.2.4 Comorbidity assessment

Comorbidity assessment was performed according to standardised definitions with review of objective assessment and confirmatory tests to verify diagnosis where possible. Below are a few examples of how comorbidity was determined among this patient population in our cohort studies.

GORD: Based on a presumptive diagnosis of GORD in the setting of a self-reported history of typical symptoms of heartburn and regurgitation or by improvement in symptoms after trial of therapy as per American College of Gastroenterology recommendations.[259] Patients were considered to have GORD where a diagnosis of GORD was recorded in the notes by a primary or secondary care physician, or in a patient taking a prescribed anti-reflux medication.

Hypertension: Based on previous guidelines of clinic blood pressure readings of >140/90mmHg on three separate occasions taking the lowest of at least two readings at each visit. Note: since 2011 guidelines, ambulatory and/or home blood pressure measurements are included in the diagnosis of arterial hypertension according to the British Hypertension and European Society of Cardiology guidelines. Patients were considered to have hypertension where a diagnosis of hypertension was recorded in the notes by a primary or secondary care physician, or in a patient taking a prescribed anti-hypertensive medication.

High cholesterol: Based on an objective fasting total cholesterol level of >5 mmol/L for healthy adults and/or >4 mmol/L in high risk patients and/or a ratio of total cholesterol to HDL above 4.The 2014 guidelines allow measurement of cholesterol in non-fasting samples. Patients were considered to have high cholesterol where a diagnosis of high cholesterol was recorded in the notes by a primary or secondary care physician AND objective evidence of cholesterol levels could be assessed, or in a patient taking a prescribed cholesterol medication for primary or secondary prevention.

COPD: Based on the presence of a significant smoking history of >10 pack years and objective confirmation of airflow obstruction according to GOLD criteria. Patients were considered to have COPD where a diagnosis of COPD was recorded in the notes in patients with a significant smoking history AND objective evidence of airflow obstruction in primary or secondary care. In patients prescribed inhaled medications without objective evidence of airflow limitation, a diagnosis of COPD was not recorded.

Osteoporosis: Based on a bone mineral density that is 2.5 SD or more below that of a "young normal" adult (T-score at or below -2.5) on DEXA scanning and/or clinical diagnosis in at-risk individuals who have sustained a low-trauma fracture according to World Health Organization guidelines. Patients were considered to have osteoporosis where a diagnosis of osteoporosis was recorded in the notes by a primary or secondary care physician AND objective evidence on DEXA scanning or in patients taking bisphosphonate treatment.

CTD: Based on assessment by an expert rheumatologist according to American College of Rheumatology guidelines incorporating history, physical examination, laboratory and radiographic findings according to individual disease.

Myocardial infarction (MI): Based on a rise and/or fall of cardiac biomarker values plus at least one of: symptoms of ischaemia, new or presumed new significant ST-segment–T wave (ST–T) changes or new left bundle branch block (LBBB), development of pathological Q waves in the ECG, imaging evidence of new loss of viable myocardium or new regional wall motion abnormality and/or identification of an intracoronary thrombus by angiography or autopsy according to the universal definition in the European Cardiac Society (ESC) guidelines. Patients were considered to have had an MI where a diagnosis was recorded in the notes AND objective evidence in the form of blood tests, ECG or imaging studies was available.

Chronic heart failure: Based on the presence of symptoms and signs of heart failure with measurement of ejection fraction on echocardiography to

determine if reduced or preserved and to determine the presence of structural heart disease or diastolic dysfunction in the presence of a preserved ejection fraction according to the European Society of Cardiology guidelines. Patients were considered to have heart failure where a diagnosis of heart failure was recorded in the notes or patients were prescribed heart failure medications AND objective evidence on echocardiography was available.

Depression: Based on the presence of at least four out of ten depressive symptoms, present for at least 2 weeks for most of every day according to ICD-10 criteria. Patients were considered to have depression where a diagnosis of depression was recorded in the notes by primary or secondary care physicians or in patients prescribed anti-depressant medications.

Solid tumour/metastatic malignancy: Based on assessment by an expert physician and/or oncologist with appropriate objective staging imaging and histopathological investigations.

Peripheral vascular disease: Based on the presence of symptoms and signs of peripheral vascular disease, objective measurement of ankle brachial pressure indices in primary care setting or confirmatory imaging investigations such as Doppler ultrasound, angiography or digital subtraction arteriography according to European Society of Cardiology guidelines. Patients were considered to have peripheral vascular disease where a diagnosis of peripheral vascular disease was recorded in the notes AND objective evidence was available.

Atrial fibrillation: Based on an irregular heart rate with ECG confirmation. Patients were considered to have atrial fibrillation where a diagnosis of atrial fibrillation was recorded in the notes by a primary or secondary care physician AND objective ECG evidence was available.

Chronic kidney disease (CKD): Based on objective reduced eGFR values for staging of chronic kidney disease as per national and international guidelines.

Diabetes mellitus: Based on plasma glucose criteria, with fasting glucose >7.0 mmol/L, random or 2-h glucose post-oral glucose tolerance test > 11.1 mmol/L, or HbA1C ≥6.5% according to the American Diabetes Association guidelines. Patients were considered to have diabetes mellitus where a diagnosis of diabetes mellitus was recorded in the notes by a primary or secondary care physician AND objective blood glucose levels were available.

Cerebrovascular accident (CVA)/Transient ischemic attack (TIA): Based on the presence of symptoms and signs with confirmatory imaging findings on CT brain, MRI brain, Doppler USS neck or other investigations according to the American Heart Association/ American Stroke Association guidelines. Patients were considered to have a CVA/TIA where a diagnosis was recorded in the notes, patients were prescribed anticoagulant medications AND objective evidence on imaging was available.

RA: Based on assessment by an expert rheumatologist according to American College of Rheumatology guidelines incorporating history, physical examination, laboratory and radiographic findings with four of seven of the diagnostic criteria present, one of which must have been present for a minimum of 6 weeks.

Pulmonary hypertension (PH): Based on an increase in mean pulmonary arterial pressure ≥25 mmHg at rest as assessed by echocardiography or right heart catheterisation where performed according to the ESC/ERS guidelines for the diagnosis and treatment of PH, 2015. Patients were considered to have pulmonary hypertension where a diagnosis was recorded in the notes AND objective evidence on imaging was available.

Thromboembolic disease: Based on objective confirmation of deep vein thrombosis on Doppler ultrasound or pulmonary embolism on CT pulmonary angiography (CTPA). Patients were considered to have thromboembolic disease where a diagnosis was recorded in the notes, patients were prescribed anticoagulant medications AND objective evidence on imaging was available.

Overt aspiration: Based on objective confirmation of aspiration on barium swallow imaging studies. Patients were considered to have overt aspiration where a diagnosis was recorded in the notes AND objective evidence on imaging was available.

Leukaemia: Based on assessment by an expert oncologist according to World Health Organisation guidelines incorporating history, physical examination, full blood count and film, imaging, bone marrow biopsy and cytogenetic abnormality confirmation.

Lymphoma: Based on assessment by an expert oncologist according to World Health Organisation guidelines incorporating history, physical examination, full blood count and film, imaging and histopathological confirmation.

Iron deficiency anaemia: Based on objective low iron stores and a haemoglobin level two standard deviations below normal as per national and international guidelines.

Cognitive impairment: Based on a clinical diagnosis by a primary or secondary care physician whereby acquired cognitive deficits in more than one area of cognition interfere with normal activities of daily living and represent a decline from a previously higher level of functioning. Patients were considered to have cognitive impairment where a diagnosis was recorded in the notes, deficits on structured memory tests were noted, and/or patients were prescribed medications to treat dementia.

3.2.5 High resolution computed tomography

HRCT images were acquired on a 64-slice multi-detector CT scanner (Somatom Sensation Cardiac 64, Siemens, Erlangen, Germany). Inspiratory spiral and expiratory sequential scans were performed at an initial collimation of 5mm, and reconstructed at 1.0mm thin slices at section intervals of 10mm. Scanning parameters included a kVp of 120 (dose-adjusted), 40mAs (care-dose), rotation time of 0.5s and a pitch of 1.4. Intravenous contrast media was not administered and scans were performed with patients positioned supine.

All scans were reported by radiologists with expertise in HRCT imaging at the time of scanning and reviewed by a specialist pulmonary physician.

Radiological severity of bronchiectasis was assessed using a modified Reiff score (MJM), which assesses the number of lobes involved (with the lingula considered to be a separate lobe, making a total of 6 lobes) and the degree of dilatation (tubular = 1, varicose = 2, and cystic = 3). The maximum score is 18 and the minimum score is 1.

Subsequent independent review for confirmation and scoring of disease severity according to the modified Bhalla score, validated for use in bronchiectasis, was performed by a pulmonary physician (MJM) and expert thoracic radiologist (JD) in studies outlined in Chapters 8 and 10, with calculation of inter-rater variability assessed using Cohen's kappa statistics with linear weighting.[66] The extent of bronchiectasis, severity of bronchial dilatation, bronchial wall thickness, presence of mucus plugging in large and small airways, and decrease in parenchymal attenuation were scored for each lobe, with the lingula considered a separate lobe. Total lung scores for each abnormality were defined as the mean score from all lobes for each HRCT feature. The proportion of cystic versus varicose or cylindrical bronchiectasis was also recorded along with the total number of lobes involved. Lobar predominance was assessed by calculating the mean scores for all HRCT features per lobe. A combined HRCT total score for all HRCT features across all lobes was subsequently derived from summing the individual scores, range 0-78 (Table 3-3).

Table 3-3 Modified Bhalla high resolution computed tomography scoring system

HRCT abnormality	Grade	Interpretation
Presence and extent of bronchiectasis	0	No disease
	1	Bronchiectasis affecting one or part of one bronchopulmonary segment (localised)
	2	Bronchiectasis in more than one bronchopulmonary segment (extensive)
	3	Generalised cystic bronchiectasis
Bronchial dilatation relative to adjacent pulmonary artery	0	Normal
	1	100–200% arterial diameter
	2	200–300% arterial diameter
	3	>300% arterial diameter
Bronchial wall thickness relative to adjacent pulmonary artery	0	Normal
	1	<50% arterial diameter
	2	50–100% arterial diameter
	3	>100% arterial diameter
Presence of mucus plugging within the large airways	0	None
	1	Present
Presence of mucus plugging within the centrilobular bronchiole	0	None
	1	Present
Extent of decreased attenuation	0	Normal
	1	≤50% lobar volume
	2	>50% lobar volume

3.2.6 Pulmonary function tests

Pulmonary function tests (PFTs) were performed at presentation and subsequent follow-up visits allowing calculation of Forced Expiratory Volume (FEV1), Forced Vital Capacity (FVC) and the FEV1/FVC ratio using a Sensormedics V-max 22 device (Table 3-4). Values were expressed as a percentage predicted for age, sex, height and ethnicity employing European Respiratory Society (ERS)/American Thoracic Society (ATS) reference ranges (Figure 3-3).[361, 362]

Table 3-4 Definitions of pulmonary function tests

Forced Expiratory Volume in One Second (FEV1)	The volume of air that can be forcibly exhaled from the lungs in the first second of a forced expiratory manoeuvre, expressed in Litres at body temperature and ambient pressure saturated with water vapour (BTPS).
Forced Vital Capacity (FVC)	The volume of air that can be forcibly and maximally exhaled out of the lungs until no more can be expired, expressed in Litres at BPTS.
FEV1/FVC ratio	This indicates what percentage of the total FVC was expelled from the lungs during the first second of forced exhalation and can be classified as normal, obstructive or restrictive (Figure 3-3)

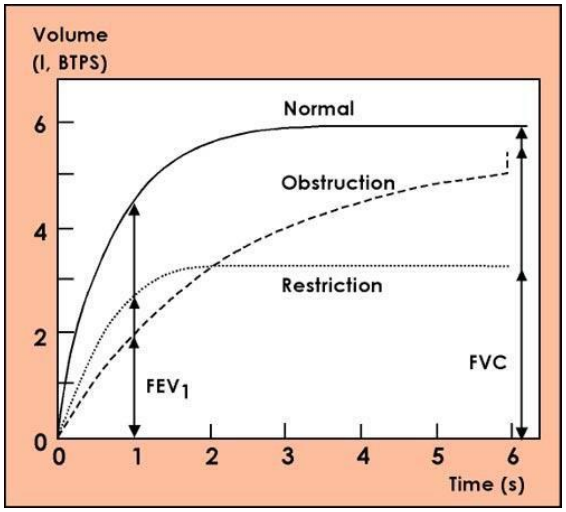

Figure 3-3 Volume-time graph demonstrating normal, obstructive and restrictive airways

During these tests, patients were seated. A mouthpiece and nose clip were positioned to prevent escape of airflow during expiration. After a few breaths enabling the patient to relax, the patient was asked to take a maximal breath in, followed by a hard, fast breath out to full expiration. To achieve accurate, reproducible tests, expiration must be both forceful and prolonged. This test was repeated for a minimum of three and a maximum of eight times as per ERS/ATS recommendations to ensure precision and reproducibility, with the highest of three technically satisfactory measurements recorded. [361, 362] Grading of FEV1/FVC obstruction was performed according to GOLD criteria whereby FEV1% ≥ 80% predicted was categorised as mild disease, 50-79% predicted as moderate disease, 30-49% predicted severe disease and very severe disease <30% predicted.

3.2.7 Bronchoscopy and clinical microbiology

Bronchoscopy and bronchoalveolar lavage (BAL) for bacterial, mycobacterial, and fungal cultures was routinely performed in the initial diagnostic workup of bronchiectasis patients with suggested spontaneous early-morning sputum culture every 6 months for microbiological surveillance on follow-up.

Bronchoscopy was performed using a 4.9mm external diameter flexible fibreoptic bronchoscope (Olympus BF45.5, Tokyo, Japan) in accordance with established BTS guidelines.[363] Bronchoscopy was performed with the patient in a semi-recumbent position. Patients were pre-medicated with 2-4mg intravenous midazolam and 2.5-5 µcg of intravenous alfentanil according to BTS guidelines for diagnostic flexible bronchoscopy in adults to achieve adequate sedation and improve bronchoscopic tolerance. Local anaesthesia was administered by way of 10% lignocaine spray to the oropharynx followed by 1 mL aliquots of topical 4% lignocaine to the vocal cords and tracheal lumen via the bronchoscope, up to a maximum of 7 mg/kg body weight. Supplemental oxygen was administered throughout the procedure. Nasal intubation was generally preferred but the oral route was used if this was unattainable. Photographs of the larynx and vocal cords were taken prior to intubation of the main bronchus to allow assessment and validation of the reflux finding score (RFS). The patients' vital signs and pulse oximetry were monitored throughout. If the patient became unstable or expressed any unwillingness to proceed, the procedure was terminated accordingly. Patients were encouraged to remain for a two-hour period of observation post-procedure as per standard practice.

BAL was performed according to standardised techniques from the lingula or right middle lobe with additional representative samples from the most affected lobe. The latter were subsequently used in the analysis to provide a more accurate assessment of potential differences in the intra-lung heterogeneity of the microbiome by comparing samples from the most affected lobe in bronchiectasis patients versus non-bronchiectatic lungs of

chronic bronchitis patients and healthy volunteer controls. 1 x 60 mL aliquots of normal saline at room temperature were introduced via the bronchoscope and aspirated using a closed sterile collecting system with the returns pooled. A 5-10 mL sample was sent for routine microbiological analysis with the remainder processed within 2-4 hours of sample collection according to a validated standard operating procedure. Cell differentials were assessed on cytospin preparations using appropriate staining techniques. Cells and cell supernatant were subsequently frozen and stored for further analysis.

All microbiology samples were processed in an Irish Clinical Pathology Accreditation (CPA)-accredited laboratory to routine diagnostic standards using standard and select supplementary media, in accordance with the BTS guidelines on microbiological profiling in bronchiectasis. Samples were analysed by trained staff using appropriate containment and safety procedures in accordance with Galway University Hospital standard operating procedures. Sensitivity testing was carried out using the agar disc diffusion method according to methods of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[364] Isolates were tested against multiple anti-microbial agents including amikacin, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem, piperacillin-tazobactam, ticarcillin-clavulanic acid and tobramycin. BAL was routinely sent for NTM and fungal cultures in all new patients.

Chronic infection was defined by the isolation of potentially pathogenic bacteria in sputum culture on ≥ 2 occasions, at least 3 months apart during a 1-year period.[50, 51] The predominant pathogen was the organism grown most frequently over the study period. Polymicrobial infection was defined as chronic infection with ≥ 2 pathogens on follow-up.[51] Isolation was defined as the presence of the pathogen on a single occasion without chronic infection. [51] Patients who were unable to provide sputum samples due to absence of a productive cough were classified as not being chronically infected for the purposes of analysis.

3.2.8 Bronchiectasis severity

The Bronchiectasis Severity Index (BSI) is a bronchiectasis-specific disease severity and clinical prediction tool, derived from a large Edinburgh-based cohort in the UK, to predict future risk of mortality, hospital admissions, exacerbations, and QoL in patients with bronchiectasis (Table 3-5).[19] This was subsequently validated in four independent cohorts to demonstrate its utility and generalisability internationally.

My role in the development and validation of the BSI was to collect and collate all data required to validate the BSI in a single UK centre whilst on fellowship and complete an overall meta-analysis of all five centres to demonstrate that the BSI successfully predicted mortality, exacerbations and hospitalisations. I subsequently presented this work as an oral presentation at the Chest World Congress 2014.

In our cohort of 126 patients in a quaternary bronchiectasis centre in Newcastle-upon-Tyne, the total mortality and hospitalisation rates were 13.5% and 45.2% respectively with a mean exacerbation rate per year of 2.7. There were 17 mortalities over a mean follow up of 40 months, 16 of whom had been categorised as having severe bronchiectasis. Significantly, there were zero mortalities or hospitalisations in the mild subgroup. The score was consistent across the disease spectrum suggesting a higher risk of mortality, hospitalisation and exacerbations in patients with higher baseline BSI scores.[19]

The BSI is a prognostic score designed to predict mortality and hospitalisation rates over a 4-year period. The score is divided into mild (0-4), moderate (5-8) and severe (≥ 9) with estimated prognostic indicators for each outcome as outlined below. The score can be accessed online at the following web address: www.bronchiectasisseverity.com.

Table 3-5 Bronchiectasis Severity Index

Severity Marker	Score	Severity Marker	Score
Age		**Hospitalisation**	
<50	0	Hospitalised for severe	5
50-69	2	exacerbations in previous 2	
70 – 79	4	years	
80+	6		
FEV1		**Exacerbations**	
>80%	0	3 or more outpatient	2
50-80%	1	exacerbations per year	
30-49%	2	Less than 3 outpatient	
<30%	3	exacerbations per year	0
BMI		**mMRC dyspnoea score**	
>18.5	0	0 – 2	0
<18.5	2	3	2
		4	3
Microbiology		**Radiological severity**	
P. aeruginosa	3	**score**	1
Colonised (other)	1	<3 lobes involved	2
Not colonised	0	≥ 3 lobes involved or cystic bronchiectasis	

Predicted 1 and 4 year outcomes associated with the BSI:

0-4: Mild bronchiectasis:

- 1-year outcomes: 0 - 2.8 % mortality rate, 0 - 3.4 % hospitalisation rate
- 4-year outcomes: 0 - 5.3 % mortality rate, 0 - 9.2 % hospitalisation rate

5-8: Moderate bronchiectasis

- 1-year outcomes: 0.8 - 4.8 % mortality rate, 1.0 - 7.2 % hospitalisation rate
- 4-year outcomes: 4 % - 11.3 % mortality rate, 9.9 - 19.4 % hospitalisation rate

≥ 9: Severe bronchiectasis

- 1-year outcomes: 7.6 % - 10.5 % mortality rate, 16.7 - 52.6 % hospitalisation rate
- 4-year outcomes: 9.9 - 29.2 % mortality, 41.2 - 80.4 % hospitalisation rate

3.2.9 Health-related quality of life

Quality of life (QoL) was assessed using the respiratory-specific St. George's Respiratory Questionnaire (SGRQ) and the disease-specific Quality Of Life Bronchiectasis (QOL-B) questionnaire (v3.1) with minimum clinically important differences (MCIDs) of 4 and 8 points respectively.[76, 78, 365] A systematic review of HrQoL questionnaires used in bronchiectasis suggests that QOL questionnaires assess a unique aspect of health not captured by objective measures.[81] The SGRQ and QOL-B are the most widely used and validated scores in bronchiectasis literature.

The SGRQ is the most widely validated patient-reported outcome measure with which to assess respiratory-specific QoL. It was originally designed and validated for use in COPD, and has since been validated for use in bronchiectasis, idiopathic pulmonary fibrosis, asthma, and CF and used in numerous clinical trials.[76, 365-368] A total score is calculated which summarises the impact of the disease on overall health status based on individual components for symptoms, activity and impact. Scores are expressed as a percentage of overall impairment where 100 represents worst possible health status and 0 indicates best possible health status. Normal ranges are described. The SGRQ has independently demonstrated good psychometric properties that support construct validity, including adequate internal consistency, test-retest reliability with good reproducibility over a 2-week interval, convergent validity with established measures, and responsiveness to spontaneous changes in health over a 6 month follow-up period. Potential disadvantages of this score include its length, its predominant use in research settings, and the suggestion that it may not be as responsive to change as a disease-specific tool.

The Quality of Life-Bronchiectasis (QOL-B), a disease-specific, self-administered, patient-reported outcome measure assessing symptoms, functioning and health-related QoL for patients with bronchiectasis, contains 37 items on 8 scales (Respiratory Symptoms, Physical, Role, Emotional and Social Functioning, Vitality, Health Perceptions and Treatment Burden).[78,

369] Each QOL-B scale has independently demonstrated good psychometric properties that support construct validity, including adequate internal consistency, test-retest reliability with good reproducibility over a 2-week interval, convergent validity with established measures, and responsivity to open-label antibiotic treatment. Potential disadvantages of this score include its length and lack of total score which complicates interpretation of data.

3.3 Gastro-oesophageal reflux investigations

For the purpose of our cohort studies, GORD was defined according to a self-reported history of typical symptoms of heartburn and regurgitation or improvement in symptoms after a trial of therapy as per ACG recommendations.[259] Patients were considered to have GORD when a diagnosis of GORD was recorded in the notes by a primary or secondary care physician, or in a patient taking a prescribed long-term anti-reflux medication.

For our case-control study (Chapter 8), a multimodal approach to the assessment of GORD was undertaken incorporating validated reflux symptom and QoL questionnaires, HRCT assessment of hiatal hernia presence and size, visual assessment of laryngopharyngeal reflux at bronchoscopy using the validated RFS, high resolution oesophageal manometry and combined 24h pH-impedance studies, and BAL biomarkers of gastric and duodenal reflux – pepsin and bile acids, respectively. Information was also collected at a cellular level to quantify potential mechanisms of injury in cultured primary bronchial epithelial cells (PBECs) derived from bronchoscopic brushings of bronchiectasis patients in unstimulated, bile acid-stimulated and combined cultures of bile acid and azithromycin to assess the attenuating effect of a subclinical dose of azithromycin in bile acid-mediated injury (Chapters 9 and 10).

3.3.1 Selection of questionnaires and patient-reported outcomes

Several questionnaires with varying characteristics have been developed for the assessment of GORD, with a few having modest diagnostic utility (~65–70%) for symptom-based diagnosis of GORD but which could not be

recommended as stand-alone diagnostic instruments.[370] To identify questionnaires and patient-reported outcome measures suitable for the symptomatic evaluation of GORD and extra-oesophageal reflux disease in adults with chronic lung disease, our group performed a systematic search of the English-language literature from January 1980 to May 2013 prior to study commencement using the Ovid-Medline database. Instruments were evaluated based on their development, psychometric properties, ease of administration, recall period, presence of normal ranges and utility across a spectrum of diseases. Two reviewers (MJM and RJ) independently screened all citations based on the title and abstract and assessed study eligibility based on full texts. Any disagreement was resolved through multidisciplinary discussion until a consensus was reached.

The following search string was used within Medline: [gastro-esophageal reflux OR GERD OR gastro-oesophageal reflux OR GORD] AND extra-oesophageal reflux AND laryngopharyngeal reflux AND [questionnaire OR scale OR patient reported outcome OR instrument OR measure OR index] AND [symptoms OR diagnosis OR evaluation] AND [validity OR reliability or responsiveness OR psychometric properties]. The references of relevant review articles were also screened. Our *a priori* exclusion criteria consisted of instruments requiring administration by an interviewer, those with extended recall periods and those relating to reflux surgery.

A total of 26 tools were identified and evaluated. Amongst 20 tools specific to GORD, 3 were found to be suitable for use in chronic lung disease: the GORD Symptom Assessment Scale (GSAS), the Reflux Disease Questionnaire (RDQ) and the Gastro-oEsophageal Reflux Disease-Questionnaire (GERD-Q).[371-373] The GERD-Q, a self-administered 6-item questionnaire designed to standardise symptom-based diagnosis and evaluation of treatment response in patients with GORD, was the most attractive due to its brevity, short recall period, rigorous developmental methodology, multi-lingual validation, and consistent reliability and responsiveness.

Similarly, three questionnaires were found to adequately score the symptoms of extra-oesophageal reflux: the Reflux Symptom Index (RSI), the Supra-Esophageal Reflux Questionnaire (SERQ) and the Hull Airway Reflux Questionnaire (HARQ).[374-376] Amongst these, the RSI offered the most balanced analysis of symptoms, was the most widely validated, and was shown to demonstrate good correlation with the RFS. The RSI is a 9-item self-administered outcomes questionnaire for evaluating symptoms of laryngo-pharyngeal reflux (LPR) – defined as the regurgitation of gastric contents into the larynx and pharynx. Each item is scored between 0 (no problem) and 5 (severe problem), with a maximum total score of 45. An RSI of greater than 13 is considered to indicate LPR. Assessment of respiratory symptoms and QoL are determined by the specific patient population with chronic lung disease. As the SGRQ has been extensively validated across a spectrum of lung diseases, this made it the most suitable instrument for assessing respiratory QoL in a mixed aerodigestive service.

For the current study therefore, the GERD-Q was used to assess typical symptoms of reflux, the RSI to assess extra-oesophageal reflux symptoms and the SGRQ to assess respiratory QoL.

Although not yet validated in a respiratory cohort, we also decided to incorporate the CReSS tool to compare the utility of this instrument with the GERD-Q and RSI in our study population.[377] The CReSS questionnaire is a composite tool developed by Ear, Nose and Throat (ENT) surgeons at the Freeman hospital, Newcastle, UK which assesses the symptoms of oesophageal, pharyngeal and upper airway reflux in three separate categories. The score was derived from a combination and item reduction analysis of the GORD symptom assessment scale (GSAS) score for the assessment of classical GORD and the RSI for the assessment of extra-oesophageal reflux, resulting in a 34-item questionnaire with a Likert scale of 0-5 to record symptoms as experienced in the previous month. Hence, the total score can range from 0-170 with higher scores indicating a greater symptom burden. The CReSS score was demonstrated to have good internal reliability with a Cronbach's α-coefficient of 0.93. Whilst extremely useful from a research

perspective, application in routine clinical practice is somewhat limited due to its length and complexity.

The panel of questionnaires chosen for our study was combined and tested in a sample of five staff from the university. The median time for completion of all five questionnaires was six minutes and free-text feedback suggested that they were acceptable as a group. Questionnaires and a breakdown of the qualitative systematic review can be provided on request.

3.3.2 Hiatal hernia scoring system on HRCT

Grading of the presence and size of a hiatal hernia was independently assessed on HRCT by a pulmonary physician (MJM) and thoracic radiologist (JD) according to the following:

- Grade 0 - No hernia
- Grade 1 - Oesophageal junction above level of diaphragm associated with a small (<2cm) portion of gastric fundus
- Grade 2 - Oesophageal junction above level of diaphragm associated with a larger (2- 5cm) portion of gastric fundus
- Grade 3 - Oesophageal junction above level of diaphragm associated with a large (> 5cm) portion of gastric fundus/body.

Calculation of the kappa statistic for inter-rater comparability and comparison with high resolution manometry techniques where available were also performed.

3.3.3 Reflux Finding Score

The RFS is an 8-item clinical severity scale based on laryngoscopic findings (Table 3-6): subglottic oedema, ventricular oedema, erythema/hyperaemia, vocal cord oedema, diffuse laryngeal oedema, posterior commissure hypertrophy, granuloma or granulation tissue, and excessive, thick endolaryngeal mucus.[378] The total score can range from 0 (best) to 26 (worst). The scoring system is based on the assessment of 40 patients with LPR confirmed by pH monitoring with a mean score of 11.5 compared with

5.2 in 40 age-matched controls. An RFS of 7 gives a 95% likelihood of a diagnosis of LPR. This tool provides an additional measure in the multimodal assessment of reflux. However, its use is limited in that it has yet to be validated in large-scale randomised trials. Real-life studies show significant correlation between the RFS and RSI and suggest a combination of these tools may negate the need for more invasive, time-consuming and cost-intensive investigations in the first-line assessment of LPR.[379, 380] To assess the RFS in our study, photographs of the larynx were taken prior to intubation at bronchoscopy. A subset of anonymised images were independently reviewed by an ENT specialist to externally validate the RFS score and allow calculation of the kappa statistic for inter-rater reliability.

Table 3-6 Reflux Finding Score

Finding	
Subglottic oedema	2 = present 0 = absent
Ventricular obliteration	4 = complete 2 = partial
Erythema/hyperaemia	2 = arytenoids
Vocal cord oedema	3 = severe 2 = moderate 1 = mild
Diffuse laryngeal oedema	3 = severe 2 = moderate 1 = mild
Posterior commissure hypertrophy	3 = severe 2 = moderate 1 = mild
Granuloma/granulation	2 = present 0 = absent
Thick endolaryngeal mucus/other	2 = present 0 = absent

3.3.4 Oesophageal physiology investigations

During my European Respiratory Society/European Lung Foundation Fellowship, I received formal clinical training in oesophageal physiology

investigations in Amsterdam Medical Centre under the auspice of Prof. Aarjan Bredenoord in the use of water-perfused and solid-state catheters prior to a residential training course in Enschede on high resolution manometry and impedance analysis. I also received clinical training in Newcastle under the remit of Prof. Mike Griffin. Following a period of observation with regards to the practice and theory of conventional and high resolution oesophageal manometry and ambulatory oesophageal monitoring, I attended regular lists and performed procedures and analyses with progressively greater independence and confidence, receiving feedback on my reports. I reviewed clinical practice guidelines published by the British and American Societies of Gastroenterology, the Association of Gastro-Intestinal Physiologists and the International High Resolution Manometry Working Group to adhere to national and international standards of practice.[256, 259, 381, 382]

On return to Ireland, I facilitated the set-up of the oesophageal physiology service in Galway University Hospitals under the upper gastrointestinal surgical service. I completed a business plan and budget proposal in order to procure the required equipment. I secured on-site facilities to enable decontamination of equipment according to international standards. I completed the operational policy for the oesophageal function unit, standard operating procedures for performing, interpreting and reporting investigations and procedures for disinfecting equipment in accordance with infection prevention and control, microbiology and decontamination services, and patient information leaflets for each procedure. After setting up the service in Galway, we linked in with St. James Hospital, Dublin, to ensure consistent reporting between centres. For the purpose of the studies in this thesis, I performed and analysed all oesophageal physiology investigations in Galway University Hospitals and set-up and performed a service to enable fast-track investigation of respiratory patients requiring oesophageal assessment beyond the scope of this study. To externally validate reports, anonymous subsets of procedures were sent to an expert gastroenterologist in this area, to enable calculation of the kappa statistic to compare inter-rater reliability.

3.3.4.1 Oesophageal manometry

Oesophageal manometry is used to quantitatively assess oesophageal motility. The pressure in the oesophagus gets converted to an electrical signal by pressure transducers, following which a computer software programme amplifies and filters the signals to be displayed on the screen in an interpretable manner. Conventionally, manometry was performed using single-use water-perfused catheters consisting of 4-8 channels. The 8-channel manometry catheters consisted of 4 lateral ports spaced 5cm apart and 4 radial ports at the same level to enable characterisation of the lower oesophageal sphincter (LOS). High resolution manometry (HRM) can be performed using water-perfused or solid-state catheters consisting of up to 36 channels spaced more closely together, enabling continuous recordings of oesophageal motility and LOS function throughout the test. The large amount of data collected allows the traces to be analysed and presented, either as conventional manometric line plots or with a visual representation of the results in a spatiotemporal topography (Clouse) plot (Figure 3-4).

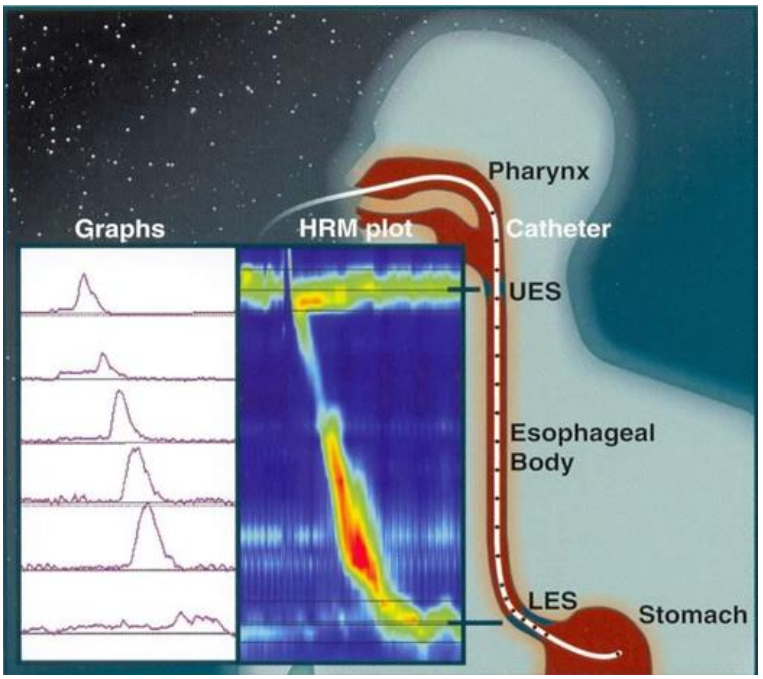

Figure 3-4 Comparison between conventional and high resolution manometry during a single swallow

Pre-procedure preparation

The 36 sensor solid state HRM catheter (used for all manometry studies in this thesis) is 4.2 mm in diameter and consists of circumferential pressure sensors 2.5 mm in length arranged at 10 mm intervals (ManoScan 360, Given Imaging Ltd). This is covered by a silicone-based thermal plastic elastomer. Each sensor detects pressure from 12 separate loci around its circumference (Figure 3-4). Using pressure transduction technology, pressures from each loci is averaged. Computer processing then collects information from all pressure sensing elements to produce circumferential pressure measurements incorporating the entire catheter length which is then displayed as a spatio-temporal plot captured in real time (Figure 3-5).

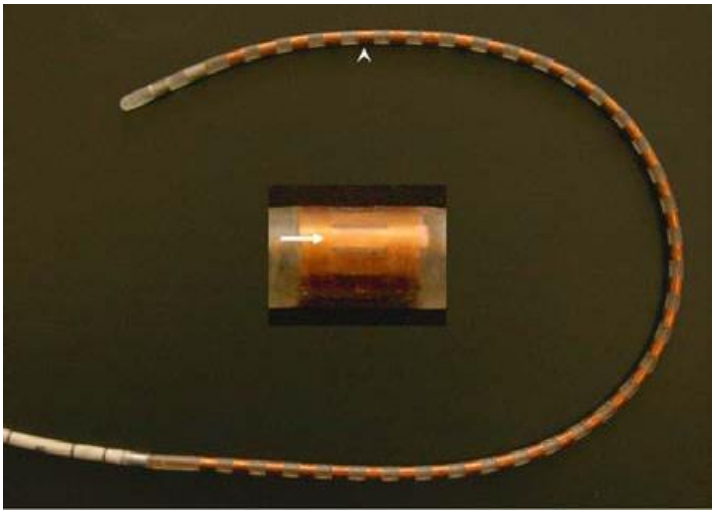

Figure 3-5 Solid-state catheter high resolution manometry sensors

Sensors are copper coloured cylinders (white vertical arrow) which are spaced at 10 mm intervals. Pressure is detected from 12 loci around each cylinder circumference (1 locus = white horizontal arrow from the magnified sensor in the centre).

Decontamination and sheathing of the HRM catheter

The HRM catheters are re-usable (up to 200 times) although their life-span can be extended (up to 400 times or longer) with ongoing service and maintenance. It is essential, therefore, that disinfection techniques do not cause damage to the sensitive silicone coating or sensors on the catheter. In

order to avoid this, only personnel trained in equipment-specific decontamination techniques could disinfect the catheter. Sheathing with a Manoshield™ (Sierra Scientific Instruments) pre-procedure provides another protective barrier that not only acts as a physical barrier between the patient and catheter, but also prolongs catheter durability. The catheter was stored in its designated box at all times after cleaning and in between patient use. Disinfection was always performed using personal protective equipment in the designated disinfectant room. Following discussion with infection control, microbiology and the decontamination coordinator in Galway University Hospitals, a triple disinfection process was initiated, using the Tristel chemical biocidal wipe system before and after the Tristel Stella™ bath immersion system to disinfect catheters prior to and after every use.

Tristel's patented chemical biocidal wipe system incorporates three individually packaged sachets and a foam pump. Decontamination with Trsitel wipes was a 3-step process during which the length of the catheter would be carefully wiped in a unidirectional (proximal to distal) fashion with gloves changed prior to every step:

1. Pre-clean wipe - composed of a low-foaming surfactant system combined with triple enzymes which produce an ultra-low surface tension suitable for cleaning of any hard surface to remove organic matter which may have deposited on the catheter surface.
2. Sporicidal, bactericidal, mycobactericidal, virucidal and fungicidal foam pump directly applied to the second wipe to activate the disinfectant to kill any organism within 30 seconds of contact.
3. Rinse wipe - this final wipe was composed of de-ionised water and a low-level antioxidant to remove and neutralise any remaining chemical residue.

The Tristel Stella™ bath immersion system is an automated system that provides manual soaking in the context of a fully automated washer disinfector and drainage device, eliminating potential risk of over-exposure of instrument to chemical solution. One activated sachet of high-level disinfectant solution Tristel Fuse added to five litres of water produces a

chlorine dioxide (ClO_2) solution that is sporicidal, bactericidal, mycobactericidal, fungicidal and virucidal within a contact time of only 5 minutes to eliminate risk of cross-contamination between patients. This system has the added advantage of inbuilt tracing and traceability features to assist the decontamination audit process. By incorporating the use of Tristel wipes before and after the Tristel Stella™ bath immersion system, each probe was thoroughly disinfected in accordance with local and international policies and procedures.

Calibration

After entering patient details, the prepared catheter was connected to the Manoscan system. The sheathed portion of the catheter was then inserted into the calibration chamber and sealed proximally. Sensors were then interrogated as the machine sequentially increased the compartmental pressure within the chamber up to 300 mmHg before dropping back to atmospheric pressure. The response characteristics of each sensing element ideally should be accurate to within 1 mmHg. Defective sensors were highlighted by the software and these were then masked manually. For studies in this thesis, catheters with ≥ 2 defective sensors were replaced.

Catheter insertion

The procedure was explained to the patient in detail and written informed consent obtained outlining possible risks of the procedure. Patients were required to be alert and un-sedated for the procedure. Only Xylocaine 1% was used to locally anaesthetise the nostril (2-3 sprays) and pharynx (5-6 sprays). Catheter insertion was performed with the patient in an upright position either sitting on a chair or on the edge of a treatment bed. Once the tip of the catheter passed into the nasopharynx, the patient was encouraged to tilt their head forward and touch their chest with their chin whilst sipping water from a straw and swallowing at regular intervals to help the catheter progress through the cricopharyngeus and into the oesophagus and stomach to an appropriate distance from the nares (70 cm for conventional manometry and 55 cm for HRM). Oesophageal anatomical landmarks could be clearly visualised on the monitor as the catheter progressed down the oesophagus. The catheter was

considered to be correctly positioned when both the upper and lower sphincters could be recognised and two pressure sensors were located in the stomach. The position of the LOS was confirmed by deep inspiration to highlight the diaphragm pressure inversion point (PIP). Once finalised, the catheter was taped to the side of the face and neck as it curled behind the ear to reduce catheter movement and pharyngeal irritation. Position from the nares was then recorded as a reference for automated calculations.

HRM procedure

All studies presented in this thesis were performed by the investigator. Tests were performed in the supine position according to validation of the Chicago HRM classification. Prior to initiation, study participants were instructed to inform the investigator of any symptoms they were to experience as soon as they occurred so that they could be marked directly onto the HRM trace. Once the patient was deemed comfortable after a short adaptation period, patients were asked not to swallow for a minimum period of 30 seconds to enable assessment of the LOS resting (baseline) pressure, the LOS and upper oesophageal sphincter (UOS) margins and the potential presence of a hiatal hernia.

Following this, the standard evaluation of oesophageal motility with the administration of ten 5ml boluses of water given to the patient via a syringe was performed. Patients were requested to swallow each bolus 'in one go' and to withhold further swallows for a period of 20 seconds thereafter allowing the previous peristaltic wave to terminate and the LOS to return to baseline pressure. The start and end of every swallow was manually framed on the screen as the swallows progressed. If more than one swallow was noted and/or if other events that could interfere with analysis were identified (e.g. cough, vomit, sniff, laughter), the swallow frame was deleted. A minimum of 10 swallows was required for analysis. Additional testing with free drinking or multiple water swallows (MWS) was performed whereby patients were asked to drink 200 mL of water freely through a straw without stopping. Drinking directly from the cup was discouraged as larger volumes per swallow reduced the total number of swallows available for analysis.

Furthermore, using a straw reduced movement of the head and neck during free drinking. If less than 200ml was swallowed, the volume consumed was documented; although if they were able and willing, the process was repeated. After completion of MWS, patients were asked not to inhibit their swallows as instructed during the 5 mL water protocol. Symptoms during or after completion were subsequently recorded and the catheter was removed and allowed to hang at room temperature/pressure for a few seconds to zero the catheter and enable the software to apply sensor-specific thermal compensation to the entire manometric data set.

HRM analysis

Proprietary software (ManoView™ ESO v3.0 Given Imaging Ltd,) was used to analyse all HRM data. Each swallow frame was interrogated and extended or reduced in order to incorporate only one swallow per frame. For 5 mL water swallows, any frame that did not comprise only one, uninterrupted swallow was excluded from analysis. To facilitate comparison between swallows, a 30 mmHg isobaric contour was used to define peristaltic integrity. The isobaric contour is a line on the HRM plot which circumscribes all pressurised segments such that the pressure within is equal to or greater than a pre-specified pressure. The qualitative and quantitative characteristics of every swallow were then described in turn after manual adjustment of oesophageal and LOS markers. Specifically, the position of the PIP was confirmed and adjusted if necessary; the principle LOS marker was placed at the maximum pressure point while the upper and lower LOS margins were placed at the respective borders of the LOS. All markers were adjusted manually for every swallow to ensure accuracy. By interpolating pressure data across the LOS, a 6 cm virtual sleeve (the e-sleeve) was derived from HRM data, thus providing a single measurement across the LOS. This function reduces the potential for inaccuracy that could arise from axial movement of the oesophagus by allowing for uninterrupted measurement of the maximum pressure along the length of the virtual sleeve. The e-sleeve markers (or lollipops) were placed at either end of the LOS margins. A typical spatiotemporal plot with the axial graph of a healthy volunteer highlighting

the isobaric contour at 30 mmHg and showing the essential landmarks is presented in Figure 3-6.

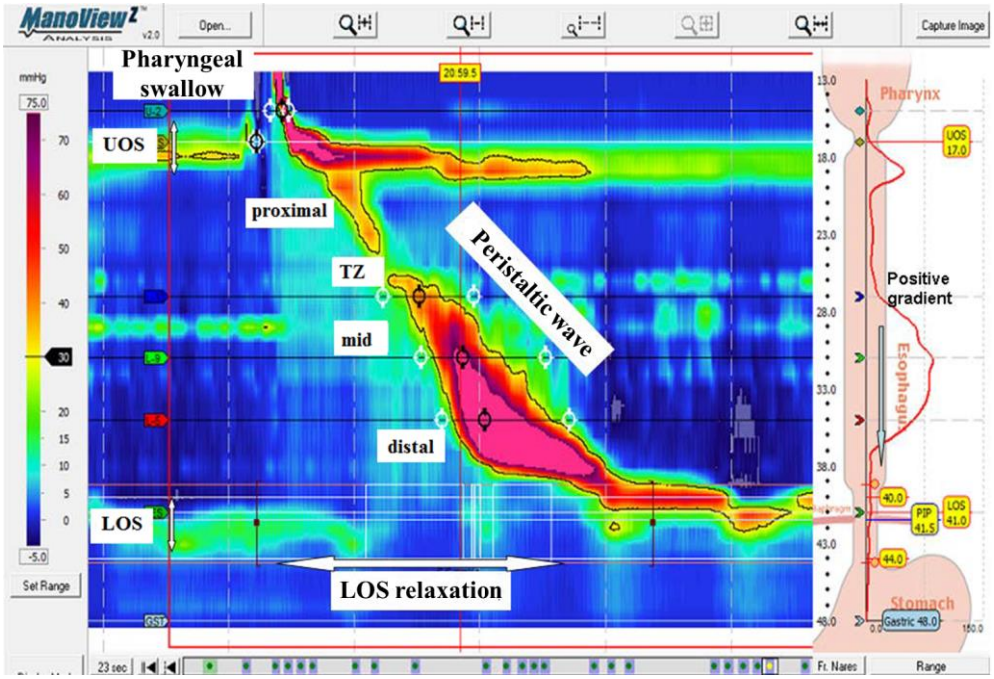

Figure 3-6 High resolution manometry swallow margins and landmarks

HRM of a normal swallow with pressure data presented as a spatiotemporal plot. A 30 mmHg contour (black line) is superimposed on the image. Important landmarks are highlighted. The axial graph on the right shows the direction of flow relative to the pressure gradient. This trace is from a healthy volunteer presented in Chapter 8. (UOS: Upper oesophageal sphincter; LOS: Lower oesophageal sphincter; TZ: transition zone).

Oesophageal peristalsis

With the advancement in technology from conventional to high resolution techniques, an international consensus process has evolved over recent years to define oesophageal motility disorders using HRM, Clouse plots, and standardised metrics. The Chicago Classification, intended to be applied to HRM studies performed in a supine position with 5 mL water swallows and for patients without previous oesophago-gastric surgery, was first published in 2009 and subsequently updated in 2012 and 2015. For the purposes of this thesis, Chicago classification v3.0 was used.[381]

Pressure topography metrics in the Chicago Classification v3.0 are described in Table 3-7 and visualised in Figure 3-7.

Table 3-7 Pressure topography metrics based on the Chicago Classification

Integrated Relaxation Pressure (IRP), mmHg	The mean pressure of maximal deglutitive relaxation measured over 4 seconds in the 10 seconds following UOS relaxation. Contributing times can be contiguous or non-contiguous (i.e. interrupted by diaphragmatic contraction). Referenced to gastric pressure. Equates to LOS relaxation pressure in conventional manometry. Normal value: IRP-4 < 15 mmHg for solid state catheters
Distal Contractile Integral (DCI), mmHg/s/cm	Amplitude x duration x length of the distal oesophageal contraction exceeding 20 mmHg from the transition zone to the proximal margin of the LOS. Equates to peristaltic amplitude in conventional manometry. Normal value: 450-8000 mmHg/s/cm
Contractile Deceleration Point (DCI), position	The inflection point along the 30 mmHg isobaric swallow contour (or pressure greater than intrabolus pressure in instances of compartmentalized pressurization) at which propagation velocity slows, demarcating peristalsis from ampullary emptying. The CDP must be localized within 3 cm of the proximal margin of the LOS.
Distal Latency (DL), seconds	Interval between UOS relaxation and the CDP Normal value: ≥ 4.5 s
Peristaltic Breaks, cm	Gaps in the peristaltic contraction between the UOS and LOS. Large break (>5cm)

Measurements are analysed in reference to normal values with each individual swallow characterised in terms of the following:

- Contraction pattern (not scored for DCI < 450 mmHg/s/cm)
    - Premature contraction (DL < 4.5 s)
    - Fragmented contraction (large break)
    - Intact (none of the above)

- Contraction vigour
    - Failed peristalsis (DCI < 100 mmHg/s/cm)
    - Weak peristalsis (DCI 100-450 mmHg/s/cm)
    - Normal peristalsis (DCI 450-8000 mmHg/s/cm)
    - Hypercontractile (DCI ≥ 8000 mmHg/s/cm in ≥ 20% of swallows)
    - Ineffective (failed or weak)
- Intra-bolus oesophageal pressurisation
    - Pan-oesophageal pressurisation: uniform pressurisation of > 30 mmHg extending from the UOS to the LOS (DCI should not be calculated in these patients).
    - Compartmentalised oesophageal pressurisation : pressurisation of ≥ 30 mmHg extending from the contractile front to the LOS
    - OGJ pressurisation: pressurisation restricted to zone between LOS and crural diaphragm (CD) in conjunction with LOS-CD separation (hiatal hernia)
    - Normal pressurisation: no bolus pressurisation ≥ 30 mmHg

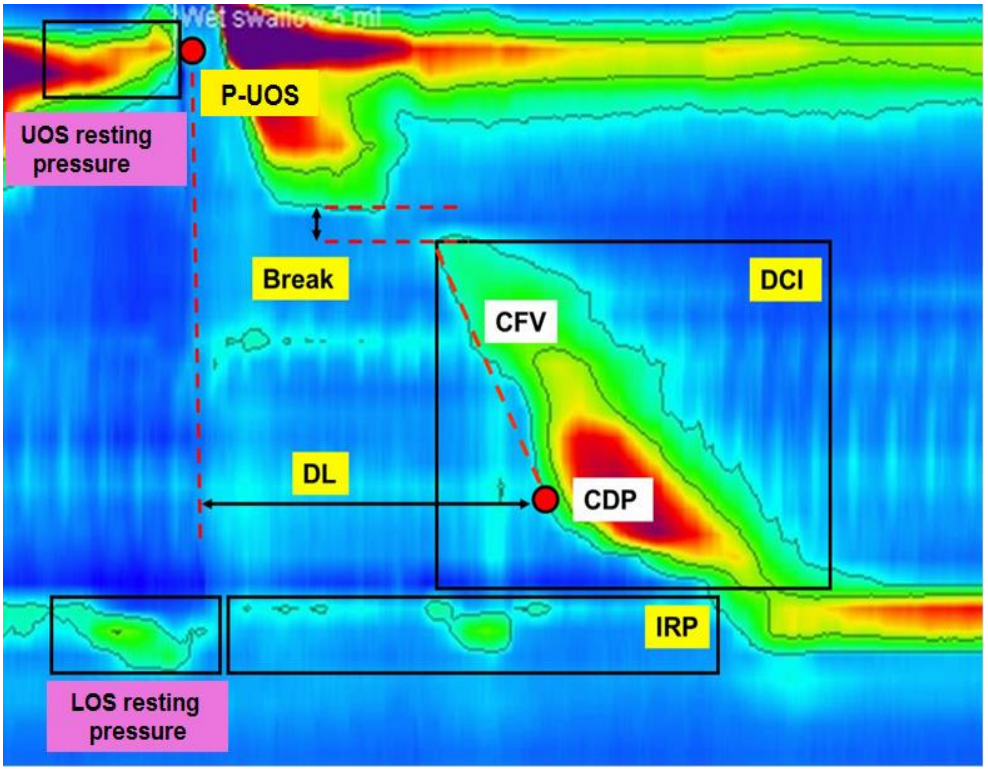

Figure 3-7 Diagramatic representation of high resolution manometric findings according to the Chiacago Classification

The presence or absence of a hiatal hernia, easily discernible on HRM with a double band at the expected position of the LOS as demonstrated in Figure 3-8, is also reported. This is important in terms of optimising catheter positioning 5 cm (plus hernia size) above the LOS. The individual characteristic of each swallow is used to compute an overall diagnosis as defined by the Chicago classification algorithm (Figure 3-9). Standard measurements of oesophageal location and length, as determined from the point of nasal insertion of the catheter, are also provided.

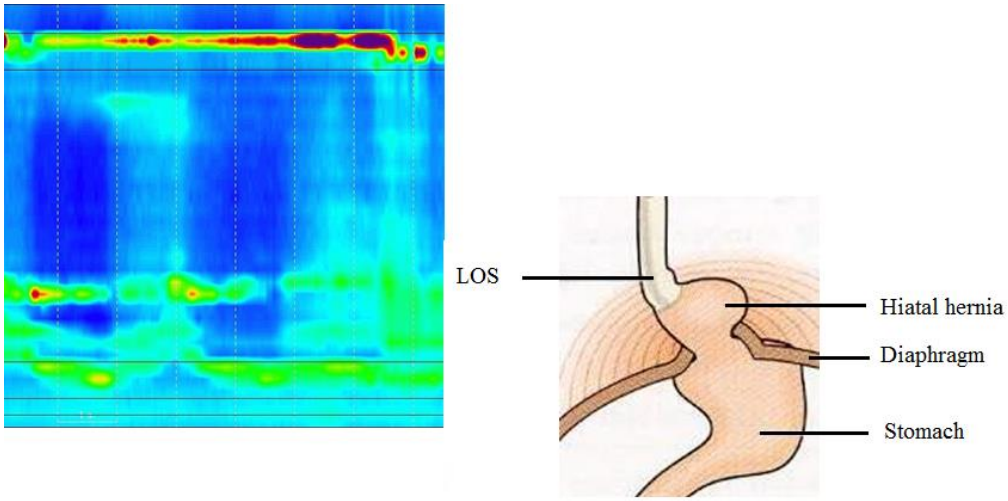

Figure 3-8 High resolution manometry showing the presence of a large hiatal hernia

A large hiatal hernia is manifest by separation between the lower oesophageal sphincter and the crural diaphragm showing the sphincter and part of the stomach present above the diaphragm.

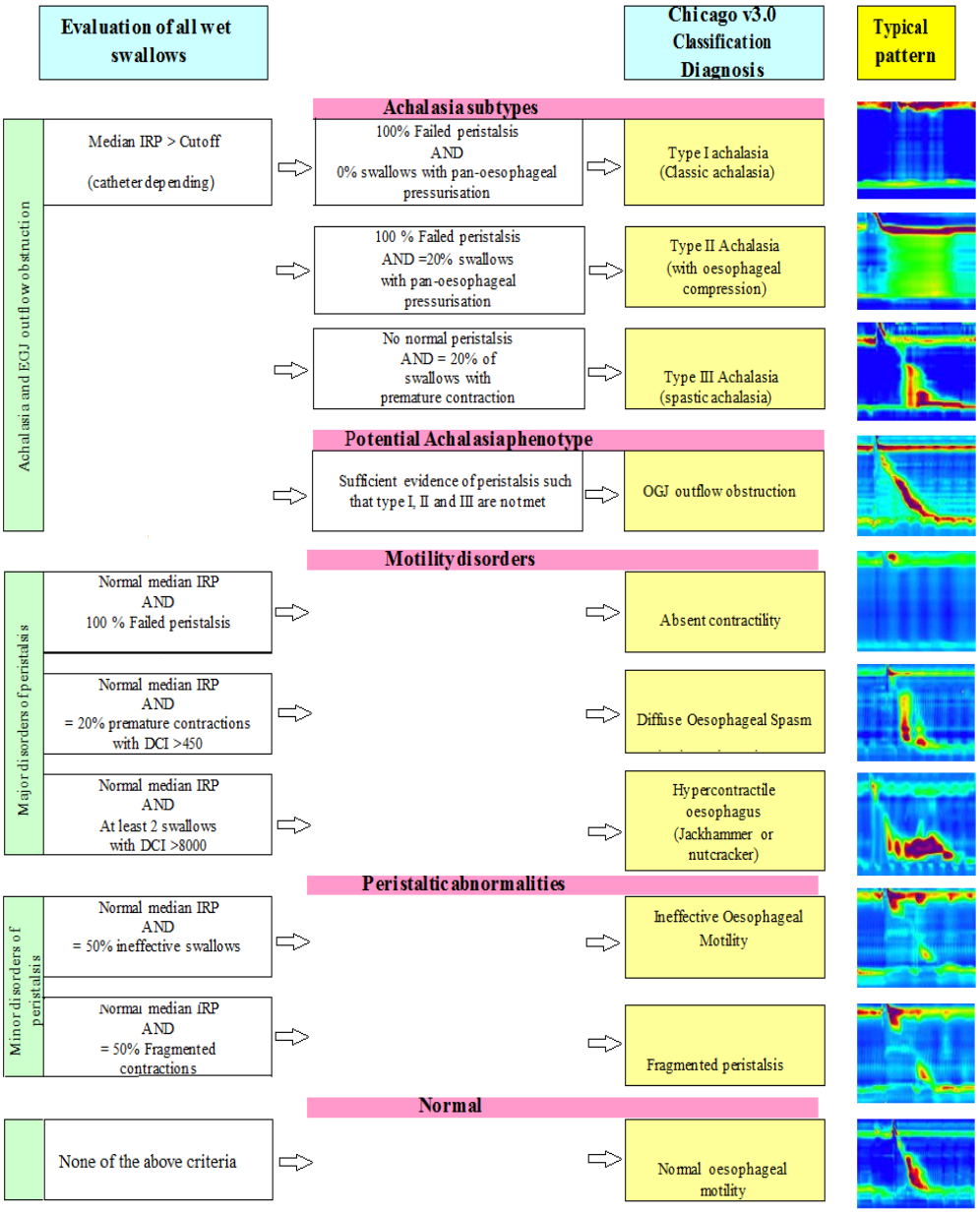

Figure 3-9 Diagramatic representation of the Chicago Classification

Hierarchical analysis of oesophageal motility based on the Chicago Classification v3.0. Reproduced with permission from Laborie/MMS, Enschede, Netherlands.

3.3.4.2 Ambulatory pH-impedance studies

Combined 24-hour ambulatory multichannel intraluminal impedance is a technology that measures changes in oesophageal intraluminal resistance and bolus transit. It consists of a catheter with several metal rings whereby changes in resistance between the rings are detected (Figure 3-10). Gas causes an increase in resistance and liquids cause a decrease. The direction of these changes allows the direction of bolus movement to be determined (Figure 3-10; Figure 3-11). This device also has a pH probe that allows reflux events to be classified into acidic (pH<4), weakly acidic (pH 4–7) or non-acidic (pH>7) following established criteria.[263]

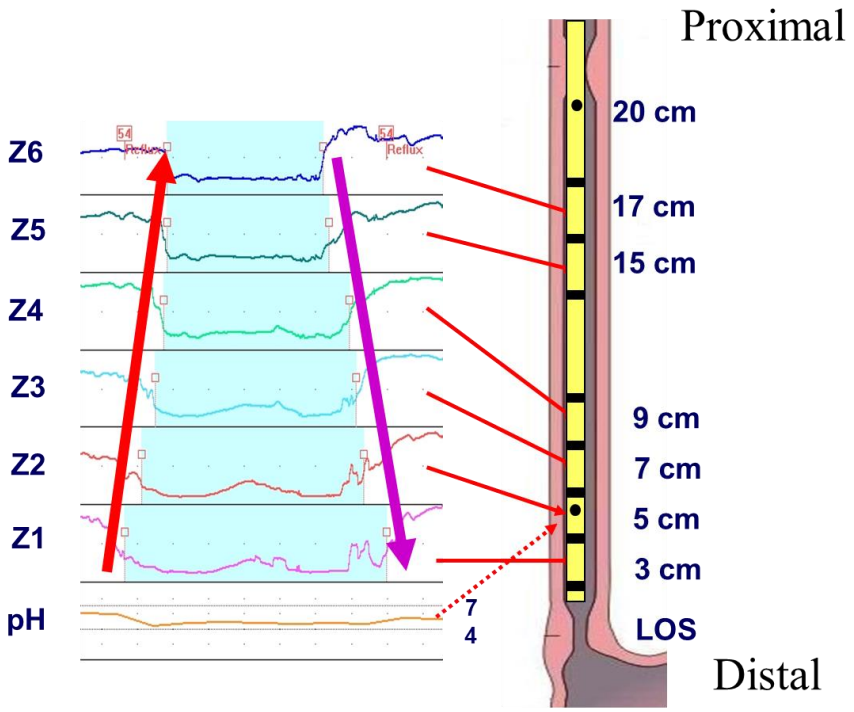

Figure 3-10 Graphical depiction of a weakly acidic liquid reflux event

The graph demonstrates a progressive decrease in resistance from ring 1 (lowest) to 6 (highest) representing movement of liquid. The direction of changes allows the direction of bolus movement to be determined which in this case is proximally from the stomach representing a liquid reflux event. The pH does not drop below 4 representing a weakly acid liquid reflux event.

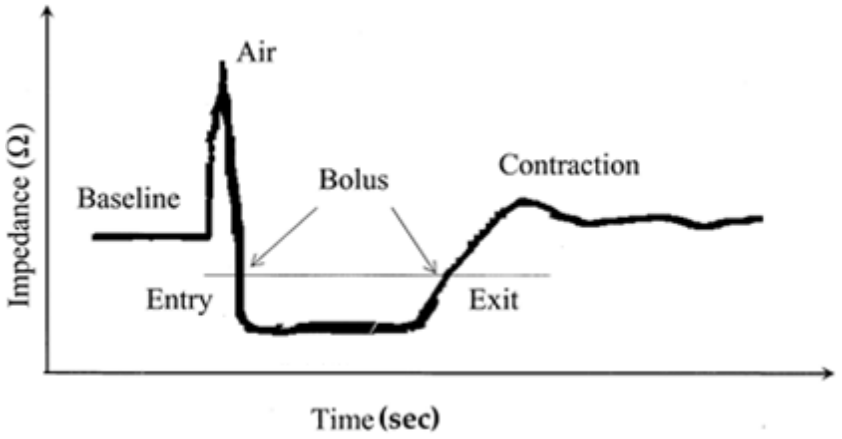

Figure 3-11 Enhanced graphical depiction of intraluminal impedance

This is a graphical representation of intraluminal impedance of the oesophageal wall in ohms, starting at baseline and progressing through the presence of air and the presence of a bolus of fluid. Impedance is increased in the presence of air and decreased in the presence of fluid.

pH-impedance procedure

Ambulatory pH-impedance was performed using the Given Imaging Ohmega device and disposable Pharsiflex catheters. The Ohmega device is a portable recording box to which the catheter is attached. The catheter measures 1.9 mm in diameter and consists of 6 metal impedance rings at 3, 5, 7, 9, 15 and 17 cm, with single or dual pH probes. All studies were performed on an out-patient basis after an overnight fast. All studies were performed off proton pump inhibitor treatment for a minimum of 7-14 days prior to procedure.

Prior to intubation, the catheter was connected to the Ohmega device and calibrated in a standardised manner in de-ionised water followed by buffer solutions of pH 4.0 and pH 7.0 for ten minutes at room temperature. After lower oesophageal sphincter (LOS) location by oesophageal manometry, the pH-impedance catheter was passed transnasally under topical anaesthesia and positioned in the oesophageal body to record pH at 5 cm and impedance at 3, 5, 7, 9, 15 and 17 cm proximal to the LOS. This position is considered to be an optimal depth to monitor distal oesophageal acid exposure while preventing slippage associated with head movement, swallowing, or catheter

migration into the stomach. Once the catheter is positioned and taped to the nose to limit its movement, the recording of data is initiated.

During pH-impedance monitoring, patients were encouraged to maintain their usual daily routines, eating habits and sleep schedule, record timing of symptoms and indicate meal times and position (supine versus upright) using event markers on the data-logger. Between meals, participants were asked to abstain from frequent snacks, beverages with a pH < 5, and to avoid gum chewing if possible. Participants were provided with a diary card to record with precision all food intake (nature, quantity and timing). Typically, ambulatory data is monitored for 24 hours although information obtained in 16 hour studies has been deemed to be as accurate and better tolerated by patients. After a monitoring period of at least 16 hours to be eligible for inclusion, patients returned to have the catheter removed. The Ohmega box was then connected to a compatible computer containing specific Given Imaging software and uploaded for analysis. The trace was reviewed manually and the electronic diary was verified with the paper diary and edited appropriately. Meal times, fluid ingestions, and artifacts were excluded to avoid confounding of calculations with a general visual inspection of the study to search for catheter displacement. On completion of manual review, an automatic analysis and summary of the pH-impedance events and symptom score correlations was produced.

pH-metry analysis

Ambulatory pH monitoring provides an assessment of the frequency and duration of reflux events. Standard analysis of reflux events undertaken in our studies include measures of:

- Total number of acid reflux episodes over specified study duration
- Mean number of reflux events per hour
- Total oesophageal acid exposure time (AET)
- Upright and recumbent AET – subdivisions of the total AET
- DeMeester score

A drop in oesophageal pH below 4 is the most discriminative threshold to define a reflux episode.[383] By cumulative summation of time when oesophageal pH is below 4, the AET, defined as the percentage of time that the pH drops below 4 over the study duration, can be derived. AET has been shown to be the single most robust and reproducible diagnostic marker of pathological GORD on pH monitoring; it can be reliably extracted from automated analysis and it has been shown to predict treatment response from medical and surgical reflux therapy.[243, 370] Classification based on original findings by Johnsson *et al*. and the Montreal definition and classification of GORD were used in this study.[253, 266] Total, upright and recumbent AET are calculated and reported separately as body position, activity and state of consciousness are known to influence intra-gastric pressure, LOS resting pressure, bolus clearance and salivary neutralisation that may lead to differences in physiological amounts of GOR in various body positions (Table 3-8).[263] When a dual probe pH catheter is used, proximal oesophageal AET can also be reported (Table 3-8).[263] More recently, the Lyon Consensus has proposed an AET > 4% as abnormal, with > 6% considered definitively pathological and 4-6% suggestive of the requirement for further investigations to provide confidence in the presence of pathologic acid burden.[243, 384] The DeMeester score is a composite parameter taking six individual metrics, total AET, upright AET, recumbent AET, number of reflux episodes, reflux episodes with pH < 4 for ≥ 5 minutes, and duration of longest reflux episode, according to a weighted formula dependent on the deviation of each of these variables from normal values.[265] The DeMeester score has been widely validated for assessing the severity of reflux disease using the 24-hour catheter-based system with an agreed upper limit of normal of 14.72.[265] The AET is the most specific of the individual components of the DeMeester score and is generally favoured as the metric used to designate oesophageal acid burden in the clinical setting.[384]

Table 3-8 Normal values of catheter-based oesophageal pH-monitoring

Time pH < 4.0 (%)	Proximal (%) – 20cm above manometric defined border of LOS	Distal (%) – 5cm above manometric defined border of LOS
Total period	< 0.9	< 4.2
Upright period	< 1.2	< 6.3
Recumbent period	< 0.0	< 1.2

pH-impedance analysis

Impedance analysis was compared to published normal ranges. The following parameters were recorded and analysed:

- Total number (and percentage) of reflux episodes (NRE) distally subdivided according to pH into acid (pH<4), weakly acid (pH 4-7), or non-acid (pH > 7) and liquid, gaseous or mixed
- Total number (and percentage) of reflux episodes (NRE) proximally subdivided according to pH into acid (pH<4), weakly acid (pH 4-7), or non-acid (pH > 7)
- Bolus exposure time [reflux time (min) and reflux percent time]
    - Total (normal <1.4%)
    - Acid (pH < 4) (normal <1.1%)
- Bolus clearance time: mean delay between detection of liquid and detection of gas at the most distal channel (normal < 44 seconds)

Data were downloaded and analysed using dedicated software and subsequently reviewed manually and compared to normal data (Table 3-9) with external validation of a subset by an experienced investigator blinded to the basal condition of the overall patients and healthy volunteers. Analysis included identification, enumeration and characterisation of individual reflux events, measure of oesophageal exposure to volume and acid, and bolus clearance times. Reflux episodes were characterised by pH-metry as acid (pH<4), weakly acid (pH 4-7), or non-acid (pH > 7) according to established criteria. Proximal reflux was defined as reflux events reaching the impedance

ring located 15 cm above the LOS. Patients with more than 17 of these events were deemed to have significant proximal reflux. Total and acid bolus exposure time in minutes and percentage was recorded to determine detection of liquid at the most distal channel within the oesophagus over a 24-hour period. Liquid reflux was defined as a retrograde 50% drop in impedance starting distally (at the level of the LOS) and propagating to at least the next two more proximal impedance measuring segments. Only liquid reflux lasting at least 3 s were taken into account. Gas reflux was defined as a rapid (3 kX/s) increase in impedance >5000, occurring simultaneously in at least two oesophageal measuring segments in the absence of swallowing. Mixed liquid–gas reflux was defined as gas reflux occurring immediately before or during a liquid reflux. Gas reflux events without liquid (belches) were considered separately and were not characterised by pH. The recent Lyon GORD Consensus proposed that >40 reflux episodes per 24 hours is abnormal with >80 being definitively abnormal.[243] Additional impedance parameters such as baseline impedance and post-reflux swallow-induced peristaltic wave (PSPW) also have potential as reflux metrics, but outcome data are currently limited.[385]

Table 3-9 Normative pH-impedance thresholds in healthy controls

pH-impedance	Number of healthy controls	Acid exposure time (% total time with pH < 4.0) per 24 hours	Number of impedance-detected reflux episodes per 24 hours
Shay e*t al.*, 2004[386]	60	6.3	73
Zerbib *et al*., 2005[387]	62	5.0	75
Tutuian *et al*., 2006[263]	20	N/A	42
Savarino *et al*., 2008[388]	48	4.2	54
Zerbib *et al*., 2013[389]	46	5.8	53
Kawamuru *et al*., 2016[390]	42	3.3	85

Measurements of reflux-symptom association

Both pH and combined pH-impedance monitoring provide analysis of the temporal association between symptoms and reflux episodes. Two symptom association analyses have been described for clinical reporting and research. These are routinely incorporated into commercial ambulatory pH proprietary software. By convention, an association is assumed if a reflux event precedes a symptom event within a two minute time window.[391]

1. Symptom Index (SI)

The Symptom Index (SI) is the percentage of symptom events preceded by a reflux episode. A symptom association ≥ 50% is considered to be positive suggesting pathological reflux, i.e. a large proportion of the patient's symptoms are considered to be reflux-related.[392] A potential disadvantage of the SI is that the number of reflux episodes is not considered leaving open the possibility of chance association.[393]

2. Symptom Association Probability (SAP)

Symptom Association Probability (SAP) is performed by dividing the 24 hour period into 2 minute segments and determining whether or not a symptom occurred 2 minutes prior to every episode of reflux recorded. It uses a 2x2 contingency table (Fisher exact test) comparing symptoms (positive/negative) and reflux events (positive/negative) to calculate the probability that the relationship observed between symptoms and reflux is not brought on by chance. The p-value is then subtracted from 1 and multiplied by 100 to provide an SAP value as a %. So a p-value of 0.05 is equivalent to an SAP of 95% (1-0.05=0.95 x 100). By statistical convention, an SAP of ≥ 95% is considered positive, suggestive of pathological reflux.[393, 394]

In summary, SI is a measure of 'effect size' and SAP is a measure of probability. As such, the two metrics are complementary, measure different things and cannot be compared with each other.[243] The combination of a positive SI and positive SAP provides the greatest evidence of a clinically relevant association between reflux episodes and symptoms. Both the SI and

SAP are predictive of the effect of medical and surgical anti-reflux therapy, independent of AET.[243, 384, 395]

3.3.5 Biomarkers of gastric and duodenal microaspiration

The detection of pepsin and bile acids, as markers of gastric and duodenal reflux, respectively, in saliva, sputum, tracheal aspirates or BAL fluid have been proposed as surrogate markers of reflux aspiration.[268, 269] Pepsin is a gastric enzyme that degrades food proteins into peptides. Pepsin and bile acids have been detected in BAL in a diverse range of respiratory pathologies, and more recently, pepsin has been noted in sputum and exhaled breath condensate (EBC) in individuals with bronchiectasis.[274, 275] It is considered that the enzymatic activity of pepsin in biological samples of sputum or BAL is a more accurate test for reflux-related aspiration than 24-h oesophageal pH monitoring. Bile acid aspiration has been associated with pulmonary injury with dose dependent cytotoxicity ranging from alteration of cellular cationic permeability to disruption of the cellular membrane.[396] Correlations between the presence of bile acids with increased sputum inflammatory markers, reduced lung function and longer duration of therapy have been noted in CF.[397] The advantages of pepsin and bile acids is that they are not normally found in the lung, suggesting that these biological markers are reliable in assessing the effect of pulmonary microaspiration in lung disease severity. Assays can also be used to evaluate reflux events without interrupting therapy with H2-blockers or proton pump inhibitors (PPI).[398] Further details relating to the methods of detection of pepsin and bile acid biomarkers are provided in section 3.4.11 and 3.4.12.

3.3.6 Excluded GORD investigations

Review of the literature suggests that other investigations such as endoscopy or barium swallows, in the context of chronic lung disease, are less likely to yield information for the diagnosis of extra-oesophageal reflux or airway reflux than oesophageal physiology investigations as patients often have atypical symptoms.[243, 384] Endoscopy has high specificity but very low sensitivity for GORD diagnosis, as oesophageal mucosa is normal in up to

70% of patients with symptomatic typical GORD.[399] Thus, endoscopy is appropriate only in the presence of alarm symptoms, such as dysphagia or unintentional weight loss, multiple risk factors for Barrett's oesophagus (>50 years of age, male sex, prolonged reflux symptoms, obesity) or failure to respond to therapy.[400] Supplemental endoscopic tools such as narrow-band imaging and confocal laser endo-microscopy provide limited additional benefit in identifying mucosal damage consistent with reflux. However, their use remains restricted to research given intrinsic limitations such as high costs, time-consuming procedures and weak inter- and intra-observer agreement.[370] Similarly, the use of barium radiography in diagnosing GORD is also not recommended due to the dynamic nature of the test.[401] Data comparing radiographic diagnosis of GORD with that from reflux testing demonstrates that radiographic findings do not correlate with the prevalence or extent of reflux seen on ambulatory pH-impedance monitoring.[401] Thus, barium radiography alone cannot be used to diagnose GORD, although radiography may be useful in defining relevant anatomy. Pharyngeal assessments were not included as there is no consensus regarding definition of pharyngeal reflux and methods for testing remain unclear and not validated.[389, 402] Measurement of airway and pharyngeal pH is therefore not recommended in current GORD guidelines due to considerable disagreement between available techniques.[384]

According to the Lyon classification of GORD, for the interpretation of oesophageal test results in the context of GORD, any one conclusive finding provides strong evidence for the presence of GORD (Figure 3-12). While normal endoscopy does not exclude GORD on its own, it provides strong evidence against GORD when combined with AET <4% and <40 reflux episodes on pH-impedance monitoring off PPI. When evidence is inconclusive or borderline, adjunctive or supportive findings can add confidence to the presence or absence of GORD but are not enough to diagnose GORD.

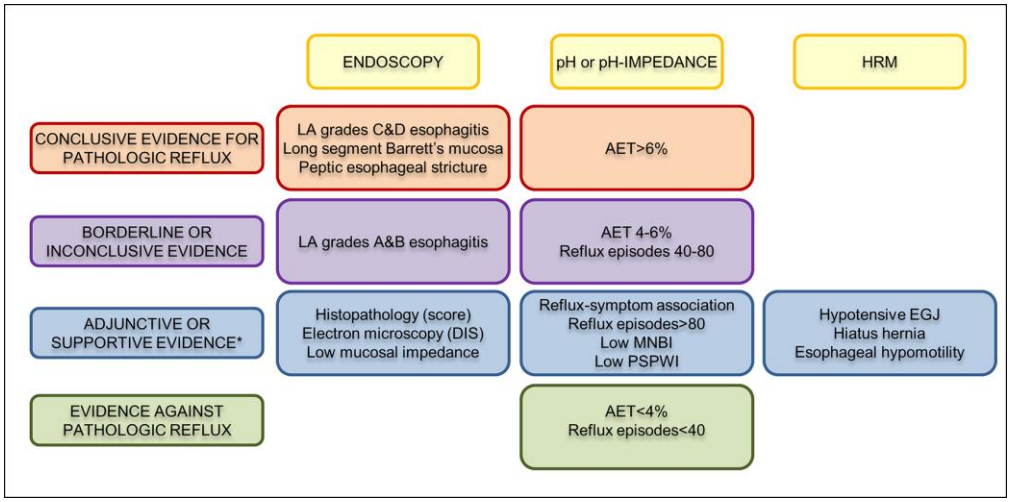

Figure 3-12 The Lyon Classification of GORD

Evidence for the presence of abnormal reflux using pH-impedance measurements is demonstrated by an AET > 4% or number of reflux episodes >40, with > 6% considered definitively pathological, and > 4-6% suggestive of the requirement for further investigations to provide confidence in the presence of pathologic acid burden, such as a positive reflux-symptoms association score. Adapted from Gyawali et al., Gut 2018.

3.4 Laboratory sample processing

3.4.1 BAL processing

BAL fluid was collected and stored in a class 2 safety cabinet for processing within 2-4 hours from the point of collection and freeze-thaw cycles were avoided. The total volume was measured and recorded to enable subsequent estimation of cell count. The samples were centrifuged at 1000 revolutions per minute (rpm) at 4°C for 5 minutes. BAL supernatant was decanted, taking care not to disturb the cell pellet, and re-centrifuged at a higher speed of 2500 rpm at 4°C for a further 5 minutes, then divided into 1 mL aliquots in micro-centrifuge tubes for storage at -80°C. The cell pellets were subsequently combined and mixed with Dulbecco's Phosphate Buffered Saline (PBS) to give an opaque solution to allow estimation of the total and differential cell counts. The final volume was then adjusted to give a concentration of 0.5 million cells/mL and the cell suspension was re-centrifuged at 1000 rpm at 4°C for a further 5 minutes. This supernatant was subsequently discarded and the cells were re-suspended in Dulbecco's PBS to give a concentration of 2–3 million cells/mL. 1 mL aliquots were retained and centrifuged at 3000 rpm for 4 minutes. The supernatant was again discarded and the cell pellets were stored at -80°C.

3.4.2 BAL total and differential cell counts

Cell count and differential per mL was performed on stained cytospin preparations, counting a minimum of 500 cells using an improved Neubauer chamber haemocytometer at 100x magnification (Figure 3-13). 10 μL of undiluted BAL was pipetted onto the chamber and secured with a glass cover slip. To obtain the total cell count per mL, the number of cells in the four large squares and the central square was determined under direct light microscopy (40x), equating to the number of cells per 0.1 μL. This number was subsequently multiplied by 10^4 to give the total number of cells per mL. Cell counts were performed twice and an average taken to improve accuracy of results.

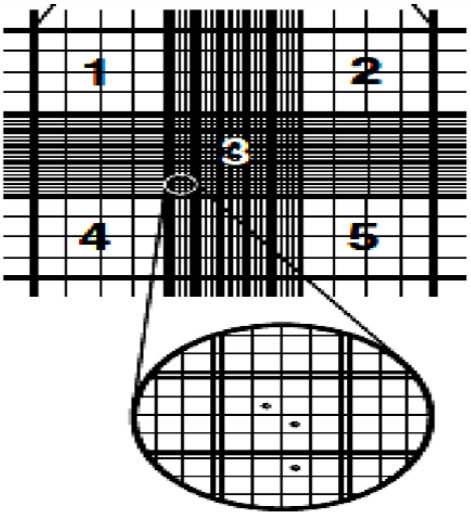

Figure 3-13 Cell counts using the improved Neubauer chamber haemocytometer

For differential cell counts, 100µL of re-suspended cells were placed into a cytospin cartridge with attached Superfrost Plus microscope slide (ThermoFisher Scientific) and centrifuged at 200 rpm for 6 minutes. The slides were allowed to dry overnight before being stained using the Diff-Quik method with immersion 6 times in methanol, 5 times in eosin, and 3 times in methylene blue prior to being allowed to dry. To obtain the differential cell count, a minimum of 500 inflammatory cells (macrophages, neutrophils, lymphocytes or eosinophils) were identified in three different regions of the slide under a light microscope (40x), and the number of neutrophils in each count was noted. Neutrophils were expressed as a percentage of the total inflammatory cell count.

3.4.3 BAL Protein assay

Protein concentrations of BAL were measured using the Pierce BCA protein assay kit (Pierce, Thermo Fisher) as per manufacturer's guidelines. A 12 point, 2-fold serial dilution of the BCA standard was performed to produce a standard curve of protein concentrations ranging from 0- 2000µg/mL. The working reagent was prepared by mixing 50 parts of BCA Reagent A with 1 part of BC Reagent B (50:1, Reagent A:B). 100 µL of standards and samples were added to a 96 well plate in triplicate followed by 100 µL of the working

reagent. The plate was mixed thoroughly on a plate shaker for 30 seconds, then subsequently covered and incubated at 37°C for 30 minutes before being cooled to room temperature. The absorbance of samples was measured at 560 nm on a plate reader (PerkElmer, Victor Wallace, Multilabel Plate Reader), and the protein concentrations of the samples quantified against the BCA standard curve.

3.4.4 Primary cell lines

Immortalised airway epithelial cell lines originating from human neoplasms or produced *in vitro* by physical or chemical mutagenesis or introduction of viral oncogenes, have been a tremendous asset to basic research as well as to the pharmaceutical and biotechnology industries.[403] Advantages of cell lines include their widespread availability, especially when compared to the scarcity of primary tissue and cells, homogeneity in terms of biochemical, electrophysiological, and growth characteristics, and the presence of matched isogenic control cell lines.[403, 404] However, although immortalised cell lines are valuable in the early stages of high throughput screening, they may have inherent limitations. The process of immortalisation may generate phenotypic, epigenetic, cellular or karyotypic instability and have major effects on cellular differentiation, morphology, or function compared to the situation *in vivo*.[403, 404]

The *ex vivo* culture of primary bronchial epithelial cells (PBECs) derived from bronchoscopic bronchial brushings of individual bronchiectasis patients provide a valuable but technically and logistically challenging source of cells that is likely to recapitulate more accurately the behaviour of bronchial epithelial cells *in vivo* and represent an invaluable tool to elucidate molecular signalling regulation in bronchiectasis. Due to the scarcity of such cells, preliminary experiments in this study were performed in a range of immortalised cell lines, primarily in human bronchial epithelial (HBE) cells as per below with limited experiments in pulmonary type II alveolar A549s (derived from adenocarcinomic human alveolar basal epithelial cells) and Calu-3 cells (ATCC HTB-55, derived from human lung adenocarcinoma).

3.4.4.1 16-HBE cell line

The human bronchial 16HBE14o epithelial cell line (gifted from Dr. Dieter Gruenert, University of California, USA) is an SV40 large T antigen-transformed immortalised cell line derived from a 1 year old male heart-lung patient with demonstrable high viability over successive passages. The transformed cells that retained differentiated morphology and function of normal human airway epithelia were maintained in Eagle's minimum essential medium (Gibco, UK) and alpha modification growth medium (Sigma-Aldrich, UK), supplemented with 10% foetal calf serum (FCS), 100 UI/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, UK). Following cell count and differential cell count, the remaining cells were seeded into collagen-coated (Vitrogen 100; Cohesion Technologies, Palo Alto, CA, USA) T75cm^2 flasks (Corning, Schipol, Netherlands) for further passage suspension and placed in a CO_2 incubator (37°C/5% CO_2) with medium replacement every 48 hours. Once confluent, PBECs were passaged using trypsin/ethylene diamine tetra-acetic (EDTA) (Sigma-Aldrich) which was neutralised using an equal volume of Roswell Park Memorial Institute RPMI (RPMI 1640 medium, Gibco) supplemented with 10% FCS. PBECs were then transferred in culture medium to T175 cm^2 collagen-coated tissue culture flasks (Corning, Schipol, Netherlands) for further passage then to 96-well plates (Corning, Schipol, Netherlands) for stimulation experiments in submerged culture.

3.4.4.2 *Ex-vivo* PBEC isolation and culture

Protected bronchial brushings (n=2) were obtained from subsegmental bronchi of large airways using a standard single-sheathed nylon cytology brush (5 fr; Wilson-Cook, Winston-Salem, NC, USA) according to standardised techniques. The brushes were detached on completion of the procedure and dispersed into 5 mL RPMI medium with 500 µL 1% penicillin, streptomycin and amphotericin based on methods previously described for transport to the laboratory for local processing within 1-2 hours or international shipping to the UK for processing within 24-48 hours.[403, 405]

On arrival at the laboratory, the suspended samples were centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the ensuing cell pellet was re-suspended in 5 mL of bronchial epithelial cell basal medium (BEBM), Lonza, San Diego, CA, USA) supplemented with bronchial epithelial cell growth medium (BEGM) Singlequots, (Lonza, San Diego, CA, USA), and 500 µL of 1% penicillin (Sigma-Aldrich, UK) and 1% streptomycin (Sigma-Aldrich, UK). The Singlequots consisted of 2 mL of Bovine Pituitary Extract (BPE), 500 µL of insulin 5 mg/ml, hydrocortisone 0.5 mg/mL, gentamycin 0.5 µg/mL, amphotericin 0.5 µg/mL, retinoic acid 0.1 µL/mL, transferrin 10 mg/mL, epinephrine 0.5 mg/mL, human epithelial growth factor (hEGF) 0.5 µg/mL, and tri-iodothyronine 0.5 mL. All PBEC work was carried out under strict sterile conditions in the laminar flow hood.

A 100 mL aliquot was taken for cell count and differential under Brightfield Light Microscopy, and the remaining cell suspension was transferred to a T25cm^2 plate pre-coated with collagen (Vitrogen 100; Cohesion, Palo Alto, CA, USA) and placed in a CO_2 incubator (37°C/5% CO_2). A further 5 mL of supplemented medium was added after the first 48 hours and the medium was subsequently exchanged every 48 hours until PBECs reached 80-95% confluence. PBEC cultures were carefully observed daily to ensure that the cells were growing satisfactorily and to look for any evidence of infection. Once confluent, the growth medium was removed from the flask and PBECs were passaged using trypsin (EDTA) (Sigma-Aldrich, UK) to detach and lift the adherent epithelial cells. The level of trypsinisation was determined by direct visualisation under light microscopy. After it was ascertained that a sufficient number of cells had been lifted from the flask, the trypsin was neutralised using an equal volume of RPMI supplemented with 10% FCS. The ensuing solution was decanted, transferred to a test tube and centrifuged at 1000 rpm for 5 minutes at 4°C to form a cell pellet. PBECs were re-suspended in 10 mL of culture medium with gentle mixing, following which the cells were seeded into Vitrogen (Cohesion)-coated T75cm^2 flasks (Corning, Schipol, Netherlands) for further passage; to eight chamber slides (Lab-Tek, Nunc, Naperville, IL, USA) for immunohistochemical analysis; to

24, 48, or 96-well plates (Corning, Schipol, Netherlands) for stimulation experiments in submerged culture; on to a semi-permeable membranes (Transwel inserts, Corning, Schipol, Netherlands), for air-liquid interface (ALI) culture; or alternatively, reserved for cryopreservation.

3.4.5 Characterisation of PBECs

To confirm epithelial characteristics, PBECs were seeded into eight-chamber slides (Lab-Tek, Nunc) in 200 µl aliquots and allowed to grow to 80-95% confluence. PBECs were then rested for 24 hours with the replacement of growth medium for resting medium, following which supernatants were removed and slides fixed in 100% ice cold acetone for 10 minutes and allowed to air dry.

PBECs were characterised in terms of their morphology (light microscopy and haematoxylin and eosin staining) and cytokeratin staining (immunohistochemistry). A representative proportion of cells were stained for cytokeratin using monoclonal mouse anti-human cytokeratin antibodies (PCK26 and CD324; BD Biosciences, USA and DakoCytomation, Ely, UK) at 1:50 dilutions with fluorescein isothiocyanate-conjugated secondary reagents, mounted in fluorescence mounting medium (DakoCytomation) and examined using confocal microscopy. Cells were subsequently counterstained with Haematoxylin and Eosin (H&E) to ensure an epithelial cell phenotype.

3.4.6 Cryopreservation and reconstitution of PBECs

Cell pellets were generated using the trypsinisation method previously described, then re-suspended in 1 mL of freezing media (80% BEBM, 10% FCS and 10% dimethyl sulfoxide (DMSO), (Sigma-Aldrich, UK)). The cell mixture was subsequently transferred to sterile cryotubes (Thermo Fisher, Loughborough, UK) for placement in an isopropranolol cell freezer (Thermo Fisher), which slowly decreases the temperature to −80◦C. After 24 hours, tubes were transferred to a liquid nitrogen cell freezer for long-term storage at -180◦C.

Cryotubes containing 1 mL cell suspensions were removed from the liquid nitrogen cell freezer and rapidly rewarmed in a 37°C water bath for 10 minutes. Once defrosted, the suspension was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and 5mL of complete BEGM pre-warmed to 37°C was slowly added and the cells re-suspended. The resultant cell suspension was then seeded in a T25cm^2 tissue culture flask pre-coated with 0.5% collagen (Vitrogen 100, Cohesion, Palo Alto, CA, USA). Cells were incubated at 37°C for 24 hours in a 5% CO2 atmosphere with the media changed after 24 hours. After 48 hours, the samples were checked under the microscope and the media changed until cells reached confluency.

3.4.7 Cell viability assays

Cell viability may be judged by morphological changes such as changes in membrane permeability or physiological changes inferred from the exclusion of certain dyes or the uptake and retention of others.[406] For the purpose of this study, two methods for assessing cell viability were employed: (a) CellTiter-Blue Assay, and (b) MTT assay.

The CellTiter-Blue Assay (Promega, USA) is based on cellular reduction of resazurin to the absorption product resorufin. This assay works on the principle that viable cells are able to metabolise and reduce the dye, whereas the capacity to reduce the dye of dead cells rapidly diminishes once their membranes are compromised. Cells were incubated, under experimental conditions, for 48 hours, before being assessed using the Titre-blue Assay (Promega, USA). The medium in the 48 or 96-well plates was discarded and cells in 4 or 8 wells were killed using 200 µl ice-cold methanol for 5 minutes. 100 µl of CellTiter-Blue was added to each well followed by 500 µl of resting (serum-free) medium. The plate was incubated for 2-4 hours, after which the samples were plated out in 96-well plates. The ratio of live to dead cells was used to generate a standard curve, divided into five regions: 100% live cells (resting medium); 25:75% dead:live cells; 50:50%; 75:25% dead:live cells; and 100% dead cells (positive control). Absorbance measurements were used to monitor results. As the absorption maximum for resazurin is 600nm and

573 nm for resorufin according to manufacturer's data, absorbance was measured at 560 nm and 600 nm was used as the reference wavelength.

Behind the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay is the principle that mitochondrial activity is equal for the majority of viable cells and that a reduction or increase in the viable cell count can therefore be linearly correlated to mitochondrial activity. When formazan crystals are reduced from the tetrazolium salt MTT, this is seen to reflect the level of mitochondrial activity within the cells that can be solubilised for homogenous measurement. As a result, measuring the formazan concentration reflected in optical density (OD) using a plate reader at 570 and 690 nm can be used to identify changes in cell viability.[407] The MTT assay is mainly used to assess cell viability in the presence of a drug or toxin. 50 μL of cells were added to a 96 well plate followed by 100 μL of MTT solution (Sigma-Aldrich) at 100 μg/mL in complete RPMI medium. The plate was then incubated in a tissue culture incubator (5% CO2) for 2 hours, after which time the media and MTT solution was removed and the cells had adhered to each individual well. 100 μL of DMSO was then added to each well, and the plate was incubated in room temperature on an orbital mixer for 30 minutes. Absorbance values were read in a Victor Wallace plate reader at a 550 nm wavelength.

The MTT detection assay is considered the most sensitive, has fewer steps, is the fastest to perform, and has the least amount of interference whereas the resazurin reduction assay offers a less expensive alternative.

3.4.8 Enzyme-linked immunosorbent assay techniques

Commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Duoset, USA) were used according to manufacturer's instructions to measure individual inflammatory markers (Table 3-10; Figure 3-14). The assays were carried out as detailed in the individual kit protocols according to the generic process described below:

- Coating with capture antibody: 100 μL of capture antibody (R&D Systems, USA) diluted to a working concentration of 1 in 100 in

reagent diluent ((0.1% bovine serum albumin (BSA), 0.05% Tween 20 in Tris buffered saline), (20mM Trizma base, 150 mM NaCl, pH 7.2-7.4, 0.2 µm filtered)) was coated on a 96-well plate (Maxisrop, Nunc). The plate was covered with a plate sealer and incubated overnight at room temperature.

- Wash step: The contents of the plate were discarded and wells washed three times using 300 µL wash buffer per well (0.05% Tween 20 in phosphate buffer solution (PBS), pH 7.2-7.4). The plate was dried by inversion and by patting the plate with force on paper towels.
- Blocking step: The plate was then blocked by adding 300 µL of block buffer (1% BSA in PBS) to each well and incubated at room temperature for 1 hour, following which the aspiration and wash stages were repeated.
- Standards and samples: After the plate was blotted dry, 100 µL of samples or standards diluted in reagent diluent were added to each well to create a standard curve for data extraction. The plate was then covered with an adhesive strip and incubated for 2 hours at room temperature. Aspiration and wash were again repeated.
- Detection antibody: 100 µL of the detection antibody diluted in reagent diluent was then added to each well. This was again covered with an adhesive strip and the plate incubated for two hours at room temperature, following which aspiration and wash were again repeated.
- Development: 100 µL of streptavidin horseradish peroxidase (HRP) diluted in reagent diluent was added to each well and incubated for 20 minutes out of direct light at room temperature. Following this, 100 µL of substrate solution (1:1 mixture of Colour Reagent A (hydrogen peroxide) and Colour Reagent B (tetramethylbenzidine)) was then added to each well. The plate was covered and incubated away from direct light for a further 20 minutes at room temperature. The addition of 50 µl stop solution (2M sulphuric acid) was required for each well and the plate was placed on the plate shaker for 1 minute at 100 rpm to ensure thorough mixing.

- Analysis: The optical density of the contents of each well was immediately determined using a microplate reader at 570 nm and 450 nm to correct for optical imperfections in the plate.
- Determination of cytokine and chemokines in sample from standard: The average optical density was derived for the standard and sample wells with subtraction of blanks from each mean value. GraphPad (V5.0) was used to create a standard curve with a 4-parameter logistic curve fit and the sample values interpolated from the standard curve based on the lower limit of detection for each cytokine or chemokine.

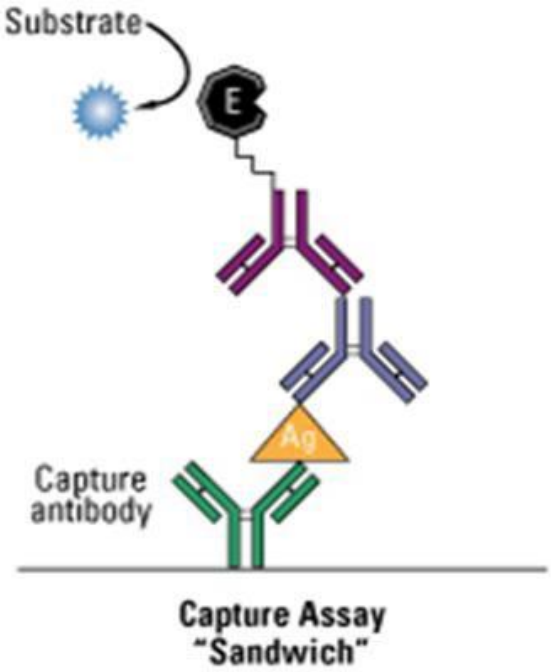

Figure 3-14 Schematic of the sandwich ELISA technique

The basic capture antibody is immobilised to the assay plate. The antigen (Ag) binds to the capture antibody and is further detected by the detection antibody, hence the 'sandwich' with the antigen stuck between two antibodies. The secondary antibody conjugated with horse-radish peroxidise (HRP) binds to the primary antibody. The substrate solution reacts with the HRP to produce a colour change proportional to the amount of inflammatory marker present in the samples. A spectrophotometer is then used to give quantitative values for colour strength to provide results. (Picture modified from Thermo Fisher Scientific UK).

Table 3-10 Composition and concentrations of reagents and antibodies used in ELISA throughout study experiments

Protein name	Capture antibody origin and concentration	Detection antibody origin and concentration	Reagent diluent	Substrate	Detection range	Supplier number
IL-8/ CXCL-8	Mouse anti-human IL-8 4µg/mL	Biotinylated goat anti-human IL-8 20ng/mL	0.1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/mL	R&D Duoset DY208
IL-6	Mouse anti-human IL-6 2µg/mL	Biotinylated goat anti-human IL-6 50ng/mL	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	9.38-600 pg/mL	R&D Duoset DY206
GM-CSF	Mouse anti-human GM-CSF 2µg/mL	Biotinylated mouse anti-human IL-8 0.5µg/mL	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	15,62-1000 pg/mL	R&D Duoset DY215
MMP9	Mouse anti-human MMP9 1µg/mL	Biotinylated goat anti-human MMP9 12.5ng/mL	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/mL	R&D Duoset DY911
Pro-collagen	Mouse anti-human Pro-Collagen 4µg/mL	Biotinylated goat anti-human Pro- Collagen 100ng/mL	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/mL	R&D Duoset DY6220
TGFβ1	Mouse anti-human TGFβ1 2µg/mL	Biotinylated chicken anti-human TGFβ1 antibody 100ng/mL	1% BSA in PBS (0.05% Tween20 in TBS), pH 7.2-7.4	TMB	31.3-2000 pg/mL	R&D Systems DY240
VEGF	Mouse anti-human VEGF antibody 2µg/mL	Biotinylated goat anti-human VEGF 100ng/mL	1% BSA in PBS, pH 7.2-7.4	TMB	31.3-2000 pg/mL	R&D Duoset DY293B

IL: interleukin; CXCL: chemokine ligand; BSA: bovine serum albumin; PBS: phosphate buffer solution; TBS: tris buffered saline; TMB- 3,3',5,5'-Tetramethylbenzidine; GM-CSF: granulocyte-macrophage colony stimulating factor; MMP: matrix metalloproteinase; TGF: transforming growth factor; VEGF: vascular endothelial growth factor.

3.4.9 Cytokine expression profiling

The Bio-Plex 200 system (Bio-Rad Life Science, California, USA), a Luminex-based multiplex analysis system that permits the simultaneous analysis of up to 100 different biomolecules (i.e., proteins, peptides, or nucleic acids) in a single microplate well, was used for analysis of Galway BAL, HBE and PBEC experiments. Each assay was performed on the surface of a 6.5 μM polystyrene bead. Beads were filled with different ratios of two fluorescent dyes, resulting in an array of 100 distinct spectral addresses. We utilised the Bio-Plex Human Cytokine 10-Plex A Panel (Bio-Rad) according to the manufacturer's instructions, with the following cytokines assayed: IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF-α and VEGF. A work flow of the multiplex assay is provided below (Figure 3-15).

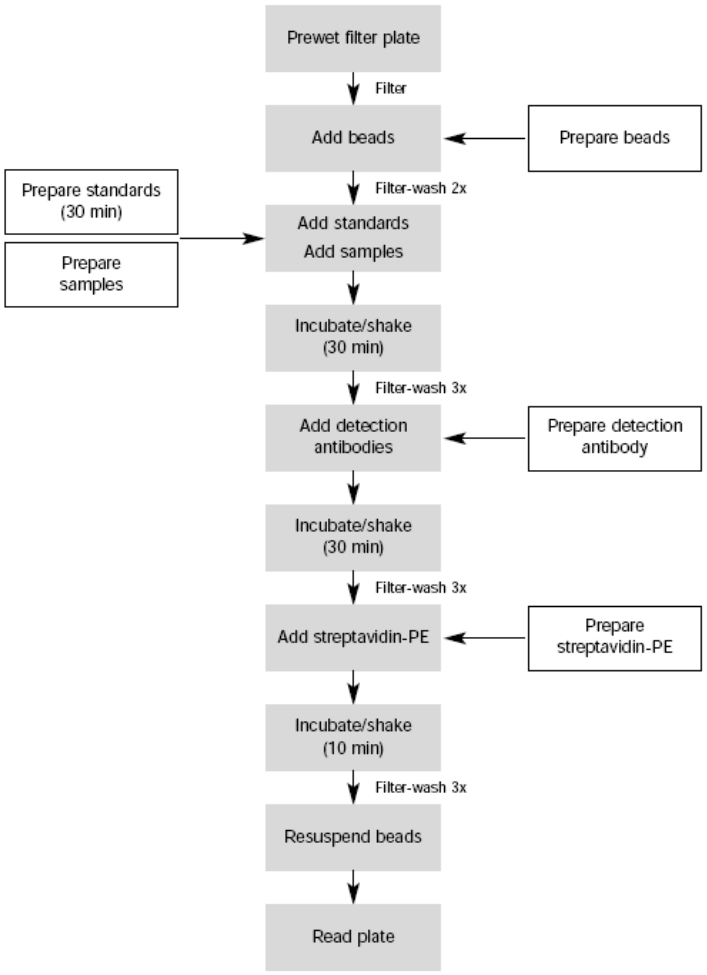

Figure 3-15 Bio-Plex cytokine assay workflow

Briefly, a standard curve using the high photomultiplier tube setting and starting at a 10-fold lower concentration of cytokine than recommended for serum or plasma samples was used. The standard vial from the Bio-Rad human cytokine 10-plex kit containing 500,000 pg of lyophilised recombinant multiplex standard was reconstituted in 500 µL of standard diluent to 44,000 pg/mL. This was vortexed gently for 5 seconds and then incubated on ice for 30 minutes. A serial dilution to produce a standard curve ranging from 0 pg/mL to 25,000 pg/mL was performed as demonstrated in Figure 3-16 below.

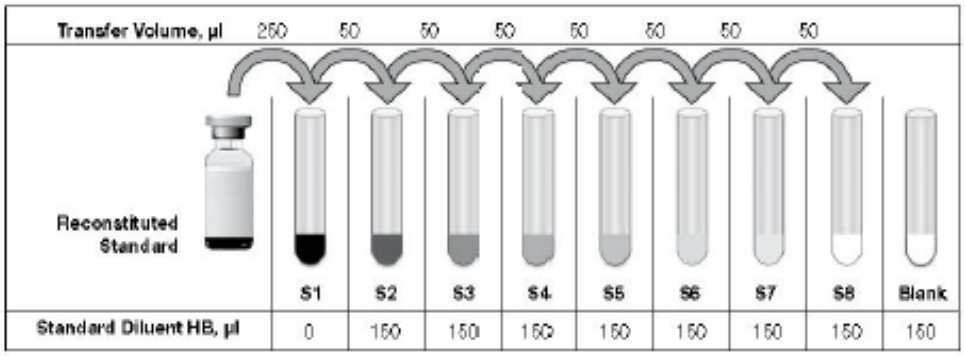

Figure 3-16 Performance of serial dilutions creation of a standard curve

Undiluted samples were thawed and vortexed during standard and plate preparation. The anti-cytokine bead stock solution was prepared by adding 200 µL assay buffer to the coupled beads following vortex at medium speed for 20 seconds to allow bead re-suspension. 50 µL of antibody coated fluorescent beads per well was then added to the 96-well plates provided. The plate was then washed 3 times with Bio-Plex wash buffer. A 50 µL volume of each sample, control or standard was subsequently added to the 96 well plates which was covered with a plastic adhesive plate sealer and incubated for 30 minutes at room temperature. The plate was washed a further 3 times with Bio-Plex wash buffer prior to the addition of 25 µL of detection antibody to each well and a further incubation period of 30 minutes at room temperature. After 3 plate washes, 50 µL of streptavadin-PE was added to each well with alternate 10 minute incubation and washing steps. The beads in each well were then re-suspended in 125 µL of Bio-Plex Assay Buffer and

the plate was subsequently read on the Bio-Plex 200 suspension array at a low RP1 target setting (used to maximise assay sensitivity when the expected concentrations may be less than 3,200 pg/mL) using a high throughput fluidics system. Analysis was performed on the Bio-Plex 200 Manager Software, v3.0.

3.4.10 Proteomics analysis

High resolution label-free shotgun proteomics was employed to investigate BAL protein profiles in bronchiectasis patients (with and without GORD) versus chronic bronchitis patients versus age, sex and ethnicity-matched healthy volunteers using nano-flow liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Protein concentrations of BAL supernatants were quantified using Pierce 660 protein assay. 50 µcg of BAL protein from each sample was added to an equal volume of acetonitrile before incubating at 100°C for 15 minutes. The samples were dried down in a centrifugal vacuum and resuspended with 50mM ammonium bicarbonate (pH 8.5) to a final concentration of 1 mg/mL. Samples were then reduced and alkylated before subjecting to nano-flow-LC-MS/MS analysis according to previous reports.[408] Protein identification and label-free quantification were carried out using Maxquant (version 1.4.1.2) against Uniprot-human database (version 2014-07-09). The fixed modification was carbamidomethylation on cysteine, and variable modifications included oxidation on methianone and N-terminal acetylation. The false discovery rate (FDR) for protein quantification was set to 1% at protein level employing the Banjamini-Hochberg method. Data visualisation was carried out using SIMCA-P (v13.0.3). For statistical analysis, the data was $\log^2$ transformed before subjecting to unpaired t-test using Perseus (v1.5.4.1). Principal component analysis (with the dataset log transformed, mean-centred and unit variance scaled) was performed with missing values replaced from normal distribution and corrected p-values of <0.05 were considered statistically significant.

3.4.11 Pepsin analysis

Total pepsin in BAL was measured in triplicate using a locally developed indirect ELISA made up of porcine pepsin (Sigma-Aldrich, UK) (Figure 3-17). 100 µL of samples or standards diluted in phosphate buffered saline (PBS) were coated on to a 96-well plates (Maxisorp, Nunc). The plates were covered with a plate sealer and incubated overnight at room temperature. The following day, each well was aspirated and washed with 300 µL wash buffer (0.05% Tween 20 in PBS), repeating the process twice for a total of three washes. The plate was then blotted dry on clean paper towels and blocked with 1% BSA in PBS for 2 hours at room temperature, following which the aspiration and wash stages were repeated. After the plate was blotted dry, 100µL of primary antibody solution (monoclonal anti-pepsin antibody, Biodesign International, USA) diluted to a working concentration of 1 in 2000 in reagent buffer (0.1% BSA and 0.05% Tween 20 in PBS) was added to the wells. The plates were then covered and incubated for a further 2 hours at room temperature with the aspiration and wash stages again repeated followed by two changes of PBS. 100 µL of secondary antibody (horseradish peroxidase conjugated polyclonal anti-goat/-sheet antibody, Sigma-Aldrich, UK) diluted to a concentration of 1 in 5000 with antibody diluent was then added to each well. The plate was again covered and incubated for a further 2 hours at room temperature. The wash step was repeated followed by the addition of 100 µL of substrate solution (2,2'- Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid) to each well. The plate was covered and incubated for 20 minutes at room temperature, avoiding direct light. 100 µL of stop solution (1% sodium dodecyl sulphate) was subsequently added to each well and the absorbance measured at 405 nm using a microplate reader (Bio-Tek EL808). Negative controls were carried out for all standards and sample dilutions by omitting the primary antibody. The lower limit of detection of the assay was 10 ng/mL.

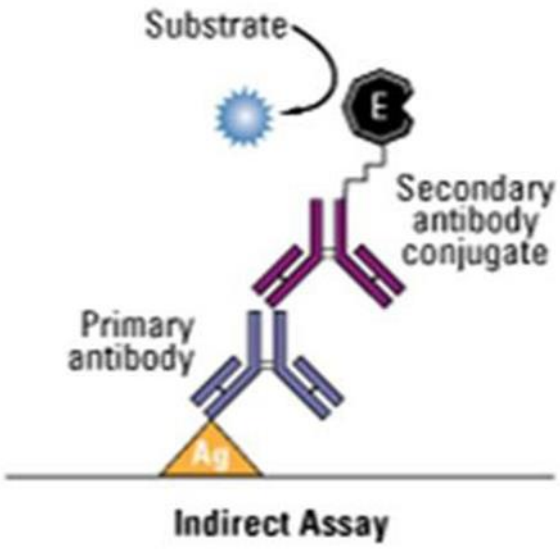

Figure 3-17 Schematic of the indirect pepsin ELISA technique

The pepsin antigen (Ag) immobilised by direct absorption to the assay plate is bound by the primary unlabelled antibody which is further detected by the secondary antibody conjugated with horse-radish peroxidise (HRP). The substrate solution reacts with the HRP to produce a colour change proportional to the amount of pepsin present in the samples. A spectrophotometer is then used to give quantitative values for colour strength. (Picture modified from Thermo Fisher Scientific UK).

3.4.12 Bile acid analysis

Tandem mass spectrometry is a technique that allows the analysis of metabolites and proteins in blood and other bodily fluids. Since the detection of bile acid salts using standard spectrophotometric based approaches has been shown within the group to be somewhat limited, a more sensitive tandem mass spectrometry method incorporating a modified extraction based protocol was used. This was performed at a nationally accredited external laboratory, blinded to the study - Sheffield Children's Hospital, UK, where the lower limit of detection of assay sensitivity has been shown to be as low as 1 nmol/L.

Conjugates and isomers of cholic acid and lithocholic acid in BAL were measured using a Waters Acquity TQ Detector tandem mass spectrometer with direct flow injection. Samples were extracted using a 500 mg SPE

cartridge on a C18SPE column (Supelco LC-18), pre- washed with 10 ml methanol, then 10 mL distilled H2O prior to sample loading. 400 µL of sample was then added to 10 mL of distilled water containing 150 µL of deuterated taurocholate (internal standard). The column was subsequently washed again with 10 mL deionised water and 2 mL hexane. The bile salts were eluted with 10-20 mL methanol and reduced to dryness by rotary evaporation. The extract was then reconstituted in 1 mL of 90% acetonitrile and centrifuged at 13,000 rpm for 5 minutes. Following this, 30 µL of BAL supernatant was injected directly onto the tandem mass spectrophotometer with 50% acetonitrile acting as a running buffer. The bile acid salts were measured using negative ion mode and multiple reaction monitoring scans, providing a sensitivity of 1 nmol/L.[409]

3.5 Ethical approval

Study protocols were approved by local and international research ethics committees (C.A. 771; C.A. 1221; C.A. 1228) and performed according to the Declaration of Helsinki. Patient information leaflets for individual investigations were completed and incorporated into routine clinical practice for bronchoscopy and oesophageal investigations. All participants provided written informed consent prior to being enrolled in the study.

3.6 Handling of missing data

A complete-case analysis was performed in all observational studies according to STROBE recommendations. This method of handling missing data includes only participants with complete data on all waves of data collection in the analysis, and is based on the assumption that any missing data is completely at random and therefore the complete cases are representative of those with missing data. This method can potentially introduce bias if the missing data are not missing completely at random or if there is a large proportion of missing data, which may reduce the precision of the analysis. Where possible, sensitivity analyses were conducted in relation to assumptions about the missing data.

For biological data, samples below the lower limit of detection were analysed in two ways: using a complete-case analysis whereby all data with results below the lower limit of detection were excluded and by setting the lower limit of detection to zero in variables where <5% were below the lower limit of detection. Variables with a significant amount of data below the lower limit of detection were excluded. The subsequent chapters are therefore based on a complete-case analysis which, although this can potentially reduce model precision of estimate parameters, it removes the introduction of bias in model estimates and therefore makes the results more meaningful and generalisable.

3.7 Statistical analysis

All analyses were computed using SPSS® v21.0 for Windows platform and GraphPad Prism v5.0. The distribution of all data was tested for normality using the Shapiro-Wilk test. Normally and non-normally distributed data were expressed as mean ± standard deviation (SD) or median ± interquartile range (IQR) and 95^{th} percentile, respectively. Comparisons between multiple groups were performed for normally distributed measures using ANOVA (with Bonferroni's post hoc adjustment) or Welch's robust test (with Tamhane's post hoc adjustment) according to the homogeneity of their variances, which was tested with Levene's statistic. In data with non-Gaussian distribution, the non-parametric Kruskal-Wallis test (with Dunn's post hoc adjustment) was used for multiple group comparisons, Mann-Whitney for unpaired differences between two groups and the Wilcoxin Signed rank test for paired differences between two groups. Differences in proportions were compared using the Chi squared or Fisher exact test. For comparison of severity scores, the area under the receiver operator curve (AUC) was used. Effects estimates were pooled using a random effects meta-analysis to determine discrimination and calibration of scores with statistical heterogeneity assessed between cohorts using the Higgins I^2 test. Weibull parametric survival analysis was used to model the prediction of 5-year mortality in candidate comorbidity scores with variables included in the model based on a backward stepwise approach. The rounded averaged β-coefficient was used to award "points" for each variable in the resultant

prediction scores. Kaplan-Meier curves were used to indicate survival analysis. Bivariate correlations between parameters were identified with Pearson's and Spearman's rank tests, for normally and non-normally distributed data, respectively. The correlation coefficient is a statistical measure of the strength of the relationship between paired data. The closer the value is to 1, the stronger the relationship. Correlation is an effect size; the strength of the correlation can therefore be described using the following guide for the absolute value Spearman's rho: ≤ 0.19 - very weak; 0.2-0.4 weak; 0.40-0.59 moderate; 0.60-0.79 strong; ≥ 0.80 very strong. Inter-rater variability was assessed using Cohen's kappa statistics with linear weighting. Fully adjusted multivariable Cox proportional hazards regression and negative binomial regression analysis was used to determine hazard ratios (HR), incidence risk ratios (IRR) and 95% confidence intervals (CIs) for end-points of exacerbations, hospitalisations and treatment effects, by the presence of GORD in the FRIENDS and EMBARC cohorts. For proteomics analysis, data was $\log^2$ transformed before subjecting to unpaired t-test using Perseus (v1.5.4.1). Principal component analysis (with the dataset log transformed, mean-centred and unit variance scaled) was performed with missing values replaced from normal distribution. We defined statistical significance as a two-tailed $p<0.05$. All analyses were performed using SPSS version 21 (SPSS, Chicago, IL, USA) for Windows platform and Graph Pad Prism Version 5 (Graph Pad Software, Inc. San Diego, CA, USA).

Chapter 4 - Multidimensional Severity Assessment in Bronchiectasis

Publications relevant to this chapter:

McDonnell MJ, Aliberti S, Goeminne PC, Dimakou K, Zucchetti SC, Davidson J, Ward C, Laffey JG, Finch S, Pesci A, Dupont LJ, Cowman S, Fardon TC, Skrbic D, Obradovic D, Loebinger MR, Rutherford RM, De Soyza A*, Chalmers JD*. Multidimensional severity assessment in bronchiectasis: an analysis of seven European cohorts. *Thorax* 2016; 71:1110-1118.

Chalmers JD, Goeminne P, Aliberti S, McDonnell MJ, Lonni S, Davidson J, Poppelwell L, Salih W, Pesci A, Dupont LJ, Fardon TC, De Soyza A, Hill AT. The Bronchiectasis Severity Index: An International Derivation and Validation Study. *Am J Respir Crit Care Med.* 2014; 189(5): 576-585

4.1 Introduction

Bronchiectasis is a heterogeneous, poorly understood, multidimensional disease with recent UK and German data demonstrating increasing prevalence and hospitalisation rates.[1, 26, 250] Management is challenging as there are no licensed therapies. Given the majority of available treatments are antibiotic-based, it is important for antimicrobial stewardship to target treatments to severe patients at risk of complications and avoid over-treatment of mild patients at low future risk.[1, 410-414]

Two composite disease-specific prognostic indices have been developed for bronchiectasis to aid clinical decision making: the Bronchiectasis Severity Index (BSI) and the FACED score.[19, 20] Both attribute points according to age, value of forced expiratory volume in 1 second (FEV_1) % predicted, presence of chronic colonisation by *Pseudomonas aeruginosa*; radiological extension and type of bronchiectasis, and degree of dyspnoea. The BSI also considers body mass index (BMI), exacerbation frequency, prior hospitalisation for severe exacerbation, and chronic colonisation with bacteria other than *P. aeruginosa*. Both scores classify patients into low, moderate and high-risk groups albeit using different thresholds. The BSI was derived from a large Edinburgh-based study in the UK, and was subsequently validated in four independent international cohorts.[19] The FACED score was derived from a retrospective Spanish study and has since been independently evaluated in a single centre UK cohort of 74 patients for long-term prediction of mortality and in 651 patients in six historical cohorts of Latin American patients.[20, 73] FACED was developed specifically to predict mortality while the BSI was developed to predict mortality, severe exacerbations, frequency of exacerbations and quality of life (QoL).

The data currently available suggests that both scores can predict future mortality in bronchiectasis. Bronchiectasis is not, however, a disease whose impact is primarily measured in terms of mortality. Outcomes other than mortality are likely to be more important in terms of patients' priorities, clinical decision making, healthcare utilisation and socioeconomic costs.[8,

26, 415-417] Clinicians face two major challenges in the management of bronchiectasis: 1) identifying patients with a high symptom burden or those at risk of frequent exacerbations or rapid lung function decline who may benefit from aggressive treatment, and 2) identifying low risk patients that could be suitable for non-specialist follow up or simpler treatment regimes.[102, 418] No therapies have been developed that can reduce mortality in bronchiectasis, but existing and developing therapies are designed to improve QoL, reduce symptoms, reduce exacerbations and slow disease progression. Therefore for clinical trials design and subsequent "real world" decision-making, these are the key outcomes to identify.[9, 10, 224] The concept of "severe" bronchiectasis should therefore reflect patients with impaired QoL, severe symptoms, frequent exacerbations and progressive disease.

There are limited published data on predictors of outcomes other than mortality in bronchiectasis. This study aimed to evaluate the predictive ability of the two bronchiectasis tools, the BSI and FACED score, in assessing clinically relevant disease outcomes across multiple European cohorts.

4.2 Methods

4.2.1 Study population

7 European centres participating in the European Bronchiectasis registry project contributed to the study (Figure 4-1). Detailed descriptions of these cohorts have previously been published.[29, 51, 95, 419, 420] All cohorts used a standardised protocol and provided data in a standardised case report form. Inclusion criteria were consecutive adult patients with a HRCT-confirmed diagnosis and clinical history consistent with bronchiectasis. Patients were excluded if they had active malignancy, cystic fibrosis, or a primary diagnosis of pulmonary fibrosis/sarcoidosis with secondary traction bronchiectasis. Patients were assessed and managed according to a standardised protocol based on the British Thoracic Society (BTS) guidelines.[9] Ethical approval was obtained from each individual centre's Research Ethics Committee. In order to ensure statistical independence, the

derivation cohorts of the BSI and FACED scores were not considered for inclusion in the present analysis.[19, 20]

4.2.2 Clinical assessments and calculation of severity scores

Patients were followed up at outpatient clinic assessments. The underlying aetiology of bronchiectasis was determined following testing recommended by BTS guidelines.[9] The BSI and FACED scores were calculated using baseline data as described in the methodology section.[19, 20] Patients were classified according to severity cut-offs described in original publications as mild, moderate or severe.

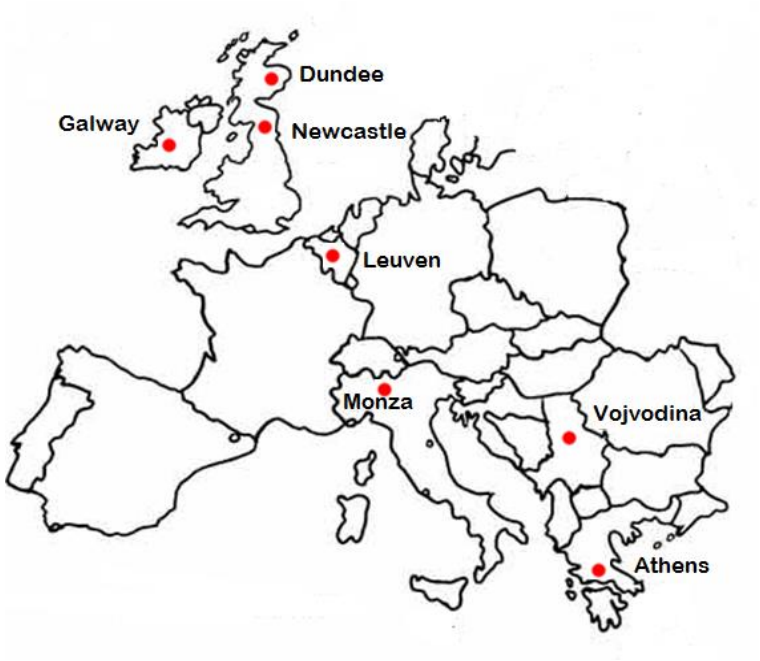

Figure 4-1 International cohorts involved in study synthesis

These include Dundee, Scotland: n=494; Galway, Ireland: n=280; Monza, Italy: n=250; Leuven, Belgium: n=190; Athens, Greece: n=159; Newcastle, England: n=126; and Vojvodina, Serbia: n=113, totalling 1,612 patients.

4.2.3 Study endpoints

Mortality: Data on all-cause mortality were collected for up to 5 years in keeping with follow-up periods of the original BSI and FACED derivation cohorts.

Exacerbations: An exacerbation was defined as the requirement for antibiotics in the presence of one or more symptoms of increasing cough, increasing sputum volume, worsening sputum purulence, worsening dyspnoea, increased fatigue/malaise, fever, and haemoptysis.[9, 25] Exacerbations were recorded for 12 months after severity score calculation.

Hospitalisation for severe exacerbations: Severe exacerbations were defined according to BTS guidelines as unscheduled hospitalisations or emergency department visits for exacerbations or complications as recorded from patient histories and verified using administrative databases. [9, 25] Hospital admissions were recorded up to 5 years follow-up post-score calculation.

FEV_1% predicted decline: This was analysed for up to 4 years of follow-up.

QoL: This was assessed by the Quality Of Life Bronchiectasis (QOL-B) questionnaire (version 3.1) and the St. Georges Respiratory Questionnaire (SGRQ), with minimum clinically important differences (MCIDs) of 8 and 4 points respectively.[76, 78]

Symptoms and function: Cough symptoms were evaluated using the Leicester cough questionnaire (LCQ) (MCID of 1.3 units).[77] Exercise capacity was evaluated with the six minute walking test distance (6MWD) which has an MCID of 84 m.[421]

4.2.4 Definition of validity

Validity of each scoring system was evaluated in terms of discrimination, the degree to which groups are different from each other, and calibration, the degree to which the scores perform as expected across different healthcare systems. Good discrimination and calibration are required for a score to be considered valid.

For mortality and hospitalisations, a scoring system was considered to be valid if the area under the receiver operator characteristic curve (AUC) exceeded 0.7. A value of > 0.7 is generally regarded as clinically useful and was therefore chosen as a cut-off for valid prediction. [19, 20] AUC evaluates discrimination. As mortality was the endpoint in both the BSI and FACED derivation studies, we also evaluated calibration compared to the original derivation. Scores were considered valid if there was no statistically significant difference between pooled event rates in the validation cohorts and the original predicted event rate.[422] For QoL and symptom scores, scores were considered to be valid if the differences between the 3 groups exceeded the reported MCIDs.[76-78, 421]

4.2.5 Statistical analysis

Simple descriptive statistics were used to compare baseline data according to data distribution. Mean differences were compared using T test (or ANOVA for more than two groups) and medians using the Mann Whitney U test or Kruskal Wallis test as appropriate. The AUC was used for discrimination analysis and differences between AUC values were compared using the method of DeLong *et al*.[423] For calibration analysis, odds ratio's (OR) and 95% confidence intervals (CI) were calculated comparing event rates in the validation cohorts versus the original derivation cohorts. Exacerbations and hospital admissions during follow-up were evaluated by Poisson regression analysis with data presented as rate ratio (RR) with 95% CI.

Effect estimates were pooled using a random effects meta-analysis to determine overall discrimination and calibration. Statistical heterogeneity between cohorts was assessed using the Higgins I^2 test.

Analyses were conducted using SPSS V21 for Windows (SPSS, Chicago, Illinois, USA), Graph Pad Prism V6 (Graph Pad Software, Inc. San Diego, California, USA), and Metadisc V1.4 (Hospital Universitario Ramon y Cajal, Spain).

4.3 Results

4.3.1 Baseline characteristics

7 international cohorts were included comprising 1,612 patients. The characteristics of each cohort are shown in Table 4-1. Cohorts were heterogenous in keeping with the fact they derive from different healthcare systems. However, all cohorts had a female predominance. The average annual rate of exacerbations ranged from 1-3/year. The cohorts were primarily classified as moderate to severe bronchiectasis based on mean BSI scores (6.0-9.7); however, in contrast, the majority were considered to be mild bronchiectasis according to the FACED score (mean 1.5-2.3), (Figure 4-1). Data for mortality, hospitalisations and exacerbations were available in all 7 cohorts. Additional data was available for lung function decline, 6MWT, QoL and LCQ from 2 cohorts (Dundee and Monza) comprising 744 patients.

Table 4-1 Baseline characteristics of European cohorts

Characteristics		Dundee, Scotland	Galway, Ireland	Monza, Italy	Leuven, Belgium	Athens, Greece	Newcastle, England	Vojvodina, Serbia
n		494	280	250	190	159	126	113
Study dates		2011-2015	2008-2015	2011-2015	2006-2012	2010-2015	2009-2013	2010-2015
Age	mean (SD)	65.3 (12.9)	60.5 (14.6)	65.1 (12.2)	66.4 (16.0)	59.3 (16.2)	59.1 (14.5)	62 (13.0)
Female	n (%)	300 (60.7)	188 (67.1)	147 (58.8)	97 (51.0)	101 (64.0)	75 (59.5)	80 (70.8)
FEV_1 % pred.	mean (SD)	71.6 (24.7)	80.3 (25.9)	79.2 (27.5)	69.3 (25.3)	70.1 (24.9)	64 (26.9)	64.8 (26.2)
BMI	mean (SD)	25.9 (5.2)	27.1 (5.6)	23.7 (4.4)	23.9 (4.3)	24.6 (3.4)	26.2 (5.1)	25.1 (4.9)
Chronic *Pseudomonas*	n (%)	63 (12.8)	39 (13.9)	54 (21.6)	16 (8.4)	58 (36.5)	13 (10.3)	1 (1%)
Reiff score	mean (SD)	4.4 (3.0)	3.4 (3.0)	5.5 (2.7)	4.5 (1.3)	4.8 (2.5)	2.8 (1.4)	4.7 (2.4)
MRC dyspnoea score	mean (SD)	2.3 (1.1)	2.0 (1.0)	2.0 (1.3)	2.3 (1.2)	2.4 (1.5)	2.5 (1.1)	2.5 (1.4)
Exacerbations/year	mean (SD)	2.1 (2.6)	2.9 (1.3)	1.9 (2.0)	1.9 (2.1)	2.4 (1.5)	3.4 (1.7)	1 (1.25)
LTOT	n (%)	8 (1.6)	9 (3.2)	35 (14.0)	10 (5.3)	26 (16.4)	0 (0)	12 (10.6%)
Prior hospitalisations	n (%)	118 (23.9)	62 (22.1)	34 (13.6)	55 (28.9)	83 (52.2)	75 (59.5)	15 (13.3%)
BSI	mean (SD)	7.3 (4.4)	6.8 (4.5)	7.3 (4.1)	7.6 (4.6)	9.1 (5.4)	9.7 (4.9)	6.0 (3.7)
FACED	mean (SD)	2.1 (1.6)	1.5 (1.5)	2.3 (1.6)	1.9 (0.9)	2.1 (1.8)	1.6 (1.6)	2.3 (1.6)
Endpoints evaluated		Mortality Hospitalisation Exacerbations Symptoms Quality of life FEV_1 decline	Mortality Admissions Exacerbations	Mortality Admissions Exacerbations Symptoms Quality of life FEV_1 decline	Mortality Admissions Exacerbations	Mortality Admissions Exacerbations	Mortality Admissions Exacerbations	Mortality Admissions Exacerbations

4.3.2 Mortality

Outcomes across each of the cohorts are shown in Table 4-2. Patients were followed up for 5-years. There was a clear difference in classification of the scores, with the BSI identifying 16.7-38.9% of patients as low risk versus 52.6-72% with FACED. A much larger number of patients were identified as having severe bronchiectasis with the BSI, 21.2-63.5%, compared to 3.6-13.2% with FACED (Figure 4-2).

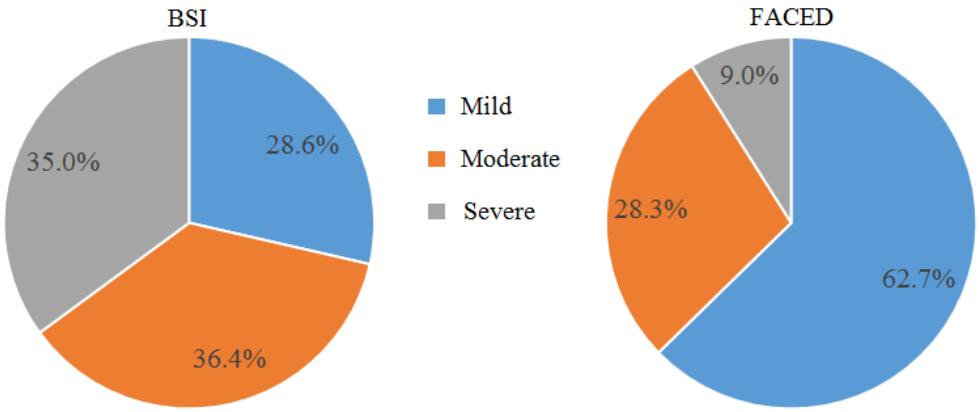

Figure 4-2 Classification of disease severity according to the Bronchiectasis Severity Index and FACED scores on meta-analysis

With the BSI, the proportion of deaths per group increased sequentially across risk strata, with zero deaths in the low risk mild subgroup in 4 of the 7 cohorts involved, and only 11 deaths in the low risk group overall (2.4%), compared to 57 deaths in the "mild" bronchiectasis group according to FACED (5.6%), where you would generally expect mortality to be low. Mortality rates were higher for patients classed as severe using FACED (35.1%) compared to the BSI (21.6%). However, although there was a step-wise increase in the percentage mortality across risk strata, the actual number of patient deaths was higher in the moderate FACED group compared to the severe group at 81 and 51 respectively (Figure 4-3).

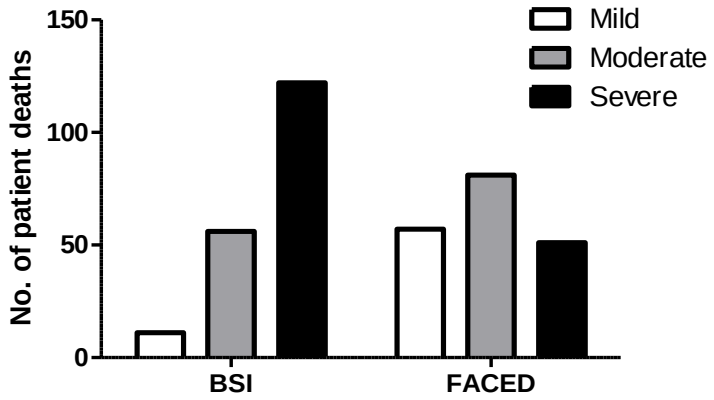

Figure 4-3 Absolute number of patient deaths on meta-analysis according to disease severity across the seven cohorts

The scores had very different characteristics to identify patients at high risk of death. The FACED score had poor sensitivity ranging from 5-56% with a pooled sensitivity from meta-analysis across all 7 cohorts of 28% (22-36%). In contrast, the BSI was more sensitive (ranging from 40-100% sensitive, pooled value 65% (57-71%) but less specific (range 41-83%, pooled value 70% (66-72%)) compared to FACED 93% (92-95%).

Comparing AUC scores for mortality, the BSI had a numerically higher AUC in the Dundee, Galway, Monza, Athens, Newcastle and Voljvodina cohorts; the FACED score had a numerically higher AUC in Leuven. The only statistically significant difference was a superior AUC for the BSI in the Newcastle cohort. All AUC values for BSI were above 0.7 and six AUC values were above 0.7 for FACED, suggesting both scores are valid in terms of discrimination for mortality (Table 4-3).

In the calibration analysis, both scores were well calibrated in terms of identifying patients at low risk of death. For identifying severe patients, however, the FACED score failed calibration with the pooled OR suggesting that the true mortality rate is 70% lower than that predicted by FACED [OR 0.33 (0.23, 0.48), $p<0.0001$], (Table 4-4).

Table 4-2 Overall distribution of patients, numbers of deaths and numbers of hospital admissions for severe exacerbations in mild, moderate and severe groups across the seven cohorts

		Overall patient distribution			Numbers of deaths			Numbers of hospital admissions		
		Mild n (%)	**Moderate n (%)**	**Severe n (%)**	**Mild n (%)**	**Moderate n (%)**	**Severe n (%)**	**Mild n (%)**	**Moderate n (%)**	**Severe n (%)**
Dundee, Scotland (n=494)	**BSI**	136 (27.5)	211 (42.7)	147 (29.8)	1 (0.7)	13 (6.2)	28 (19.0)	3 (2.2)	24 (11.4)	75 (51.0)
	FACED	303 (61.3)	145 (29.4)	46 (9.3)	12 (4.0)	15 (10.3)	15 (32.6)	44 (14.5)	45 (31.0)	13 (28.3)
Galway, Ireland (n=280)	**BSI**	109 (38.9)	92 (32.9)	79 (28.2)	8 (7.3)	11 (12.0)	25 (31.6)	2 (1.8)	6 (6.5)	30 (38.0)
	FACED	217 (77.5)	53 (18.9)	10 (3.6)	23 (10.6)	19 (35.8)	2 (20.0)	18 (8.3)	15 (28.3)	5 (50.0)
Monza, Italy (n=250)	**BSI**	67 (26.8)	104 (41.6)	79 (31.6)	0 (0)	3 (2.9)	11 (13.9)	10 (14.9)	27 (25.9)	55 (69.6)
	FACED	135 (54.0)	88 (35.2)	27 (10.8)	3 (2.2)	4 (4.5)	7 (25.9)	40 (29.6)	34 (38.6)	18 (66.7)
Leuven, Belgium (n=190)	**BSI**	51 (26.8)	63 (33.2)	76 (40.0)	2 (3.9)	16 (25.4)	26 (34.2)	6 (11.8	18 (28.6)	34 (44.7)
	FACED	100 (52.6)	65 (34.2)	25 (13.2)	9 (9.0)	19 (29.2)	16 (64.0)	23 (23.0)	22 (33.8)	13 (52.0)
Athens, Greece (n=159)	**BSI**	36 (22.6)	43 (27.0)	80 (50.3)	0 (0)	0 (0)	9 (11.3)	1 (2.8)	7 (16.3)	27 (33.8)
	FACED	104 (65.4)	35 (22.0)	20 (12.6)	0 (0)	4 (11.4)	5 (25.0)	17 (16.3)	9 (25.7)	9 (45.0)

Table 4-2 (continued) Overall distribution of patients, numbers of deaths and numbers of hospital admissions for severe exacerbations in mild, moderate and severe groups across the seven cohorts

		Overall patient distribution			Numbers of deaths			Numbers of hospital admissions		
		Mild n (%)	**Moderate n (%)**	**Severe n (%)**	**Mild n (%)**	**Moderate n (%)**	**Severe n (%)**	**Mild n (%)**	**Moderate n (%)**	**Severe n (%)**
Newcastle, England (n=126)	**BSI**	21 (16.7)	25(19.8)	80 (63.5)	0 (0)	1 (4.0)	15 (18.8)	0 (0)	5 (20.0)	52 (65.0)
	FACED	91 (72.2)	27 (21.4)	8 (6.3)	6 (6.6)	7 (25.9)	3 (37.5)	37 (40.7)	15 (55.6)	5 (62.5)
Vojvodina, Serbia (n=113)	**BSI**	41 (36.3)	48 (42.5)	24 (21.2)	0 (0)	12 (25.0)	8 (33.3)	0 (0)	1 (2.1)	12 (50)
	FACED	60 (53.1)	44 (38.9)	9 (8.0)	4 (6.7)	13 (29.5)	3 (33.3)	2 (3.3)	10 (22.7)	1 (11.1)
Pooled analysis (n=1,612)	**BSI**	461 (28.6)	586 (36.4)	565 (35.0)	11 (2.4)	56 (9.5)	122 (21.6)	22 (4.8)	88 (15.0)	285 (49.6)
	FACED	1,010 (62.7)	457 (28.3%)	(9.0%)	57 (5.6)	81 (17.7)	51 (35.1)	181 (17.9)	150 (32.8)	64 (44.1)

Table 4-3 Area under the receiver operator characteristic curve values for mortality and hospital admissions across seven European cohorts

Cohort	Scores	AUC- mortality	AUC hospital admissions
Dundee, Scotland (n=494)	**BSI**	0.78 (0.71-0.85)	0.84 (0.80-0.88)*
	FACED	0.76 (0.70-0.83)	0.68 (0.63-0.73)
Galway, Ireland (n=280)	**BSI**	0.73 (0.64-0.81)	0.87 (0.80-0.93)
	FACED	0.71 (0.62-0.80)	0.79 (0.73-0.86)
Monza, Italy (n=250)	**BSI**	0.86 (0.75-0.96)	0.79 (0.73-0.85)*
	FACED	0.77 (0.64-0.90)	0.62 (0.55-0.70)
Leuven, Belgium (n=190)	**BSI**	0.73 (0.65-0.81)	0.71 (0.63-0.78)
	FACED	0.78 (0.70-0.86)	0.65 (0.57-0.74)
Athens, Greece (n=159)	**BSI**	0.93 (0.87-0.98)	0.76 (0.67-0.84)*
	FACED	0.87 (0.80-0.94)	0.62 (0.51-0.74)
Newcastle, England (n=126)	**BSI**	0.82 (0.73-0.91)*	0.80 (0.72-0.87)*
	FACED	0.68 (0.52-0.84)	0.56 (0.46-0.66)
Vojvodina, Serbia (n=113)	**BSI**	0.75 (0.65-0.85)	0.97 (0.93-1.00)*
	FACED	0.74 (0.64-0.85)	0.69 (0.58-0.81)
Pooled analysis (n=1,612)	**BSI**	0.76 (0.74-0.78)	0.82 (0.78-0.84)*
	FACED	0.76 (0.74-0.78)	0.65 (0.63-0.67)

** indicates $p<0.05$ comparing BSI to FACED.*

Table 4-4 Calibration analysis of the BSI and FACED scores in high and low risk cohort groups

BSI							
Low Risk Cohort	**Event rate**	**Control rate**	**Odds ratio**	**High Risk Cohort**	**Event rate**	**Control rate**	**Odds ratio**
Dundee, Scotland	0.7%	2.6%	0.28 [0.03, 2.39]	Dundee, Scotland	19.0%	23.3%	0.77 [0.46, 1.31]
Galway, Ireland	7.3%	2.6%	2.95 [0.94, 9.24]	Galway, Ireland	31.6%	23.3%	0.76 [0.39, 1.46]
Monza, Italy	0%	2.6%	0.25 [0.01, 4.60]	Monza, Italy	13.9%	23.3%	0.53 [0.26, 1.09]
Leuven, Belgium	3.9%	2.6%	1.52 [0.29, 8.06]	Leuven, Belgium	34.2%	23.3%	1.71 [0.96, 3.05]
Athens, Greece	0%	2.6%	0.46 [0.03, 8.58]	Athens, Greece	11.3%	23.3%	0.42 [0.19, 0.90]
Newcastle, England	0%	2.6%	0.79 [0.04, 14.76]	Newcastle, England	18.8%	23.3%	1.52 [0.85, 2.72]
Vojvodina, Serbia	0%	2.6%	0.41 [0.02, 7.53]	Vojvodina, Serbia	33.3%	23.3%	1.64 [0.66, 4.09]
Pooled analysis	I^2=8%		1.09 (0.49-2.45), p=0.8	**Pooled analysis**	I^2=62%		0.93 (0.62-1.39), p=0.7
FACED							
Low Risk Cohort	**Event rate**	**Control rate**	**Odds ratio**	**High Risk Cohort**	**Event rate**	**Control rate**	**Odds ratio**
Dundee, Scotland	4.0%	4.3%	0.92 [0.39, 2.18]	Dundee, Scotland	32.6%	62.1%	0.30 [0.15, 0.60]
Galway, Ireland	10.6%	4.3%	2.66 [1.23, 5.72]	Galway, Ireland	20.0%	62.1%	0.15 [0.03, 0.75]
Monza, Italy	2.2%	4.3%	0.51 [0.14, 1.88]	Monza, Italy	25.9%	62.1%	0.21 [0.08, 0.54]
Leuven, Belgium	9.0%	4.3%	2.22 [0.87, 5.63]	Leuven, Belgium	64.0%	62.1%	1.08 [0.45, 2.64]
Athens, Greece	0%	4.3%	0.10 [0.01, 1.76]	Athens, Greece	25.0%	62.1%	0.20 [0.07, 0.59]
Newcastle, England	6.6%	4.3%	1.58 [0.56, 4.48]	Newcastle, England	37.5%	62.1%	0.37 [0.08, 0.54]
Vojvodina, Serbia	6.7%	4.3%	1.60 [0.48, 5.29]	Vojvodina, Serbia	33.3%	62.1%	0.30 [0.07, 1.27]
Pooled analysis	I^2=42%		1.36 (0.79-2.33), p=0.3	**Pooled analysis**	I^2=37%		0.33 (0.23-0.48), p<0.0001

4.3.3 Hospital admissions

The BSI appeared to be superior to FACED in terms of predicting hospital admissions (Table 4-3; Figure 4-4(a)). Rates of hospitalisation in mild patients according to BSI were 0-14.9% and increased proportionately across mild, moderate and severe risk groups. Hospitalisation rates were much more variable for "mild" patients according to FACED, with rates from 3.3-40.7%, and in the Dundee and Vojvodina cohorts, there was a paradoxically higher percentage of hospital admissions in the "moderate" group compared to "severe", (Figure 4-4(b)).

Comparing AUC values for hospitalisations, BSI showed statistically significant superiority in 6 of 7 cohorts when analysed individually. FACED was only predictive of hospital admissions in the Galway cohort using the cut-off of 0.7 for valid discrimination.

Rates of hospital admissions comparing the mild and severe groups with the moderate group for each severity score are shown in Table 4-5. Notably, the rate ratio (RR) for low risk patients using the BSI was numerically lower than FACED in all 7 cohorts. Similarly, RR for hospitalisation in the severe group was numerically higher in 5 of 7 cohorts.

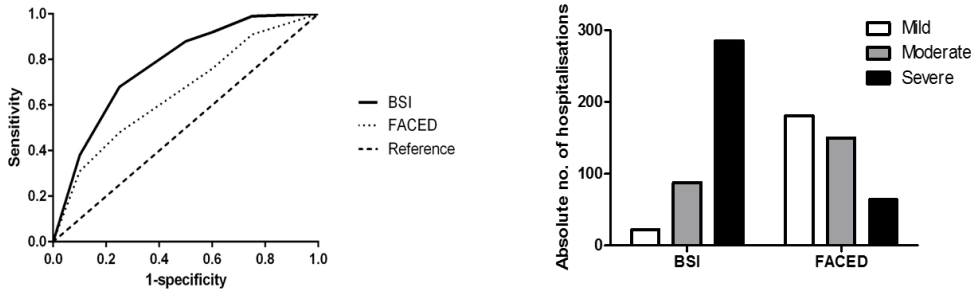

Figure 4-4 Comparative analysis of hospitalisations according to BSI and FACED scores

(a) ROC curve analysis of hospitalisations according to BSI AUC 0.82 (0.78-0.84) and FACED AUC 0.65 (0.63-0.67) with $p<0.05$ comparing BSI to FACED; (b) Absolute number of patient hospitalisations on meta-analysis according to disease severity of mild, moderate or severe groups across the seven cohorts.

4.3.4 Exacerbations

Exacerbation rates were strongly linked to clinical severity scores. Using Poisson regression, low risk patients determined by the BSI, had fewer exacerbations in all cohorts, and differences were statistically significant in 4 out of 7 cohorts (Table 4-5). Severe patients according to the BSI had significantly more exacerbations than the moderate group across all cohorts. In contrast, FACED was less consistent for predicting exacerbations. Paradoxically, we observed numerically more exacerbations in the moderate versus severe FACED group in the Newcastle and Vojvodina cohorts, while "mild" patients had numerically more exacerbations than moderate patients in the Monza and Athens cohorts suggesting the FACED score did not reliably and consistently identify patients at high risk of exacerbations. Differences between mild and moderate FACED groups were only statistically significant in 2 out of 7 cohorts (Galway and Vojvodina) and differences between moderate and severe were only statistically significant in 4 out of 7 cohorts. In the pooled analysis, the differences between mild and moderate FACED groups were not statistically significant, while overall differences between severe and moderate were significant (Table 4-6).

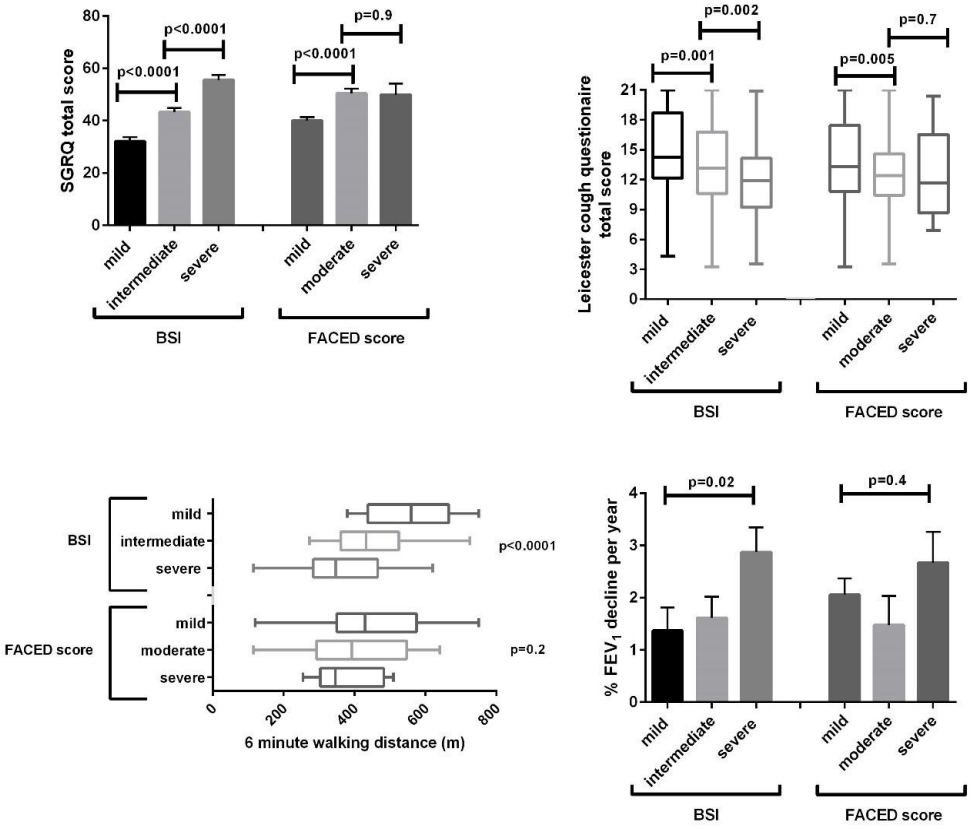

Figure 4-5 Bar graphs showing quality of life, respiratory symptoms, exercise capacity and lung function decline.

For SGRQ and FEV1 decline, bars show mean with SEM. For Leicester Cough Questionnaire and 6-minute walking distance, mean, SD and range are shown. BSI: Bronchiectasis severity; SGRQ: St. George's Respiratory Questionnaire.

Table 4-5 Hospitalisations meta-analysis data showing rate ratios of hospitalisations for severe exacerbations during follow up according to different severity scores

Cohort	Scores	Rate Ratio - Mild	Rate Ratio – Moderate	Rate Ratio – Severe
Dundee, Scotland (n=494)	**BSI**	0.19 (0.06-0.64) *	1.00 (reference)	4.46 (2.82-7.07) *
	FACED	0.47 (0.31-0.71) *	1.00 (reference)	0.91 (0.49-1.69)
Galway, Ireland (n=280)	**BSI**	0.28 (0.06-1.39)	1.00 (reference)	5.82 (2.42-14.0) *
	FACED	0.29 (0.15-0.58) *	1.00 (reference)	1.77 (0.64-4.86)
Monza, Italy (n=250)	**BSI**	0.50 (0.26-0.97) *	1.00 (reference)	4.45 (3.08-6.42) *
	FACED	0.75 (0.52-1.08)	1.00 (reference)	3.69 (2.55-5.34) *
Leuven, Belgium (n=190)	**BSI**	0.56 (0.29-1.30)	1.00 (reference)	1.68 (0.93-3.04)
	FACED	0.68 (0.38-1.22)	1.00 (reference)	1.54 (0.77-3.05)
Athens, Greece (n=159)	**BSI**	0.17 (0.02-1.39)	1.00 (reference)	2.07 (0.90-4.76)
	FACED	0.64 (0.28-1.43)	1.00 (reference)	1.75 (0.70-4.41)
Newcastle, England (n=126)	**BSI**	0 (no events)*	1.00 (reference)	3.25 (1.30-8.14)*
	FACED	0.75 (0.41-1.41)	1.00 (reference)	1.18 (0.47-2.95)
Vojvodina, Serbia (n=113)	**BSI**	0 (no events)*	1.00 (reference)	24.5 (3.2-188.4)*
	FACED	0.15 (0.03-0.67)	1.00 (reference)	0.49 (0.06-3.82)
Pooled cohort (n=1, 612)	**BSI**	0.41 (0.27-0.62)*	1.00 (reference)	3.71 (2.95-4.66)*
	FACED	0.55 (0.41-0.75)*	1.00 (reference)	1.58 (0.92-2.71)

Table 4-6 Exacerbation rates meta-analysis data showing rate ratios of exacerbations during follow up according to different severity scores

Cohort	Scores	Rate Ratio - Mild	Rate Ratio – Moderate	Rate Ratio - Severe
Dundee, Scotland (n=494)	**BSI**	0.66 (0.53-0.82)*	1.00 (reference)	2.12 (1.84-2.50)*
	FACED	0.91 (0.77-1.06)	1.00 (reference)	1.44 (1.15-1.80)*
Galway, Ireland (n=280)	**BSI**	0.70 (0.55-0.88)*	1.00 (reference)	1.44 (1.16-1.78)*
	FACED	0.74 (0.59-0.92)*	1.00 (reference)	1.25 (0.82-1.92)
Monza, Italy (n=250)	**BSI**	0.76 (0.59-0.99)*	1.00 (reference)	1.38 (1.11-1.70)*
	FACED	1.06 (0.86-1.31)	1.00 (reference)	1.64 (1.24-2.17)*
Leuven, Belgium (n=190)	**BSI**	0.78 (0.52-1.18)	1.00 (reference)	2.08 (1.52-2.84)*
	FACED	0.86 (0.64-1.14)	1.00 (reference)	1.51 (1.05-2.16)*
Athens, Greece (n=159)	**BSI**	0.67 (0.34-1.33)	1.00 (reference)	1.92 (1.21-3.04)*
	FACED	1.20 (0.73-1.96)	1.00 (reference)	2.36 (1.33-4.21)*
Newcastle, England (n=126)	**BSI**	0.47 (0.32-0.71)*	1.00 (reference)	1.29 (1.02-1.65)*
	FACED	0.81 (0.63-1.04)	1.00 (reference)	0.95 (0.64-1.41)
Vojvodina, Serbia (n=113)	**BSI**	0.14 (0.05-0.38)*	1.00 (reference)	2.61 (1.69-4.04)*
	FACED	0.22 (0.13-0.37)*	1.00 (reference)	0.93 (0.49-1.77)
Pooled cohort (n=1, 612)	**BSI**	0.63 (0.52-0.78)*	1.00 (reference)	1.73 (1.42-2.12)*
	FACED	0.78 (0.60-1.01)	1.00 (reference)	1.40 (1.16-1.68)*

4.3.5 Quality of life bronchiectasis questionnaire

QoL and symptom data were available in 2 cohorts (744 patients) which were pooled for analysis. Using the QOL-B, statistically significant differences were observed for respiratory symptoms, physical functioning and role functioning domains between mild/moderate and moderate/severe groups using the BSI. All differences were above the MCID (Table 4-7).

There were also significant differences for BSI in the vitality domains across both mild/moderate and moderate/severe groups but at levels below the MCID. Health perceptions were not significantly different between mild/moderate groups but were statistically and clinically different between moderate/severe groups. FACED demonstrated less discrimination in terms of QOL-B domains. There were clinically and statistically significant differences between the mild/moderate FACED groups for physical functioning but the only other difference exceeding the MCID was for role functioning.

Similar data were observed for the SGRQ (Figure 4-5). The differences between mild, moderate and severe groups for the BSI were significant ($p<0.0001$) with a difference of 11 points between mild/moderate groups, and 12 points between moderate/severe groups, both above the MCID of 4 points. There was a 10-point difference between the mild/moderate FACED groups ($p<0.0001$) but no difference between moderate/severe groups, $p=0.9$. We further validated these findings in the only other published validation study from the Royal Brompton Hospital (74 patients).[73] In this cohort, there were clear differences between BSI groups in terms of SGRQ score, with mean differences of 9 and 11 points comparing mild/moderate and moderate/severe respectively ($p=0.003$). In contrast there were no statistically significant differences between FACED groups, and the between-group difference for moderate/severe was below the MCID, $p=0.2$ (Figure 4-6).

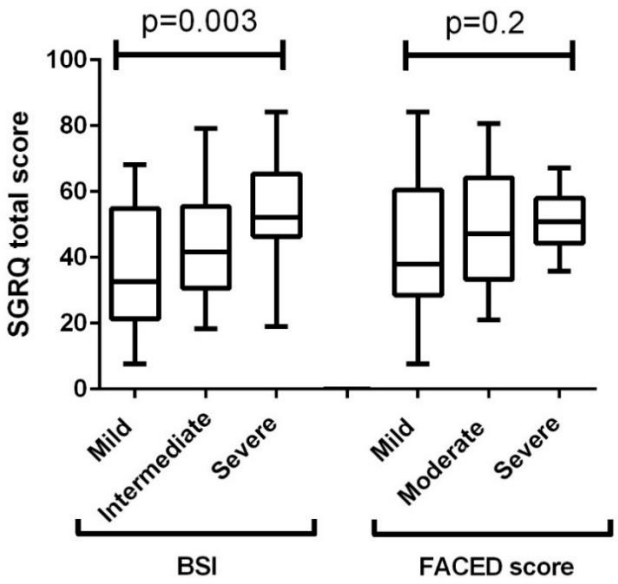

Figure 4-6 Comparative analysis of quality of life between the BSI and FACED scores in validation cohort

Bar graphs showing quality of life using the SGRQ total score across BSI and FACED groups in the Ellis study. SGRQ: St. George's Respiratory Questionnaire.

4.3.6 Symptoms and function

As above, symptom and functional data were only available in 2 cohorts (744 patients). Cough severity, evaluated by the LCQ was different between BSI groups ($p<0.0001$ by ANOVA) with mean differences of 1.5 and 1.4 between groups, both above the stated MCID of 1.3. There was a significant difference between mild/moderate FACED groups, but this did not reach the MCID (mean difference 1.1, $p=0.005$); there was no difference between moderate/severe groups (mean difference 0.26, $p=0.7$).

Data for 6MWD were available for 471 patients. The differences in 6MWD between groups for the BSI were 115m (mild/moderate) and 83m (moderate/severe), the latter just failing to reach the MCID. For FACED, differences were 53m (mild/moderate) and 21m (moderate/severe), neither of which were statistically significant or above the MCID ($p=0.2$).

There was a weak but statistically significant association between BSI and annual FEV1 decline (Figure 4-5), driven by a higher rate of decline in the severe group as there was no difference between mild/moderate groups (p=0.7). FACED did not predict lung function decline with the lowest rate of decline observed in the moderate group.

Table 4-7 Quality of life-Bronchiectasis (QOL-B) domains

		Mean difference (mild *vs.* moderate)	**p-value**	**Mean difference (moderate *vs*. severe)**	**p-value**
Respiratory symptoms (MCID=8)	**BSI**	**-10.4 (3.0)**	0.0006	**-8.8 (2.6)**	0.0009
	FACED	-6.3 (2.5)	0.01	-2.9 (3.4)	0.4
Physical Functioning (MCID=10)	**BSI**	**-14.8 (4.8)**	0.002	**-20.7 (3.9)**	<0.0001
	FACED	**-20.1 (3.7)**	<0.0001	-4.5 (4.6)	0.3
Vitality (MCID=10)	**BSI**	-7.4 (3.2)	0.02	-6.8 (2.9)	0.02
	FACED	-7.5 (2.6)	0.005	-2.2 (3.6)	0.5
Role Functioning (MCID=8)	**BSI**	**-11.1 (4.0)**	0.006	**-13.9 (3.5)**	0.0001
	FACED	**-12.6 (2.2)**	<0.0001	**-8.8 (4.0)**	0.03
Health perceptions (MCID=8)	**BSI**	-3.5 (3.5)	0.3	**-8.6 (2.9)**	0.003
	FACED	-7.7 (2.7)	0.005	5.2 (4.0)	0.2
Emotional Functioning (MCID=7)	**BSI**	-5.0 (3.3)	0.1	-6.1 (3.2)	0.05
	FACED	-2.2 (2.9)	0.5	-1.2 (4.0)	0.8
Social Functioning (MCID=9)	**BSI**	-5.0 (4.0)	0.2	-7.7 (3.7)	0.03
	FACED	-7.6 (3.2)	0.02	1.6 (4.8)	0.7
Treatment Burden (MCID=9)	**BSI**	-6.6 (4.9)	0.2	-5.8 (3.7)	0.1
	FACED	-3.6 (3.6)	0.3	-2.2 (4.6)	0.6

Values show mean difference with standard deviation for between-group differences. Differences that are statistically significant and above the minimum clinically important difference (MCID) are shown in bold.

4.4 Discussion

This study is the first multicentre study critically appraising whether prognostic indices can predict clinically meaningful outcomes broader than simply, mortality. If bronchiectasis severity tools are to be used in clinical practice to guide escalation of therapy, they need to predict outcomes that are relevant to these decisions.[9] This prospective international observational study suggests that the BSI is superior to FACED in predicting clinically important disease-related outcomes, including hospital admissions, exacerbations, QoL, respiratory symptoms, 6MWD and lung function decline in bronchiectasis.

The primary outcome in most intervention trials in bronchiectasis has been exacerbations or QoL; these are also considered to be the most important triggers for changes in treatment. [1, 9, 410-414] According to our analysis, the BSI consistently stratified patients as having low, moderate and high risk of exacerbations and severe exacerbations requiring hospitalisation, while there were lesser differences between FACED risk groups for exacerbations. Furthermore, FACED had a very poor overall ability to predict hospitalisations with AUC scores below 0.7 in 6 of the 7 included cohorts. It is perhaps not surprising that FACED predicts exacerbations poorly, as although risk factors for exacerbations in bronchiectasis have not been well defined, data from COPD shows that the strongest predictor of future exacerbations is a previous history of exacerbations, and anecdotally the same is true in bronchiectasis, with the recent identification of the frequent exacerbator phenotype which remains consistent over time and shows high disease severity, poor quality of life, and increased mortality during follow-up.[24, 93] The BSI incorporates prior history of mild and severe exacerbations, while the FACED score does not.

The BSI was also a valid predictor of respiratory symptoms and physical functioning using the QOL-B, as well as passing validity testing against the SGRQ, the 6MWD and the LCQ. This is consistent with multiple studies published over the past 18 months where the BSI has been correlated with the

SGRQ, the COPD assessment test - another measure of symptoms, the capsaicin cough sensitivity, impulse oscillometry, the 6MWD and activity measured using accelerometers.[424-427] Finally, Dente *et al.* published a significant association between BSI and sputum neutrophilic inflammation, while no relationship with FACED was found.[428] In the present analysis, the BSI accurately categorised different severities of bronchiectasis according to these parameters whereas, although sometimes showing trends towards differences in these parameters the FACED score did not pass validity for the majority of QOL-B domains, the 6MWD or the LCQ.

Our analysis also suggests a relationship between the predictive ability of the BSI and lung function decline, which again was not evident with FACED. There have been few studies of lung function decline in bronchiectasis. In their analysis of 76 patients, Martinez-Garcia *et al.* found *P. aeruginosa* colonisation and severe exacerbations to be the strongest predictors.[325] It is perhaps therefore not surprising that the BSI predicts lung function decline as it awards a high proportion of points to *P. aeruginosa* colonisation (3 points) and hospitalisation for severe exacerbations (5 points), while FACED awards 1 point to *P. aeruginosa* colonisation and does not consider severe exacerbations. Our results are therefore in keeping with previous studies.

There are clearly advantages and disadvantages to both scores. The BSI is slightly more complex than the FACED score, requiring the measurement of 8, rather than 5, clinical parameters with a variable weighting that awards different points for each. Evidence suggests that clinicians may find weighted scores more difficult to apply leading to an under-utilisation in clinical practice and that scores should be recalibrated according to local practices.[429] However, in the current era of telemedicine and online medical calculators that assign a total on inputting the relevant data in sequential order, this is potentially a concern of the past.[430-432] Although FACED is an acronym that may be easier for clinicians to remember, the score is subject to the same limitations, also awarding different weight to different variables, while having a lower accuracy for the majority of clinical outcomes.

Our analysis identified several potential limitations of the FACED score. It failed calibration analysis, suggesting that its prediction performance varies across different healthcare settings and requires local recalibration before use. We further confirmed this finding by incorporating the cohort of Ellis *et al*,[73] into the analysis, which independently confirms the failure of calibration. FACED consistently had a sensitivity of <50% for prediction of mortality and hospitalisations. The FACED score appears to prioritise specificity over sensitivity. This is potentially problematic for clinical decision making as it is counter-intuitive to say that only a quarter of patients dying of bronchiectasis or being hospitalised for severe exacerbations, have "severe" bronchiectasis. There are some circumstances where a high specificity, i.e. a high confidence that a high-risk patient will die, is desirable, the most obvious being in assessment of mortality risk in potential lung transplant recipients, and the FACED score appears to be well adapted to this. However, in view of these limitations and the finding that the "severe" FACED group was not associated with increased exacerbations or differences in most other morbidity parameters, we suggest that FACED should not be viewed as a severity assessment tool, but rather as a mortality risk tool and therefore the terms mild, moderate and severe be replaced with low, intermediate and high risk of mortality when describing the FACED score.

It is widely accepted that prognostic model development is a three-stage process, comprising derivation (creating the rule), validation (applying the rule to new populations of patients to confirm its accuracy) and impact analysis (applying the rule and determining if it can improve clinical outcomes for patients).[433]. Our results suggest the BSI is superior in identifying patients at low risk of mortality, hospitalisation, exacerbations and morbidity who may benefit from primary care or nurse-led follow-up, which have the potential to either improve access or reduce healthcare costs and improve patient satisfaction.[434] The BSI is also more sensitive in identifying patients at high risk of mortality, hospitalisation and exacerbations who may benefit from more aggressive treatment early on in their disease course to reduce associated complications, as well as closer follow-up in specialist bronchiectasis clinics. Our results do not, however,

prove that implementation of the BSI may improve clinical outcomes and an impact analysis is now required.

A great strength of the present study is the inclusion of multiple cohorts across Europe. However, limitations of the study must be acknowledged. At the time of this study, the E-FACED score had not been developed and further comparative analyses of the same may have yielded different results. These scores have mainly been evaluated in European cohorts with similar demographics. Further validation would be desirable in populations significantly different from the original derivation studies, for example in the USA where there is a high prevalence of NTM bronchiectasis or in Asian populations, where the overall prevalence of bronchiectasis is suspected to be higher than in Caucasians.[15, 435, 436] A large amount of additional data is likely to be generated by ongoing international registry projects which incorporate calculation of the BSI.[14, 15]

4.5 Conclusion

In conclusion, the BSI accurately predicts mortality, hospital admissions, exacerbations, QoL, respiratory symptoms, 6MWD and lung function decline in bronchiectasis, providing a clinically relevant evaluation of disease severity.

Chapter 5 - Comorbidities and the Risk of Mortality in Patients with Bronchiectasis

Publications relevant to this chapter:

McDonnell MJ, Aliberti S, Goeminne PC, Restrepo MI, Finch S, Pesci A, Dupont LJ, Fardon TC, Wilson R, Loebinger MR, Skrbic D, Obradovic D, De Soyza A, Ward C, Laffey JG, Rutherford RM, Chalmers JD. Comorbidities and the risk of mortality in patients with bronchiectasis: an international multicentre cohort study. *Lancet Respir Med*. 2016; 4(12):969-979.

5.1 Introduction

Bronchiectasis is a chronic airway disease showing an increasing prevalence over the past decade with an associated growing morbidity and mortality worldwide.[248] As a complex multi-component disease, bronchiectasis is characterised by chronic systemic inflammation that frequently co-exists with comorbidities, which may be causative, synergistic, or coincidental, depending on the manner in which they interact.[437]

In addition to known aetiologies of bronchiectasis, several other diseases may occur at any stage of bronchiectasis and are likely major contributors to increased hospitalisations, healthcare utilisation and socioeconomic costs.[438] These include cardiovascular disorders, gastro-oesophageal reflux disease (GORD), psychological illnesses, pulmonary hypertension, cognitive impairment, and lung, oesophageal and haematological malignancies.[29, 244, 274, 291, 315, 439-442] A few studies have explored bronchiectasis-related comorbidities and suggest that, compared with age and sex-matched controls, some comorbidities are more likely to coexist with bronchiectasis[29, 244, 274, 291, 315, 439-442] and have a relevant impact on different outcomes, such as exercise capacity,[439] exacerbation frequency,[291, 315] lung function,[29, 442] health-related quality of life,[291, 315, 440, 442] healthcare utilisation,[315] and mortality.[244, 441] Few studies have systematically evaluated the prevalence and role of comorbidities in bronchiectasis; several were performed in single centres with small patient numbers,[274, 291, 439, 441, 442] or utilised retrospective databases or cross-sectional designs,[29, 244, 315, 440] limiting the applicability of their findings. However, none have systematically evaluated how comorbidities impact on prognosis in a prospective study.

It is suggested that individual comorbidities and aetiologies of bronchiectasis, such as chronic obstructive pulmonary disease (COPD) and rheumatoid arthritis (RA), confer an increased severity and mortality compared to other aetiologies despite targeted treatment of underlying conditions.[443, 444] Recent literature has also shown that in approximately 30-40% of patients

with bronchiectasis, the primary cause of death is attributed to non-respiratory disease.[49] However, current guidelines fail to provide clear recommendations on how comorbidities should be identified, assessed and treated within the context of bronchiectasis.[9] Neither of the two prognostic scoring indices recently developed to estimate mortality in patients with bronchiectasis, the bronchiectasis severity index (BSI) or the FACED score, were planned to systematically evaluate the prevalence and role of comorbidities.[19, 20]

In view of this lack of data, we designed a study which aimed to determine not only the prevalence of individual comorbidities in bronchiectasis patients but also the strength of association between the number and nature of comorbidities and risk of death over time. We further aimed to develop a disease-specific comorbidity index and explore if this could provide additional prognostic information to that provided by the BSI.

Our hypothesis was that multiple comorbidities would be a common finding across national cohorts, that these would contribute significantly to mortality and that it was practicable to apply a standardised assessment to assess the role of comorbidities in the mortality of patients with bronchiectasis.

5.2 Methods

5.2.1 Data collection

This study included data from four databases of prospectively enrolled outpatients with bronchiectasis in Dundee (UK), Galway (Ireland), Leuven (Belgium) and Monza (Italy). Consecutive patients aged ≥18 years were enrolled on the basis of a radiological diagnosis made on high-resolution computed tomography (HRCT) and a clinical history consistent with bronchiectasis. Patients with cystic fibrosis or traction bronchiectasis due to pulmonary fibrosis were excluded in all four cohorts. Data from each cohort was collected independently following individual ethics approval and collated for statistical analysis. Standardised assessment and diagnostic work-up according to the 2010 British Thoracic Society (BTS) guidelines was

performed at each site as detailed in the methodology chapter.[9] Enrolment into the study required that all variables required to calculate clinical prediction scores and the key relevant outcomes of mortality, hospitalisations and exacerbations on follow-up were available. Exacerbations and hospitalisations were defined according to BTS guidelines, and mortality, evaluated at the end of the 5-year follow-up period, was confirmed in 100% of participants.[9] This cohort is entirely independent from the cohorts used to derive the BSI or FACED scores. [19, 20]

5.2.2 Comorbidities

All comorbidity diagnoses were systematically recorded according to standardised definitions and were retrospectively obtained on full review of hard copy or electronic records, patients' prescriptions and review of confirmatory tests where available. The 19 conditions included in the Charlson Comorbidity Index (CCI) were included in data collection plus any other identified comorbidity.[338] Conditions that had completely resolved, e.g. pneumonia, were excluded. Objective confirmation of diagnoses was sought in each case where possible. Self-reported diagnoses consisted of GORD, depression and anxiety as per standard practice internationally.

5.2.3 Statistical Analysis and Derivation of Clinical Prediction Tool

Continuous data are presented as mean and standard deviation (SD) or median and interquartile range (IQR) where appropriate, and categorical data as frequencies and percentages. The Mann Whitney U and chi-squared test were used for comparison of numerical and categorical data, respectively. For comparisons of more than two groups, one-way analysis of variance or the Kruskal-Wallis test were used as appropriate. Weibull parametric survival analysis was used to model the prediction of 5-year mortality. Three candidate comorbidity scores were considered and compared to the CCI, BSI and FACED scores: (a) The Comorbidity count - a simple sum of the number of comorbidities per individual patient; (b) The Bronchiectasis Comorbidity Index (BCI) - a weighted comorbidity score without those conditions regarded as potential underlying aetiologies of bronchiectasis; (c) The

Bronchiectasis Aetiology Comorbidity Index (BACI) - a weighted comorbidity score including conditions regarded as underlying aetiologies.

Based on Peduzzi's modelling, a maximum of 13 variables could be incorporated into the multivariable models in order to comply with statistical norms based on the number of outcomes in our cohort.[445] Comorbidities with <1% prevalence or those with significant collinearity were excluded. Variables were included in the model using a backward stepwise approach requiring a $p<0.2$ for retention in the model. All models were adjusted for age and gender. These variables were then formed into prediction tools using the rounded averaged β-coefficient to award "points" for each variable as previously described.[19] The sum of the points intends to capture the individual or combination of diseases affecting each patient. The performance of the resulting models for mortality was assessed using the area under the receiver operator characteristic curve (AUC) with the exception of the UK validation cohort which had a much longer median follow up of 19 years, whereby Kaplan-Meier analysis was performed to avoid favouring fixed effects at the expense of modifiable risk factors that may increase short-term risk but not necessarily guarantee long-term risk. We subsequently tested the predictive ability of the optimal model to determine future disease outcomes using Spearman's rho correlation and explored if it could add further prognostic information when used alongside the BSI and FACED mortality index. Some endpoints, such as quality of life, were only available in 2 cohorts (Dundee and Monza). Such analyses were only conducted in patients with available data – no imputation or other methods of handling missing data were used. For all analyses, $p< 0.05$ was considered statistically significant. All analyses were performed using SPSS Version 21 (SPSS, Chicago, IL, USA) for Windows platform and Graph Pad Prism Version 5 (Graph Pad Software, Inc. San Diego, CA, USA). The reporting of this study conforms to STROBE and TRIPOD recommendations.[446, 447]

5.2.4 Validation cohorts

The derived index was subsequently validated in two independent cohorts. One was a historical cohort of patients recruited for the validation of the

SGRQ in bronchiectasis.[49] This cohort was selected as a prospective study with the longest duration of follow-up available in the literature to date, where data on comorbidities was systematically collected. The other validation cohort consisted of prospectively recruited bronchiectasis patients in Serbia in Eastern Europe, enabling further assessment of the generalisability of the score.

5.3 Results

5.3.1 Characteristics of the cohort and comorbidities

The demographic and baseline characteristics of the 986 patients included in the analysis are summarised in Table 5-1 and are consistent with other published series in terms of older age, female predominance and bacterial colonisation rates. The cohort consisted primarily of Caucasian females with a median FEV1% predicted of 75% (54-95) and a median FEV1/FVC% predicted of 70% (59-79) demonstrating moderate airflow limitation. The median BSI was 6 (4-10) and all BSI tertiles (mild, moderate and severe) were evenly represented. Mortality, n (%) at 1, 2, 3 and 5 years of follow-up were 37 (3.7), 47 (4.8), 85 (8.6) and 122 (12.4), respectively. No patients received lung transplantation during follow-up.

A total of 81 comorbidities were reported in this cohort. The median (IQR) number of comorbidities was 4 (2-6) per subject for the whole cohort with a range of 0-20; males had significantly more comorbidities than females, median 4 (2-6) for males and 3 (2-5) for females, $p=0.005$. The median number of comorbidities was higher for non-survivors compared with survivors [6 (4-9) vs 3 (2-5) respectively, $p<0.0001$]. A significant association was also observed between the median number of comorbidities and the BSI score (low risk: 3; intermediate risk: 3; high risk: 4; $p<0.0001$).

Table 5-1 Patient characteristics of derivation cohort

Patient characteristics	Derivation Cohort (n=986)
Demographic variables	
Age, Years, Median (IQR)	67 (57-74)
Female, n (%)	589 (59.7)
Body Mass Index, Median (IQR)	24.6 (21.2-27.8)
Smokers / Ex-smokers, n (%)	379 (38.4)
Clinical status	
MRC dyspnea score, Median (IQR)	2 (1-3)
Exacerbations in previous year, Median (IQR)	2 (1-3)
≥ 1 hospitalisation in previous year, n (%)	224 (22.7)
Lung function	
FEV_1 % predicted, Median (IQR)	75 (54-95)
Radiology status	
Reiff score, Median (IQR)	4 (2-6)
Microbiological status	
Pseudomonas colonisation, n (%)	122 (12.4)
Other colonisation, n (%)	229 (25.3)
Disease severity	
BSI score, Median (IQR)	6 (4-10)
BSI 0-4 (mild), n (%)	312 (31.6)
BSI 5-8 (moderate), n (%)	351 (35.6)
BSI ≥ 9 (severe), n (%)	323 (32.8)
Comorbidities	
No. of comorbidities, Median (IQR)	4 (2-6)
Range	0-20

The distribution of the most prevalent (>1%) and significant comorbidities is shown in Figure 5-1. There is a heavy tailed distribution, ranging from 34% to less than 1%. 26 comorbidities had a significantly higher prevalence in non-survivors compared with survivors (the majority are shown here by the presence of asterisks). Full details are shown in Table 5-2.

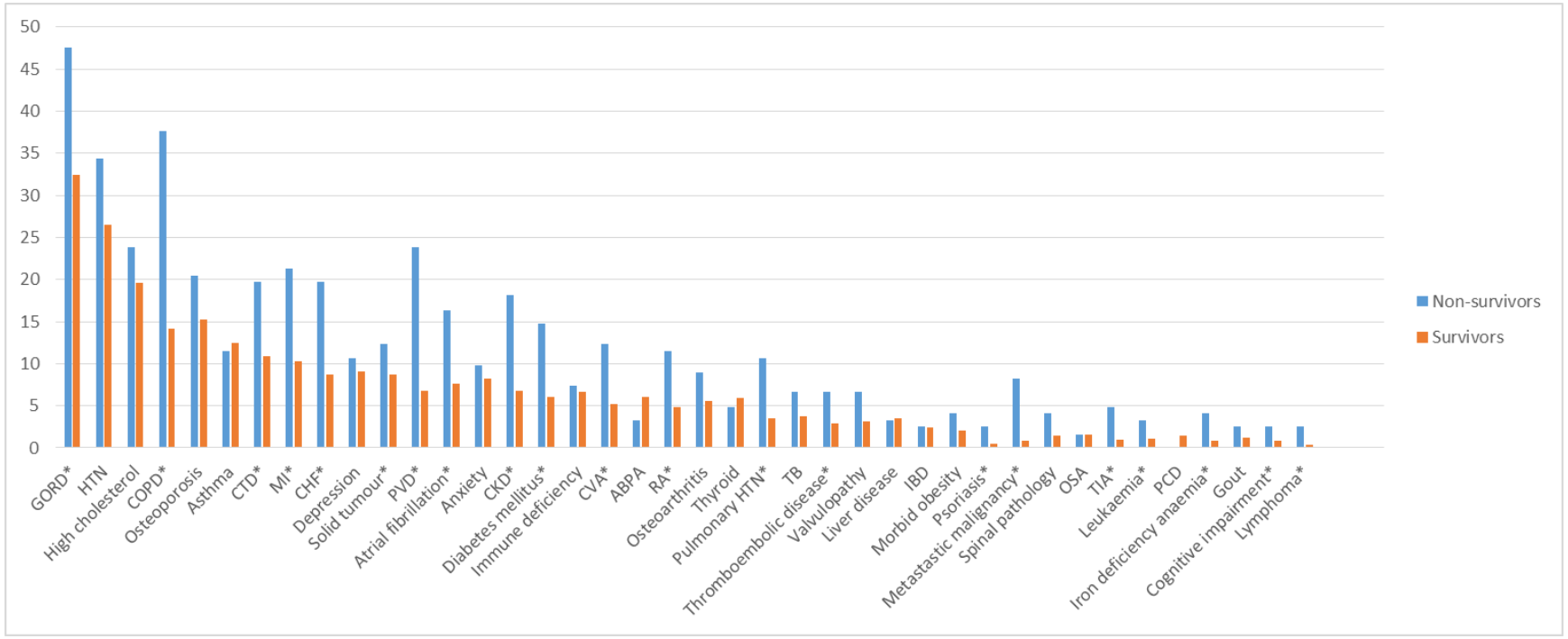

Figure 5-1 Comorbidites in order of overall prevalence among survivor and non-survivor bronchiectasis patients in the derivation cohort.

Comorbidities with a significantly higher prevalence in non-survivors compared with survivors regardless of their absolute prevalence are marked with an asterisk. GORD: Gastro-oesophageal reflux disease; HTN: hypertension; COPD: Chronic obstructive pulmonary disease; CTD: Connective tissue disease; MI: myocardial infarction; CHF: Chronic heart failure; PVD: Peripheral vascular disease; CKD: Chronic kidney disease; CVA: Cerebrovascular attack; RA: Rheumatoid arthritis; ABPA: Allergic bronchopulmonary aspergillosis; TB: Tuberculosis; OSA: Obstructive sleep apnoea..

Table 5-2 Comorbidities prevalence for the full cohort and prevalence comparison between survivors and non-survivors

	Comorbidity prevalence	**Total cohort %**	**Survivors %**	**Non-survivors %**	**P value**
		n = 986	n = 864	n = 122	
1	*GORD	34.3	32.4	47.6	**0.001**
2	HTN	27.5	26.5	34.4	0.080
3	High cholesterol	20.1	19.6	23.8	0.281
4	*COPD	17.1	14.2	37.7	**<0.001**
5	Osteoporosis	15.9	15.2	20.5	0.147
6	Rhinosinusitis	13.1	13.5	9.8	0.315
7	*Asthma	12.4	12.5	15.5	0.183
8	*CTD	12	10.9	19.7	**0.010**
9	*MI	11.7	10.3	21.3	**0.001**
10	*CHF	10	8.7	19.7	**<0.001**
11	Depression	9.3	9.1	10.7	0.496
12	*Solid tumour	9.1	8.7	12.3	**0.028**
	*Lung cancer	1.4	0.9	4.9	**0.004**
	*Oesophageal cancer	0.8	0.5	3.3	**0.010**
13	*PVD	8.9	6.8	23.8	**<0.001**
14	*PUD	8.9	6.8	23.8	**<0.001**
15	*Atrial fibrillation	8.7	7.6	16.4	**0.003**
16	Anxiety	8.4	8.2	9.8	0.372
17	*CKD	8.2	6.8	18.1	**<0.001**
18	*Diabetes Mellitus	7.1	6.0	14.8	**0.002**
19	Immunodeficiency	6.8	6.7	7.4	0.704
20	*CVA	6.1	5.2	12.3	**0.007**
21	ABPA	5.8	6.1	3.3	0.298
22	*RA	5.7	4.9	11.5	**0.007**
23	Osteoarthritis	5.6	5.6	9	0.151
24	Thyroid disorder	5.6	5.9	4.9	0.836
25	*Pulmonary hypertension	4.4	3.5	10.7	**0.001**
26	TB	4.1	3.8	6.6	0.151
27	Childhood infection	4	3.9	4.1	0.808
28	Valvulopathy	3.5	3.1	6.6	0.066
29	*Thromboembolic disease	3.4	2.9	6.6	**0.028**
30	Liver disease	3.4	3.5	3.3	1.000
31	Sarcoidosis	2.5	2.7	0.8	0.346
32	IBD	2.4	2.4	2.5	1.000
33	Morbid obesity	2.3	2.1	4.1	0.191
34	Overt aspiration	1.9	1.8	2.5	0.721

	Comorbidity prevalence	Total cohort %	Survivors %	Non-survivors %	P value
		n = 986	n = 864	n = 122	
35	*Psoriasis	0.7	0.5	2.5	**0.045**
36	*Metastatic malignancy	1.8	0.9	8.2	**<0.001**
37	Spinal problems	1.8	1.5	4.1	0.061
38	OSA	1.6	1.6	1.6	1.000
39	Pulmonary nodules	1.5	1.6	0.8	0.710
40	*TIA	1.5	1	4.9	**0.006**
41	*Leukaemia	1.4	1.1	3.3	**0.021**
42	BPH	1.4	1.0	2.5	0.177
43	PCD	1.3	1.5	0	0.388
44	*Iron deficiency anaemia	1.3	0.9	4.1	**0.015**
45	Gout	1.3	1.2	2.5	0.211
46	Cataracts	1.3	1.3	1.6	0.669
47	*Cognitive impairment	1.1	0.8	2.6	**0.010**
48	*Lymphoma	1.1	0.4	2.6	**0.006**
49	Vasculitis	1.1	1	1.7	0.635
50	PMR	1.1	1.2	0.8	1.000
51	Recurrent cystitis	1.1	0.9	2.5	0.145
52	A1AT deficiency	1	1.2	0	0.621
53	Diverticular disease	1	0.8	2.5	0.116
54	Gallstones	0.9	1	0	0.611
55	Congenital disorders	0.8	0.8	0.8	1.000
56	Other psychological disorder	0.6	0.7	0	1.000
57	Epilepsy	0.6	0.5	1.6	0.163
58	Postural hypotension	0.6	0.5	1.6	0.163
59	*Multiple myeloma	0.6	0.3	2.5	**0.028**
60	Coeliac disease	0.5	0.3	1.7	0.118
61	Pernicious anaemia	0.5	0.3	1.6	0.118
62	Parkinson’s disease	0.5	0.4	0.9	0.484
63	Pneumothorax	0.4	0.2	1.6	0.077
64	Hemochromatosis	0.4	0.5	0	1.000
65	Fibromyalgia	0.4	0.2	1.6	0.077
66	Primary renal disease	0.4	0.2	1.6	0.077
67	Migraine	0.4	0.3	0.8	0.411
68	Other neurological disorders	0.4	0.2	1.6	0.077
69	Glaucoma	0.4	0.3	0.8	0.411
70	Haemangioma	0.4	0.5	0	1.000
71	AIDS	0.2	0	0.2	1.000

	Comorbidity prevalence	Total cohort %	Survivors %	Non-survivors %	P value
		n = 986	n = 864	n = 122	
72	Cardiomyopathy	0.2	0.1	0.8	0.232
73	*AAA	0.2	0	1.6	**0.015**
74	Ovarian problems	0.2	0.2	0	1.000
75	Syphilis	0.2	0.2	0	1.000
76	Asbestosis	0.1	0.1	0	1.000
77	Spinal muscular atrophy	0.1	0.1	0	1.000
78	Myasthenia gravis	0.1	0.1	0	1.000
79	Pancreatitis	0.1	0.1	0	1.000
80	Tracheomalacia	0.1	0.1	0	1.000
81	Swyer James McLeod	0.1	0.1	0	1.000

GORD: gastro-oesophageal reflux disease; HTN: hypertension; COPD: chronic obstructive pulmonary disease; CTD; connective tissue disease; MI: myocardial infarction; CHF: congestive heart failure; PVD: peripheral vascular disease; PUD: peptic ulcer disease; CKD: chronic kidney disease; CVA: cerebrovascular attack; ABPA: allergic bronchopulmonary aspergillosis; RA: rheumatoid arthritis; TB: tuberculosis; IBD: inflammatory bowel disease; OSA: obstructive sleep apnoea; TIA: transient ischaemic attack; BPH: benign prostatic hyperplasia; PCD: primary ciliary dyskinesia; PMR: polymyalgia rheumatica; A1AT: Alpha$_{-1}$ anti-trypsin deficiency; AIDS: acquired immunodeficiency syndrome; AAA: abdominal aortic aneurysm.

5.3.2 Comorbidity scores

The Comorbidity Count

In its simplest form, the comorbidity count, i.e. the sum of the number of comorbidities per patient, was significantly associated with mortality, with a hazard ratio (HR) of 1.17, 95% CI 1.12-1.23 on univariate analysis, suggesting that an increase of 1 comorbidity in the count equates to a 17% increase in mortality. When adjusted for BSI, the HR (95% CI) was still significant at 1.13 (1.08-1.18).

The Bronchiectasis Aetiology Comorbidity Index (BACI)

The comorbidities included in the BACI are shown in Table 5-3. COPD, connective tissue disease, inflammatory bowel disease and asthma were all included in the final model predicting mortality and are recognised aetiologies

of bronchiectasis that may be associated with poorer outcomes. Overall, the HR (95% CI) for death conferred by a one-point increase in the BACI score was 1.18 (1.14-1.23), p<0.0001. Interestingly, this is higher than the adjusted HR for the BSI of 1.10 (1.06-1.14), p<0.0001 suggesting that the BACI has independent prognostic value comparable to the BSI.

Table 5-3 Derivation of the Bronchiectasis Aetiology Comorbidity Index (BACI) and points allocation

Comorbidity	Hazard Ratio	95% CI	P value	Points
Metastatic malignancy	6.69	3.53-12.68	<0.0001	**12**
Haematological malignancy	2.85	1.17-6.97	0.02	**6**
Chronic obstructive pulmonary disease	2.22	1.53-3.23	<0.0001	**5**
Cognitive impairment	2.21	0.82-6.01	0.12	**5**
Inflammatory bowel disease	2.01	0.75-5.40	0.17	**4**
Liver disease	1.94	0.80-4.72	0.14	**4**
Connective tissue disease	1.78	1.19-2.68	0.005	**3**
Iron deficiency anaemia	1.78	0.80-2.68	0.16	**3**
Diabetes	1.76	1.10-2.80	0.02	**3**
Asthma	1.65	1.00-2.73	0.050	**3**
Pulmonary hypertension	1.58	0.88-2.84	0.12	**3**
Peripheral vascular disease	1.50	1.00-2.25	0.052	**2**
Ischaemic heart disease	1.31	0.91-1.89	0.14	**2**

The Bronchiectasis Comorbidity Index (BCI)

As a sensitivity analysis, we evaluated a model excluding the above conditions thought to be associated with bronchiectasis, producing similar results (Table 5-4). The HR for the BCI was comparable at 1.17 (1.12-1.23), confirming the importance of comorbidities in bronchiectasis prognosis.

Table 5-4 Derivation of the Bronchiectasis Comorbidity Index (BCI) and points allocation

Comorbidity	**Hazard Ratio**	**95% CI**	**P value**	**Points**
Metastatic malignancy	5.21	2.83-9.58	<0.0001	**10**
Iron deficiency anaemia	2.52	1.15-5.55	0.02	**6**
Liver disease	2.21	0.91-5.37	0.08	**5**
Haematological malignancy*	1.87	0.79-4.45	0.16	**4**
Diabetes mellitus	1.77	1.13-2.79	0.01	**3**
Solid tumour	1.60	1.00-2.57	0.048	**3**
Pulmonary hypertension	1.56	0.87-2.80	0.14	**3**
Peptic ulcer disease	1.49	0.85-2.59	0.16	**2**
Peripheral vascular disease	1.44	0.97-2.15	0.07	**2**
Gastro-oesophageal reflux disease	1.31	0.96-1.79	0.09	**2**
Ischaemic heart disease	1.31	0.91-1.87	0.14	**2**

**Although haematological malignancy can be a cause of bronchiectasis, we retained this in the model where haematological malignancy was not considered by the clinician as the underlying aetiology after testing.*

5.3.3 Comparison of comorbidity scores to predict 5-year mortality

Comparative AUC (95% CI) scores for the BACI and BCI with the widely validated BSI can be seen in Figure 5-2 (a). The BACI has the highest overall predictive ability in this cohort to predict 5-year survival with an AUC score of 0.79 (0.75-0.83) *vs.* 0.74 (0.69-0.78) for the BCI, 0.78 (0.73-0.84) for the BSI and 0.71 (0.66-0.75) for the FACED score, respectively. The CC and CCI (Figure 5-2(b)) showed AUC scores of 0.72 (0.67-0.76) and 0.74 (0.69-0.78) respectively, which were inferior to the BACI (p=0.0001 on comparing AUC values), suggesting that a specific comorbidity index for bronchiectasis is appropriate.

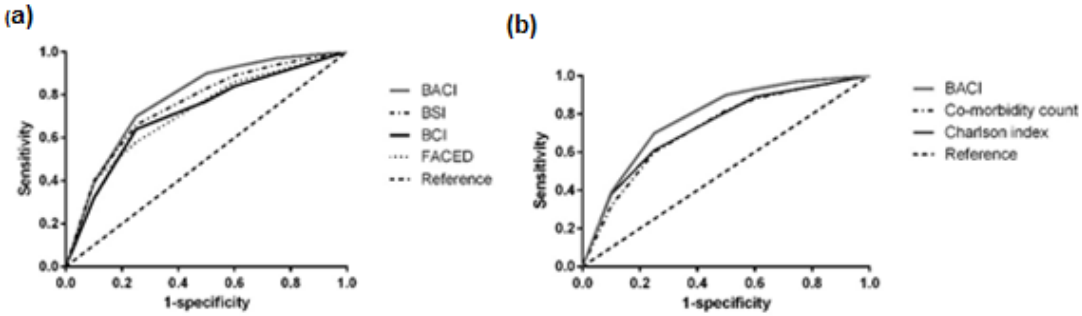

Figure 5-2 Receiver operator characteristic curves for 5-year mortality rate

(A) The performance of the BACI, BCI without causes, widely validated BSI, and the FACED scores using AUC. (B) Performance of the BACI in relation to the comorbidity count and the widely validated Charlson Comorbidity Index using AUC scores. AUC = area under the receiver operator characteristic curve; BACI = Bronchiectasis Aetiology Comorbidity Index; BCI = Bronchiectasis Comorbidity Index; FACED = 5-component mortality score.

The BACI performed consistently better than all scores in predicting 2, 3 and 5-year mortality in this cohort, with AUC scores of 0.75, 0.76 and 0.79 respectively, indicating that the score works similarly for annual prediction as for longer term prediction.

The AUC was used to identify the level of the BACI with the greatest predictive value for death in patients with bronchiectasis. Patients were classified into tertiles designated no high-risk comorbidities (for patients with

a score of zero, n=402), intermediate risk comorbidities (for patients with >1 and <6 points, n=398) and high-risk comorbidities (for patients with a score ≥ 6 points, n=186). The relationship between these risk groups and mortality and morbidity are shown in Figure 5-3.

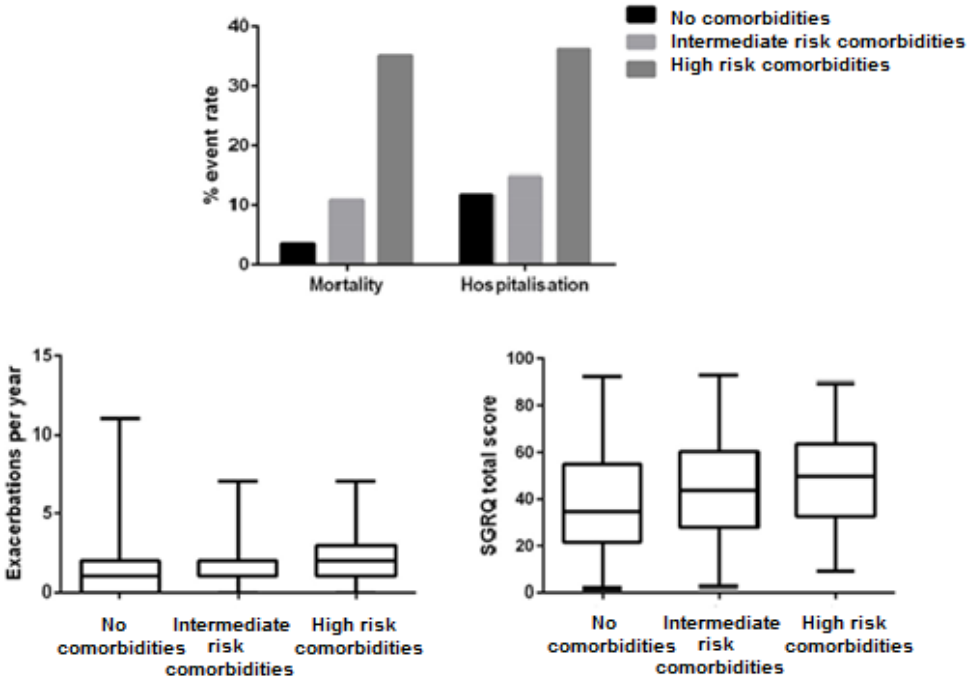

Figure 5-3 Performance of the BACI in predicting mortality, hospital admissions, exacerbation frequency and quality of life

All comparisons between groups for mortality and hospital admissions were statistically significant t p<0.0001. Between group comparisons for exacerbations were statistically significant at p=0.03. Correlation between the BACI and SGRQ assessing quality of life was significant at p=0.0008. No comorbidities (score of 0), intermediate risk comorbidities (score between 1 and <6), high risk comorbidities (score ≥ 6) according to BACI.

The sensitivity and specificity values for the BACI, BCI and the BSI are shown in Table 5-5. The BACI no comorbidity group had the highest negative likelihood ratio suggesting that it can identify patients at lowest risk of death. All scores showed relatively high negative predictive values and relatively low positive predictive values for mortality prediction.

Table 5-5 Predictive characteristics for 5-year mortality rate for derived clinical prediction tools

Organisms	Positive Likelihood Ratio	Negative Likelihood Ratio	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
BACI						
Group 2 and 3 *vs*. Group 1	1.61 (1.47-1.75)	0.26 (0.16-0.42)	88.5 (81.5-93.6)	44.9 (41.6-48.3)	18.5 (15.4-21.9)	96.5 (94.2-98.1)
Group 3 *vs*. Group 1 and 2	3.80 (3.01-4.81)	0.54 (0.45-0.66)	53.3 (44.0-62.4)	86.0 (83.5-88.2)	34.9 (28.1-42.3)	92.9 (90.9-94.6))
BCI						
Group 2 and 3 *vs*. Group 1	1.57 (1.42-1.74)	0.35 (0.23-0.53)	83.6 (75.8-89.7)	46.9 (43.5-50.3)	18.2 (15.1-21.6)	95.3 (92.8-97.1)
Group 3 *vs*. Group 1 and 2	4.68 (3.27-6.69)	0.73 (0.65-0.83)	32.0 (23.8-41.0)	93.2 (91.3-94.8)	39.8 (30.0-50.2)	90.7 (88.6-92.5)
BSI						
Moderate or severe *vs*. mild	1.37 (1.26-1.48)	0.31 (0.18-0.52)	89.3 (82.5-94.2)	34.6 (31.4-37.9)	16.2 (13.5-19.2)	95.8 (92.9-97.8)
Severe vs. moderate or mild	2.18 (1.83-2.59)	0.53 (0.42-0.67)	62.3 (53.1-70.9)	71.4 (68.3-74.4)	23.5 (19.0-28.5)	93.1 (90.9-94.9)

BACI: Bronchiectasis Aetiology Comorbidity Index; BCI: Bronchiectasis Comorbidity Index; BSI: Bronchiectasis Severity Index.

5.3.4 The BACI, BSI and mortality

Comparable with previous studies, we found that the BSI was a significant predictor of death in patients with bronchiectasis. Kaplan-Meier survival curves of the BACI groups stratified according to BSI severity show the positive predictive contribution of the BACI to the BSI in Figure 5-4. All comparisons between groups were statistically significant (mild: p=0.004; moderate: p<0.0001; severe: p<0.0001). Across all BSI strata, survival is numerically lower in intermediate and high risk groups compared to those with no significant comorbidities. The difference between groups becomes much more evident as disease severity increases.

A prediction model incorporating both the BSI and the BACI was superior to either model alone for the prediction of 5-year mortality in this cohort with an AUC (95% CI) of 0.83 (0.79-0.87).

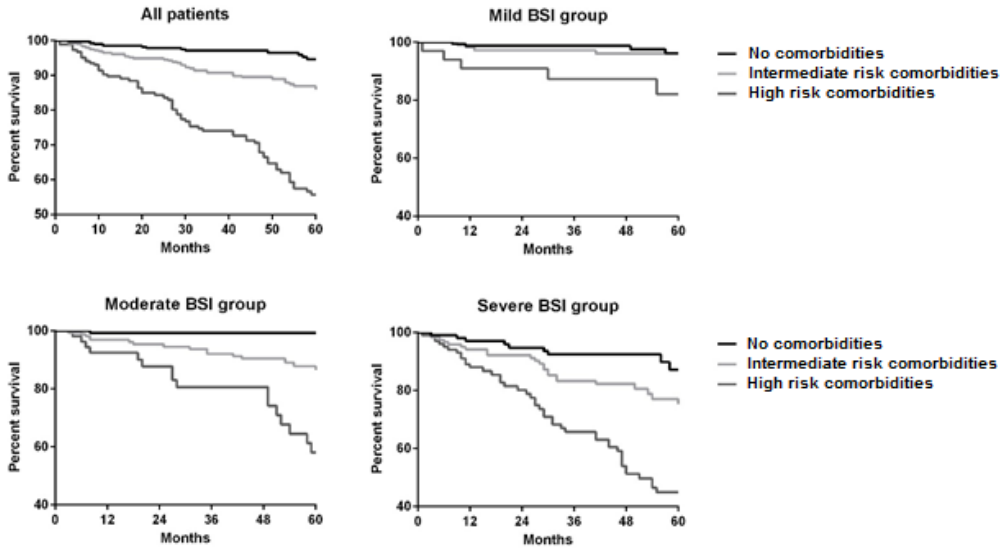

Figure 5-4 Kaplan-Meier survival curves representing survival probability at 5 years of follow-up

Kaplan-Meier survival curves according to the BSI for (a) all patients; (b) patients with mild disease (BSI 0-4 points); (c) patients with moderate disease (BSI 5-8 points); and (d) patients with severe disease (BSI ≥ 9 points). No comorbidities (BACI score of 0); intermediate risk comorbidities (BACI score between 1 and <6); high risk comorbidities (BACI score ≥ 6).

5.3.5 The BACI and other disease outcomes

Significant correlations of the BACI with a number of baseline demographic variables and important clinical outcomes were noted. The BACI correlated with both the BSI and FACED disease severity scores as well as lung function, radiological scores, dyspnoea scores, prior exacerbations and hospitalisations. Of note, it also predicts subsequent exacerbations and hospitalisations on follow-up and is independently correlated with *Pseudomonas aeruginosa* colonisation, offering further predictive potential in the clinical setting and suggesting that comorbidities may influence pulmonary outcomes (Table 5-6).

Table 5-6 Correlation of the BACI with clinical parameters and severity indices

Patient characteristics (n=986)	Spearmans Rho	p-value
BSI	0.23	**<0.0001**
FACED	0.24	**<0.0001**
Age	0.20	**<0.0001**
Male gender	0.20	**<0.0001**
Smoking history	0.33	**<0.0001**
Reiff radiological score	0.08	**0.008**
MRC dyspnoea score	0.31	**<0.0001**
LTOT	0.23	**<0.0001**
Prior exacerbations	0.12	**0.0002**
Prior hospitalisations	0.13	**<0.0001**
FEV1 %	-0.26	**<0.0001**
P. aeruginosa colonisation	0.078	**0.01**
Exacerbations on follow-up	0.11	**0.0006**
Hospitalisations on follow-up	0.22	**<0.0001**

5.3.6 Independent validation cohorts

The Serbian validation cohort consisted of 113 patients, mean age 62 years (13) at diagnosis, 70% female. 5-year mortality was 17.7%. The AUC for predicting 5 year mortality in the Serbian cohort was 0.74 (95% CI 0.63-0.86). The UK validation cohort included 88 patients, mean age (SD) 51 years (12.1) at enrolment, with 57% female. Mortality at 20 years was 40.9%. The BACI was significantly associated with mortality at 20 years, p=0.004 (Kaplan-Meier), (Figure 5-5).

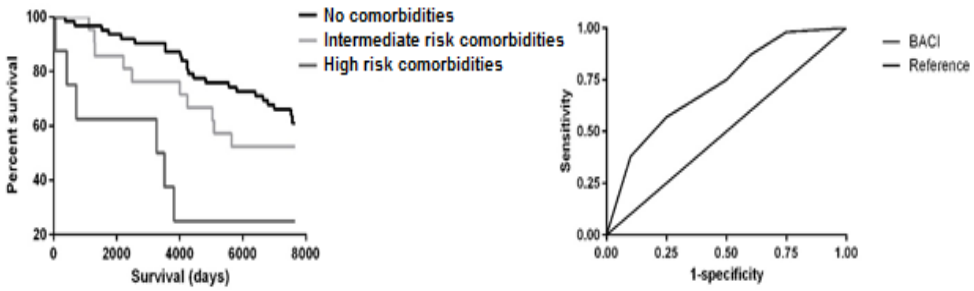

Figure 5-5 Validation of the BACI in two independent cohorts

(a) Kaplan-Meier survival values representing survival probability in BACI groups at 20 years in a UK population. Kaplan-Meier analysis was used to avoid favouring fixed effects at the expense of modifiable risk factors that may increase the short-term risk but not necessarily guarantee long-term risk. (b) Sensitivity versus 1-specificity plots showing AUC of BACI score in a Serbian cohort. AUC = area under receiver operator characteristic curve. BACI = Bronchiectasis Aetiology Comorbidity Index.

5.4 Discussion

The present study is the first multicentre international observational study to systematically describe the prevalence and associations of comorbidities on mortality in patients with bronchiectasis. We derived a new disease-specific comorbidity risk index (the BACI) to help predict which patients with bronchiectasis are at increased risk of death independently of their baseline physiological state. The BACI accurately stratified the risk of mortality and hospitalisations whilst demonstrating that comorbidities contribute to exacerbation frequency and impaired quality of life. The BACI may be a

useful clinical predictive tool, when used independently, or in conjunction with the BSI, to risk-stratify patients and assist clinical decision making and personalised medicines approaches in bronchiectasis.

This is one of the largest cohort studies performed to date in bronchiectasis and is in keeping with other derivation studies in bronchiectasis and other comorbidity derivation tools. In bronchiectasis, the BSI and FACED consisted of 608 and 397 patients respectively in their derivation cohorts.[19, 20] The Charlson Comorbidity Index, which is perhaps one of the most widely utilised comorbidity assessment tools worldwide, consisted of 604 patients in their derivation cohort.[338] Therefore, our sample size of 986 compares favourably with previous cohorts.

Most respiratory diseases have disease-specific assessment tools, designed to identify patients at high risk of complications who may benefit from early treatment intensification. There is accumulating evidence that patients with bronchiectasis, similar to COPD, are prone to developing other important diseases, over and above what can be expected in an age and sex-matched general population, including cardiovascular disease, pulmonary hypertension and lung cancer, among others.[244, 439, 441] Patients with bronchiectasis can be regarded as having a so-called “double hit” because many patients might already have an underlying aetiology that led to the development of bronchiectasis and could potentially increase the likelihood of developing further complications. For example, patients with rheumatoid arthritis and bronchiectasis might be given immunosuppressive treatments that increase the likelihood of complications, or patients with COPD-associated bronchiectasis may be at increased risk of lung cancer due to synergistic effects of airway inflammation and smoking.[448]

Systemic inflammation has been proposed as a potential explanation of the mechanistic pathway relating bronchiectasis with its comorbidities, in part due to the ageing process, which is strongly associated with an increased likelihood of developing multiple chronic conditions.[449] The association between biomarkers of systemic inflammation and outcomes in

bronchiectasis, including comorbidities, has not been well documented. In COPD, studies have demonstrated that elevated baseline inflammatory markers are associated with an increased risk of myocardial infarction, diabetes mellitus, lung cancer and pneumonia with the "inflamed comorbids" having the lowest survival in COPD populations.[58] Addressing this knowledge gap may allow us to identify pathway-specific treatment targets that could be beneficial in the treatment of multi-diseased bronchiectasis patients. Statins and macrolides have both been shown in randomised controlled trials (RCTs) to modify disease prognosis and improve clinical outcomes in bronchiectasis, owing to their anti-inflammatory effects; the development of new selective anti-inflammatory agents may hold promise for the future.[237, 410]

81 different comorbidities were identified during the 5-year follow-up of the patients in the four cohorts of this study. As expected, not all comorbidities were equally prevalent and there were highly varying strengths of association with mortality. Healthcare providers are often limited in their assessment of patients due to time constraints and high patient numbers, therefore guidance that could identify comorbidities at highest risk of worse outcomes could optimise patient care. Our results show that, of the 81 comorbidities identified, 26 differed significantly between survivors and non-survivors. This is far higher than the 15 identified in the derivation of the COmorbidity TEst (COTE) in COPD.[56] The 13 comorbidities associated with the highest risk of death on multivariate analysis were incorporated into the BACI. Similar to those in COPD, these could constitute a core of "red flag" comorbidities that healthcare providers should pay increased attention to in guiding a targeted personalised screening and treatment approach in patients with bronchiectasis.[56] Some diseases (such as cardiovascular disease, pulmonary hypertension, cognitive impairment, depression and anxiety, and lung, oesophageal and haematological malignancies), have previously been shown in small single-centre retrospective studies to be more prevalent in bronchiectasis patients; therefore inclusion of these disorders in the BACI is consistent with this available literature.[29, 244, 274, 291, 315, 439-442] However, the increased risk of death conferred by iron deficiency anaemia,

diabetes mellitus and peripheral vascular disease in this population is less well described. These findings therefore raise the possibility of a shared common biological pathway among these diseases, which requires further exploration.

Although hypertension, high cholesterol and osteoporosis were in the top five most prevalent comorbidities, the direct risk of mortality conferred by these conditions was not significant. Whether this is because they are all treatable or they are risk factors for other potentially more harmful diseases, such as myocardial infarction, is unclear. However, selected solid tumours, such as lung and oesophageal cancer, conferred a significant increased risk of death with prevalence rates of 5% *vs.* 1% and 3.5% *vs.* 0.5% in non-survivors and survivors ($p=0.004$ and $p=0.01$), respectively. Haematological malignancies, including lymphoma and leukaemia, were also associated with a significantly increased mortality risk in this patient population. These findings have previously been demonstrated in a nationwide cohort study of >53,000 bronchiectasis patients in Taiwan compared to >215,000 age and sex-matched controls whereby a 2.5 fold increased risk of lung cancer and a 2-fold increased risk of oesophageal and haematological malignancies was demonstrated.[244]

A novel finding in this study was the relatively high prevalence of peripheral vascular disease (9%) and its strong independent association with risk of death, the mechanism of which remains unclear. Diabetes and iron deficiency anaemia have both been described in COPD, the former possibly linked to overuse of inhaled corticosteroids in this patient population but more likely, both support the systemic inflammation hypothesis due to repeated infection, inflammation and chronic immune activation.[58, 450] Correction of anaemia could improve symptoms of fatigue and dyspnoea, thereby improving patients' QoL and exercise capacity, reducing hospitalisations and improving overall survival. Anxiety and depression have been reported to be highly prevalent among bronchiectasis patients correlating with quality of life measures.[315] In COPD, anxiety is an independent risk factor for mortality but no association of depression or anxiety with mortality was identified in this patient cohort.[56]

This study confirms that patients with bronchiectasis are frequently afflicted by comorbidities that contribute to disease progression, many of which confer an independent risk of death and may be missed unless specifically searched for. Although the data may be somewhat intuitive, our finding that COPD, inflammatory bowel disease, connective tissue diseases and asthma are associated with a higher mortality risk may inform decisions about which patients with bronchiectasis should be followed up more closely. Health care providers caring for these patients should routinely screen for the comorbidities outlined in the BACI because there may be effective interventions or changes in management that could reduce the risk of death. Further follow up studies are needed as with the development of any score to substantiate the use of the BACI, and determine how this score may affect clinical practice. Further exploration into the relation between high BACI scores and lung or systemic inflammation would also be interesting, in light of the association between exacerbations and Pseudomonas colonisation in comorbid patients.

The BACI is a quantitative risk stratification and comorbidity index for clinicians and researchers to quantify and prioritise comorbidities in bronchiectasis. Our data demonstrate that measurements of comorbidities as captured by the BACI improve the prognostic accuracy for mortality, particularly when used in conjunction with the BSI.[19] The BACI captures diseases not included in the CCI and carries independent prognostic value relating to future disease outcomes such as future exacerbations, hospitalisations and *Pseudomonas* colonisation. Combining the BACI and BSI equips healthcare workers and researchers to better stratify patients and provides a platform for comparative effectiveness research.

This study has several limitations. First, there is the potential for missed or as yet, undiagnosed comorbidities. For example, we experienced a somewhat lower prevalence of depression and anxiety in our cohort compared with studies that utilised the Hospital Anxiety and Depression Score to assess psychological wellbeing. However, in clinical practice, depression and anxiety are diagnosed upon history-taking and therefore this should not

influence the results of the study. Similarly, there is no objective assessment for GORD, as we rely heavily on questionnaires for diagnosis in the clinical setting, often only resorting to the gold-standard 24h pH-impedance studies in refractory cases due to cost constraints. We may also have underestimated the prevalence of other conditions, such as pulmonary hypertension, in patients who had not had an adequate work-up for the same but who may in fact, still have co-existing disease. There may have been variation between diagnostic criteria used in diagnosing comorbidities due to changes in guidelines throughout the study time period and variation in clinical practice between primary and secondary care and different healthcare institutions.

Second, although a small number of patients in our derivation cohort had undergone transplant assessment, none of the patients in our derivation or validation cohorts had received a transplant therefore we are unable to comment on the utility of the score in this patient population.[228] Comorbidity assessment is routine in the assessment of lung transplant candidates in order to determine suitability. The BACI score may highlight comorbidities that could negate transplant, e.g. metastatic malignancy, or perhaps delay transplant, e.g. iron deficiency anaemia where additional treatment may be needed beforehand. However, the BACI would not be considered in isolation in the assessment of transplant suitability and, as with any clinical prediction tool, it needs to be considered in the context of all other available information.

Third, with regards to our validation cohorts, we were unable to account for potential recruitment bias in the patients recruited to the UK cohort, who were younger and had fewer comorbidities that patients recruited to the Serbian cohort. Nevertheless, it is reassuring to see that the BACI works well in different cohorts of different durations across different healthcare systems.

Finally, our derived score is relatively complex, awarding different points for each comorbidity. To aid calculation of the score, an online calculator is accessible at http:\\www.bronchiectasisseverity.com. This assigns a total on

inputting the relevant data in sequential order and can therefore be completed in a very short space of time in the clinical setting.

The BACI requires validation in developed countries such as the US and developing countries to further substantiate its use, and further studies determining how this score may impact clinical practice are now needed. In support of our findings, however, our large representative derivation cohort was made up of almost 1000 patients of varying severity across different healthcare systems in four European countries, with external validation in two independent cohorts, one with 19-years follow-up and one from Eastern Europe, which should make these results generalisable to many bronchiectasis clinics worldwide.

5.5 Conclusion

Comorbidities in bronchiectasis are common and significantly contribute to disease burden and mortality. Surprising links with certain comorbidities may provide new insights into the underlying pathogenesis of this disease. We have derived a disease-specific bronchiectasis aetiology and comorbidity assessment tool for predicting future risk of mortality in bronchiectasis. Greater focus is needed to identify, assess and manage comorbidities in bronchiectasis in both clinical and research settings. Future interventions and treatment approaches should consider multiple comorbidities in these patients in order to maximise outcome and reduce the illness burden associated with this disease.

Chapter 6 - Hiatal Hernias are Correlated with Increased Disease Severity in Bronchiectasis

Publications relevant to this chapter:

McDonnell MJ, Ahmed A, Wall D, Bruzzi J, O'Mahoney M, Breen D, O'Regan A, Gilmartin JJ, Rutherford RM. Prevalence of hiatal hernias in non-cystic fibrosis bronchiectasis and associations with disease severity. *Respirology* 2015; 20(5):749-57.

6.1 Introduction

Bronchiectasis is an umbrella term for patients suffering from repeated episodes of bronchitis and recurrent chest infections with associated structural dilatation of the airways visible on high resolution computed tomography (HRCT). Bronchiectasis has numerous aetiologies but despite intensive investigation, up to 50% of patients have no causative factor identified.[183]

Gastro-oesophageal reflux disease (GORD) has been shown to be associated with disease severity in several chronic respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), idiopathic pulmonary fibrosis (IPF) and bronchiolitis obliterans post-lung transplantation.[93, 312, 451-453] It has been suggested that GORD may play a role in the development and severity of bronchiectasis.[269] Prevalence studies of reflux in bronchiectasis, utilising questionnaire and/or 24-hour oesophageal pH monitoring, have shown symptomatic and clinically silent reflux in 26-75% of patients.[6] Symptomatic GORD in bronchiectasis has been associated with reduced lung function, an increased exacerbation frequency and reduced quality of life.[291]

The relationship between hiatal hernias (HH) and GORD has been extensively investigated over the past few decades. HH occurs when part of the stomach protrudes into the thoracic cavity through the oesophageal hiatus of the diaphragm due to disruption of the anti-reflux barrier at the gastro-oesophageal junction. This is an anatomically complex area made up of the lower oesophageal sphincter (LOS), the crural diaphragm and the anatomical flap valve.[256, 317] In patients with a HH, the flap valve disrupts and the LOS moves above the crural diaphragm, causing the high-pressure zone to lose its synergistic configuration, and both the LOS and diaphragm sphincters to become appreciably weaker, compromising oesophageal acid clearance and facilitating the development of reflux.[256]

An increased prevalence of HH has been reported in asthma and IPF.[454-456] To our knowledge, there have been no studies to date looking at the

prevalence rate of HH in bronchiectasis patients. We therefore estimated the prevalence of HH on HRCT among a well-defined cohort of bronchiectasis patients and compared clinical indices in HH-positive (HH+) and HH-negative (HH-) patients to determine potential associations with independent and composite markers of disease severity.

6.2 Methodology

6.2.1 Study population

A retrospective observational cross-sectional cohort study of 100 consecutive patients referred for investigation of bronchiectasis in Galway University Hospitals, Ireland over an 18 month period was performed. All suspected bronchiectasis patients in our institution undergo a dedicated diagnostic clinical work-up including history, examination, detailed aetiological screening bloods, spirometry, bronchoscopy, bronchoalveolar lavage (BAL) and HRCT according to methodologies previously described.[9, 34]

Data was collected on baseline demographics including gender, age at diagnosis, body mass index (BMI), smoking status, medical comorbidities (determined by the Charlson Comorbidity Index), GORD symptoms and proton pump inhibitor (PPI) treatment, aetiology of bronchiectasis, Medical Research Council Dyspnoea (MRCD) score, number of exacerbations and hospitalisations over the prior year, baseline microbiological status on BAL, baseline pulmonary function and baseline radiological involvement. Ethical approval was granted by our local Research Ethics Committee. Individual patient consent was not required as this was a retrospective research study based on routine diagnostic investigations and bronchiectasis work up.

6.2.2 Diagnosis of bronchiectasis

The diagnosis of bronchiectasis was defined as patients with daily mucopurulent sputum production plus dilated and thickened airways on HRCT.[9] Aetiology was defined according to methodologies previously described; a diagnosis of idiopathic bronchiectasis was made if all

aetiological screening tests were negative and no associations with other known diseases was found.[9, 34]

CT images were acquired on a 64-slice multi-detector CT scanner (Somatom Sensation Cardiac 64, Siemens, Erlangen, Germany). Inspiratory spiral and expiratory sequential scans were performed at an initial collimation of 5mm, and reconstructed at 1.0mm thin slices at section intervals of 10mm. Scanning parameters included a kVp of 120 (dose-adjusted), 40mAs (care-dose), rotation time of 0.5s and a pitch of 1.4. Intravenous contrast media was not administered and scans were performed with patients positioned supine.

All scans were reported by radiologists with expertise in HRCT imaging at the time of scanning. Subsequent independent review for confirmation and scoring of disease severity according to the modified Bhalla score, validated for use in bronchiectasis, was performed by an expert thoracic radiologist (Figure 6-1).[66] The extent of bronchiectasis, severity of bronchial dilatation, bronchial wall thickness, presence of mucus plugging in large and small airways, and decrease in parenchymal attenuation were scored for each lobe, with the lingula considered a separate lobe, making a total of 6 lobes. Total lung scores for each abnormality were defined as the mean score from all lobes for each HRCT feature. The proportion of cystic versus varicose or cylindrical bronchiectasis was recorded along with the total number of lobes involved. Lobar predominance was assessed by calculating the mean scores for all HRCT features per lobe. A combined HRCT total score for all HRCT features across all lobes was subsequently derived from summing the individual scores.

6.2.3 Evaluation of the presence of a hiatal hernia

The presence of HH was determined by evaluation of the oesophageal junction in relation to the diaphragm using recognised anatomical definitions.[457] Where “present”, each HH was classified according to gastric fundus size as small (<2cm), moderate (2-5cm) and large (> 5cm). “Absent” HH was graded as 0.

6.2.4 Pulmonary function tests

Pulmonary function tests (PFTs) were performed using a Sensormedics V-Max 22 device allowing calculation of Forced Expiratory Volume (FEV1), Forced Vital Capacity (FVC), and FEV1/FVC ratio. Values were expressed as percentage predicted for age, sex, height and ethnicity employing standard European Respiratory Society (ERS)/ American Thoracic Society reference ranges.[361] All PFTs were carried out by pulmonary physiologists trained according to the recommendations of the ERS.[362]

6.2.5 Relationship between hiatal presence and disease severity

To analyse the relationship between HH and disease severity, statistical comparisons of known individual clinical markers of severity between HH+ and HH- patients were performed. Factors that have been shown to be associated with disease severity in individual studies include extent of overall bronchiectasis and bronchial wall hypertrophy on HRCT, chronic colonisation by *Pseudmonas aeruginosa*, exacerbation frequency, and high concentrations of pro-inflammatory markers in sputum or serum.[47, 325, 458] Two composite severity scores for bronchiectasis have recently been developed: the Bronchiectasis Severity Index (BSI) and the FACED scoring system, both of which can be utilised to determine a potential relationship between the presence of HH and disease severity.[19, 20]

6.2.6 Statistical analysis

Statistics were computed using SPSS® v21.0, for Windows platform and GraphPad Prism v5.0. Mean and standard deviation were used for continuous parametric data, median and interquartile range for continuous non-parametric data, and frequencies and percentages for categorical data. Patients were divided into HH-positive (HH+) and HH-negative (HH-) groups. Subgroup analyses were performed using the Chi-squared test or unpaired t-test depending on data distribution. Chi-squared test was used for clinical characteristics including gender, cystic versus cylindrical disease and microbial colonisation. Group mean spirometric values and age were normally distributed and compared using the unpaired t-test. HRCT scores

(total and by section) were not normally distributed. Group means were compared by the Mann–Whitney U-test. A p-value <0.05 was considered to be statistically significant.

The reporting of this observational study conforms to the recommendations of STROBE.[446]

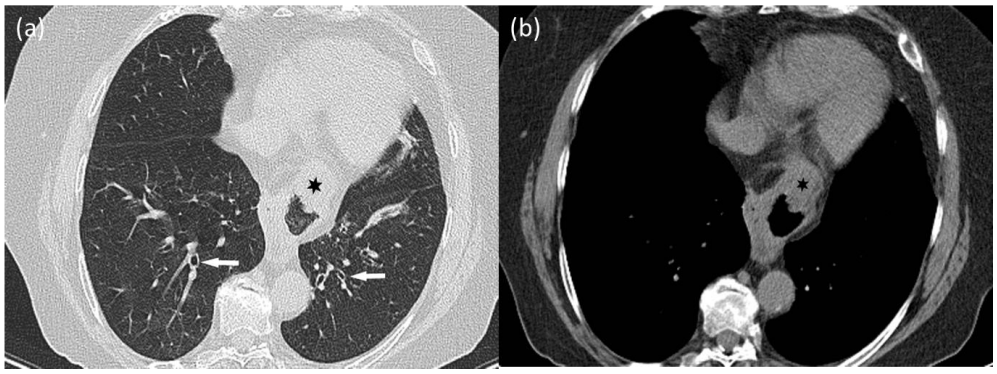

Figure 6-1 Axial computed tomography images

Axial computed tomography (CT) images of the lower thorax in (a) lung and (b) mediastinal soft tissue windows demonstrating mild cylindrical bronchiectasis and peri-bronchial wall thickening (arrows) in association with a large hiatal hernia (asterix)

6.3 Results

6.3.1 Study population

A total of 81 patients (55 females, 68%) were found to have confirmed bronchiectasis on HRCT imaging, with mean (SD) age 62.6 (12.4) years, FEV1 85.8% (27.5) predicted and FVC 96.5% (41.7) predicted. Of these, 29 (35.8%) were confirmed to have HH+ on imaging (24 small, 1 moderate, and 4 large; Figure 6-2). Baseline characteristics for total cohort and comparisons between HH+ and HH- subgroups are shown in Table 6-1. HH+ patients had a trend towards higher BMI (p=0.07) and a significantly larger proportion of HH+ patients had reflux symptoms for which they had been prescribed a PPI (HH+ 62.1% *vs.* HH-28.8%, p<0.01) compared with HH- patients.

Table 6-1 Patient characteristics

Baseline data	Total (n=81)	HH+ (n=29)	HH- (n=52)	p-value
Age, yr (SD)	62.6 (12.4)	64.4 (9.7)	61.6 (13.6)	0.29
Females, n (%)	55 (67.9)	21 (72.4)	34 (65.4)	0.51
BMI (SD)	26.9 (5.7)	28.5 (4.8)	26.1 (6.0)	0.07
Smokers/Ex-smokers, n (%)	45 (55.6)	17 (58.6)	28 (53.8)	0.68
Charlson Comorbidity Index ≥ 3, n (%)	63 (77.7)	23 (79.3)	15 (28.8)	0.58
Reflux on PPI	33 (40.7)	18 (62.1)	15 (28.8)	**<0.01****

*HH+ presence of a hiatal hernia on independent expert radiological review; HH- absence of a hiatal hernia on independent expert radiology review; BMI: body mass index. **p<0.01.*

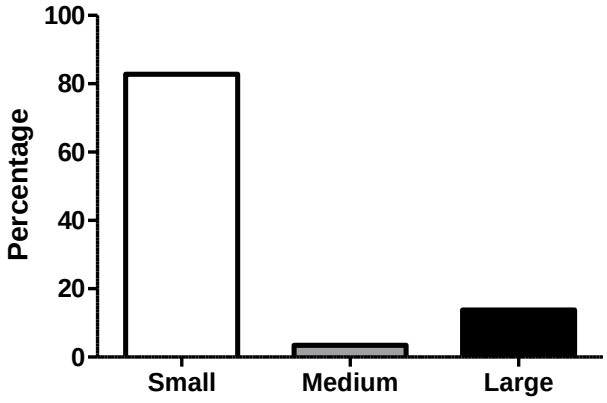

Figure 6-2 Prevalence of hiatal hernias among bronchiectasis patients according to hiatal hernia size

Hiatal hernias were graded according to the following: small (<2cm), moderate (2-5cm) and large (> 5cm).

6.3.2 Aetiology

Of the 81 patients in our cohort, the majority had idiopathic bronchiectasis (28.3%), closely followed by post-infectious (27.2%) secondary to childhood infections, bacterial pneumonia, pulmonary tuberculosis (TB) and non-tuberculous mycobacterial (NTM) infection. COPD and asthma accounted for the next largest groups at 16.0% and 8.6% respectively. Aspiration confirmed on barium studies, secondary to previous cerebrovascular events, oesophageal malignancy, myasthenia gravis and post-cardiac arrest, accounted for 7.4% of cases. Aside from ABPA, where there were 3 (10.3%) HH+ patients compared to zero HH- patients (p=0.04), there were no statistically significant differences in aetiology between the two patient subgroups (Table 6-2).

Table 6-2 Aetiology of bronchiectasis in the study population and according to the presence of a hiatal hernia.

Aetiology, n (%)	Total (n=81)	HH+ (n=29)	HH- (n=52)	p-value
Idiopathic	23 (28.3)	10 (34.4)	13 (25.0)	0.44
Post-infectious	22 (27.2)	7 (24.1)	15 (28.8)	0.79
- Childhood infection	3 (3.7)	1 (3.4)	2 (3.8)	1.00
- Pneumonia	7 (8.6)	2 (6.9)	5 (9.6)	1.00
- Pulmonary TB	9 (11.1)	4 (13.8)	5 (9.6)	0.71
- NTM infection	3 (3.7)	0	3 (5.8)	0.55
Asthma	7 (8.6)	3 (10.3)	4 (7.8)	0.69
COPD	13 (16.0)	3 (10.3)	10 (19.2)	0.36
ABPA	3 (3.7)	3 (10.3)	0	**0.04***
Alpha-1-antitripsin deficiency	1 (1.2)	1 (3.4)	0	0.36
Immune deficiency	3 (3.7)	0	3 (5.8)	0.55
Aspiration	6 (7.4)	2 (6.9)	4 (7.7)	1.00
Primary ciliary dyskinesia	1 (1.2)	0	1 (1.9)	1.00
Connective tissue disease	2 (2.4)	0	2 (3.8)	0.54

*TB: tuberculosis; NTM: Non-tuberculous mycobacteria; COPD: chronic obstructive pulmonary disease; ABPA: allergic bronchopulmonary aspergillosis. *p<0.05.*

6.3.3 Pulmonary function

Lung function data was available in 77 patients but was unattainable in 4 patients (2 HH+ and 2 HH-; Table 6-3). On comparing the remaining patients by the presence of HH, a significant reduction in FEV1% predicted was observed in HH+ patients (HH+ 75.4(24.5), HH- 90.4(25.5); p=0.02), (Table 6-3). Comparisons across the spectrum of lung function severity showed that patients with severe airflow limitation were more likely to be HH+ on HRCT than those with minimal airflow limitation (p<0.0001).

Table 6-3 Differences in pulmonary function in bronchiectasis patients with and without a hiatal hernia

Pulmonary function	Total (n=77/81)	HH+ (n=27)	HH- (n=50)	p-value
FEV1%, mean (SD)	85.7 (26.7)	75.4 (24.5)	90.4 (25.5)	**0.02***
FVC%, mean (SD)	105.7 (28.2)	101.1 (20.9)	109.9 (26.3)	0.15
Ratio%, mean (SD)	68.9 (11.3)	66.5 (10.3)	71.0 (9.8)	0.06

*FEV1%: forced expiratory volume in 1 second; FVC: forced vital capacity; ratio: FEV1%/FVC%. A ratio of <70% represents obstructive airways disease. HH+ presence of a hiatal hernia on independent expert radiological review, HH- absence of a hiatal hernia on independent expert radiology review. *p<0.05.*

6.3.4 Radiological disease

HH+ patients were found to have an increased frequency of cystic bronchiectasis compared with HH- patients (HH+ 31.0%. HH- 11.5%; p=0.03) as well as an increased number of bronchiectatic lobes affected (HH+ 2.62 (1.21), HH- 2.17 (0.94); p=0.03). There was no significant difference in total HRCT score (HH+ 16.7 (14.2), HH- 12.6 (9.12); p=0.12).

A breakdown of the modified Bhalla score per individual HRCT feature and per lobe is demonstrated in Table 6-4. The extent of bronchiectasis and the extent of decreased parenchymal attenuation was significantly different

between the two groups (HH+ 6.2 (4.7), HH- 4.5 (3.1); p=0.04) and (HH+ 1.0 (1.8), HH- 0.2 (0.5); p=0.03) with a trend towards increased bronchial wall thickness (HH+ 5.0 (4.2), HH- 3.8 (2.7); p=0.07). However, there was no predilection for any particular lobe.

Table 6-4 HRCT parameters including breakdown of modified Bhalla score according to individual HRCT features and lobar involvement

HRCT parameters	Total (n=81)	HH+	HH-	p-value
Cystic, n (%)	15 (18.5)	9 (31.0)	6 (11.5)	**0.03***
No. lobes involved, mean (SD)	2.33 (1.47)	2.62 (1.54)	2.17 (1.42)	**0.03***
Total HRCT score, mean (SD)	14.1 (11.3)	16.9 (14.1)	12.6 (9.1)	0.12
Individual HRCT features (score out of 13 for all lobes)				
Extent of bronchiectasis	5.1 (3.8)	6.2 (4.7)	4.5 (3.1)	**0.04***
Bronchial wall dilatation	4.3 (3.4)	5.0 (4.2)	3.8 (2.7)	0.07
Bronchial wall thickening	3.5 (2.8)	3.9 (3.3)	3.2 (2.5)	0.87
Extent of mucus plugging in small airways	0.3 (0.7)	0.3 (0.7)	0.3 (0.7)	1
Extent of mucus plugging in large airways	0.6 (1.0)	0.4 (1.0)	0.6 (1.0)	0.42
Extent of decreased attenuation	0.5 (1.2)	1.0 (1.8)	0.2 (0.5)	**0.03***
Lobar involvement on HRCT (score out of 13 per lobe)				
Right upper lobe	1.4 (2.5)	1.9 (3.0)	1.1 (2.2)	0.19
Right middle lobe	2.9 (3.0)	3.2 (3.5)	2.7 (2.7)	0.54
Right lower lobe	2.9 (3.0)	2.9 (3.2)	3.0 (3.0)	0.89
Left upper lobe	1.3 (2.5)	1.7 (3.1)	1.0 (2.1)	0.23
Lingula	2.1 (2.7)	2.9 (3.2)	1.7 (2.4)	0.06
Left lower lobe	3.5 (3.4)	4.2 (3.9)	3.1 (3.1)	0.17

*HRCT: high resolution computerised tomography scanning. HH+ presence of a hiatus hernia on independent expert radiological review, HH- absence of a hiatus hernia on independent expert radiology review. *p<0.05*

6.3.5 Microbiology

BAL samples were culture positive in 41 (50.6%) of patients (HH+ 48.3%, HH- 51.9%). There was a significantly lower frequency of *Haemophilus influenzae* in HH+ patients (HH+ 10.3%, HH- 30.7%; p=0.046). The most commonly identified *organisms* of the total cohort were *H. influenzae* 23.4%, Methicillin Sensitive *Staphylococcus aureus* (MSSA) 12.3%, *Streptococcus pneumoniae* 8.2%, Coliforms 6.2%, *P. aeruginosa* 4.9%, Methicillin Resistant *Staphylococcus aureus* (MRSA) 2.5%, and one patient each with *Aspergillus fumigatus, Candida albicans, Acinobacter* and *Moraxella catarrhalis*.

6.3.6 Disease severity scores

Increased disease severity scores were observed in HH+ patients compared to HH- patients with both the BSI and the FACED bronchiectasis severity scores: BSI (HH+ 4.93 (1.65), HH- 3.25 (2.13); p<0.001) Table 6-5 and Figure 6-3). Sub-analysis of HH+ patients with and without PPI treatment showed no differences in treatment with PPIs on severity scores: BSI (HH+ on PPI 4.94 (1.63), HH+ not on PPI 4.91 (1.76); p=0.96);, FACED ((HH+ on PPI 2.09 (1.64), HH+ not on PPI 2.27 (1.49); p=0.59).

Table 6-5 Disease severity scores

Composite severity scores	Total (n=81)	HH+ (n=29)	HH- (n=52)	p-value
BSI, mean (SD)	3.85 (2,12)	4.93 (1.65)	3.25 (2.13)	**0.0004*** **
FACED, mean (SD)	1.64 (1.48)	2.21 (1.52)	1.32 (1.43)	**0.0097** **

*BSI: bronchiectasis severity index; FACED: acronym for 5 dichotomised clinical variables. **p<0.01; ***p<0.001.*

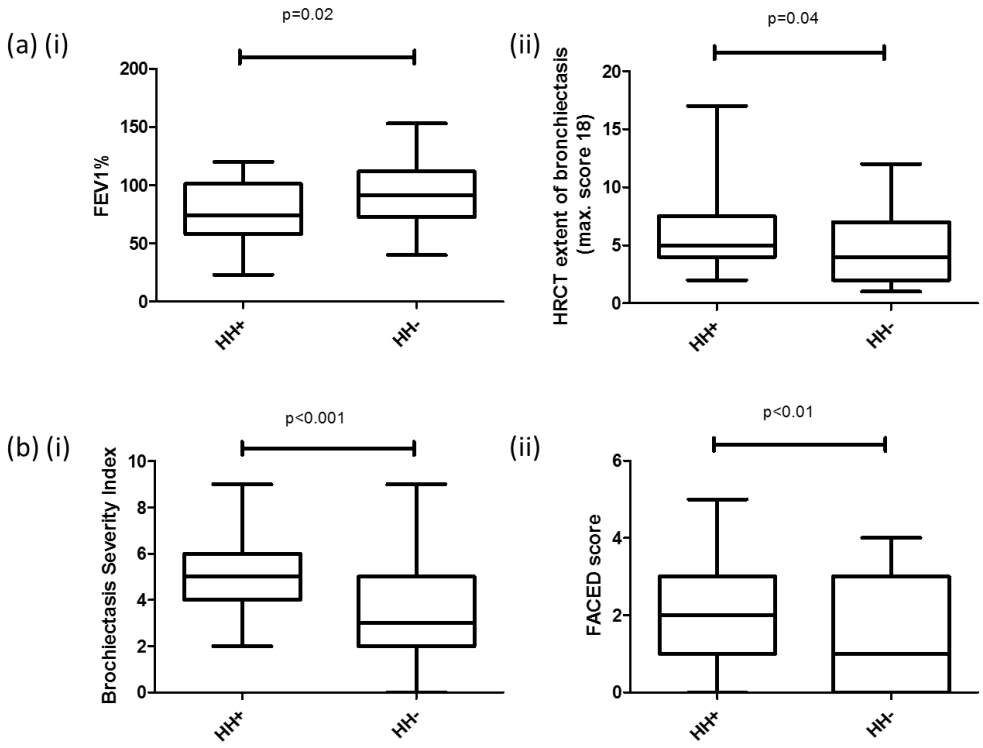

Figure 6-3 Bronchiectasis cohort with and without hiatal hernia as determined by an independent blinded expert thoracic radiologist on high-resolution computed tomography.

*Individual markers of disease severity include: (i) forced expiratory volume in 1 second (FEV1) % predicted and (ii) radiological extent of bronchiectasis throughout all lobes according to the modified Bhalla score. *p<0.05. Bronchiectasis severity scores include: (i) Bronchiectasis Severity Index (BSI) and (ii) FACED score. **p<0.01 ***p<0.001. Bars indicate median values. Box plots indicate median, and 25th–75th percentiles; whiskers indicate 5th–95th percentile.*

6.4 Discussion

In the past decade, there has been great interest in the potential role of HH and GORD in the pathogenesis of obstructive airway and parenchymal lung diseases. To our knowledge, the present study is the first to explore the association of HH on HRCT and markers of disease severity in bronchiectasis. We found a prevalence of HH in over a third of our patients (36%) compared with a background 10-20% prevalence in the general population.[256, 457] We also observed significant statistical associations between the presence of HH and increased GORD symptoms, reduced lung function, increased extent and severity of radiological disease, and increased

composite bronchiectasis severity scores. The majority of HHs were of small size; however, much larger patient numbers would be required to determine a correlation between HH size and disease severity.

Age, gender, smoking, obesity, positional and physiological changes in respiratory mechanics and medications are listed as potential contributing factors for HH and GORD.[256, 317, 457, 459, 460] In this cohort, age, gender, smoking status, medical comorbidities, BMI and medications were not found to statistically differ between HH+ and HH- patients, perhaps supporting the hypothesis that changes in respiratory mechanics in bronchiectasis may be contributory to the development of HH and associated increased disease severity.[461] As all patients enrolled in this study were undergoing diagnostic evaluation for bronchiectasis, they were all treatment naïve, thereby negating the potential confounding effects of medications such as azithromycin in reducing the effects of GORD and HH. Sub-analysis of HH+ patients with and without PPI treatment showed no effect of PPI treatment on bronchiectasis disease severity, suggesting that PPIs do not fully counteract the effects of HH and GORD.

Aetiology was only found to be significant between the two subgroups in ABPA patients. Whether or not this has any clinical relevance remains unclear. ABPA affects asthmatic patients who are known to have increased prevalence of HH and GORD. However, no association was noted with asthma alone and HH. It has been suggested that GORD-derived bile may be a host determinant contributing to chronic respiratory infection, driving the switch from acute to persistent infection; however, the effects of bile on aspergillus have yet to be elucidated.

Regarding structural changes in the airway, HH+ patients demonstrated a significantly increased frequency of cystic bronchiectasis compared with HH- patients as well as an increased number of bronchiectatic lobes affected. HH+ patients were also shown to have increased extent of bronchiectasis and decreased parenchymal attenuation with a trend towards increased bronchial wall thickness in the modified Bhalla score components. No differences in

relation to pattern of distribution or presence of airway thickening were noted hence aspiration into the right lower lobe is unlikely to be responsible. Unfortunately, we can only speculate as to how and why these changes may occur; further mechanistic work in cellular and microbial models are needed to better understand the effects of GORD in the lungs.

There are two possible explanations for the lower frequency of *H. influenzae* in HH+ patients. Studies in non-encapsulated *H. influenzae* have demonstrated that expression of urease enhances viability in acid environments, suggesting an increased likelihood of survival in HH+ patients.[462] Proton pump inhibitors may also be taken more frequently in patients with HH which may alter the microbiological flora in gastric juices and subsequent refluxate.

Multi-detector CT scanning (MDCT) has been shown in other lung diseases to be capable of demonstrating the presence of HH with good intra-observer agreement.[66] Positive aspects of our study include the rigour involved in confirming the diagnosis of bronchiectasis with functional testing, lower airway BAL microbiological sampling and detailed immunophenotyping at baseline. A further advantage is that HRCT is routinely performed in bronchiectasis patients, potentially allowing for a diagnosis of HH without recourse to further imaging or invasive oesophageal tests. The critical question, however, is whether the presence of HH on HRCT alone is a strong indicator of reflux? Our results showed a significantly larger proportion of HH+ patients with GORD compared to HH- patients, which supports an association between the presence of HH and GORD symptomatology. There is good evidence that the presence of a HH correlates with reflux on oesophageal testing in patients with GORD due to loss of integrity of the LOS.[256, 317, 457, 459, 460] There is also evidence from other lung diseases that GORD can lead to structural damage in the airway and lungs.[312, 397, 453]

In bronchiectasis, Mandal *et al.* demonstrated that nearly three-quarters of bronchiectasis patients reported airway reflux utilising the Hull Airway

Reflux Questionnaire.[291] Moreover, patients with airway reflux coughed more severely and had significantly higher sputum cytokine levels. The presence of GORD in this study, however, was not confirmed by oesophageal physiology tests. [291] Lee *et al.* subsequently demonstrated an increased prevalence of distal and proximal GORD in a small number of bronchiectasis patients using dual chamber 24h pH-monitoring, but no significant associations of reflux with symptom scores, sputum pepsin measurements or radiological or pulmonary markers of disease severity were found in this cohort.[274]

Our study is somewhat limited by its cross-sectional, retrospective design with limited longitudinal data. We did not include validated symptom questionnaires or formal assessments of reflux, relying on history alone. GORD may also be present in the absence of a HH or with very small HHs not detectable by MDCT. We recognise that statistically significant associations do not prove cause and effect and that the present study is only hypothesis-generating. Indeed, HH may be caused by the underlying respiratory disease as severe patients have more hyperinflated lungs potentially altering the diaphragm-oesophageal interface. More severe airflow obstruction may also have a siphoning effect on gastric juices into the oesophagus as more negative inspiratory pressure is generated whilst breathing. More severe coughing may also contribute to the formation of a HH due to abrupt spikes in intra-abdominal pressure.[257] From this study, it appears that the presence of a HH may have some impact on disease development and severity in bronchiectasis, but further prospective longitudinal studies are needed to determine the potential impact of HH and GORD on disease progression over time. As a next step, we suggest combined use of questionnaire, pH impedance, and BAL markers to assess GORD and aspiration in bronchiectasis patients with and without HHs on HRCT. Significant GORD may be very rare in the absence of a HH, and the presence of a HH on HRCT alone may strongly signify GORD as a potential causal agent in a subgroup of bronchiectasis patients. Targeted therapy such as acid reduction, pro-kinetic agents and/or surgical correction could then be considered.[307-309, 321, 463, 464]

A recent study turned this question on its head, retrospectively reviewing HRCT scans of 4,388 patients in a single institution to determine the incidence of bronchiectasis hypothesised to be caused by the compression of a HH on bronchi. A total of 98 (2.2%) HH cases were detected. The rate of HH according to small, moderate and large size were 45 (45.9%), 9 (9.2 %), and 44 (44.9), respectively. 9 (9%) patients (8 female, 89%) were found to have confirmed bronchiectasis anatomically adjacent to the HH. The rate of HH accompanied by bronchiectasis was similar among males and females. Bronchiectasis rate, was 12 times (OR: 12.34, 95% CI: 1.48-103.03, p = 0.009) higher in those with a large HH compared to those with a small or moderate HH. The authors concluded that the presence of a HH may lead to the development of bronchiectasis due to external compression other than lymphadenopathy or a tumour; however, further studies are required to substantiate these findings.[465]

6.5 Conclusion

In conclusion, we demonstrated a higher than background prevalence of HH among our cohort of bronchiectasis patients. The presence of HH correlated with increased GORD symptoms, reduced lung function and increased extent and severity of radiological disease. Which is the chicken or the egg requires further clarification and study though it is highly likely that these forces act in a bi-directional manner contributing to each other. Further investigation into the impact of the presence of HH and GORD in bronchiectasis using a multimodal assessment approach is therefore recommended.

Chapter 7 - Impact of Gastro-Oesophageal Reflux Disease on Mortality and Exacerbations in Bronchiectasis

Publications relevant to this chapter:

McDonnell MJ, Rutherford RM, Ward C, De Soyza A, Laffey JG, O'Toole D, Aliberti S, Goeminne PC, Chalmers JD. Impact of gastro-oesophageal reflux disease on mortality and exacerbations in bronchiectasis. *In submission* 2020.

7.1 Introduction

It is widely accepted that comorbidities are common in and contribute significantly to the morbidity and mortality associated with bronchiectasis.[23] Gastro-oesophageal reflux disease (GORD) is a common comorbidity in bronchiectasis with a reported prevalence of 34-74% using symptom questionnaires and 11-75% using oesophageal pH-monitoring.[28] Patients with co-existing bronchiectasis and GORD have reportedly been shown to have increased bronchiectasis severity, manifest by increased symptoms, exacerbations, hospitalisations, radiological extent, chronic infection, reduced pulmonary function and worse quality of life.[28, 269] The association of GORD with mortality has yet to be determined.

To further elucidate the relationship between GORD with exacerbations, hospitalisations and mortality in bronchiectasis, we combined and analysed data from 4 well-characterised longitudinal prospective observational cohorts in Europe containing 5-year follow-up data. This was used to achieve the following aims: (1) to determine factors associated with the presence of GORD in bronchiectasis patients, and (2) to evaluate the relationship between GORD, exacerbations, hospitalisations and mortality in bronchiectasis.

7.2 Methods

7.2.1 Study design and participants

Participants aged 18 years or older with a primary diagnosis of bronchiectasis supported by a consistent clinical history (cough, chronic sputum production and/or recurrent respiratory infections), and computed tomography chest imaging demonstrating bronchiectasis affecting one or more lobes, were included. Patients with cystic fibrosis, traction bronchiectasis due to interstitial lung disease and active non-tuberculous mycobacterial disease were excluded. Ethical approval was granted by individual ethics committees at each participating centre. Diagnostic work-up and assessment was performed according to the 2010 British Thoracic Society (BTS) guidelines.[9]

A core dataset consisting of demographics, previous medical history, comorbidities, radiological, functional, laboratory and microbiological findings were recorded at each site. Self-reported history of a physician's diagnosis of GORD and proton pump inhibitor (PPI) acid suppression therapy was used as the primary exposure variable. Disease severity was evaluated using the Bronchiectasis Severity Index (BSI).[19] Enrolment into the study required that patients were clinically stable and free from antibiotic therapy for a minimum of 4 weeks prior to baseline data collection and that all variables needed to calculate the BSI and the key relevant outcomes of mortality, exacerbations and hospital admissions on follow-up were available.

7.2.2 Study outcomes

Longitudinal outcomes were evaluated for up to 5 years of follow-up, including exacerbations, hospitalisations and mortality. Exacerbations and hospitalisations were defined according to BTS guidelines as: the requirement for antibiotics in the presence of one or more symptoms of increasing cough, increasing sputum volume, worsening sputum purulence, worsening dyspnoea, increased fatigue/malaise, fever, and haemoptysis; and unscheduled hospitalisations or emergency department visits for exacerbations or complications as recorded from patient histories and verified using pharmacy and administrative databases.[9]

7.2.3 Statistical analysis

Continuous data are presented as mean and standard deviation (SD) or median and interquartile range (IQR), and categorical data as frequencies and percentages. The Mann Whitney U and chi-squared test were used for comparison of numerical and categorical data, respectively. For comparisons of more than two groups, one-way analysis of variance or the Kruskal-Wallis test were used as appropriate. Binary logistic regression was used to determine independent factors associated with GORD. All-cause mortality, exacerbations, and hospital admissions were analysed as separate primary end points, by the presence of GORD, using multivariable Cox proportional hazards regression and negative binomial regression analysis to determine

hazard ratios (HR), incidence risk ratios (IRR) and their 95% confidence intervals (CI). Variables included in the model were those determined to be clinically significant in impacting on outcomes. For all models, adjustments were made for BSI, sex, aetiologies, comorbidities and treatment. Kaplan–Meier curves were used to illustrate survival data. All analyses were performed using SPSS version 21 (SPSS, Chicago, IL, USA) for Windows platform and Graph Pad Prism Version 5 (Graph Pad Software, Inc. San Diego, CA, USA).

7.3 Results

7.3.1 Patient characteristics and follow-up data

A total of 1,543 patients from 4 European countries (UK, Ireland, Belgium and Italy) were included, of whom 355 (23%) reported a diagnosis of GORD. During the 5-year follow-up, at time of analyses, 376 (24.4%) required at least one hospitalisation and 182 (12%) patients died during follow-up.

Baseline characteristics of the patients overall and by GORD status are reported in Table 7-1. GORD was more prevalent among patients who were older, smokers, and with asthma, COPD or CTD underlying aetiologies or with common comorbidities such as chronic renal failure. The presence of GORD was associated with a higher frequency of exacerbations and higher BSI scores.

Exacerbation rate during follow-up was median (IQR) 2 (0-3) events per patient per year and was higher in patients who self-reported a physician's diagnosis of GORD than those who did not (2 (1-3) *vs.* 2 (0-3); $p<0.0001$). The proportion of patients hospitalised for a severe exacerbation also differed by GORD status but did not reach statistical significance (98 (27.6%) *vs.* 278 (23.4%); $p=0.1$). 5-year follow-up mortality was significantly higher in patients with GORD compared to those without (109 (33%) in GORD-positive patients *vs.* 79 (6%) in GORD-negative; $p<0.0001$), (Figure 7-1).

Table 7-1 Baseline characteristics of bronchiectasis patients and summary follow-up data stratified by presence or absence of GORD

	Total (n=1543)	**GORD-positive (n=355)**	**GORD-negative (n=1188)**	**p value**
Demographics				
Age, yr, median, IQR	66 (57-74)	67 (58-74)	66 (56-74)	**0.04**
Female, n (%)		221 (62.3%)	728 (61.3%)	0.7
BMI, kg/m^2, median (IQR)	24.9 (21.8-27.9)	24.8 (21.8-28.1)	25.0 (21.7-27.8)	0.8
Smokers and ex-smokers, n (%)	696 (45.1)	181 (51.0%)	515 (43.4%)	**0.01**
Aetiologies				
Idiopathic, n (%)	601 (39.0)	110 (31.0%)	491 (41.3%)	**0.0004**
Post-infectious, n (%)	358 (23.2)	67 (18.9%)	291 (24.5%)	**0.03**
Asthma, n (%)	127 (8.2)	39 (11.0%)	88 (7.4%)	**0.03**
COPD, n (%)	327 (21.2)	100 (28.2%)	227 (19.1%)	**0.0002**
Connective tissue disease, n (%)	99 (6.4)	43 (12.1%)	56 (4.7%)	**<0.0001**
ABPA, n (%)	88 (5.7)	11 (3.1%)	71 (6.0%)	**0.03**
IBD, n (%)	34 (2.2)	8 (2.3%)	26 (2.2%)	0.9
Immunodeficiency	81 (5.2)	16 (4.5%)	65 (5.5%)	0.5
Comorbidities				
Ischaemic heart disease, n (%)	287 (18.6)	68 (19.2%)	219 (18.4%)	0.8
Stroke, n (%)	80 (5.2)	18 (5.1%)	62 (5.2%)	0.9
Diabetes, n (%)	146 (9.5)	40 (11.3%)	106 (8.9%)	0.2
Chronic Renal Failure, n (%)	88 (5.7)	34 (9.6%)	54 (4.5%)	**<0.0001**
Haematological malignancy, n (%)	27 (1.7)	5 (1.4%)	22 (1.9%)	0.6
Solid tumor, n (%)	116 (7.5)	33 (9.3%)	83 (7.0%)	0.1
Functional status				0.3
FEV1 % >80% predicted	589 (38.2)	150 (42.3%)	439 (45.4%)	
FEV1 % 50-80% predicted	540 (35.0)	133 (37.5%)	407 (34.3%)	
FEV1 % 30-49% predicted	246 (15.9)	51 (14.4%)	195 (16.4%)	
FEV1 % < 30% predicted	68 (4.4)	21 (5.9%)	47 (4.0%)	

	Total (n=1543)	**GORD-positive (n=355)**	**GORD-negative (n=1188)**	**p value**
Clinical status				
Exacerbations in the previous year, median (IQR)	2 (0-3)	2 (1-3)	2 (0-3)	**<0.0001**
At least one hospitalisation in the previous year, n (%)	376 (24.4)	98 (27.6%)	278 (23.4%)	0.1
Disease severity				
BSI score, median (IQR)	6 (4-10)	7 (4-11)	6 (4-10)	**<0.0001**
Mild BSI score (0–4), n (%)	471 (30.5)	90 (25.4%)	381 (32.1%)	
Moderate BSI score (5–8), n (%)	574 (37.2)	130 (36.6%)	444 (37.4%)	
Severe BSI score (>9), n (%)	498 (32.3)	135 (38.0%)	363 (30.6%)	
Bacteriology				
H. influenzae	337 (21.8)	53 (14.9%)	284 (23.9%)	**<0.0001**
P. aeruginosa	177 (11.5)	50 (14.1%)	127 (10.7%)	0.08
S. aureus	94 (6.1)	19 (5.4%)	75 (6.3%)	0.5
M. catarrhalis	84 (5.4)	4 (1.1%)	80 (6.7%)	**<0.0001**
Enterobacteriaceae	117 (7.6)	18 (5.1%)	99 (8.3%)	**0.04**
Baseline therapy				
Long term oral antibiotics	412 (26.7)	113 (31.8%)	299 (25.2%)	**0.01**
Long term inhaled antibiotics	93 (6.0)	20 (5.6%)	73 (6.1%)	0.7

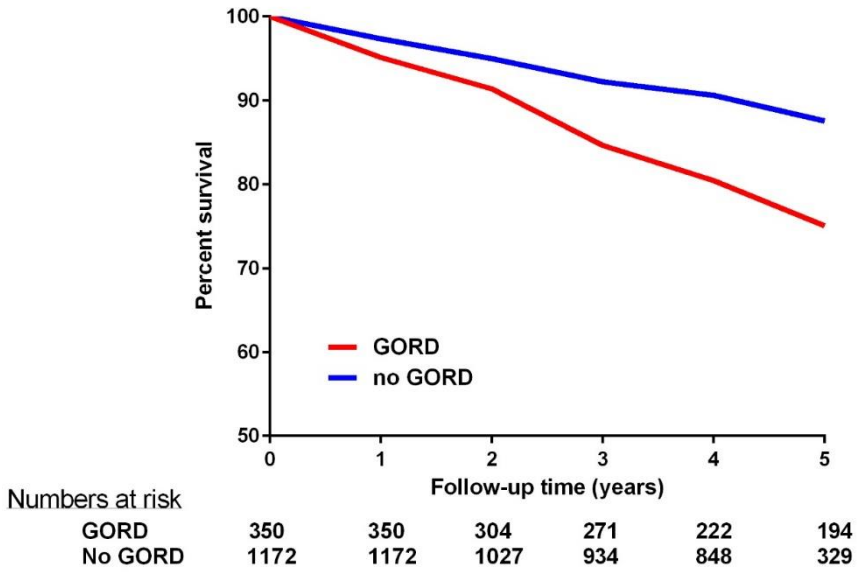

Figure 7-1 Kaplan-Meier survival curve and univariate Cox proportional hazard regression analysis for mortality in bronchiectasis with and without GORD

7.3.2 Factors associated with GORD

Factors unconditionally associated with GORD for all patients are shown in Table 7-2. Self-reported GORD was significantly more likely to be reported in older patients (OR 1.01, 95% CI 1.00-1.02; p=0.04) with a higher number of baseline exacerbations (OR 1.96, 95% CI 1.32-2.91; p=0.001). Sex, BMI and smoking status were not statistically significant between groups. Associations with certain underlying aetiologies were noted with GORD less likely to occur in patients with idiopathic bronchiectasis (OR 0.73, 95% CI 0.55-0.98; p=0.04) and significantly more likely to occur in patients with asthma (OR 1.86, 95% CI 1.21-2.86; p=0.004) or connective tissue disease (OR 2.15, 95% CI 1.35-3.43; p=0.001). Similarly, GORD was almost twice as likely to occur in patients with chronic renal failure as a comorbidity (OR 1.73, 95% CI 1.06-2.83; p=0.03) and significantly less likely to be found in patients with baseline microbiological growth of *Haemophilus influenzae* (OR 0.64, 95% CI 0.46-0.91; p=0.012), *Moraxella Catarrhalis* (OR 0.19, 95% CI 0.07-0.54; p=0.002) and *Enterococci sp.* (OR 0.51, 95% CI 0.30-0.89; p=0.016), (Table 7-2). No associations between GORD and bacterial growth of *Pseudomonas aeruginosa* or *Staphylococcus aureus* were noted.

Table 7-2 Factors associated with self-reported GORD in a bronchiectasis population: univariate analyses

Variable	Odds ratio for GORD	95% confidence interval	p-value
Demographic variables			
Age	1.01	1.00 – 1.02	**0.04**
Gender (male vs female)	0.94	0.72 – 1.24	0.67
MRCD score	1.06	0.93 – 1.20	0.39
Aetiologies			
Idiopathic	0.73	0.55 – 0.98	**0.04**
Post-infectious	0.78	0.56 – 1.08	0.13
ABPA	0.53	0.27 – 1.07	0.08
Asthma	1.86	1.21 – 2.87	**0.004**
COPD	1.23	0.87 – 1.74	0.25
CTD	2.15	1.35 – 3.43	**0.001**
IBD	0.98	0.42 – 2.29	0.95
Functional status			
FEV1% predicted			0.22
FEV1 >80%	1.05	0.55 – 2.00	0.89
FEV1 50-79%	1.02	0.56 – 1.90	0.95
FEV1 30-49%	0.68	0.36 – 1.30	0.25
FEV1 < 30%			
Radiiological status			
Radiology score	1.00	0.96 – 1.05	0.97
BMI <18.5	1.45	0.89 – 2.37	0.14
Smoking status	1.20	0.91 – 1.58	0.20
Exacerbations in the previous year			**0.005**
≤ 1 / year	1.45	0.96 – 2.24	0.007
2 / year	1.88	1.27 – 2.78	**0.002**
≥ 3 / year	1.29	0.82 – 2.02	0.28
4 or more	1.96	1.32 – 2.91	**0.001**
Bacteriology chronic infection			
H. influenzae	0.64	0.46 – 0.91	**0.012**
S. aureus	0.67	0.38 – 1.17	0.161
M. catarrhalis	0.19	0.07 – 0.54	**0.002**
Enterobacteriaceae	0.51	0.29 – 0.88	**0.016**
P. aeruginosa	1.33	0.84 – 2.10	0.22
Comorbidities			
IHD	1.01	0.73 – 1.42	0.91
CVA	0.96	0.54 – 1.72	0.90
Diabetes	1.25	0.82 – 1.91	0.31
Chronic renal failure	1.73	1.06 – 2.83	**0.029**
Dementia	1.40	0.49 – 4.03	**0.006**
Solid tumour	1.21	0.76 – 1.92	0.42

Variable	Odds ratio for GORD	95% confidence interval	p-value
Haematological malignancy	0.63	0.23 – 1.72	0.36
Baseline therapy			
Oral long-term antibiotic therapy	1.34	0.99-1.81	**0.06**
Nebulised long term antibiotic therapy	0.97	0.47-1.62	0.66

7.3.3 GORD and mortality analysis

There were 182 (12%) deaths during the 5-year follow-up period; 109 (31%) in the GORD-positive group and 73 (6%) in the GORD-negative group; p<0.0001.

A univariate Cox proportional regression analysis of the impact of GORD on mortality showed a higher mortality in the GORD-positive group versus the GORD-negative patients (HR 2.45, 95% CI 1.73-3.46; p<0.0001), (Figure 7-1). Adjusting for BSI, sex, aetiologies, comorbidities and treatment, this risk remained significant (HR 1.60, 95% CI 1.17-2.19; p=0.003) (Table 7-3). Other variables contributing to an increased mortality were age (HR 1.05; 95% CI 1.05-1.08; p<0.001), sex (HR 1.24; 95% CI 1.03-1.76; p=0.03), MRCD score (HR 1.30; 95% CI 1.07-1.36; p=0.002), lower FEV1% predicted, exacerbation frequency (HR 1.09; 95% CI 1.03-1.15; p=0.03), and comorbidities (solid tumour and haematological malignancy: HR 1.99, 95% CI 1.27-3.10; p=0.002 and HR 2.94, 95% CI 1.44-5.98, p=0.003, respectively).

Table 7-3 Multivariate Cox regression analysis of factors associated with survival in bronchiectasis

Variable	Hazard ratio for death	95% confidence interval	p value
GORD	1.60	1.17 – 2.19	**0.003**
Age	1.05	1.05 - 1.08	**<0.001**
Sex	1.24	1.03 – 1.76	**0.03**
MRCD score	1.30	1.07 – 1.36	**0.002**
FEV1 % predicted			**<0.001**
FEV1 >80%	0.32	0.18 – 0.50	**<0.001**
FEV1 50-80%	0.38	0.22 – 0.65	**<0.001**
FEV1 30-49%	0.76	0.42 – 1.67	0.17
Radiology score	1.01	0.96 – 1.06	0.74
BMI <18.5	1.34	0.80 – 2.30	0.26
Smoking status	1.20	0.87 – 1.62	0.28
Exacerbations in the previous year	1.09	1.03 – 1.15	**0.003**
H. influenzae chronic infection	0.55	0.34 – 0.89	**0.016**
P. aeruginosa chronic infection	1.15	0.73 – 1.81	0.55
IHD	1.25	0.87 – 1.79	0.22
CVA	1.44	0.87 – 2.37	0.16
Diabetes	1.27	0.85 – 1.90	0.24
Chronic renal failure	1.39	0.81 – 2.16	0.15
Dementia	1.73	0.83 – 3.58	0.14
Solid tumour	1.99	1.27 – 3.10	**0.002**
Haematological malignancy	2.94	1.44 – 5.98	**0.003**
Oral long term antibiotic therapy	0.94	0.68 – 1.31	0.71
Nebulised long term antibiotic therapy	0.68	0.37 – 1.27	0.23

We hypothesised that mortality associated with GORD may be exacerbation-dependent, therefore a separate analysis of mortality was made dividing the study population into 4 categories: 1) GORD-negative patients with less than 2 exacerbations per year; 2) GORD-negative patients with 2 or more exacerbations per year; 3) GORD-positive patients with less than 2 exacerbations per year; and 4) GORD-positive patients with 2 or more

exacerbations per year. The number of patients and deaths per group are described in Table 7-4. Survival curves per group are presented in Figure 7-2.

Table 7-4 Study population (number of patients and deaths per group) stratified according to presence of GORD and number of exacerbations

Total, n (%)	**1, 543 (100%)**	**Deaths per group, n (%)**
GORD-negative patients with less than 2 exacerbations per year, n (%)	520 (33.7)	16 (3.1)
GORD-negative patients with 2 or more exacerbations per year, n (%)	668 (43.3)	57 (8.5)
GORD-positive patients with less than 2 exacerbations per year, n (%)	111 (7.2)	30 (27.0)
GORD-positive patients with 2 or more exacerbations per year, n (%)	244 (15.8)	79 (32.4)

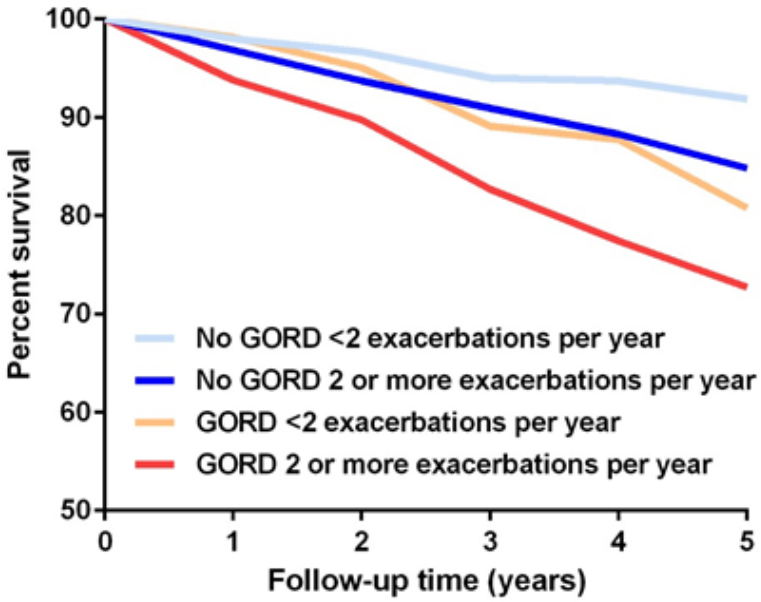

Figure 7-2 Kaplan-Meier log-rank test survival curve showing comparison between bronchiectasis patients with and without GORD according to their exacerbation frequency

Bronchiectasis patients were divided into 4 groups: 1) GORD-negative patients with less than 2 exacerbations per year; 2) GORD-negative patients with 2 or more exacerbations per year; 3) GORD-positive patients with less than 2 exacerbations per year; 4) GORD-positive patients with 2 or more exacerbations per year.

A multivariate analysis was performed using the same variables (Table 7-5). Having GORD with less than 2 exacerbations per year had no significant impact in terms of mortality (HR 1.49, 95% CI 0.80-2.76; p=0.21). However, patients with 2 or more exacerbations per year had a significantly increased mortality (HR 1.63: 95% CI 1.05-2.55; p=0.031), an effect that was magnified in the presence of GORD (HR 2.60, 95% CI 1.63-4.14; p<0.001).

Table 7-5 Multivariate Cox regression analysis of factors associated with survival after stratification by presence of GORD and number of exacerbations

Variable	Hazard ratio for death	95% confidence interval	p value
Groups			
GORD-negative patients with <2 exacerbations per year	1.00	reference	n/a
GORD-negative patients with ≥2 exacerbations per year	1.63	1.05 – 2.55	**0.031**
GORD-positive patients with <2 exacerbations per year	1.49	0.80 – 2.76	0.21
GORD-positive patients with ≥2 exacerbations per year	2.60	1.63 – 4.14	**<0.001**
Age	1.05	1.02 – 1.06	**<0.001**
Gender (male vs female)	1.31	0.96 – 1.79	0.09
MRCD score	1.32	1.15 – 1.52	**<0.001**
FEV1% predicted			
FEV1 >80%	0.30	0.17 – 0.55	**<0.001**
FEV1 50-80%	0.35	0.21 – 0.59	**<0.001**
FEV1 30-49%	0.67	0.40 – 1.11	0.12
Radiology score	1.01	0.96 – 1.05	0.76
BMI <18.5	1.34	0.80 – 2.29	0.25
H. influenzae chronic infection	0.56	0.35 – 0.91	**0.018**
P. aeruginosa chronic infection	1.17	0.74 – 1.85	0.51
IHD	1.31	0.92 – 1.87	0.13
CVA	1.40	0.86 – 2.30	0.18
Diabetes	1.21	0.82 – 1.80	0.34

Variable	Hazard ratio for death	95% confidence interval	p value
Chronic renal failure	1.57	1.02 – 2.43	**0.043**
Dementia			
Solid tumour	1.91	1.23 – 2.97	**0.043**
Haematological malignancy	2.82	1.38 – 5.76	**0.004**
Oral long-term antibiotic suppressive therapy	0.97	0.70 – 1.34	0.86
Nebulised long term antibiotic suppressive therapy	0.76	0.42 – 1.37	0.35

7.3.4 Risk of exacerbations and hospital admissions with GORD

Patients with GORD had a much higher probability of exacerbations in both univariate analysis (IRR 1.35 (95% CI 1.16-1.56; p<0.0001) and in the fully adjusted model incorporating BSI, sex, aetiologies, comorbidities and treatment (IRR 1.21, 95% CI 1.04-1.21; p=0.015). GORD was not associated with increased hospitalisations on univariate (IRR 0.92, 95% CI 0.70-1.22; p=0.58) or multivariate (IRR 0.78, 95% CI 0.57-1.07; p=0.20) analysis.

7.4 Discussion

In this large cohort of bronchiectasis patients, we found that a self-reported diagnosis of GORD at baseline was associated with an almost 2-fold increase in all-cause mortality and a 20-40% increased risk of exacerbations during a 5-year follow-up period. However, self-reported history of GORD was not associated with severe exacerbations requiring hospitalisation.

There is ample epidemiological evidence demonstrating an increased prevalence of GORD as a frequent comorbidity in numerous chronic airway and parenchymal respiratory conditions, but any causal relationship between GORD and chronic respiratory disease remains controversial and unclear. Given the somewhat unexpected reverse association with idiopathic disease in this study, it is likely that GORD contributes to bronchiectasis disease severity at a synergistic level rather than being significant as a single aetiological disease entity. Defining GORD as an aetiology is extremely

difficult as current definitions do not take into account the effects of airway reflux or microaspiration and therefore likely underestimate the impact associated with co-presence of these conditions. Microaspiration of gastric refluxate into the lungs causing direct intra-pulmonary epithelial injury is the current prevailing hypothesis, potentially triggering an inflammatory response in excess of the normal state in genetically predisposed individuals contributing to underlying respiratory disease progression.[28, 273] What is inflammatory to one individual's respiratory tract may not be to another. Evidence of this variation is demonstrated in daily clinical practice with, for instance, susceptibility of smokers to developing COPD or patients developing occupational asthma. The magnitude of the reflux volume (proximal *vs.* distal), its frequency, and the composition and constituents of the refluxate (pH, liquid *vs*. gaseous, gastric (pepsin) or duodenal (bile acid) predominant, microbiology, food particles) is also contributory. The problem is made even more complex by the varying reflux presentation, poor correlation between GORD symptoms, endoscopic findings and pH studies, and the paucity of standardised patient-reported questionnaires that can aid clinical diagnosis.

GORD and bronchiectasis likely act in a bi-directional manner. It is generally assumed that the respiratory tract is a passive recipient of noxious refluxate, but bronchiectasis may also worsen or precipitate GORD through a number of mechanisms contributing to the vicious vortex of disease.[35] Patients with obstructive lung disease such as bronchiectasis are hyperinflated with descent of the diaphragm, thus lowering the resting pressure of the lower oesophageal sphincter and predisposing to reflux.[273] There may also be a greater trans-diaphragmatic pressure after eating when hyperinflation is evident as demonstrated by the early satiety of patients with severe COPD.[466] As airflow obstruction becomes more severe, a greater negative intra-thoracic pressure has to be created in order to inspire and this may also have a siphoning effect of gastric contents into the oesophagus.[29]

Other potential mechanisms of GORD in bronchiectasis include coughing which can involve sudden dynamic contracture of the abdominal muscles

with an associated spike in intra-abdominal pressure which could provoke reflux. It seems intuitive that patients with chronic cough are more likely to develop a hiatal hernia. Interestingly, an increased prevalence of HH with worse disease outcomes has been demonstrated in IPF, bronchiectasis and more recently, in asthma patients.[29, 467, 468] These conditions have the most end-organ damage of reflux-related diseases possibly due to greater refluxate associated with a hiatal hernia and cough being a very dominant symptom. Conversely, the presence of a hiatal hernia has also been reported to lead to bronchiectasis with a single centre study evaluating HRCT scans for hiatal hernias demonstrating a bronchiectasis rate of 12.3 (95% CI: 1.5-103) in large hiatal hernia groups compared to small and moderate groups.[465] Lengthening of the oesophagus in hyperinflation may also create a traction force on the stomach leading to development of a hiatal hernia. Inhaled anti-cholinergic agents may have a negative effect on oesophageal motility and gastric emptying and may contribute to tissue laxity and subsequent development of a hiatal hernia.[316] Swallowing dysfunction may result from a range of pathologies, including neurological impairment, vocal cord injury, surgery, and radiation and is often overlooked as an aetiological cause of bronchiectasis.[28, 242] Altered autonomic tone of the lower oesophageal sphincter as a consequence of medication such as salbutamol or theophylline or use of gastro-irritant medication such as steroids and/or non-steroidal anti-inflammatories may be attributable to the increased association of GORD in asthma and connective tissue diseases.[316, 469, 470]

Current evidence lends weight to the concept of GORD as a potential driver of exacerbations and disease progression in genetically susceptible individuals. The increased risk of exacerbations in bronchiectasis patients with GORD has been reported in a several studies to date.[291, 292, 471] Little is known about this association but it is thought that the relationship may be reciprocal with cough and hyperinflation during exacerbations resulting in more reflux, and microaspiration of refluxate resulting in further exacerbations. A hypothesis potentially explaining the risk of increased exacerbations in patients taking acid suppression therapy would be that non-

acidic reflux is important in bronchiectasis, and that acid suppression medication use by reducing acid-associated symptoms may reduce patients' presentation.[472] Non-acidic reflux may consequently be more likely to result in microaspiration as protective cough reflexes are not stimulated. This is important given the impact of exacerbations in bronchiectasis and the urgent need for novel interventions to reduce the frequency and severity of exacerbations and subsequent disease progression.[473]

Large-scale epidemiological studies of GORD in the general population have demonstrated conflicting results in relation to mortality.[474-477] Malignant complications of GORD are well recognised in terms of oesophageal cancer, head and neck cancers and lung cancer, which are all associated with an increased mortality.[478-480] However, recent studies suggest that individuals with GORD do not have an increased all-cause or overall cancer-specific mortality, except for males with severe GORD who have an increased oesophageal adenocarcinoma-specific mortality.[476] The increased mortality risk of GORD and bronchiectasis is not demonstrated in asthma, COPD or cystic fibrosis, but treatment of GORD in idiopathic pulmonary fibrosis and COPD has been associated with a survival benefit, supporting the hypothesis that treating GORD may influence chronic respiratory disease behaviour.[481, 482] Evidence of efficacy of GORD treatment in bronchiectasis is yet to be made. A retrospective study of 257 bronchiectasis patients with GORD comparing 27 patients treated with long-term PPIs compared to 230 without PPI treatment showed no significant differences in lung function after 6 months. A further retrospective review of the clinical outcomes of seven patients with GORD-related deteriorated bronchiectasis showed that active anti-reflux treatment with Stretta radiofrequency (SRF) and/or laparoscopic fundoplication was beneficial to patient symptoms and outcome.[308] Surgical management, with Nissen Fundoplication, has been successfully applied to bronchiectasis patients awaiting transplantation, with reductions in symptoms of GORD as well as of lung disease.[309, 310] Given the increased mortality and exacerbation frequency associated with GORD and bronchiectasis, adequately powered RCTs of anti-reflux therapy (medical and/or surgical) in this patient population are needed.

Acid suppression therapy only targets acid production and, therefore, non-acid reflux will persist in PPI- treated patients. Given the known association with GORD and increased exacerbations in bronchiectasis and chronic respiratory diseases, more interest in the mechanistic effect of macrolides and their potential action on gastric motility should be considered.[198, 414] Azithromycin has been shown to be a potent agonist of the hormone motilin which improves gastrointestinal motility, reducing reflux and aspiration.[281] Treatment with azithromycin has been shown to abolish or reduce the acid pocket, reduce hiatal hernia size reducing acid oesophageal acid exposure and the number of acid reflux episodes in patients with GORD.[321] Azithromycin's beneficial effect in bronchiectasis and other lung conditions may therefore be due to a reduction in the deleterious effects of unrecognised reflux and aspiration.

Population studies of both bronchiectasis and GORD as individual conditions have been associated with an increased risk of oesophageal and lung cancer with the combination of both leading to a "double hit" potentially increasing the likelihood of developing these complications.[244, 478, 480, 483] It is important to consider that there may be a synergistic effect of multiplicative conditions contributing to systemic inflammation and increased mortality; for instance, a patient with bronchiectasis secondary to RA who has co-existing asthma may be more likely to have GORD than a patient with idiopathic bronchiectasis and no other comorbidities, and will therefore likely have an increased risk of mortality due to the combined effects of asthma, RA and GORD which have all previously been associated with an increased mortality in bronchiectasis.[23] To our knowledge, no previous studies have identified any association between GORD and chronic renal failure, the mechanism of which remains unclear but is likely to support the systemic inflammation hypothesis due to repeated infection, inflammation and chronic immune activation.

Distinct microbiological differences observed in this study showed a lower prevalence of infection with *H. influenzae,* and a trend towards increased *P. aeruginosa* colonisation. Bile increases biofilm formation and quorum

sensing in *P. aeruginosa*, driving the switch from acute to persistent infection, suggesting that GORD-derived bile could be a host determinant contributing to chronic respiratory infection.[328] The potential for horizontal transmission of microorganisms between the gut-lung axis may indicate that the upper gastrointestinal tract could act as a potential reservoir of microorganisms.[484] An increased reflux burden has been found to be associated with *P. aeruginosa* in CF.[326] More recently, similar bacterial profiles of CF sputum and gastric juice samples were demonstrated, distinct from non-CF gastric juice, perhaps providing novel evidence of an aerodigestive microbiome in CF.[327] However, it is difficult to establish whether cross-infection relates to swallowing of sputum with seeding of the gastrointestinal microbiome or if reflux and aspiration into the lungs may be causative.

Our study has several limitations; firstly, our ascertainment of GORD via self-report may be limited by recall or reporter bias and there is the potential for missed or as yet, undiagnosed comorbid GORD. Secondly, there is no objective assessment for GORD, as we are often reliant on self-report physician diagnosis in the clinical setting, often only resorting to the gold-standard 24h pH-impedance studies in refractory cases due to cost constraints and lack of adequate resources. To date, few studies have used validated standardised questionnaires to determine the history of GORD making comparisons of findings between studies difficult. The gold standard for the diagnosis of GORD is 24-hour oesophageal pH-impedance monitoring, which has not yet been published in a prospective bronchiectasis population. Further studies are needed to clarify the significance of acid suppression medication use and bronchiectasis exacerbations in both the presence and absence of GORD.

7.5 Conclusion

In summary, a self-reported history of GORD is a prevalent comorbidity in bronchiectasis and is associated with an increased risk of all-cause mortality and an increased exacerbation frequency. Greater focus is needed to identify,

assess and manage GORD in bronchiectasis in both clinical and research settings to maximise outcome and reduce the illness burden associated with this disease. Adequately powered RCT evidence that treatment targeted at GORD can improve outcomes in bronchiectasis is needed.

Chapter 8 – Macrolides Attenuate Markers of Gastro-Oesophageal Reflux-Associated Airway Inflammation, Remodelling and Disease Severity in Bronchiectasis

Publications relevant to this chapter:

McDonnell MJ, O'Toole D, Das J, Aldhahrani A, Verdon B, Pearson JP, Lordan JL, De Soyza A, Bruzzi J, Huang J, Soussi N, Gominne P, Aliberti S, Polverino E, Ringhausen F, Loebinger MR, Chalmers JD, Laffey JG, Rutherford RM, Ward C. Macrolides Attenuate Gastro-Oesophageal Reflux-Associated Airway Inflammation, Remodelling and Disease Severity in Bronchiectasis. *In submission* 2020.

8.1 Introduction

Bronchiectasis is a chronic inflammatory lung disease characterised by airways inflammation, ciliary dysfunction and chronic infection, leading to permanent tissue destruction and airway remodelling in a vicious vortex of progressive insults and injury.[35] Bronchiectasis is important globally due to its increasing prevalence, substantial economic burden on health care, and associated morbidity.[4] It is extremely heterogeneous in terms of its aetiology, comorbidities, inflammatory profile, functional impairment, chronic infection status, and geographical variation, making it difficult to target treatment that will modify disease progression.[1, 23, 60, 124] Very few studies have assessed host defence dysfunction or the role of the bronchial epithelium in bronchiectasis. The bronchial epithelium forms the first line of defence to injurious external stimuli and regulates the immune functions that bridge both innate and adaptive immunity.[485, 486] Structural and functional changes in the bronchial epithelium can significantly alter the airway milieu, host defences and repair processes in bronchiectasis, driving disease severity and progression.[487]

Gastro-oesophageal reflux disease (GORD) is a common comorbidity in bronchiectasis with a reported prevalence of 34-74% using symptom questionnaires, 11-75% using oesophageal pH-monitoring and 26-70% using pepsin as a surrogate marker of pulmonary microaspiration.[28] Co-existing GORD has been associated with worse disease severity, increased exacerbation frequency, reduced quality of life (QoL) and a doubling of 5-year mortality in bronchiectasis patients.[28, 269] In recent years, the concept of airways microaspiration associated with reflux has become widely established, with 24h pH-impedance - which quantifies the type, number, phase, duration and proximal extent of each reflux episode - the current gold standard investigation of choice for diagnosing GORD.[255, 261, 262] Airway reflux may be entirely asymptomatic, with gaseous or mixed refluxate just as pathogenic to end-organs as liquid acid reflux-derived injury.[255, 259, 263-266] It is also increasingly recognised that reflux may be from the duodenum and contain pro-inflammatory bile acids which have not only been

associated with frank epithelial, pre-malignant injury in Barrett's oesophagus but are also associated with reduced microbial lung biodiversity and the establishment of chronic pathogen infections via the emergence of adaptive signalling variants in *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[328, 329, 488-491] Bronchiectasis and GORD have both been linked with an increased incidence of lung and gastric epithelial malignancy which could, in part, be due to the effects of reflux-mediated epithelial cell damage.[23]

Reflux may affect the vicious vortex at a number of levels, in terms of immune dysregulation driving airway inflammation, infection and remodelling.[35] The aim of our study is to further explore the association of GORD and bronchiectasis at a clinical and cellular level by prospectively assessing the prevalence and potential mechanism and disease associations of GORD, airway reflux and duodeno-gastro-oesophageal microaspiration in a well-defined population of bronchiectasis patients, comparing findings to age, sex, ethnicity and BMI-matched chronic bronchitis patients and healthy control volunteers.

8.2 Methodology

8.2.1 Study design and participant recruitment

Between September 2015 and April 2017, consecutive patients aged ≥18 years with a confirmed new or known diagnosis of bronchiectasis were recruited from respiratory outpatient clinics in Galway University Hospitals (GUH), Ireland, and the Royal Brompton Hospital (RBH), UK, for enrolment into this prospective parallel bicentric observational case-control study. A diagnosis of bronchiectasis was based on confirmatory high-resolution computed tomographic (HRCT) changes confirmed by a pulmonary physician and thoracic radiologist in the presence of a compatible clinical history of bronchiectasis. Patients with cystic fibrosis, traction bronchiectasis due to pulmonary fibrosis, active allergic bronchopulmonary aspergillosis (ABPA) or active non-tuberculous mycobacterial (NTM) disease were excluded. Chronic bronchitis participants consisted of age, sex, ethnicity and

body mass index (BMI)-matched patients with a clinical history of recurrent infections but no overt radiological evidence of bronchiectasis following independent expert review. All bronchiectasis and chronic bronchitis patients had to be free from antibiotic treatment for exacerbation for a minimum of 4 weeks prior to enrolment and initial data collection. Healthy control volunteers consisted of age, sex, ethnicity and BMI-matched individuals with no known or existing lung condition, who were never or ex-smokers of ≥10 years since cessation with a non-significant <10 pack-year history, and no known or existing history or symptoms of reflux.

The study protocol was approved by local and international research ethics committees and performed according to the Declaration of Helsinki. All participants provided written informed consent prior to being enrolled in the study.

8.2.2 Pulmonary investigations

All patients underwent a comprehensive diagnostic bronchiectasis work-up according to the 2010 BTS guidelines as previously described.[9, 23] Structured questionnaires incorporating patient demographics, history of pulmonary and reflux symptoms, previous medical history, comorbidities, medications, as well as baseline radiological, physiological, laboratory and microbiological findings were completed. Bronchiectasis severity was calculated using the Bronchiectasis Severity Index (BSI).[19] Quality of life was measured by the St. George's Respiratory Questionnaire (SGRQ) and the disease-specific Quality of life-bronchiectasis (QoL-B) questionnaire.[76, 78]

HRCT images were acquired on a 64-slice multi-detector CT scanner (Somatom Sensation Cardiac 64, Siemens, Erlangen, Germany) using standardised protocols as previously described. All scans were reported by radiologists with expertise in HRCT imaging at the time of scanning. Blind independent review for confirmation and scoring of disease severity according to the modified Reiff and modified Bhalla scores, both of which have been validated for use in bronchiectasis, were performed by a pulmonary

physician and a thoracic radiologist.[66] The extent of bronchiectasis, severity of bronchial dilatation, bronchial wall thickness, presence of mucus plugging in large and small airways, and decrease in parenchymal attenuation were scored for each lobe, with the lingula considered a separate lobe. Total lung scores for each abnormality were defined as the mean score from all lobes for each HRCT feature. The proportion of cystic versus varicose or cylindrical bronchiectasis was also recorded along with the total number of lobes involved. Lobar predominance was assessed by calculating the mean scores for all HRCT features per lobe. A combined HRCT total score for all HRCT features across all lobes was subsequently derived from summing the individual scores.

All patients underwent spirometry using a Sensormedics V-Max 22 device. Values were expressed as a percentage predicted for age, sex, height and ethnicity employing European Respiratory Society (ERS)/American Thoracic Society (ATS) reference ranges.[361, 362] Bronchoscopy was performed in accordance with established BTS bronchoscopy guidelines.[363] Patients were asked to discontinue any medication that may influence oesophageal motility (i.e. nitrates, calcium antagonists, domperidone, benzodiazepines and metoclopramide) and acid suppressive therapy for a minimum of 7-14 days prior to bronchoscopic and oesophageal investigations. Bronchoalveolar lavage (BAL) was obtained from the right middle lobe or lingula or most affected lobe as a standardized 3 x 60 ml procedure. BAL fluid was recovered by gentle manual suction, and divided to enable microbiological assessment, measurement of pro-inflammatory cytokines and chemokines, measurement of pepsin and bile acids, and proteomic profiling. Microbiological examinations performed on all BAL and spontaneous early-morning sputum cultures were performed for every patient during stable state. Identification of microorganisms and susceptibility testing were performed according to standard methods previously described.[51] All microbiology samples were processed in Clinical Pathology Accredited (CPA) laboratories to routine diagnostic standards using standard and select supplementary media, in accordance with the BTS guidelines on microbiological profiling in bronchiectasis. Sensitivity testing was carried out using the agar disc

diffusion method according to methods of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Isolates were tested against multiple anti-microbial agents including amikacin, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem, piperacillin-tazobactam, ticarcillin-clavulanic acid and tobramycin. Chronic infection was defined by the isolation of potentially pathogenic bacteria in sputum culture on ≥ 2 occasions, at least 3 months apart during a 1-year period, with the patient in stable state.[50, 51]

Bronchial brushings (n=2) were obtained from subsegmental bronchi using a protected specimen single-sheathed nylon cytology brush (5 fr; Wilson-Cook, Winston-Salem, NC, USA) and the brush dispersed in 5 mL of Roswell Park Memorial Institute (RPMI 1640, Sigma, UK) with 100UI/mL penicillin, 100μg/mL streptomycin (Sigma, UK) and 50μg/mL amphotericin B (Lonza, USA) based on methods previously described.[403, 405] All samples were processed within 2-4 h of collection and freeze-thaw cycles were avoided.

8.2.3 Oesophageal investigations

Validated reflux questionnaires were selected following a formal review of the literature. This led us to a prospective decision to adopt the Gastroesophageal Reflux Disease Questionnaire (GERD-Q) and the Reflux Symptom Index (RSI) to assess typical and extra-oesophageal symptoms of reflux respectively, both scores having previously been validated against gold-standard pH-impedance measurements. These were considered most attractive due to their brevity, short recall period, rigorous developmental methodology, multi-lingual validation, and consistent reliability and responsiveness with a maximum score of 18 for the GERD-Q, ≥ 8 indicative of gastro-oesophageal reflux disease, and a maximum score of 45 for the RSI, ≥ 13 indicative of extra-oesophageal reflux. The CReSS score, a composite tool derived from a combination and item reduction analysis of the GORD symptom assessment scale (GSAS) score for assessing typical GORD symptoms and the RSI for extra-oesophageal symptoms, was also employed

for validation in a respiratory cohort, with a total score ranging from 0-170, higher scores indicating a greater symptom burden.[377]

Manometry testing

High resolution oesophageal manometry data was obtained using a 4.2 mm outer diameter solid-state manometry catheter with 36 circumferential sensors spaced at 1 cm intervals (Manoscan 360; Sierra Scientific Instruments Inc, Los Angeles, CA, USA). Studies were performed after 6-8 hours fasting. The catheter was calibrated and zeroed to atmospheric pressure. It was inserted intranasally and positioned appropriately to record the pressure from the hypopharynx to the stomach. After a 5-minute period of acclimatisation and recording basal pressure for 30 seconds without swallowing, subjects underwent 10 water swallows (5 mL) followed by multiple water swallows in the supine position. They were instructed to swallow only once until the next bolus. Each swallow was allowed a 20 to 30 seconds interval.

Manometric measurements included lower oesophageal sphincter (LOS) location and length, presence and size of a hiatal hernia, LOS pressure and relaxation, contraction amplitude and duration, and coordination and propagation of velocity after swallows. Manometry was reported according to international criteria determined by the Chicago Classification v3.0.[381]

pH-impedance monitoring

All studies were performed off proton pump inhibitor treatment for a minimum of 7-14 days. Data were downloaded and analysed using dedicated software and subsequently reviewed manually with external validation of a subset by an experienced investigator blinded to the basal condition of the overall patients and healthy volunteers. GORD episodes were classified as acidic (pH<4), weakly acidic (pH 4–7) or non-acidic (pH>7) following established criteria.

DeMeester score

Number and type of reflux episodes, acid exposure (reflux time (min) and reflux percentage time) and proximal extent (reflux reaching 15 cm above the

LOS) were calculated. Total distal oesophageal acid exposure < 4.2% over 24 hours was considered normal and total number of reflux episodes < 40 was considered normal.

Pepsin analysis in BAL or sputum

BAL and sputum samples were analysed separately for the presence of pepsin in triplicate using a locally developed indirect ELISA as previously described. Negative controls for standards and sample dilutions were performed with the omission of primary antibody. The lower limit of detection of the assay was <1 ng/mL. Briefly, 100 μL of pepsin standard (pepsin from porcine gastric mucosa, Sigma) and undiluted BAL supernatants were allowed to adsorb onto a 96-well plate (Nunc MaxiSorp) in triplicate. Non-specific binding sites were blocked with 1% ELISA grade bovine serum albumin (Merck Millipore) in PBS (pH 7.4). The primary antibody was specific to porcine pepsin (Biodesign International Cat no W59117G). Secondary antibody was horse radish peroxidase-conjugated rabbit, anti-goat (Sigma). Antibodies were diluted in Tris-buffered saline (20 mM Triza base, 150 mM NaCl, pH 7.4) containing 0.1% BSA and 0.05% Tween 20. The substrate used was 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and the reaction was stopped with an equal volume of 1% SDS.

8.2.4 BAL inflammatory markers

All laboratory work was carried out under strict sterile conditions in the laminar flow hood. Differential cell counts were performed on Kwik Diff™ stain (Thermo Scientific, USA) cyto-centrifuge preparations. Cell-free BAL supernatants were prepared by centrifugation (5 min, 1000g, 21°C); aliquots were stored at -80°C. BAL cytokines and chemokines were subsequently measured on a LuminexTM system (Bio-Plex 200) using a Bio-Rad human cytokine kit (Bio-Plex cytokine assay) following the manufacturer's instructions for (IL-6, CXCL-8, IL-10, IL-17, TNF-α, IFN-γ, VEGF and GM-CSF) with individual ELISAs (R&D duosets) for MMP-9 and TGF-β. Data were collected with a minimum of 100 beads per analyte using Bio-Plex Manager Software (Bio-Rad Laboratories, Inc, Hercules, CA).

To identify potential biomarkers differentiating bronchiectasis patients with and without reflux compared to chronic bronchitis patients and healthy controls, BAL protein profiling was performed using nano-flow LC-MS/MS (liquid chromatography coupled to high-resolution tandem mass spectrometry) as previously described.[408]. Protein identification and label-free quantification were carried out using Maxquant (v1.4.1.2) against Uniprot-human database (v2014-07-09) with Andromeda software. False discovery rate for protein identification was set to 1% protein level utilising the Benjamini-Hochberg method and data visualization carried out using SIMCA P (v13.0.3).

8.2.5 Primary bronchial epithelial cell cultures

Bronchial epithelial cells were harvested from bronchoscopic bronchial brushings of study participants as described above. Epithelial cells were isolated and grown in submerged culture as previously described.[403, 405] Briefly, after bronchoscopy, suspended brushing samples were centrifuged and the ensuing cell pellet dissolved in basal epithelial growth medium (BEBM [Lonza, USA] supplemented with BEGM singlequots (Lonza, USA), 100UI/mL penicillin, 100µg/mL streptomycin (Sigma-Aldrich, UK) and 50µg/mL amphotericin B (Lonza, USA). A 100 mL aliquot was taken for cell count and differential and the remaining cell suspension was transferred to a T25 cm^2 flask pre-coated with collagen (Vitrogen 100; Cohesion Technologies, Palo Alto, CA, USA) and placed in a CO_2-enriched incubator (37°C/5% CO_2). BEGM was replaced every 48 hours until PBECs reached 80-95% confluence. Once confluent, PBECs were passaged using trypsin/ethylene diamine tetra-acetic (EDTA) (Sigma-Aldrich, UK) which was neutralised using an equal volume of RPMI supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich, UK). PBECs were then transferred in culture medium to T75 cm^2 collagen-coated tissue culture flasks (Thermo-Fisher Scientific, UK) for further expansion; to 48 or 96-well plates for stimulation experiments in submerged culture (Corning, Schipol, Netherlands); to eight chamber slides (Lab-Tek, Nunc, Naperville, IL, USA) for immunohistochemical analysis staining for the epithelial marker

cytokeratin, using monoclonal mouse anti-human cytokeratin antibodies; or reserved for cryopreservation.

8.2.6 Bile acid-azithromycin experiments

Bile acids were dissolved in methanol to prepare stock solutions (100µmol/L) and the solution diluted with resting medium to achieve a range of physiological experimental concentrations based on preliminary data in immortalised cell lines.[246] Cells were exposed to physiologically relevant concentrations of individual and combined primary (cholic and chenodeoxycholic) and secondary (deoxycholic and lithocholic) bile acids, with and without a sub-microbicidal concentration of azithromycin (Pfizer, UK) for 48 hours, as determined by previous PBEC work on lung allografts in our group.[492] Cell viability was assessed using the MTT and methylene blue assay. Supernatant levels of factors critical to driving airway neutrophilia, epithelial to mesenchymal transition (EMT) and remodelling were analysed using the Luminex platform and individual commercial ELISAs as described above.

8.2.7 EMBARC cohort

To validate our findings, we analysed epidemiological data from the EMBARC (European Multicentre Bronchiectasis Audit and Research Collaboration) bronchiectasis patient registry to determine the relationship of GORD with exacerbations, hospitalisations and mortality in bronchiectasis, and to consider the potential role of macrolides, which are known to have pro-kinetic and anti-inflammatory effects, in alleviating poorer bronchiectasis outcomes associated with GORD.[14]

8.2.8 Statistical analysis

Distribution of continuous data was assessed using the Shapiro-Wilk test. Normally and non-normally distributed data are expressed as mean ± standard deviation (SD) or median ± interquartile range (IQR) and 95^{th} percentile, respectively. Comparisons between the different patient groups were performed for normally distributed measures using ANOVA (with Bonferroni's post hoc adjustment) or Welch's robust test (with Tamhane's

post hoc adjustment) according to the homogeneity of their variances, which was tested with Levene's statistic. Non-parametric Kruskal-Wallis test (with Dunn's post hoc adjustment) was used for data with non-Gaussian distribution. Differences in proportions were compared using the Chi-squared or Fisher's exact test. Bivariate correlations between bronchiectasis severity and reflux parameters were identified with Pearson's and Spearman's rank tests, for normally and non-normally distributed data, respectively. The inter-rater variability in grading bronchiectasis on HRCT and grading pH-impedance studies was assessed using Cohen's kappa statistics with linear weighting. As reflux parameters were not normally distributed, results are reported as median and interquartile range (IQR) and 95th percentile and differences between groups were assessed using Kruskal–Wallis (BE vs CB vs HC) or Mann–Whitney tests (BE R vs BE NR) as appropriate. For proteomics analysis, data was $\log^2$ transformed before subjecting to unpaired t-test using Perseus (v1.5.4.1). Principal component analysis (with the dataset log transformed, mean-centred and unit variance scaled) was performed with missing values replaced from normal distribution. Fully adjusted multivariable Cox proportional hazards regression and negative binomial regression analysis was used to determine hazard ratios (HR), incidence risk ratios (IRR) and 95% confidence intervals (CIs) for end-points of exacerbations, hospitalisations and treatment effects, by the presence of GORD in the EMBARC cohort. We defined statistical significance as a two-tailed $p<0.05$. All analyses were performed using SPSS version 21 (SPSS, Chicago, IL, USA) for Windows platform and Graph Pad Prism Version 5 (Graph Pad Software, Inc. San Diego, CA, USA).

8.3 Results

8.3.1 Patient characteristics

93 (47 GUH and 44 RBH) patients with a definitive diagnosis of bronchiectasis, 25 with chronic bronchitis and 13 healthy volunteers were consecutively enrolled into the study. Detailed demographic and clinical characteristics of all patients and healthy volunteers are shown in Table 8-1. There were no significant differences in terms of age, sex and BMI between

groups. All healthy volunteers had normal pulmonary (bronchoscopy and pulmonary function tests) and oesophageal (high resolution manometry and pH-impedance) investigations.

Table 8-1 Demographic and clinical characteristics of bronchiectasis patients, chronic bronchitis patients and healthy volunteer controls

Variables		**Bronchiectasis**	**Chronic bronchitis**	**Healthy controls**	**p-value**
Total n. (%)		93 (100)	25 (100)	13 (100)	n/a
Demographics and comorbidities					
Age, years	median (IQR)	65 (57-72)	62 (57-64)	65 (64-67)	0.41
Sex, female	n (%)	53 (57)	15 (60)	7 (54)	0.89
Body Mass Index	median (IQR)	26 (23-29)	27 (26-28)	25 (24-29)	0.22
Smoker/ex-smokers	n (%)	43 (46)	14 (56)	5 (38)	0.52
Daily anti-reflux medications	n (%)	53 (57)	15 (60)	N/A	0.82
Disease severity					
BSI score	median (IQR)	7 (5-10)	4 (2-4)	0 (0)	**<0.0001**
E-FACED score	median (IQR)	4 (2-5)	2 (1-3)	0 (0)	**<0.001**
BACI score	median (IQR)	3 (0-5)	1 (0-3)	0 (0)	**0.002**
CCI score	median (IQR)	1 (0-2)	1 (0-1)	0 (0)	**0.007**
Radiological status					
No. lobes	median (IQR)	3 (3-4)	0 (0)	0 (0)	**<0.0001**
Clinical status					
mMRC dyspnoea scale	median (IQR)	2 (2-3)	1.5 (1-2)	0 (0)	**<0.0001**
Exacerbations in the previous year	median (IQR)	3 (3-4)	2 (2-3)	0 (0)	**<0.0001**
At least one hospitalisation in the previous year	n. (%)	21 (23)	0 (0)	0 (0)	**<0.0001**
Functional Status					

FEV_1 (% predicted)	median (IQR)	75 (54-86)	96 (85-103)	107 (99-117)	**<0.0001**
FEV1 (absolute)	median IQR	1.8 (1.3-2.5)	2.4 (2.1-2.9)	3.4 (2.9-3.7)	**<0.001**
FVC (% predicted)	median (IQR)	102 (82-117)	113 (98-113)	119 (110-128)	**0.04**
FVC (absolute)	median (IQR)	2.9 (2.2-3.9)	3.5 (3.0-4.1)	4.5 (3.3-5.3)	**0.07**
Ratio (% predicted)	median (IQR)	62 (53-72)	69 (58-77)	75 (71-80)	**0.04**
DLCO (% predicted)	median (IQR)	69 (59-83)	72 (63-85)	83 (82-97)	**0.04**
Microbiology					
Chronic infection with *Pseudomonas*	n (%)	21 (23)	0 (0)	0 (0)	**<0.0001**
Chronic infection with other pathogens	n (%)	26 (28)	0 (0)	0 (0)	**<0.0001**
Quality of Life					
SGRQ total score	median (IQR)	46 (33-57)	29 (13-34)	0 (0-1)	**<0.0001**
SGRQ symptoms	median (IQR)	64 (53-78)	39 (22-56)	0 (0)	**<0.0001**
SGRQ impact	median (IQR)	48 (39-60)	33 (20-48)	0 (0)	**<0.0001**
SGRQ activity	median (IQR)	40 (23-51)	21 (9-24)	0 (0)	**<0.0001**
HADS total score	median (IQR)	7 (4-11)	5 (2-8)	3 (0-3)	**0.005**
HADS anxiety	median (IQR)	5 (3-7)	3 (1-5)	2 (0-3)	**0.014**
HADS depression	median (IQR)	2 (1-4)	2 (0-3)	0 (0-1)	**0.002**

8.3.2 Prevalence of GORD in bronchiectasis

A multimodal assessment approach was used to determine the presence of GORD in bronchiectasis. Prevalence using questionnaires ranged from 22-44% using typical (21/93) and atypical (41/93) symptom questionnaires respectively, 69% (44/73) using oesophageal pH-impedance monitoring and 91% (85/93) using detectable pepsin as a surrogate marker of pulmonary microaspiration. Detectable pepsin was significantly associated with a

positive RSI and DeMeester score on pH-impedance ($p<0.01$ and $p=0.004$, Mann-Whitney U–test using appropriate cut-offs; $r=0.46$, $p<0.0001$ and $r=0.29$, $p=0.022$ using Spearman's correlation coefficient). Prevalence using all methods was significantly higher in bronchiectasis patients compared with chronic bronchitis patients and healthy controls in a stepwise manner (Figure 8-1 (a) and (b)).

Oesophageal manometric evaluation

Only a subset (n=51/87 GUH, participation rate 59%) completed high resolution manometry studies (29 bronchiectasis, 13 chronic bronchitis and 9 healthy controls). Patients with bronchiectasis and chronic bronchitis differed from healthy volunteers in terms of lower mean LOS basal pressures ($p<0.001$), a higher prevalence of hiatal hernias ($p<0.01$), and an increased incidence of oesophageal dysmotility ($p=0.06$) (Table 8-2). Distal oesophageal spasm was identified in 4/29 (14%) bronchiectasis patients, achalasia in 2/29 (7%) and a hypercontractile oesophagus in 1/29 (4%). No differences in upper oesophageal sphincter pressures or other manometric parameters were noted.

Oesophageal 24-h pH-impedance monitoring

Of n=95/131 (overall participation rate 73%) who completed ambulatory pH-impedance studies, 44/73 (69%) bronchiectasis patients had an abnormal distal acid exposure, compared with 4 (31%) and 0 (0%) of chronic bronchitis and healthy volunteer patients respectively; $p<0.0001$. The DeMeester score was significantly higher in bronchiectasis patients compared with chronic bronchitis patients and healthy controls: 16.4 (5.7–37.4) *vs.* 10.5 (5.9–14.9) *vs.* 4.7 (4.0–9.2) respectively; $p=0.03$), (Figure 8-1 (c)). Percentage total, upright and recumbent acid exposure time (AET) with $pH<4$ was significantly higher in bronchiectasis patients compared with chronic bronchitis patients and healthy controls (percentage total AET 9.25 (4.7–15.4, 25.6) *vs.* 3.3 (1.4–7.4, 17.3) *vs.* 0.7 (0.2–4.1, 4.2), for bronchiectasis patients, chronic bronchitis patients and healthy controls, respectively; all $p<0.001$), (Figure 8-1 (d)).

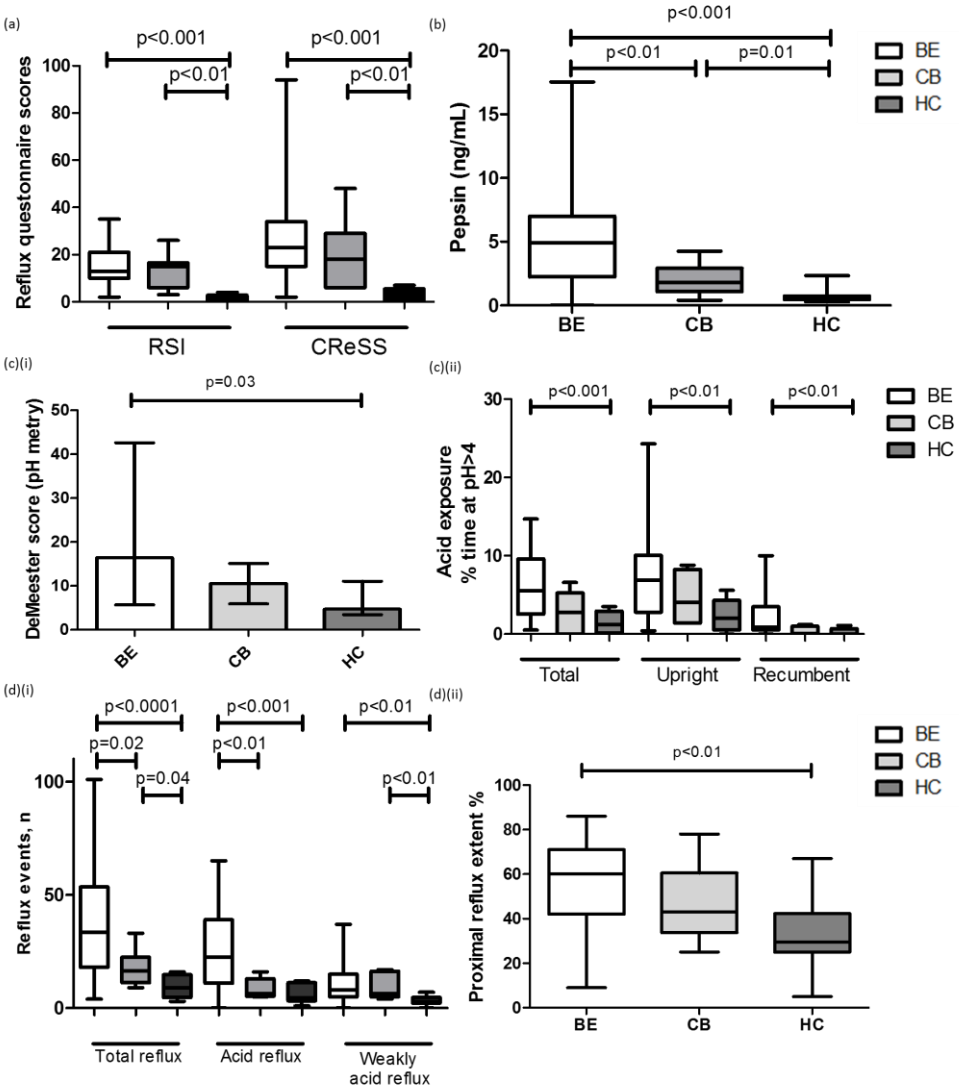

Figure 8-1 Prevalence of gastro-oesophageal reflux disease comparing bronchiectasis patients with chronic bronchitis patients and healthy volunteer controls according to different diagnostic methods

These include (a) reflux symptom questionnaires RSI and CReSS; (b) pepsin as a marker of gastric microaspiration; (c) pH-metry according to (i) total DeMeester score, and (ii) acid exposure time (AET) (% of time pH>4); and (d) pH-impedance according to combined and individual acidic and weakly acidic, and (ii) percentage of proximal exposure. Box plots indicate median and 25th-75th percentiles; whiskers indicate 5th-95th percentiles. Statistical significance was assessed using Kruskall-Wallace with Dunn's post-hoc comparison across groups.

In bronchiectasis patients, the total (both acid and weakly acidic) number of reflux episodes (median (range)) was higher (37 (18–101) than that of chronic bronchitis patients (18 (11-33); p=0.007) and healthy controls (9 (6–16);

p=0.0001) (Figure 8-1 (e)). This was also the case when considering acid and weakly acidic reflux episodes separately (p=0.03).

More reflux episodes reached the proximal oesophagus (51 (26.5–65.5); 95) in bronchiectasis patients than chronic bronchitis patients (20.5 (9.5–34.5); 62; p<0.0001) and healthy controls (9 (5–20); 32; p<0.0001), (Figure 8-1 (f)). In addition, the percentage of total reflux episodes reaching the proximal measuring site was higher in bronchiectasis (66%) than non-bronchiectasis patients (42%; p<0.0001) and healthy controls (31%; p<0.0001).

Table 8-2 Manometric parameters of bronchiectasis patients compared with chronic bronchitis patients and healthy volunteer controls

Variables		**Bronchiectasis**	**Chronic bronchitis**	**Healthy controls**	**p-value**
Basal LOS pressure (mmHg)	mean (SD)	14.3 (4-32.5)	16.5 (7-38)	22 (12-44)	**<0.001**
Manometric hiatal hernia	n (%)	16 (55)	5 (38)	0 (0)	**0.01**
Oesophageal dysmotility disorder	n (%)	7 (24)	0 (0)	0 (0)	0.06

8.3.3 Associations of GORD with disease severity in bronchiectasis

Sub-analysis of bronchiectasis patients with and without airway reflux determined by RSI ≥ 13 showed that bronchiectasis patients with GORD had higher MRC dyspnoea scores [3 (2-3) *vs.* 2 (1-2); p<0.001], higher bacterial colonisation rates with organisms other than *P. aeruginosa* [22 (42%) *vs.* 9 (22%); p=0.03], and significantly more exacerbations [4 (3-5) *vs.* 3 (2-3); p<0.0001] than bronchiectasis patients without reflux, driving higher BSI scores [7 (5-10) *vs*. 4 (2-4); p<0.01], (Table 8-3; Figure 8-2). Bronchiectasis patients with GORD were also more likely to have a higher number of other disease comorbidities aside from GORD, evidenced in higher Bronchiectasis Aetiology Comorbidity Index (BACI) scores of which GORD is not a feature, with a median of 3 (0-5) in bronchiectasis patients with GORD *vs.* 1 (0-3) in

bronchiectasis patients without GORD (p=0.002), perhaps suggesting that systemic inflammation may play a role in contributing to disease severity in bronchiectasis.

When the cohort was divided into positive/negative pH-impedance studies, positive pH-metry, as determined by a DeMeester composite score of ≥ 14.72, was associated with pepsin (r=0.55, p=0.01) and RSI (R=0.49, p=0.023), along with number of exacerbations (r=0.51, p=0,019) and 5 out of 7 components of the QOL-B with exceptions of emotional and social functioning (physical functioning r=-0.56, p=0.009; role functioning r=-0.53, p=0.014; vitality r=-0.53, p=0.013; health perception r=-0.50, p=0.021; and respiratory symptoms r=-0.52, p=0.017).

Positive pH-impedance, determined by the combined total number of acid, weakly acid and non-acid reflux events with a score >40 considered to be pathological, was associated with all other measures of GORD including GERD-Q (r=0.48, p=0.045), RSI (r=0.45, p=0.048), pepsin (r=0.46, p=0.037) and DeMeester score (r=0.85, p<0.0001), as well as number of exacerbations (r=0.51, p=0.019), SGRQ (r=0.45, p=0.039) and the same 5 out of 7 components of QOL-B (physical functioning r=-0.6, p=0.004; role functioning r=-0.58, p=0.006; vitality r=-0.58, p=0.005; health perception r=-0.55, p=0.009; and respiratory symptoms r=-0.68, p<0.001).

Using the validated BSI we observed a clear relationship between pepsin and bronchiectasis severity. Median pepsin levels were significantly lower in mild disease [4 (1-7)] compared to moderate [6 (4-8)] and severe disease [5(4-8)] (p=0.02, Kruskal-Wallis with Dunn's post-comparison) but failed to reach significance for exacerbation frequency <3/≥3 (p=0.09, Mann-Whitney U-test). Relationships were also observed between pepsin and no. of exacerbations (r=0.36, p=0.001), mMRC score (r=0.3, p<0.01), SGRQ total (r=0.59, p<0.001), HADS total (r=0.4, p=0.001) and a weaker association was observed with FEV1% (R=-0.21, p=0.03). Higher pepsin levels were also associated with higher BAL inflammatory markers including CXCL-8, VEGF and MMP-9 (r=0.45, 0.42 and 0.6 respectively, all p<0.001). There

were no significant associations with age, sex, BMI, PPI use, BAL neutrophil count or chronic microbial infection.

Table 8-3 Association of gastro-oesophageal reflux with bronchiectasis disease severity

Variables		**GORD +**	**GORD -**	**p-value**
Total n. (%)		41 (44)	52 (56)	
Demographics				
Age, years	median (IQR)	64 (57-73)	65 (56-69)	0.39
Sex, female	n (%)	30 (54)	23 (53)	1.00
Body Mass Index	median (IQR)	25 (23-30)	24 (22-28)	0.87
Aetiology				
Idiopathic	n (%)	25 (61)	3 (6)	**<0.0001**
Post-infectious	n (%)	8 (20)	13 (25)	0.62
COPD	n (%)	5 (12)	7 (13)	1.00
Asthma	n (%)	6 (15)	3 (6)	0.18
Connective tissue disease	n (%)	1 (2)	5 (10)	0.22
ABPA	n (%)	4 (10)	2 (4)	0.40
Immune deficiency	n (%)	2 (5)	2 (4)	1.00
PCD	n (%)	2 (5)	1 (2)	0.58
Haematological malignancy	n (%)	2 (5)	0 (0)	0.19
Young's syndrome	n (%)	1 (2)	0 (0)	0.44
Yellow nail syndrome	n (%)	1 (2)	0 (0)	0.44
Disease severity				
BSI score	median (IQR)	8 (6-11)	6 (4-9)	**0.04**
E-FACED score	median (IQR)	2 (1-3)	0 (0)	0.36
BACI score	median (IQR)	3 (0-5)	3 (0-3)	0.19
CCI score	Median (IQR)	1 (0-2)	1 (0-2)	0.48
Radiological status				
No. lobes	median (IQR)	3 (2-4)	2 (2-4)	0.47
Mod. Reiff score	median (IQR)	3 (3-4)	2 (2-4)	0.68
Mod. Bhalla score	median (IQR)			
Clinical status				
mMRC dyspnoea scale	median (IQR)	3 (2-3)	2 (1-2)	**<0.001**
Exacerbations in the previous year	median (IQR)	4 (3-5)	3 (1-3)	**<0.0001**

At least one hospitalisation in the previous year	n.(%)	12 (29)	9 (13)	0.47
Functional Status				
FEV_1 (% predicted)	median (IQR)	76 (54-85)	75 (56-88)	0.68
FVC (% predicted)	median (IQR)	102 (82-117)	113 (98-117)	0.96
Ratio (% predicted)	median (IQR)	62 (53-72)	69 (57-77)	0.91
DLCO (% predicted)	median (IQR)	68 (58-72)	72 (63-85)	0.57
Microbiology				
Chronic infection with *Pseudomonas*	n (%)	11 (27)	10 (19)	0.47
Chronic infection with other pathogens	n (%)	17 (42)	9 (17)	**0.02**
BAL inflammatory markers				
Pepsin	median (IQR)	5.6 (4.0-8.2)	3.7 (1.7-6.9)	**0.0006**
IL-6	median (IQR)	474 (226-929)	270 (213-1057)	0.46
CXCL-8/IL-8	median (IQR)	13434 (8136-22478)	7542 (4832-12559)	**0.02**
IL-10	median (IQR)	237 (150-427)	135 (103-165)	0.11
IL-17	median (IQR)	131 (59-132)	118 (56-239)	0.76
IFN-γ	median (IQR)	1156 (437-1939)	656 (362-1401)	0.46
VEGF	median (IQR)	5485 (3010-9899)	2802 (1850-5760)	**0.04**
MMP-9	median (IQR)	1254 (741-1617)	1085 (721-1338)	0.77
TGF-β	median (IQR)	57 (37-101)	31 (28-49)	**0.01**

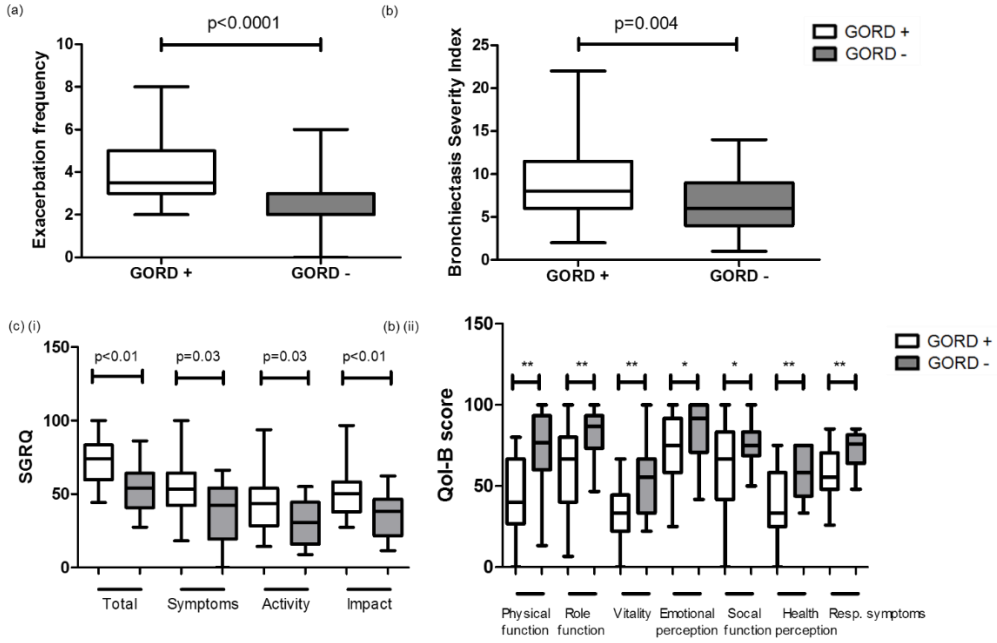

Figure 8-2 Associations with disease severity and quality of life

*Associations with disease severity and quality of life for patients with bronchiectasis and gastro-oesophageal reflux (GORD +) versus bronchiectasis without gastro-oesophageal reflux (GORD -) according to: (a) Bronchiectasis Severity Index (BSI); (b) Exacerbation frequency in the previous year; and (c) Health-related quality of life as measured by (i) St. George's Respiratory Questionnaire (SGRQ) and (ii) Quality of Life-Bronchiectasis questionnaire (QOL-B). Box plots indicate median and 25th-75th percentiles; whiskers indicate 5th-95th percentiles. Statistical significance was assessed using Kruskall-Wallace with Dunn's post-hoc comparison across groups. **p<0.01; * p<0.05.*

8.3.4 GORD is associated with NET-related proteins and epithelial injury

BAL inflammatory markers were significantly decreased in a stepwise manner between bronchiectasis, chronic bronchitis and healthy volunteer controls, and on sub-analysis between bronchiectasis patients with and without GORD (Table 8-3 and Table 8-4). When analysing in the context of bronchiectasis with and without GORD, median levels of all inflammatory markers were higher in bronchiectasis patients with GORD compared with bronchiectasis patients without GORD, with statistically significant differences observed in CXCL-8/IL-8, VEGF and TGF-β (Table 8-3).

Table 8-4 Bronchoalveolar inflammatory markers of bronchiectasis patients compared with chronic bronchitis patients and healthy volunteer controls

Variables		**Bronchiectasis**	**Chronic bronchitis**	**Healthy controls**	**p-value**
Total	n (%)	93 (100)	25 (100)	13 (100)	
IL-6	median (IQR)	407 (223-934)	151 (83-192)	35 (16-58)	**<0.0001**
CXCL-8/ IL-8	median (IQR)	11003 (6554-20076)	1284 (852-2258)	564 (533-672)	**<0.0001**
IL-10	median (IQR)	207 (122-390)	74 (53-114)	42 (29-44)	**<0.0001**
IL-17	median (IQR)	1079 (327-1678)	73 (7-118)	60 (35-80)	**<0.0001**
IFN-γ	median (IQR)	3890 (2542-7709)	1036 (847-1593)	601 (421-657)	**<0.0001**
VEGF	median (IQR)	1240 (717-1535)	440 (227-556)	353 (249-482)	**<0.0001**
MMP-9	median (IQR)	407 (223-934)	151 (83-192)	35 (16-58)	**<0.0001**
TGF-B	median (IQR)	45 (29-75)	22 (19-30)	10 (3-14)	**<0.0001**

The evaluation of candidate protein markers of airways inflammation and remodelling were complimented by an unbiased assessment of our BAL samples. BAL protein profiling was carried out in 43 bronchiectasis patients, 10 chronic bronchitis patients and 10 healthy control volunteers to explore potential biomarkers relevant to disease severity in bronchiectasis. A total of 647 proteins were identified in this sample set. Principal component analysis of BAL protein profiles revealed distinct differences between bronchiectasis patients, chronic bronchitis patients and healthy controls, with neutrophil extracellular trap (NET)-related proteins, immunoglobulins and anti-oxidative stress proteins being the predominant driving factors separating bronchiectasis patients from the other groups. Within bronchiectasis, three distinct clusters were identified; cluster 1, associated with an increased frequency of hospitalisations and characterised by higher levels of neutrophil-associated proteins and antimicrobial peptides; cluster 2, associated with GORD as characterised by high RSI, high DeMeester scores on pH-metry, high total number of combined reflux events on pH-impedance and high

pepsin levels and characterised by a higher expression of NET-related proteins responsible for neutrophil degranulation, and proteins related to epithelial injury; and cluster 3, possibly representing milder or earlier stage disease, characterised by proteins relating to metabolism and glutathione conjugation (Figure 8-3). Among differentially expressed proteins in cluster 1 were CLCA1, a modifier of calcium-activated chloride channels (CaCCs) involved in goblet cell mucus production from the respiratory tract epithelium and innate immunity; and ORM1, a key acute-phase reactant involved in inflammation and potentially immunosuppression. Cluster 2 proteins included histone, a major protein component in NETs, associated with intense inflammation, polymorphonuclear accumulation, destruction, release of cytokines and chemokines and epithelial and endothelial tissue destruction; and induced apotosis of airway epithelial cells; MNDA (myeloid nuclear differentiation antigen) involved in neutrophil apoptosis and inflammatory response, and factors involved in epithelial to mesenchymal transition in damaged cells with malignant potential, such as TLN1, OLFM4, VIM and GPI.[493-496]

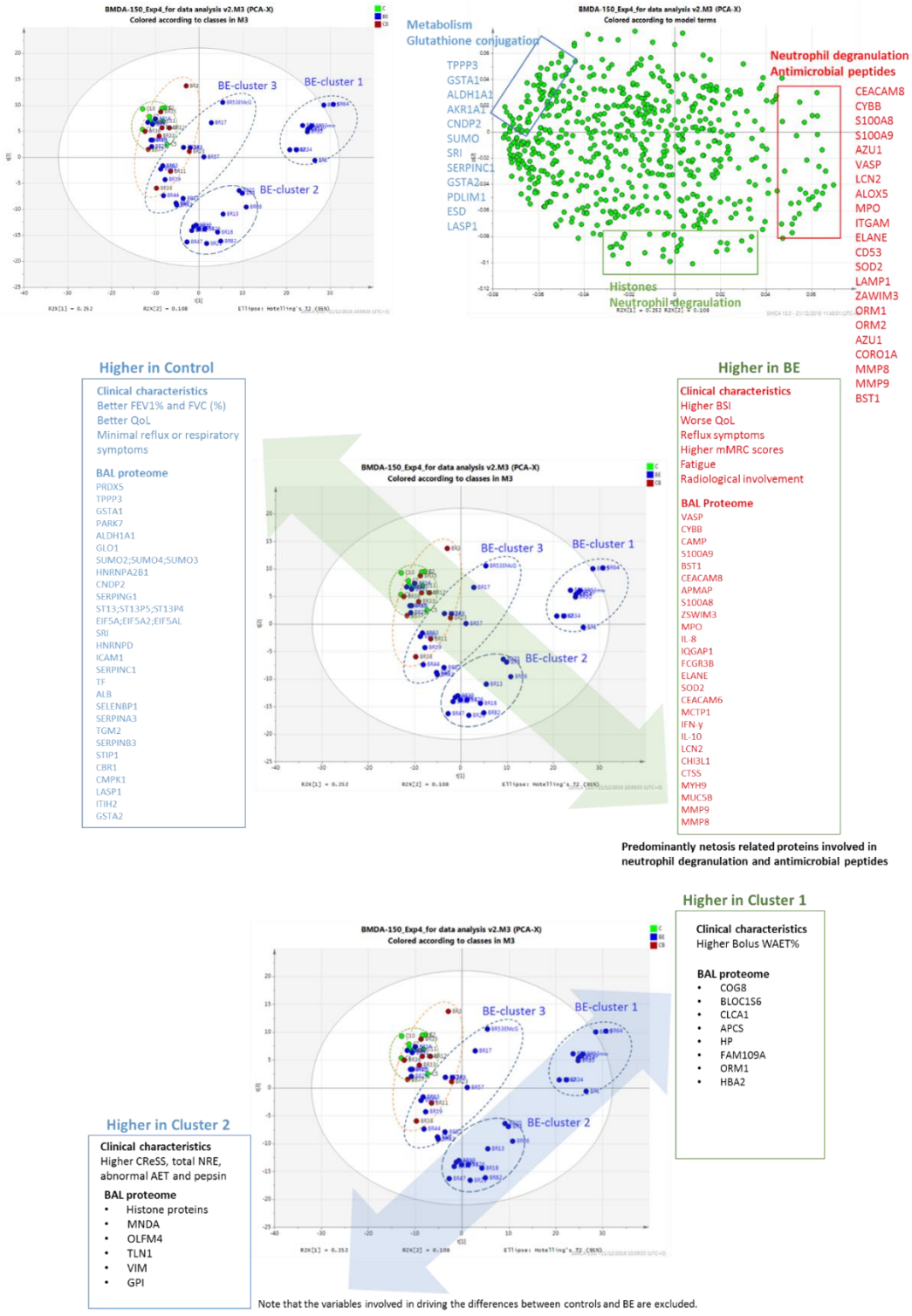

Figure 8-3 Associations of gastro-oesophageal reflux in bronchiectasis with neutrophil extracellular trap (NET)-related proteins and epithelial injury

8.3.5 Bile acids cause direct inflammation and injury in the pulmonary epithelium

Duodeno-gastroesophageal reflux and aspiration, as measured by the presence of pepsin and bile acids in the lower airways, have been shown to contribute to inflammation and lung injury in a diverse range of respiratory pathophysiologies. Based on this observation, we hypothesised that bile acids may cause lung injury by direct damage to the airway epithelium coupled with the release of inflammatory mediators involved in neutrophil recruitment and induction of acute phase reactants.

In vitro studies in 16-HBE immortalised airway epithelial cells exposed to combined physiologically achievable bile acid challenges of primary (cholic 50% and chenodeoxycholic 30%) and secondary (deoxycholic 15% and lithocholic 5%) bile acids in varying acidic milieus showed a concentration-dependent cytotoxic and inflammatory response (Figure 8-4). Cell viability was reduced and IL-8 release increased at higher combined bile acid concentrations [$F=42.61$; $p<0.0001$ and $F=7.01$, $p=0.0007$) and at lower acidic levels [$F=55.98$; $p<0.0001$ and $F=7.79$; $p<0.0001$ (2-way ANOVA with Bonferonni post-test corrections)]. This suggests a possible toxic synergism between bile acids and an acidic environment contributing to airway epithelial cell damage.

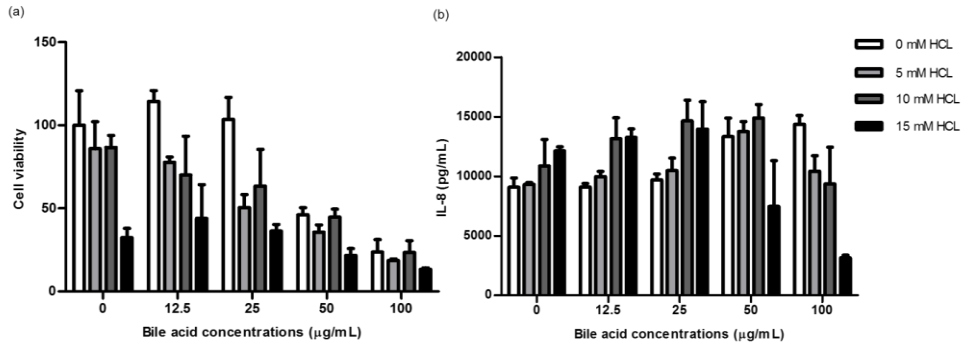

Figure 8-4 Bile acids cause direct inflammation and injury and worsen acid induced injury in the airway epithelium

(a) Cytotoxic effects of increasing concentrations of combined bile acids in non-acidic ($F=55.98$, $p<0.0001$) and acidic ($F=42.61$, $p<0.0001$) conditions on HBE-16 airway epithelial cells. (b) Pro-inflammatory effects of increasing concentrations of combined bile acids in non-acidic ($F=7.01$, $p=0.0007$) and acidic ($F=7.79$, $p<0.0001$) conditions on HBE-16 airway epithelial cells. Statistical comparisons were performed using 2-way ANOVA with Bonferonni post-test corrections (n=6 in triplicate).

Ex vivo studies of individual and combined physiologically achievable bile acids in PBECs derived from bronchiectasis patients (n=10, mean age 65, 50% female, BMI 28.6; mean BSI 6) compared with healthy controls (n=4, mean age 65, 50% female, BMI 29.9) showed significantly increased expression of numerous cytokines and chemokines (IL-6, CXCL-8/IL-8, IL-10, IFN-γ, GM-CSF, VEGF, MMP-9, pro-collagen and TGF-β) in unstimulated bronchiectasis patients compared with controls and bile acid-stimulated cells (Figure 8-5; Table 8-5 (a)).

Individual exposure of LCA in particular, with varying effects of CDCA and CA, showed a significant increase in CXCL-8/IL-8, VEGF, MMP-9, and TGF-β suggesting that it is highly toxic to epithelial cells even at small concentrations (Table 8-5 (a)). Combined bile acid challenges in bronchiectasis PBECS caused predominant increases in CXCL-8/IL-8 and VEGF at both 10 and 25 μg/mL bile acid concentrations (both $p<0.001$ for

IL8; $p<0.05$ and 0.01 for VEGF) with non -significant increased *ex vivo* production of IL-6, IL-10, IFN-γ and GM-CSF (Figure 8-5).

8.3.6 Azithromycin attenuates markers of bile-acid mediated neutrophilic inflammation, remodelling and epithelial to mesenchymal transitioning in bronchiectasis airway epithelial cells

Azithromycin has been demonstrated in several clinical trials and meta-analyses to reduce exacerbation frequency and markers of airway inflammation in bronchiectasis. Given the strong association of GORD with increased exacerbation frequency and the known pro-kinetic effects of azithromycin, we hypothesised that the beneficial effects of azithromycin in bronchiectasis may in part be due to potential inhibition of bile acid-induced airway epithelial inflammation and neutrophil activation.

Azithromycin attenuated the production of all individual LCA-induced bile acid inflammatory markers with varying effects on CDCA and CA-induced inflammation, including inhibition of pro-collagen, TGF-β, MMP-9 and VEGF, suggesting that it may have a role in reducing bile acid-mediated airway inflammation, remodelling and epithelial to mesenchymal transitioning in bronchiectasis airway epithelial cells (Figure 8-5; Table 8-5). Co-stimulation of epithelial cells with combined bile acids in the presence of azithromycin showed significant attenuation of IL-8 and VEGF. These are recognised as key candidate mediators of inflammation and disease progression in bronchiectasis (Figure 8-5).

Table 8-5 Effects of individual bile acid-mediated inflammation and injury and attenuating effects of azithromycin in neutrophilic inflammation, remodelling and epithelial to mesenchymal transition in bronchiectasis primary bronchial epithelial cells

	CXCL-8/ IL-8	TGF-β	Pro-collagen	MMP-9	VEGF
(a) Bile acid stimulation (n=6 in triplicate)					
Control	162.61 (79.21-232.93)	59.04 (34.02-73.52)	9.64 (5.07-14.53)	60.80 (34.42-157.02)	225.42 (151.67-462.06)
LCA	922.42 (301.43-1698.82)*	225.01 (53.81-1669.89)*	10.49 (7.13-17.15)	228.57 (121.73-979.14)*	471.98 (180.55-831.15)*
CDCA	366.72 (135.35-554.24)*	431.29 (18.01-510.11)	10.12 (7.50-19.42)	257.52 (76.79-897.72)*	448.02 (144.94-829.18)*
CA	279.65 (144.26-382.15)	26.38 (10.03-80.45)	9.93 (6.10-19.93)	128.96 (83.28-237.23)*	299.05 (165.51-1360.49)
(b) Azithromycin co-stimulation (n=6 in triplicate)					
Control	89.19 (65.64-105.85)	18.11 (5.21-24.82)	7.55 (7.08-13.35)	54.59 (21.45-142.37)	153.34 (50.01-212.47)
LCA	308.88 (52.82-924.99)*	26.38 (10.03-80.45)*	7.81 (6.70-15.87)*	151.82 (74.98-203.30)*	316.29 (101.21-374.68)*
CDCA	139.78 (16.56-295.97)	20.74 (16.78-25.89)	7.71 (7.42-17.46)	117.56 (21.06-423.24)*	336.05 (58.75-631.15)*
CA	146.39 (110.57-255.86)	20.65 (13.14-21.93)	7.85 (5.65-16.56)*	37.98 (16.01-115.10)*	158.31 (58.75-297.07)*

*The effect of individual bile acid [lithocholic acid (LCA, 1 µmol/L), chenodeoxycholic acid (CDCA, 10 µmol/L), and cholic acid (CA, µmol/L)] – mediated CXCL-8/IL-8, TGF-β, pro-collagen, MMP-9 and VEGF release [median (range)] and azithromycin (20 ng/mL) co-stimulation from primary bronchial epithelial cells derived from bronchiectasis patients (n=6, triplicate readings). Non-parametric Wilcoxin matched-pairs signed rank test was used to test for statistical significance with a 2-sided p-value <0.05 considered significant. *p<0.05.*

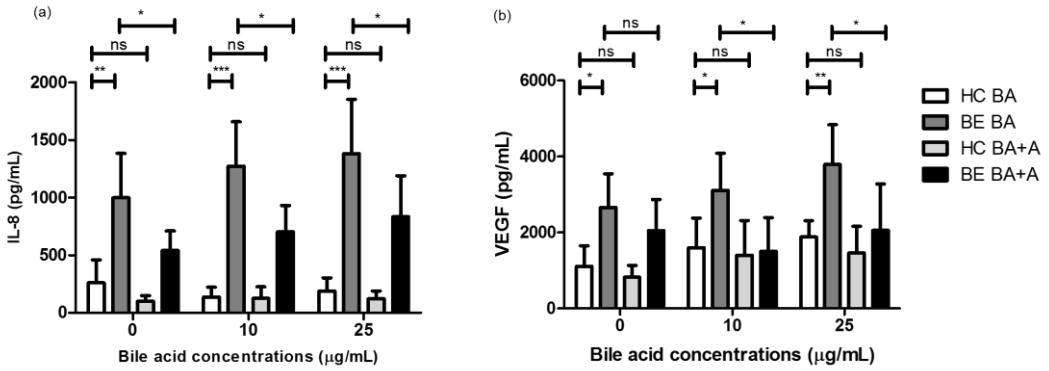

Figure 8-5 Pro-inflammatory and inhibitory effect of combined physiological bile acids with and without azithromycin co-stimulation

*Pro-inflammatory and inhibitory effect of combined bile acids (BAs) in physiological proportions at total concentrations of 0, 10 and 25 μg/mL, with and without a sub-microbicidal concentration of azithromycin (A) 10 ng/mL, on ex-vivo primary bronchial epithelial cells derived from bronchial brushings of bronchiectasis (BE) patients (n=4) and healthy controls (HC) (n=4). Statistical significance was assessed using non-parametric Wilcoxin matched-pairs signed rank test with a 2-sided p-value of <0.05 considered to be significant. ***p<0.001. **p<0.01. * p<0.05.*

8.3.7 EMBARC validation study confirms GORD-association with exacerbation frequency and beneficial effects of macrolides in GORD-positive patients

The EMBARC cohort included 8,792 patients from 5 other countries (UK, France, Spain, Germany and Italy). Patient characteristics are presented in Table 8-6. Self-reported GORD was present in 3,381 (38.5%) bronchiectasis patients followed up for a total of 18,165 person years. Outcomes were the frequency of exacerbations, hospitalisations, and all-cause mortality.

Table 8-6 Patient demographics in EMBARC validation cohort

Variables		**GORD +**	**GORD -**
Total	n. (%)	3381 (38)	5411 (62)
Demographics			
Age, years	median (IQR)	70 (63-76)	67 (55-74)
Sex, male	n (%)	1320 (39.0%)	2033 (37.6%)
Body Mass Index	median (IQR)	25.7 (22.2-29.7)	24.3 (31.3-27.8)
Ex-smokers	n (%)	1585 (46.9)	2046 (37.8%)
Current smokers	n (%)	136 (4.0)	293 (5.4%)
Aetiologies			
Idiopathic	n (%)	1059 (31.3%)	1931 (35.7%)
Post-infective	n (%)	644 (19.0%)	1061 (19.6%)
Post-TB	n (%)	118 (3.5%)	237 (4.4%)
ABPA	n (%)	89 (2.6%)	170 (3.1%)
Connective tissue disease	n (%)	97 (2.9%)	66 (1.2%)
Primary ciliary dyskinesia	n (%)	25 (0.7%)	135 (2.5%)
Asthma	n (%)	253 (7.5%)	365 (6.7%)
COPD	n (%)	327 (9.7%)	393 (7.3%)
Others	n (%)	769 (22.7%)	1053 (19.5%)
Disease severity			
BSI score	median (IQR)	7 (4-11)	6 (3-9)
Radiological status			
Mod. Reiff score	median (IQR)	3 (2-6)	3 (2-6)
Clinical status			
Sputum volume (mL)	median (IQR)	8 (0-20)	6 (0-20)
Exacerbations in the previous year	median (IQR)	2 (1-4)	2 (1-4)
At least one hospitalisation in the previous year	n.(%)	1030 (30.5%)	1181 (21.8%)
FEV_1 (% predicted)	median (IQR)	75.1 (54.0-94.0)	77.3 (56.2-96.1)
Microbiology			
P. aeruginosa	n (%)	808 (23.9%)	1047 (19.3%)
H. influenzae	n (%)	344 (10.2%)	685 (12.7%)
S. aureus	n (%)	149 (4.4%)	252 (4.7%)
M. catarrhalis	n (%)	103 (3.0%)	133 (2.5%)
Enterobacteriaceae	n (%)	192 (5.7%)	228 (4.2%)

Treatment			
Long-term macrolide treatment	n (%)	635 (18.8%)	915 (16.9%)
Inhaled corticosteroids	n (%)	2114 (62.5%)	2761 (51.0%)
Inhaled antibiotic treatment	n (%)	367 (10.9%)	408 (7.5%)
Quality of life			
QOL-B Respiratory Symptom Score	median (IQR)	55.6 (37.0-74.1)	63 (44.4-77.8)

Exacerbations

Without any adjustment, GORD was associated with a 16% increased frequency of exacerbations [IRR 1.16 (1.08-1.25), p<0.001]. GORD remained an independent predictor of future exacerbations after adjustment for BSI [IRR 1.11 (1.03-1.19), p=0.006). In a fully adjusted model incorporating age, gender, FEV1, radiological severity, *P. aeruginosa* infection and country, GORD remained an independent predictor [IRR 1.19 (1.09-1.28), p<0.0001].

GORD was also associated with an increased risk of severe exacerbations requiring hospitalisation. The unadjusted effect estimate was IRR 1.57 (1.41-1.76), p<0.0001]. The effect persisted after adjustment for BSI [IRR 1.29 (1.15-1.45), p<0.0001} and in the fully adjusted model [1.47 (1.28-1.68), p<0.0001].

Mortality

A total of 322 patients died during follow-up. On univariate analysis, GORD was associated with a significantly increased mortality [HR 1.47 (1.18-1.83), p<0.0001]. However, this relationship was no longer significant after adjustment for BSI or in the fully adjusted model [HR 1.10 (0.88-1.36), p=0.4 and HR 1.15 (0.92-1.44), p=0.2 respectively].

Impact of macrolides on GORD

Based on the preclinical data suggesting that azithromycin may protect against epithelial inflammation induced by duodeno-gastro-oesophageal reflux and aspiration, we examined whether macrolides attenuated the increased risk of exacerbations associated with GORD in the EMBARC cohort. 1,550 study patients were macrolide users. We found a significant interaction between macrolide use and the effect of GORD on the number of exacerbations and hospitalisations with all models, suggesting that macrolides play a significant role in reducing GORD-associated exacerbation frequency in bronchiectasis (Table 8-7). Since GORD was not independently associated with mortality, we did not test the modifying effect of macrolides on this outcome.

Table 8-7 Validation of association of gastro-oesophageal reflux on (a) exacerbations and (b) hospitalisations in bronchiectasis patients with and without macrolide therapy in the EMBARC bronchiectasis cohort

(a) Exacerbations

Subgroup	Macrolide non-users	Macrolide users	Interaction test
Unadjusted	1.24 (1.13-1.35); **p<0.0001**	1.00 (0.84-1.19); **p=0.98**	0.015
Adjusted for BSI	1.17 (1.07-1.28); **p=0.001**	0.96 (0.81-1.15); **p=0.68**	0.024
Fully adjusted	1.24 (1.13-1.35); **p<0.0001)**	1.01 (0.84-1.21); **p=0.93**	0.023

(b) Severe exacerbations requiring hospitalisation

Subgroup	Macrolide non-users	Macrolide users	Interaction test
Unadjusted	1.74 (1.51-2.01); **p<0.0001**	1.04 (0.79-1.37); **p=0.79**	0.0005
Adjusted for BSI	1.29 (1.10-1.51); **p=0.002**	0.91 (0.68-1.22); **p=0.52**	0.019
Fully adjusted	1.63 (1.40-1.90); **p<0.0001**	1.00 (0.75-1.33); **p=0.99**	0.0016

8.4 Discussion

This study is the first multicentre international case-control study to systematically characterise the role of GORD, airway reflux and pulmonary microaspiration in bronchiectasis using a multimodal assessment approach. We have shown that GORD and microaspiration are a frequent comorbidity in bronchiectasis with a prevalence of 44-91%. Co-presence is associated with a high burden of bronchiectasis severity using the multidimensional BSI, largely driven by an increased exacerbation frequency and reduced QoL, the effects of which may be attenuated at a clinical and cellular level by treatment with azithromycin. This work is unique in that it assesses mechanistic outcomes and disease severity associated with reflux but also measures the impact of reflux on QoL at an international level, which is arguably at least, if not more important, from a patient's perspective. Thus, GORD symptoms need to be recognised and managed in bronchiectasis, to identify and manage patients deemed most at risk of future exacerbations and their consequences.

In COPD, several studies have focussed on the importance of GORD in mediating exacerbations.[93, 473, 497, 498] The ECLIPSE study found GORD to be the second most common predictor of frequent exacerbations in COPD.[93] It is noteworthy that the ECLIPSE finding was based on a self-reported history of GORD or heartburn, which arguably might represent acid reflux rather than total reflux events present in COPD.[499] Previously published large-scale meta-analyses have demonstrated a 5-7 fold increased risk of COPD exacerbations with GORD.[497, 498] Studies and trials of PPIs have provided conflicting results in reducing reflux-related chronic lung disease, with PPIs reported to be associated with an increased prevalence of non-acid reflux and unwanted side effects such as an increased risk of pneumonia, indicating that other therapeutic options may be needed.[500, 501] The observed rate of oesophageal dysmotility in a quarter of bronchiectasis patients in this study, together with age and frailty in this patient population, arguably represent absolute/relative contraindications for surgical fundoplication indicating that careful, multi-disciplinary assessments are required if this is considered.

The mode of action of macrolides in chronic lung disease has long been a subject of controversial debate. Antibiotic effects do not seem to play a significant role, especially not against *Pseudomonas*, which is naturally resistant against macrolides. It is well known that patients with more severe bronchiectasis have higher levels of airway neutrophilic inflammation including markers such as neutrophil elastase and matrix metalloproteinases. Anti-inflammatory effects of macrolides, especially inhibitory effects on TNF-α and CXCL-8/IL-8 have been demonstrated, but have not been hugely dramatic and not more pronounced than, for example, the effects of inhaled corticosteroids in COPD.[502] In our *ex vivo* studies of bronchiectasis, azithromycin proved to have significant inhibitory effects on bile-acid medicated CXCL-8/IL-8, VEGF and EMT markers, MMP-9, pro-collagen and TGF-B, suggesting a role for azithromycin in attenuating reflux-medicated inflammation, angiogenesis and airways remodelling. This is in keeping with the proteomics findings of reflux-related epithelial injury and EMT markers higher in bronchiectasis patients with reflux including TLN1, OLFM4, VIM and GPI. EMT is the process of epithelial cells losing epithelial proteins and gaining mesenchymal markers. When tissue is damaged or invaded by pathogens, a series of signalling cascades activate the immune system, resulting in inflammatory responses that lead to EMT. When tissue is persistently damaged, this leads to chronic inflammation, increased and prolonged EMT, and increased fibroblast proliferation resulting in hyperplasia and tissue damage. EMT has been described in numerous lung conditions including asthma, IPF and CF and is of potential relevance in the mechanistic pathway of bronchiectasis.

Azithromycin has also been shown to reduce the diversity of the oropharyngeal, respiratory and gut microbiome, and reduce mucus secretion, all of which could potentially translate to a reduced exacerbation rate.[503-506] The pro-kinetic effects on gastrointestinal motility with a resultant decrease in GORD is well described for macrolides, but has always been felt to only partially explain the beneficial effects of macrolides on exacerbations. Azithromycin has been shown to reduce acid reflux episodes and oesophageal acid exposure, leading to a reduction in hiatal hernia size.[321] In COPD,

azithromycin has been demonstrated to reduce exacerbations regardless of the presence of GORD, but with a greater effect in those without GORD.[507] Analysis of our EMBARC data suggests that macrolides play a significant role in reducing GORD-associated exacerbation frequency in bronchiectasis. To our knowledge, this is also one of the first studies to characterise host defence and epithelial dysfunction in BAL and *ex vivo* PBECs of bronchiectasis patients versus chronic bronchitis patients and healthy volunteers. There are robust differences in inflammatory markers measured by ELISA across the spectrum of disease from bronchiectasis to chronic bronchitis patients and healthy volunteers. Whether chronic bronchitis in this population refers to "early" bronchiectasis manifest clinically but not radiologically, remains to be seen.

There is increasing interest in identifying phenotypes and endotypes with distinct clinical outcomes and treatment responses in bronchiectasis due its inherent heterogeneity. Proteomic analysis of these bronchiectasis patients suggested three distinct clusters with GORD being particularly associated with NET-related proteins, markers of epithelial injury and markers of EMT. The finding that reflux is associated with epithelial injury, EMT and a neutrophil-mediated inflammation may be important for other diseases where reflux plays a contributing role. It is also possible GORD may increase airway bacterial load in the lower airways leading to an increased susceptibility to frequent exacerbations. Further mechanistic work is required to determine whether GORD is primarily a driver of epithelial injury and chronic neutrophilic inflammation or, as suggested by preliminary studies in CF cell lines, if it has a direct role in the pathogenesis of chronic infection in bronchiectasis.

Strengths of this work include the use of multiple methods of clinical assessment of GORD in a multi-centre study, the inclusion of a healthy volunteer and chronic bronchitis control group, characterisation of host defence and epithelial dysfunction in BAL and PBECs across the different patient groups, confirmation of findings using multiple methods including ELISA and proteomics analysis, and validation of findings in the international

EMBARC bronchiectasis cohort. Nevertheless, the study has limitations. There are no animal models of bronchiectasis and so there is no direct method of testing whether GORD is directly involved in the pathogenesis of bronchiectasis. We used *in vivo* and *ex vivo* PBECs to study the relationship of bile-acid induced injury on cells. Expansion of our work into air liquid interface models would provide complementary information as to the effect of reflux-related injury on mucus production in bronchiectasis as would analysis of the microbiome to better delineate the effects of GORD on microbial infection. Further studies in this field to determine if treatment of reflux can improve outcomes and destabilise the components of the bronchiectasis vortex by comparing azithromycin-naïve versus treated patients should be considered. There is also a need for well-designed studies of therapy for GORD in bronchiectasis that are adequately powered for exacerbation reduction and that target the various types of reflux and oesophageal dysmotility so that we can more effectively prevent exacerbations in these patients and improve their QoL.

8.5 Conclusion

In conclusion, this study has identified a significant relationship between GORD and bronchiectasis, with reflux-related epithelial injury, inflammation, infection and remodelling all potential contributors to the vicious vortex of bronchiectasis resulting in increased disease severity and progression, manifest clinically by an increased exacerbation and hospitalisation frequency and markedly reduced QoL. We have also demonstrated attenuation of reflux-related injury with azithromycin at both a clinical and cellular level suggesting a further possible mechanism for the previously described benefit of azithromycin in bronchiectasis.

Chapter 9 – Bronchiectasis Primary Bronchial Epithelial Cell Culture Models: An International Collaboration

Publications relevant to this chapter:

McDonnell MJ, Haq I, Verdon B, Aldrahani A, Pearson JP, Lordan JL, De Soyza A, Brodlie M, Rutherford RM, Laffey JG, O'Toole D, Ward C. Primary bronchial epithelial cell culture from bronchiectasis patients: an international collaboration. *In submission* 2020.

9.1 Introduction

Bronchiectasis is a chronic debilitating lung condition characterised by dysregulated innate immunity with bronchial epithelial cells playing a pivotal role in orchestrating airway remodelling and scarring, contributing to disease progression. Previously classified as a rare orphan disease, the diagnosis of bronchiectasis is increasing worldwide with reported rates of up to 566 per 100,000 population and an incidence that has increased by 40% in the last 10 years.[26, 35] Despite having its own diagnostic code, there are few medications or therapies approved by regulatory authorities in the USA or Europe for bronchiectasis.[10, 35] Experimental models are therefore vital to elucidate mechanistic studies of airway epithelial responses in bronchiectasis, for the discovery and evaluation of potential therapeutic compounds and, in light of the recent Covid-19 pandemic, may also play a role in predicting vulnerability of potential at risk patients.

The airway epithelium forms the first line of defence to injurious external stimuli and regulates the immune functions that bridge both innate and adaptive immunity.[485, 486] The bronchial epithelium is a well-recognised source of cytokines, chemokines and growth factors which contribute to inflammatory cell recruitment and activation and the airway remodelling process of bronchiectasis.[44, 508] There is a growing body of evidence to suggest that airway epithelial dysfunction is involved in disease initiation and progression of several chronic inflammatory airways disorders.[487] Bronchiectasis is a disease ensuing from immune dysregulation, involving elements of hyper- and hypo-reactivity.[509] In bronchiectasis, the epithelium becomes damaged over time leading to an increased susceptibility to injury compared to normal airway epithelium.[510] Exposure to repeated noxious and inflammatory stimuli on a background of airway epithelial dysfunction, whether an exaggerated initial response or delay in resolution, affects epithelial regeneration and repair pathways and the ability of epithelial cells to restore barrier functions, leading to aberrant remodelling and structural damage that can further impair epithelial functions and generate a vicious vortex creating an environment conducive to further infection.[35,

487, 511-513] Structural and functional changes in bronchial epithelium can significantly alter the airway milieu, host defences and repair processes in bronchiectasis, leading to progressive lung damage and facilitating chronic and recurrent airway infections in bronchiectasis.[487]

Immortalised airway epithelial cell lines originating from human neoplasms or produced *in vitro* by physical or chemical mutagenesis or introduction of viral oncogenes, have been a tremendous asset to basic research as well as to the pharmaceutical and biotechnology industries.[403] These cell culture systems have contributed significantly to our current understanding of lung disease, facilitating advances in our knowledge of the biochemical and metabolic mechanisms underlying cell function and disease pathology. Advantages of cell lines include their widespread availability, especially when compared to the scarcity of primary tissue and cells, homogeneity in terms of biochemical, electrophysiological, and growth characteristics, and the presence of matched isogenic control cell lines.[403, 404] However, although immortalised cell lines are valuable in the early stages of high throughput screening, they have inherent limitations. The process of immortalisation may generate phenotypic, epigenetic, cellular or karyotypic instability and have major effects on cellular differentiation, morphology, or function compared to the situation *in vivo*.[403, 404] The *ex vivo* culture of primary bronchial epithelial cells (PBECs) derived from bronchoscopic bronchial brushings of individual bronchiectasis patients provide a valuable but technically and logistically challenging source of cells that is likely to recapitulate more accurately the behaviour of bronchial epithelial cells *in vivo* and represent an invaluable tool to elucidate molecular signalling regulation in bronchiectasis. The airway epithelium could therefore represent a suitable target for novel therapeutic strategies, aiming to restore barrier integrity and defences against inhaled pathogens.

We believe that the bronchial epithelium plays a pivotal role in the development and severity of bronchiectasis both as a target for injury and as a mediator of the disease process through response to injury via a combination of immune-dependent and independent mechanisms, leading to epithelial

activation, an excessive pro-inflammatory response to injury, and epithelial-to-mesenchymal interactions contributing to scarring and airway remodelling. *Ex vivo* culture of PBECs from bronchiectasis patients via bronchial brushings has not yet been described. In this paper, we describe our experience in establishing a program to culture PBECs from bronchiectasis patients to investigate the role of the bronchial epithelium in the underlying pathogenesis of this condition and to determine the feasibility of international transfer of brushings for culture after a 48-72 hour window.

9.2 Methods

9.2.1 Study design and participant recruitment

Patients aged ≥18 years with a confirmed new or known diagnosis of bronchiectasis were recruited from respiratory outpatient clinics in Galway University Hospitals (GUH), Ireland. A diagnosis of bronchiectasis was based on high-resolution computed tomographic (HRCT) changes confirmed by a pulmonary physician and expert thoracic radiologist in the presence of a compatible clinical history of bronchiectasis in accordance with British Thoracic Society Guidelines.[9] Patients with cystic fibrosis, traction bronchiectasis due to pulmonary fibrosis, active allergic bronchopulmonary aspergillosis (ABPA) or active non-tuberculous mycobacterial disease were excluded. All bronchiectasis patients had to be free from antibiotic treatment for exacerbation for a minimum of 4 weeks prior to enrolment and sample collection.

9.2.2 Ethics and consent

The study protocol was approved by the local research ethics committees in Galway and Newcastle and performed according to the Declaration of Helsinki. All participants provided written informed consent prior to study enrolment.

9.2.3 Bronchial epithelial cell isolation and culture

Bronchoscopy was performed using a 4.9mm external diameter flexible fibreoptic bronchoscope (Olympus BF45.5, Tokyo, Japan) in accordance with

established BTS bronchoscopy guidelines.[363] Patients were placed in a semi-recumbent position and pre-medicated with 2mL of intravenous midazolam and 250μcg alfentanyl plus topical 4% lignocaine applied to the vocal cords and tracheal lumen in 1 mL aliquots to a maximum dose of 7 mg/kg body weight.[363] Bronchial brushings (n=2) were obtained from subsegmental bronchi using a protected specimen single-sheathed nylon cytology brush (5 fr; Wilson-Cook, Winston-Salem, NC, USA) and the brush dispersed in 5 mL of Roswell Park Memorial Institute (RPMI 1640, Sigma, UK) with 100UI/mL penicillin, 100μg/mL streptomycin (Sigma, UK) and 50μg/mL amphotericin B (Lonza, USA) based on methods previously described.[403, 405] International transfer was arranged within a 48-72 hour door-door transfer window.

On arrival to the laboratory in the UK, samples were manually agitated to separate cells from the brush head. Brushes were removed using forceps prior to centrifugation at 1250 revolutions per minute (rpm) for 5 minutes. The supernatant was discarded and the ensuing cell pellet was re-suspended in 5 mL of bronchial epithelial growth medium (BEGM) consisting of basal epithelial based medium (BEBM; Lonza, USA) supplemented with the following SingleQuots: bovine pituitary extract, insulin, hydrocortisone, retinoic acid, transferrin, epinephrine, human epidermal growth factor, triiodothyronine, gentamicin, and amphotericin (Lonza, USA), along with 100UI/mL penicillin, 100μg/mL streptomycin (Sigma-Aldrich, UK) and 50μg/mL amphotericin B (Lonza, USA). A 100 mL aliquot was taken for cell count and differential and the remaining cell suspension was transferred to a 25 cm^2 plate pre-coated with collagen (Vitrogen 100; Cohesion Technologies, Palo Alto, CA, USA) and placed in a CO_2 incubator (37°C/5% CO_2). BEGM was replaced every 48 hours until primary bronchial epithelial cell cultures (PBECs) reached 80-95% confluence. PBEC cultures were carefully observed throughout to ensure that the cells were growing satisfactorily and to look for any evidence of infection. Once confluent, PBECs were passaged using trypsin/ethylene diamine tetra-acetic (EDTA) (Sigma-Aldrich, UK) which was neutralised using an equal volume of RPMI supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich, UK). PBECs were then

transferred in culture medium to 75 cm^2 collagen-coated tissue culture flasks (Thermo-Fisher Scientific, UK) for further passage; to eight chamber slides (Lab-Tek, Nunc, Naperville, IL, USA) for immunohistochemical analysis; to 24, 48, or 96-well plates for stimulation experiments in submerged culture (Corning, Schipol, Netherlands); on to a semi-permeable membranes for air-liquid interface (ALI) culture; or alternatively, reserved for cryopreservation.

9.2.4 Air liquid interface models

A representative proportion of submerged cultures were expanded into ALI models to enable *ex vivo* development of a muco-ciliary phenotype that is more representative of the *in vivo* pseudostratified columnar airway epithelium, allowing investigation of airway epithelial function. ALI cultures were performed by seeding reconstituted PBECs on to the apical compartment of pre-collagen coated and medium-primed Transwell (Corning) semipermeable clear polyester supports (6.5 mm diameter, 0.4 μm pore size) submerged in 100 μL BEGM aiming for a density of 60,000-80,000 cells per membrane (approximately 200,000 cells/cm^2). 600 μL of BEGM supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, USA) was added to the Transwell basolateral compartments. Cells were placed in a CO_2 incubator (37°C/5% CO_2). Once fully confluent, typically after 48 to 72 hours, cells were air lifted to enable differentiation to a pseudostratified, ciliated columnar airway epithelium. The apical medium was exchanged the following day to remove unattached epithelial cells. Basolateral BEGM was replaced with ALI differentiation medium as published previously. Essentially, this consists of a 50:50 mix of BEGM basal medium with Dulbecco's Modified Eagle's Medium (DMEM-high glucose) (Lonza, USA) supplemented with Singlequots with adjusted concentrations of retinoic acid and human epidermal growth factor and omission of gentamicin/amphotericin. Calcium chloride was supplemented with 500 μL of 1M solution. Cells were regularly visualised using light microscopy for leakage and membrane integrity. Basolateral medium was exchanged every 48 hours. The apical surface was washed weekly using phosphate buffered saline (PBS) (Sigma, UK) with resultant washings stored at -80 C for future

analysis. A summary of the tissue culture ALI process is provided in Figure 9-1.

Serial transepithelial electrical resistance (TEER) measurements were performed to assess tight junction integrity using an epithelial voltohmmeter (EVOM2TM, World Precision Instruments, Stevenage, UK) as per manufacturer's instructions. To facilitate this, 100 µL of pre-warmed PBS was added to the apical compartment of each membrane, and cultures were incubated and allowed to equilibrate at 37 ºC for 20 minutes. TEER measurement was also performed in 'blank' membranes without seeded cells to allow subtraction of the background level of resistance.

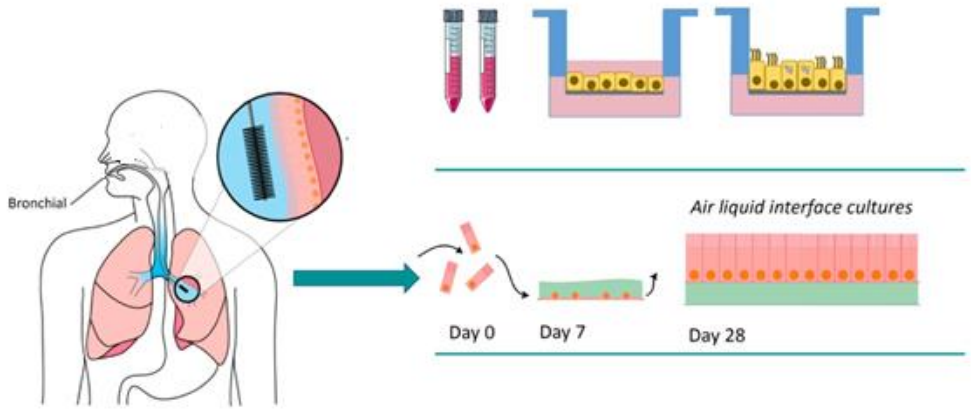

Figure 9-1 Air liquid interface culture methods

9.2.5 Ussing's chamber electrophysiological studies

Electrophysiological studies were performed using non-perfused Ussing chambers controlled by the VCC MC8 (Voltage Current Clamp MultiChannel, Physiologic Instruments, Inc.) to obtain measurement of short circuit current (I_{sc}) as an indicator of net transepithelial ion transport.[514] Snapwell semipermeable supports of differentiated ALI cultures were mounted in the Ussing chamber bathed both apically and basolaterally in 5 mL Krebs solution to eliminate potential osmotic or chemical effects. Apical and basolateral compartments were electrically isolated and separated by the polarised epithelial monolayer. 'Blank' semi-permeable supports, without cultured cells, were also included to offset the potential difference and fluid

resistance between the two chambers. Calomel voltage sensing electrodes were placed on each side of the membrane. Transepithelial potential difference was clamped to 0 mV by current injection with silver-silver chloride (Ag/Ag) electrodes to eliminate the electrical gradient. Under these conditions, the short circuit current (Isc) is a direct measure of net transepithelial ion transport across the epithelial monolayer. 3M potassium chloride salt bridges containing 3% agar were used to connect chambers to the electrodes. The chamber was maintained at 37°C in 5% CO2/95% O2, pH 7.4. A 1 second 5mV pulse was applied at 30 second intervals to monitor resistance changes applying Ohm's law. After a 20-minute period of stabilisation, relevant ion channel inhibitors and activators were added to either the apical or basolateral compartments and resultant Isc responses were recorded. For all experiments, the protocol consisted of apical addition of amiloride (100 μM, Tocris) to inhibit ENaC, followed by apical addition of forskolin (10 μM, Tocris) to activate CFTR-mediated chloride transport through adenylate cyclase stimulation and intracellular cAMP increase. CFTR is then inhibited by apical addition of $CFTR_{inh}$-172 (20 μM, Tocris). Finally, UTP (100 μM, Sigma-Aldrich) was apically added as a purinergic agonist for CaCC activation. The resultant analogue signal was digitised with a Powerlab 200 interface (AD instruments, Australia) and recorded to a computer with Scope 3 software (AD instruments, Australia). A representative Using chamber trace derived from a non-CF bronchiectasis ALI culture is shown in Figure 9-2.

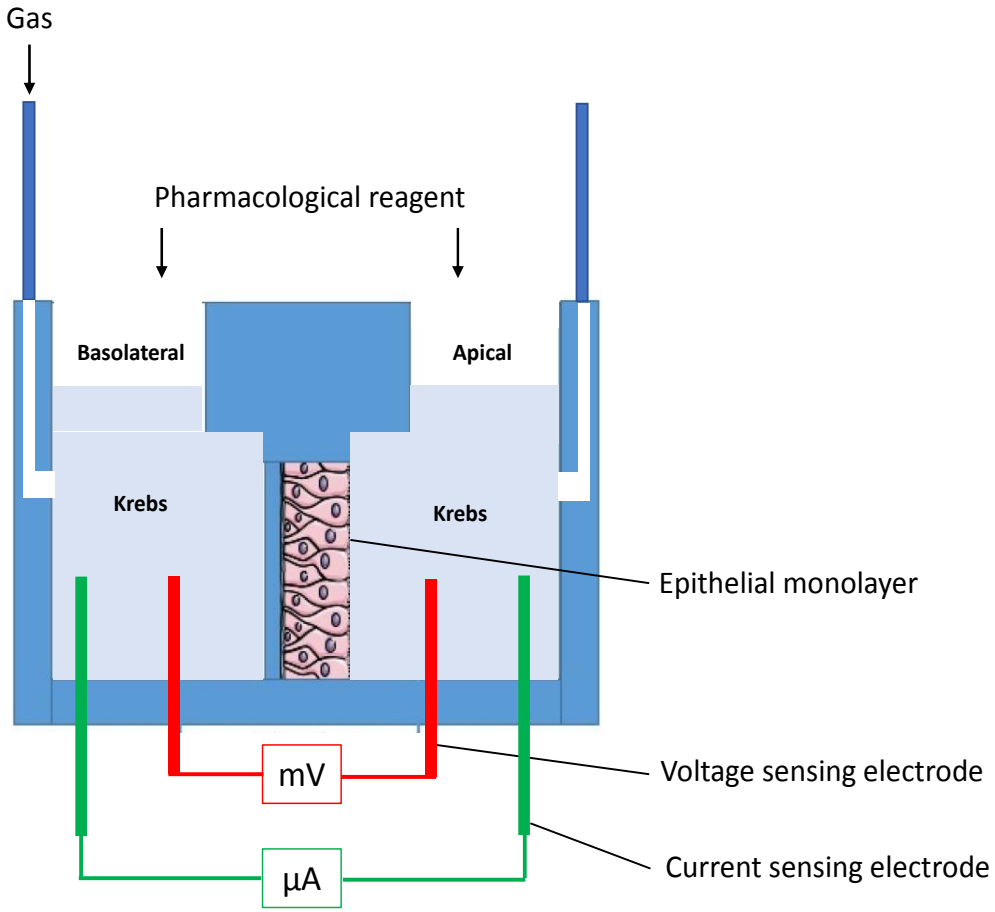

Figure 9-2 Schematic representation of Ussing chamber

PBEC ALI differentiated cultures grown on semi-permeable supports were mounted in the Ussing chamber. The epithelial monolayer was bathed apically and basolaterally with 5 mL of Krebs solution. Each chamber was connected to voltage and current sensing electrodes. The I_{sc} required to clamp the V_{TE} to 0 mV was recorded. Pharmacological reagents were added to the apical or basolateral compartments and resultant changes in I_{sc} and R_T were assessed. The resultant amiloride-sensitive Isc was calculated by subtracting the baseline Isc (determined by calculating the mean Isc values for 1 minute prior to amiloride addition) from the minimum Isc value after amiloride. Responses to forskolin, CFTRinh-172 and UTP were calculated using a similar approach.

9.2.6 Histology and immunohistochemistry

Cultured cells were characterised by their morphology under confocal microscopy and a representative proportion of paraffin embedded sections of cultured cells were stained with haematoxylin-eosin staining (Thermofisher, UK) to enable histological assessment of epithelial characteristics, Periodic Acid Schiff (Sigma-Aldrich, UK) to stain for mucin glycoproteins, and Alcian Blue Periodic Acid Schiff (Sigma-Aldrich, UK) to differentiate between acidic and neutral mucin glycoproteins. Motile cilia express α-acetylated tubulin, a feature of microtubule stability and a widely used marker of cilial assessment in airway epithelial cultures (Jain *et al*., 2010, Rymut *et al*., 2013). ALIs were subsequently immunostained for acetyl anti- α tubulin antibody (Sigma-Aldrich, UK) to assess for the presence of cilia in ALI cultures using standard techniques.

9.2.7 Cryopreservation and reconstitution of cryopreserved cells

The majority of these samples were cryopreserved on arrival and reconstituted at a later time for stimulation experiments at passage 2 and 3. Cell pellets were generated using the trypsinisation method described above. The reserved pellets were re-suspended in 1 mL of 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in FCS and transferred to sterile cryotubes (Thermo Fisher, Loughborough, UK). Tubes were placed in an isopranolol cell freezer (Thermo Fisher, UK) at room temperature, which was then stored at −80°C for 24 hours. At this point the tubes were transferred to a liquid nitrogen cell freezer for long-term storage. Cryotubes containing 1 mL cell suspensions were removed from a liquid nitrogen cell freezer and rapidly rewarmed in a 37°C water bath. Once defrosted the suspension was centrifuged at 1250 rpm for 5 minutes. The supernatant was discarded and 5mL of BEGM pre-warmed to 37°C was slowly added and the cells re-suspended. The resultant cell suspension was then seeded in a 25 cm^2 tissue culture flask and cultured as described.

9.2.8 Microbiological assessment

All bronchoalveolar lavage (BAL) microbiology samples were processed in an Irish Clinical Pathology Accredited (CPA)-laboratory to routine diagnostic standards using standard and select supplementary media with extended culture for bacterial, fungal, and NTM spp., in accordance with BTS guidelines on microbiological profiling in bronchiectasis. Samples were analysed by trained staff using appropriate containment and safety procedures in accordance with Galway University Hospital standard operating procedures. Sensitivity testing was carried out using the agar disc diffusion method according to methods of the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Isolates were tested against multiple anti-microbial agents including amikacin, ceftazidime, ciprofloxacin, colistin, gentamicin, meropenem, piperacillin-tazobactam, ticarcillin-clavulanic acid and tobramycin. Infected cell cultures, based on daily visual inspection, were discarded appropriately.

9.2.9 Statistical analysis

Comparison between successful culture and cultures that failed to grow was made using the Chi-squared test with Fisher's exact test for categorical data and Mann-Whitney U-test for non-categorical data. Significance was assumed at $p<0.05$.

9.3 Results

PBECs were successfully cultured in 14/22 (64%) bronchiectasis patients attempted (mean (SD) age 61.1 (14.7), 59% female). Brushings reached confluence at a median of 14 days post-bronchoscopic sampling and successfully underwent further passage. All PBECs were initially cultured under submerged conditions with light micrographs of cells at confluence and positive haematoxylin-eosin staining confirming typical epithelial cell morphology similar to that described previously (Figure 9-3 (a-c)). Although it is possible to perform multiple passages *in vitro*, PBECs are mortal and possess a finite capacity for regeneration prior to becoming senescent.[403, 515]

The remaining 8 brushings failed to reach confluence due to early infection of the culture with bacteria known to colonise the airways in bronchiectasis, mostly occurring within the first 4-5 days post brushing. The organisms isolated from BAL microbiology at the time of the bronchoscopic procedure are shown in Table 1. 12/22 (55%) patients were found to have positive microbiology on BAL. Of the 8 that failed to reach confluence, 6 (75%) had positive BAL microbiology suggesting that successful culture may be more likely in bronchiectasis patients with negative BAL microbiology ($p<0.01$). No other factors appeared to predict submerged culture success. All cells remained viable after storage in liquid nitrogen, facilitating further cell culture experiments at subsequent passage.

Of those expanded to the ALI model, it was possible to visualise mucus production on the surface of ALI cultures with the naked eye and on phase contrast microscopy. ALI cultures stained positive for Periodic acid-Schiff's stain, with and without the addition of Alcian Blue, demonstrating the presence of mucin glycoproteins (Figure 9-3 (d-f)). The representative image of immunofluorescent staining in a bronchiectasis PBEC ALI shows evidence of cilia in the apical aspect of the differentiated culture in 52% of surface epithelial cells confirming ciliogenesis (Figure 9-3 (f)). Airway mucin MUC5B was detected in apical washings from ALI cultures by slot-blot ELISA. These results confirm the presence of different cell types and well-differentiated mucus-secreting cells.

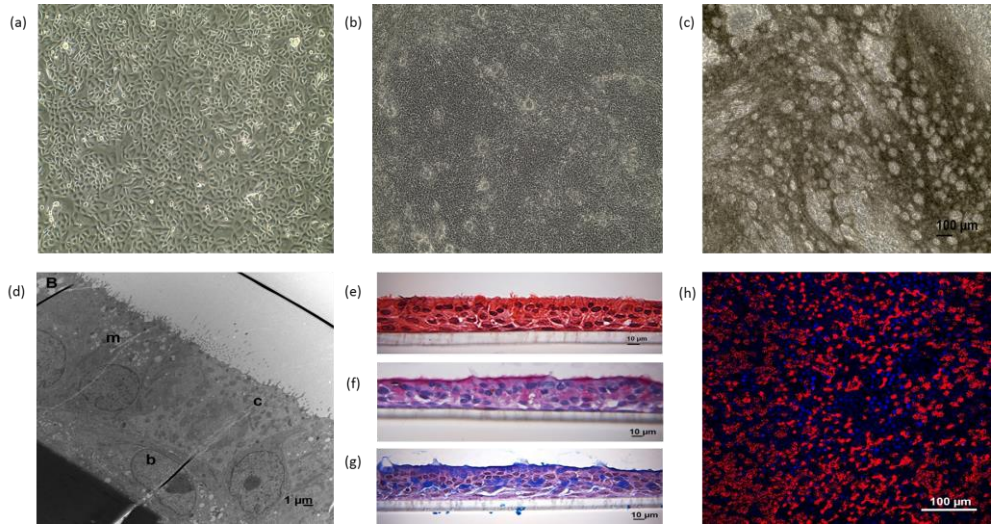

Figure 9-3 Epithelial characterisation of cultures in submerged media and air liquid interface (ALI) cultures

Representative micrographs of cultured primary bronchial epithelial cells (PBECs) from bronchial brushings of a 39-year-old female patient with post-infective bronchiectasis and BAL-positive Haemophilus influenza (HI) (a)-(h). Light micrograph of confluent PBECs in (a) submerged culture; (b) air-liquid interface (ALI) culture; (c) phase contrast appearance of ALI cultures with secretory droplets and mucus material overlying the surface of the epithelial monolayer; (d) assessment of epithelial differentiation of PBECs using transmission electron microscopy showing the presence of ciliated-c, mucus producing-m and basal-b cells in differentiated PBEC ALI in bronchiectasis-B patient; (e) Haematoxylin and eosin stain of paraffin-embedded confluent PBEC ALIs at 28 days; (f) positive periodic acid–Schiff (PAS) of paraffin-embedded PBEC ALIs demonstrating glycoprotein presence shown by the magenta staining localised to the apical epithelium and mucus secreting cells; (g) PAS staining with Alcian Blue confirming the presence of acidic mucin glycoproteins demonstrated by the blue staining; (h) immunofluorescent detection of a-acetylated tubulin (red) at the apical surface of epithelial cells with nuclei stained with DAPI (blue) in a PBEC ALI fixed in 4 % PFA after 28 days.

Ussing chamber experiments were performed in a representative proportion of bronchiectasis PBEC ALI cultures at 25 to 33 days. Normal airway electrophysiology was observed in all bronchiectasis ALI models with TEER measurements of >500 /cm^2 indicative of a polarised epithelium (Figure 9-4).[516]

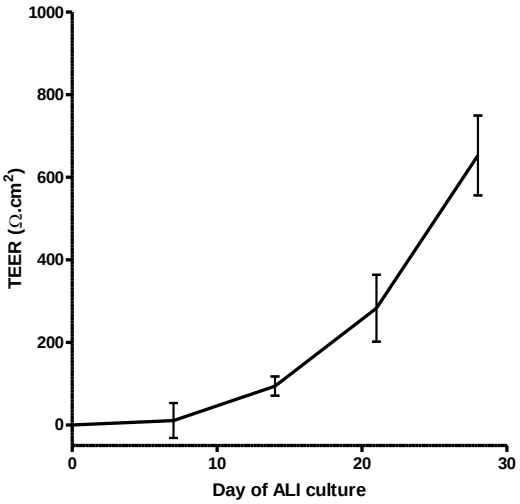

Figure 9-4 Transepithelial electrical resistance (TEER) in bronchiectasis ALI

Transepithelial electrical resistance (TEER) measurements up to D28 in a bronchiectasis air liquid interface (ALI) culture from a 37-year-old female patient with post-infective bronchiectasis and BAL-positive Haemophilus influenza (HI) demonstrating an increase in TEER over time.

Of the ALI models, cultures that took longer to reach confluence in the initial submerged expansion phase were more likely to fail at ALI suggesting that time to confluence may be a useful marker of ALI culture success that could help with early exclusion of cultures unlikely to progress during ALI. Representative comparative Ussing traces of short circuit current and transepithelial electrical resistance responses to forskolin and CFTRinh-172 in bronchiectasis and cystic fibrosis PBEC ALI cultures are demonstrated in Figure 9-5 and Figure 9-6.

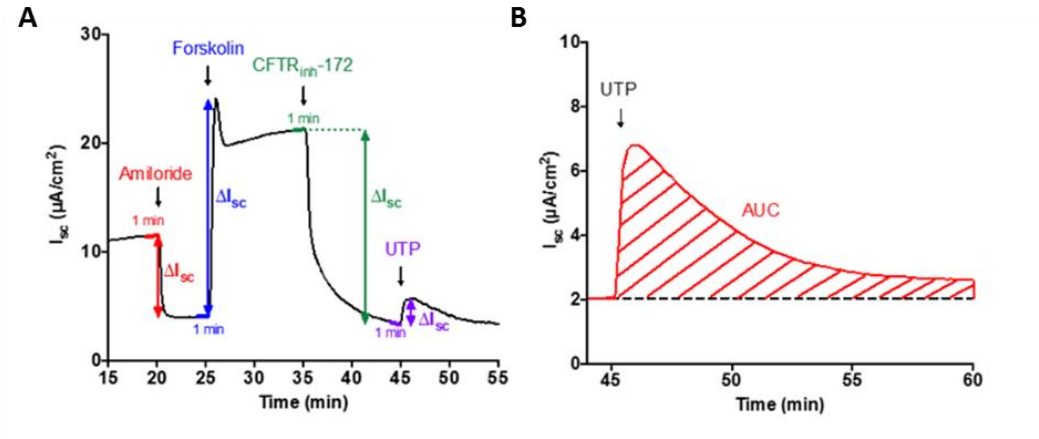

Figure 9-5 Representative Ussing trace for bronchiectasis PBEC ALI

Representative short circuit current (I_{sc}) trace for responses to amiloride, forskolin, $CFTR_{inh}$-172 and UTP from a 37-year-old male bronchiectasis patient with Young's syndrome and BAL-positive HI (A). There is an appropriate reduction in I_{sc} net ion transport following inhibition of the epithelial sodium channel (ENaC) with amiloride. The resultant amiloride-sensitive I_{sc} (red) is calculated by subtracting the minimum I_{sc} from the calculated baseline 1 minute prior to amiloride addition. Similarly, there was an appropriate increase in chloride transport following activation of the CFTR protein with the cAMP agonist forskolin. The forskolin-induced I_{sc} (blue) is obtained by subtracting the mean baseline I_{sc} 1 minute prior to forskolin addition from the maximum peak response to forskolin. Appropriate inhibition of chloride transport in response to CFTRinh-172 (green) and further activation of chloride transport by the purinergic receptor antagonist UTP (purple) were calculated using the same approach. The area under the curve (AUC) was used to assess the total UTP-induced I_{sc} response (B).

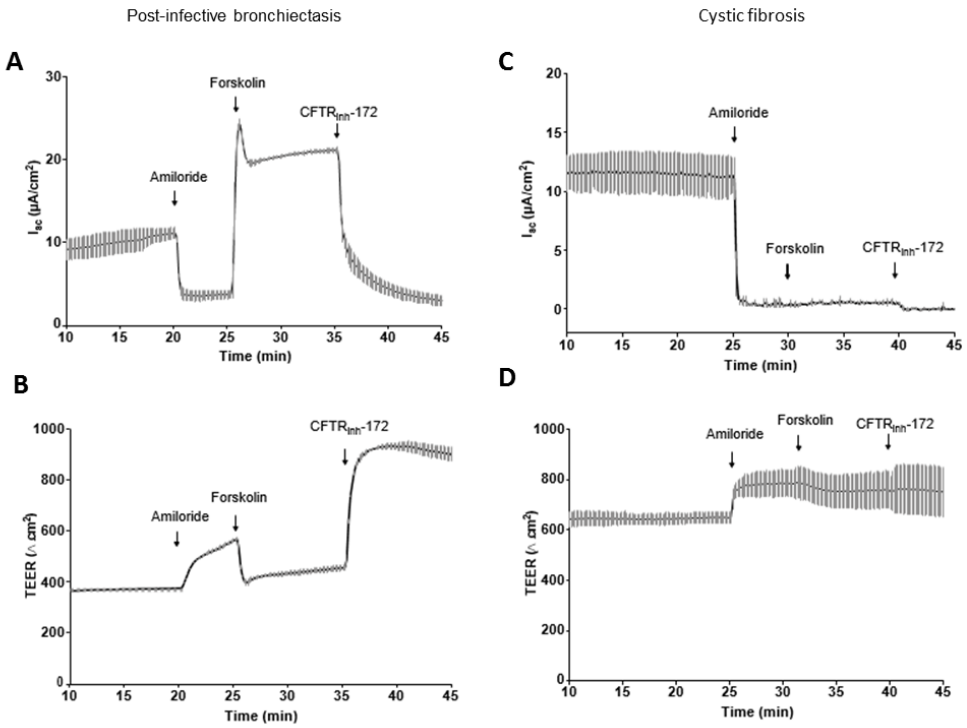

Figure 9-6 Representative comparative Ussing traces of short circuit current and transepithelial electrical resistance responses to forskolin and CFTRinh-172 in bronchiectasis and cystic fibrosis PBEC ALI cultures

PBEC ALI culture inserts derived from a post-infective bronchiectasis and cystic fibrosis patient were mounted in the Ussing chamber and bathed apically and basolaterally in 125 mM chloride Krebs. After a 20-minute period of stabilisation, 10 μM amiloride was added to the apical compartment to inhibit ENaC. 10 μM forskolin and 20 μM CFTRinh-172 were added apically at 25 and 35 minutes respectively. Resultant short circuit current (Isc) (A and C) and corresponding transepithelial electrical resistance (TEER) (B and D) were measured, with a relatively normal response in bronchiectasis and abnormal ion transport characterised by failure of cyclic adenosine monophosphate (cAMP)/forskolin–activated Cl− conductance, due to dysfunctional mutant CFTR channels in cystic fibrosis cells. Lines mean responses in 2 inserts with error bars for the SD.

9.4 Discussion

We describe the successful establishment of a program to culture PBECs from bronchial brushings of bronchiectasis patients following a transfer period of 48-72 hours using minimal antibiotic prophylaxis with both submerged culture and ALI models despite the prevalence of pathogenic microbes and copious mucopurulent secretions. To the best of our knowledge, this is the first description of a program to culture PBECs in bronchiectasis patients on an international platform with successful culture beyond an incubation period of 24 hours. This demonstrates the feasibility of international transfer of biological specimens and facilitating multicentre collaborative approaches to better understand the pathophysiology of this disease.

Successful culture resulted in establishment of phenotypically characteristic epithelial cells with loss of differentiation in submerged cultures and maintenance of the ciliated phenotype in ALI cultures. The airway epithelium not only acts as a physical barrier to prevent potential pathogens entering airway mucosa, but is also a key regulator of innate immune responses toward invading pathogens and controls ion transport to keep the airways hydrated.[405] Higher infection rates were observed in unsuccessful cultures. Organisms appeared to be derived from patients who were generally stable with presumed “chronic infection” previously termed “colonisation” and in whom a clinical diagnosis of acute infection was not made, despite positive BAL microbiology. The methodology also highlights the potential injurious role of occult infection in this patient group, re-emphasising the difficulties in deciding what represents ‘‘infection’’ in the bronchiectasis airway. As further information is gained regarding the lung microbiome demonstrating that patients show remarkable consistency in lung microbiome over time within the same patient, it is likely that the term “colonisation” will become obsolete.

The current authors believe that the use of primary cell cultures from bronchiectasis patients is an important adjunct to the use of commercially available airway epithelial cell lines in understanding the mechanisms of

chronic airway dysfunction. Patient-derived PBECs are an advance on conventional, commercially available bronchial epithelial cell lines, as they are derived directly from the group of interest. The heterogeneity of bronchiectasis perhaps presents further issues with in relation to PBEC studies and basic mechanistic studies looking differences across aetiologies may provide valuable insight into disease pathogenesis. Studies in COPD have shown that nasal epithelial cells are unable to substitute for *in vitro* bronchial epithelial cells in airway inflammation studies.[517] In the emerging era of precision medicine and targeted therapies, cultured PBECs from bronchiectasis patients represent an important resource that may be used to establish valuable novel immortalised cell lines.[516]

However, the culture of PBECs from bronchiectasis brushings is demanding, both in terms of effort and expense, and is limited by their finite life span but ultimately it yields a model system that more accurately recapitulates the situation *in vivo* than immortalised cell lines.[403] In bronchiectasis patients, there is a diverse spectrum of disease and often great heterogeneity between aetiology, radiology, microbiology and clinical phenotype. It is important to remember that PBECs are likely to reflect this inherent biological heterogeneity in terms of their function. For some experiments the homogeneity of an immortalised cell line with isogenic controls may be more preferable, for example, in the early stages of high-throughput screening. However, disease-specific PBECs are vital to confirm and validate any initial findings in an immortalised cell line prior to more advanced stages of analysis. Development of a reliable method for PBEC culture could facilitate comparison of epithelial cell responses between bronchiectasis patients and cells from patients with other neutrophilic lung diseases or healthy control volunteers, allowing dissection of the role of the bronchial epithelium in the pathogenesis of bronchiectasis.

Use of ALI models allowed electophysiological studies to investigate ion transport profiles relating to ENaC and CFTR function in Ussing chamber experiments to be performed in bronchiectasis PBEC ALIs and compared with those of CF. In this study, bronchiectasis PBEC ALIs demonstrated a

normal response to forskolin suggesting normal ion transport compared with the complete lack of response to forskolin in CF PBEC ALIs, associated with dysfunctional CFTR. It should be noted, however, that of the few functional studies performed in clinical bronchiectasis, impaired sodium transport (abnormal sweat chloride concentration or nasal potential difference measurement) has been found in approximately two thirds (69%) of patients with idiopathic bronchiectasis, suggesting abnormal ion transport that may be consistent with a CFTR-related disease.[518] This could have significant implications on patients at a prognostic and socioeconomic level and further research into this area at a clinical and cellular level is needed.

Although establishing the feasibility of a programme to harvest bronchial brushings on an international platform is exceptionally valuable for future collaborative work, this study has a few inherent limitations. The authors did not employ quantitative culture in this study. Future studies may address questions relating to quantitative microbiology along with differences in airway micro- or myco-biome, changing biofilms over time and the role of donor-derived infection. In the current study there was an overall success rate of 64% in reaching confluence and passage of PBECs. Previously published studies in this centre of primary cell culture from lung transplant and CF recipients have demonstrated success rates of 39-54%.

The authors did not specifically measure the infection rate of the culture medium which may have provided additional information to explain success or failure. This has been shown in culture studies of lung transplant patients to influence the success of culture. Previous studies of cell culture in CF explanted lungs utilised patient-specific antimicrobials based on organisms isolated in sputum pre-transplant and their relevant sensitivities balancing the relative cytotoxicity of different antimicrobials against the necessary concentration required for the desired antimicrobial effect.[403] However, microbiological growth in culture medium may simply reflect contamination of the medium in the culture hood or incubator. Secondly, the culture medium, containing standard antibiotics and antifungals, may select out organisms present in low concentrations from the brushings and encourage

their growth. Thirdly, organisms may derive from the upper airways of patients including the nasopharynx, although the use of a protected specimen brush should reduce the chance of spurious contamination. Finally, the process of epithelial brushing could identify a discrete population of micro-organisms adherent to the epithelium as a biofilm even in the presence of apparently culture negative BAL.

9.5 Conclusion

In this paper we report our initial experience in establishing an international programme to culture PBECs from bronchiectasis bronchial brushings, demonstrating feasibility even after a 48-72 hour window for international transfer, despite the potential for early, patient-derived infection. The culture of PBECs from chronically infected lungs poses technical and logistical challenges but ultimately yields a valuable cellular model to study bronchiectasis lung disease. This technique is also relevant to other lung conditions and will likely be an important tool in understanding how Covid-19 affects airway epithelial cells in the future. Determining the host innate immune response in affected *ex vivo* PBECs could predict the subsequent course of the disease and identify those more likely to progress to ARDS.[519] To the best of our knowledge, this is the first description of a program to culture PBECs in bronchiectasis patients on an international platform. This provides an important model to facilitate future mechanistic studies to elucidate pathogenic mechanisms and investigate potential therapeutic targets in bronchiectasis and other lung diseases.

Chapter 10 - Discussion and Conclusions

10.1 Introduction

Bronchiectasis is a heterogeneous, poorly understood, multidimensional disease, associated with significant morbidity, premature mortality, escalating public health costs and profound reductions in quality of life (QoL). Comorbidity is a frequent finding in these patients, often with synergistic effects on disease severity and resultant poorer clinical outcomes. The cause and effect relationship between gastro-oesophageal reflux disease (GORD) and bronchiectasis is likely bi-directional, contributing to worse overall outcomes; therefore it is essential that low-cost, simple, population-wide interventions are found to reduce the severity and progression of bronchiectasis disease. In this thesis, I examined the contribution and mechanism of GORD, airway reflux and pulmonary microaspiration to bronchiectasis using a multimodal assessment approach examining clinical endpoints of exacerbations, hospitalisations, QoL and mortality, and surrogate outcomes, such as bronchoalevolar lavage (BAL) inflammatory markers and the use of *ex vivo* primary cell culture models, to determine the effects of GORD and potential therapeutic effects of azithromycin on the development and severity of bronchiectasis.

10.2 Summary of findings contributing to current knowledge

10.2.1 Systematic review of GORD in bronchiectasis

I completed a systematic review of the literature exploring the association between GORD, airway reflux, *Helicobacter Pylori* infection and pulmonary microaspiration with bronchiectasis clinical and treatment outcomes. I developed the research question, designed the electronic search strategy, completed abstract and full title selection and review, extracted data, collated results and drafted the manuscript. This systematic review, using standardised methodology, identified eighteen eligible cohort studies. There is a distinct paucity of randomised clinical trials looking at the relationship between GORD and bronchiectasis to date. Due to differences in study design,

measurement of GORD and reported outcomes, a decision was made *a priori* not to perform meta-analysis or generate quantitative summary estimates but to focus on a qualitative synthesis of findings. Despite differences in study design, size and duration of follow-up, a consistent association between the presence of GORD and adverse bronchiectasis outcomes was observed. Similarly, potential treatment options of GORD in bronchiectasis remain somewhat controversial with a lack of randomised controlled trials hampering progress in this area. The evidence base is therefore insufficient to make firm guideline recommendations in relation to GORD in bronchiectasis highlighting the need for further studies in this area, including those deliberated in subsequent Chapters of this thesis.

10.2.2 Multidimensional severity assessment in bronchiectasis

In order to establish best outcome measures to use for clinical studies in bronchiectasis and to better define the effect of GORD on bronchiectasis outcomes, I conducted a meta-analysis comparing two validated scoring systems assessing multidimensional disease severity in bronchiectasis across seven European Cohorts in the FRIENDS bronchiectasis database. I co-developed the research question, collated the databases, performed and interpreted statistical analyses and drafted the manuscript with oversight from Prof. James Chalmers. I presented this work at an oral presentation at the European Respiratory Society and was subsequently invited to speak at the Thorax Highlights Symposium at the British Thoracic Society following publication. This analysis showed that, although both scores were equally capable of predicting 5-year mortality, the Bronchiectasis Severity Index (BSI) was superior to the FACED score in assessing disease severity including exacerbations, hospitalisations, lung function decline, exercise capacity and QoL. This was therefore included in all subsequent analyses to determine the effects of GORD on bronchiectasis disease severity and has since been incorporated in observational and ongoing trial designs to better identify patients most likely to benefit from treatment.

10.2.3 Comorbidities and the risk of mortality in bronchiectasis

Given the few studies assessing the effects of comorbidities in bronchiectasis, I designed a multicentre cohort analysis using data from four European centres to determine the effects of comorbidities on mortality in bronchiectasis. I developed the research question, collated the databases, performed statistical analyses with oversight from Prof. James Chalmers and drafted the manuscript. I presented this work as an oral presentation at the European Respiratory Society and the manuscript was subsequently published in *The Lancet: Respiratory Medicine*. The average patient was determined to have four comorbid conditions. An independent relationship was demonstrated between the number of comorbidities and long-term mortality. 26 of 81 comorbid conditions identified were independently associated with mortality, GORD being among these. This study is particularly relevant for having been the first to demonstrate the link between comorbidities and aetiologies, which were then used to construct the Bronchiectasis Aetiology and Comorbidity Index (BACI). The BACI predicted 5-year mortality, hospital admissions, exacerbations, and QoL for all strata of bronchiectasis severity as assessed by the BSI. *P. aeruginosa* chronic infection was also linked to comorbidities, perhaps due to an enhanced underlying systemic inflammatory response. Comorbidities predicted mortality risk with a higher accuracy than markers of bronchiectasis severity, with a combined BSI/BACI score having the highest prognostic potential in terms of predicting mortality and other outcomes of disease severity, emphasising the importance of incorporating aetiologies and comorbidities into a multidimensional assessment of patients with bronchiectasis.

10.2.4 Associations between hiatal hernias and disease severity in bronchiectasis

An increased prevalence of hiatal hernias has been demonstrated in several chronic lung diseases. The relationship between hiatal hernias and GORD has been extensively investigated over the past few decades. This study was the first to explore the prevalence and potential association of hiatal hernias with

disease severity in bronchiectasis. I actively contributed to all study components including the development of the research question under the supervision of Dr. Robert Rutherford, data collection, statistical analysis and interpretation, and writing of the manuscript. The presence of a hiatal hernia in bronchiectasis patients correlated with increased disease severity characterised by decreased lung function, increased extent and severity of radiological disease, and an increased BSI score. Given bronchiectasis is a radiological diagnosis, assessing an individual's HRCT for the presence of a hiatal hernia could render important prognostic and therapeutic information in all bronchiectasis patients.

10.2.5 Associations between GORD, disease severity and mortality in bronchiectasis

Based on the findings of the systematic review and above studies, I completed analyses of a prospective cohort study exploring the association between GORD, disease severity and mortality in bronchiectasis. Few studies have looked at the impact of GORD on mortality in bronchiectasis and whether or not this is something that should be looked for specifically in these patients. Based on the positive findings in the Galway cohort presented at the European Respiratory Society, I did a further analysis comprising participants from three other European clinical centres in the FRIENDS database to validate these findings. For this chapter, I contributed actively to all components including: development of the research question, collection of all prospective data from the Galway cohort, design of the statistical analysis plan, collation and interpretation of statistical analyses and writing of the manuscript. In this large prospective cohort study, the presence of GORD was associated with an increased rate of exacerbations and hospitalisations and a 2-fold increase in mortality among patients with bronchiectasis. Further studies are required in larger population cohorts to confirm or refute these findings. If a relationship with mortality is confirmed, it is imperative to obtain adequately powered RCT evidence for treatment targeted at GORD to improve outcomes in bronchiectasis.

10.2.6 Macrolides attenuate gastro-oesophageal reflux-associated airway inflammation, remodelling and disease severity in Bronchiectasis

Taken together, cohort studies in chapters 4, 5 and 6 suggest that GORD is associated with an increased risk of poor outcomes in bronchiectasis. This study aimed to further explore the association of GORD and bronchiectasis at a clinical and cellular level in the first multicentre international case-control study in bronchiectasis aimed at systematically characterising the role of GORD, airway reflux and pulmonary microaspiration in bronchiectasis using a multimodal assessment approach, comparing findings to age, sex, ethnicity and BMI-matched chronic bronchitis patients and healthy control volunteers. I co-designed the study with oversight from Dr. Robert Rutherford, Prof. Chris Ward, Prof. John Laffey and Dr. Daniel O'Toole. I acquired skills in primary cell culture of *ex vivo* bronchial epithelial cells (PBECs) in Newcastle and translated them into the laboratory in Galway. I trained in oesophageal physiology techniques in Newcastle and Amsterdam and subsequently developed the oesophageal physiology laboratory in Galway after successfully completing a business case, budget proposal, operational policies and patient information leaflets to establish the service. I collected all data from the Galway cohort including performing all procedures, culturing PBECs and all laboratory analyses. I performed and interpreted statistical analyses and drafted the manuscript with oversight from all supervisors. This study is the first described to utilise e*x vivo* cultures of PBECs from bronchiectasis patients via bronchial brushings and the first to assess proteomic analysis of BAL in bronchiectasis patients.

In this study, we confirmed that that the presence of GORD, airway reflux and microaspiration is associated with a high burden of bronchiectasis severity, reflux-related epithelial injury, inflammation, infection and remodelling, all potential contributors to the vicious vortex of bronchiectasis resulting in increased disease severity and progression, manifest clinically by an increased exacerbation and hospitalisation frequency and markedly reduced QoL. We also demonstrated unique novel data in characterising the inflammatory profile of bronchoalveolar lavage in bronchiectasis patients using different techniques, demonstrating distinct differences between

bronchiectasis patients, chronic bronchitis patients and healthy controls, with neutrophil extracellular trap (NET)-related proteins, immunoglobulins and anti-oxidative stress proteins being the predominant driving factors separating bronchiectasis patients from the other groups. Further novel data includes the attenuation of reflux-related injury with macrolides at both a clinical and cellular level suggesting a further possible mechanism for the previously described benefit of macrolides in bronchiectasis. Thus, GORD symptoms need to be recognised and managed in bronchiectasis, to identify and manage patients deemed most at risk of future exacerbations and their known consequences.

10.2.7 Primary bronchial epithelial cell culture from bronchiectasis patients: an international collaboration

The cell culture investigations and proteomic analysis in the previous chapter suggest that the bronchial epithelium plays a pivotal role in the development and severity of bronchiectasis both as a target for injury and as a mediator of the disease process through response to injury via a combination of immune-dependent and independent mechanisms. In this paper, we describe our experience in establishing the feasibility of a programme to harvest bronchial brushings on an international platform with success using submerged and air-liquid interface (ALI) models. Cell cultures in the UK were processed by Prof. Chris Ward and his laboratory group. I co-designed the study, collected bronchoscopic samples in Galway, facilitated data analysis and drafted the manuscript. I was awarded a prize for best abstract in pulmonary infections at the American Thoracic Society for this work which ultimately yields an important cellular model to facilitate future collaborative mechanistic studies to further elucidate pathogenic mechanisms and investigate potential therapeutic targets in bronchiectasis with application in other lung diseases.

10.3 Linking it all together and potential future studies

Linking back to the original over-arching hypothesis that GORD, airway reflux and microaspiration of duodeno-gastro-oesophageal contents into the lung contributes to bronchial epithelial cell damage, stimulation of cytokine

production and an airway inflammatory and remodelling response that drives disease severity and progression in the bronchiectasis vicious vortex, this body of work has provided significant evidence to support this at both a phenotypic and endotypic level with relevance to potential treatment-related disease-modifying effects.

Within the respiratory community, there has been a marked reluctance to accept GORD and airway reflux as a potential cause of respiratory disease. The focus over the last half decade has shifted from intrinsic to extrinsic pathophysiology, a balance which needs to be redressed to understand some of the otherwise inexplicable phenomena described in "idiopathic" or intractable respiratory patients.[520] Despite the wealth of epidemiological data which has repeatedly demonstrated strong associations between GORD and respiratory diseases, this is often frequently dismissed as two common diseases co-existing together. The failure of large-scale trials of primarily anti-acid anti-reflux medication has thus been taken as proof that reflux is not a factor in the development and progression of respiratory disease. However, failure to appreciate the non-acid component of potentially pathogenic gaseous reflux and damaging components of duodeno-gastro-oesophageal microaspiration, is largely responsible for this confusion. [520] We have demonstrated GORD, airway reflux and/or microaspiration prevalence rates of up to 91% depending on methodology used. Similar to other chronic lung conditions, approximately a third of bronchiectasis patients have a demonstrable hiatal hernia utilising high resolution CT imaging or manometry, with oesophageal dysmotility present in a significant proportion likely rendering them unsuitable for potential surgical intervention.[29, 467, 468] As in the ECLIPSE study in COPD whereby GORD was considered to be a major cause of further exacerbations, we have shown that classic GORD is associated with increased disease severity manifest by an increased exacerbation frequency, increased hospitalisations and reduced QoL in bronchiectasis.[93] A 2.5 fold increased mortality risk was observed with the presence of GORD in the FRIENDS cohort; however, no observed increased mortality was found in the larger EMBARC cohort. Contrary to popular belief, PPI use for the treatment of typical GORD was not associated with an

increased hospitalisation rate although further large registry studies are needed to confirm or refute these findings. In the most recent systematic review and meta-analyses of GORD in COPD incorporating 13,245 COPD patients from 10 observational studies, GORD was associated with a more than 5-fold increased risk of exacerbations (OR: 5.37; 95% CI 2.71–10.64).[498] Exacerbations are acute events that accelerate and sustain the vicious vortex of bronchiectasis, irrespective of the cause that is responsible for the exacerbation.[521] Identifying the aetiology of the exacerbation and finding biomarkers capable of predicting future exacerbation risk and/or early detection of exacerbation development and response to therapy are of paramount importance.

Of huge significance in the case control study, bronchiectasis patients were clearly shown to have a dysregulated immune response at baseline compared with chronic bronchitis patients and healthy volunteers, largely driven by neutrophil extracellular trap (NET)-related proteins, immunoglobulins and anti-oxidative stress proteins on proteomic analysis. Data is only just emerging in this area based on analysis of sputum proteomics and microbiome data. These results utilise BAL which is much more specific to airways diseases and are the first globally to demonstrate these findings. The application of emerging technologies of proteomics, metabolomics, and genomics to well-characterised groups of bronchiectasis patients such as those with co-existing GORD in the EMBARC-BRIDGE and other projects may enable us to better understand pathophysiological mechanisms and identify targets for future therapies. By recognising the clinical and biological complexity of bronchiectasis and the effects of GORD and other associated comorbidities, we may be able to pave the way towards a more precise, safe and effective therapy in these patients aligned with causal mechanistic disease pathways, to deliver a tailored personalised treatment approach.

Both *in vitro* and *ex-vivo* primary cell culture models of duodeno-gastro-oesophageal reflux show that microaspiration of bile acids could potentially lead to direct inflammation and injury characterised by neutrophilic inflammation, epithelial to mesenchymal transition and airways remodelling.

It is suspected that prolonged exposure of the mucosa to damaging or noxious stimuli contributes to the development of chronic infection due to bacterial persistence via modulation of the lung microbiome and respiratory pathogen biofilm formation, contributing to increased infection and bronchiectasis disease severity and chronicity.[328, 329, 489] Mucins are one of the main constituents of the mucus that covers epithelial surfaces.[522] MUC5B appears to be the airway mucin most responsive to stimulation by inflammatory mediators and is the predominant airway mucin in COPD.[523] IL-8, IL-6 and IL-1β have been shown to up-regulate MUC5B and in murine models of cystic fibrosis, neutrophil elastase appears to drive mucus hyper-secretion.[524-526] Further mechanistic work utilising ALIs to assess the effect of reflux-mediated injury on mucin hypersecretion and co-stimulatory effect with bacteria may further demonstrate and improve our understanding of the complexity of the interdependent aspects of the bronchiectasis vicious vortex.

The novel link identified between macrolides and the attenuation of GORD-mediated inflammation, exacerbations and hospitalisations in cellular models and clinical datasets may be due to a combination of anti-inflammatory and pro-motility properties which could have relevance to other neutrophilic airway conditions extending the previously reported benefits of macrolides on reducing bronchiectasis outcomes. Studies of azithromycin in COPD suggest that patients with GORD have a shorter time to first exacerbation, more frequent exacerbations and more hospitalisations than those without GORD, and that taking azithromycin daily for 1 year reduced exacerbations in COPD patients with or without GORD but was more effective in participants without GORD.[507] This is in contrast to our data in bronchiectasis where azithromycin clearly attenuates reflux-related exacerbations. Perhaps the lack of effect in COPD may be related in part, to previously observed effects of reduced effectiveness in smokers.[527] It is likely, however, that the effect of azithromycin is somewhat underestimated as these epidemiological studies did not assess for the presence of airway reflux or microaspiration. The suggestion that GORD, airway reflux and microaspiration might underlie the effect observed in the macrolide trials in

bronchiectasis is biologically plausible. If this hypothesis is correct, it offers the potential for broader application of azithromycin to non-acid reflux disease. However, more confirmatory data are needed assessing the effectiveness of azithromycin in objectively reducing GORD, airway reflux and/or microaspiration or reflux-related exacerbations in azithromycin-naïve bronchiectasis patients before we accept an effect on GORD as the reason why azithromycin has a clinically significant benefit in the reduction of bronchiectasis exacerbations in symptomatic bronchiectasis.

10.4 Potential impact

This collection of work has highlighted the potential role of GORD, airway reflux and microaspiration as important contributors in bronchiectasis disease severity, with particular relevance to an increased exacerbation frequency and reduced QoL. Clinicians should therefore routinely assess for the presence of reflux in their multidimensional assessment of bronchiectasis patients, particularly among the 'frequent exacerbators'. There are a range of therapeutic options available including medical, endoscopic and surgical techniques. Many patients may already be on a PPI as per their primary care provider but this may not be enough to negate the non-acid effects of airway reflux and microaspiration. Whilst macrolides are commonly given to bronchiectasis patients with ≥ 3 or more exacerbations per year to reduce their exacerbation frequency, our pre-clinical and clinical studies suggest that macrolides play a significant role in reducing GORD-associated exacerbation frequency in bronchiectasis and therefore should always be considered in the treatment armentarium frequent exacerbators, particularly those with a confirmed diagnosis or objective evidence of reflux. Should this fail to improve, referral to an appropriate specialist for endoscopic or surgical options should be considered according to patient preferences.

10.5 Future work

Further work on the effects of GORD on the microbiome is already underway with analysis of BAL samples in bronchiectasis patients with reflux versus those without to identify any potential microbial differences. There is

growing evidence to suggest that crosstalk between the gut-lung axis may be responsible for maintaining host homeostasis and disease development. Diversity in the gut microbiota decreases during ageing leading to an imbalanced state, or dysbiosis, which has been shown to contribute to immune dysfunction and generalised inflammation with links to various chronic lung and other conditions such as arthritis, obesity, cardiovascular disease and type 2 diabetes, all common comorbidities among bronchiectasis patients.[23, 528] This tri-directional relationship between the gut, lung and immune system is likely to influence disease severity and progression in bronchiectasis patients and a grant application comparing these differences in bronchiectasis is currently underway. Further work assessing the inter-relationship between the gut-lung microbiome, mycobiome and virome in bronchiectasis, and the effects of azithromycin treatment on the same, particularly in those with GORD, airway reflux or microaspiration where this relationship is of particular relevance, could pave the way for new approaches in our understanding and management of bronchiectasis and other chronic respiratory diseases.

10.6 Overall conclusion

In conclusion, these studies provide novel observational clinical and translational evidence of bronchiectasis disease severity and the associations of GORD, airway reflux and pulmonary microaspiration with increased airways inflammation, epithelial injury, increased disease severity and reduced QoL. These findings have and will likely contribute further to British and European Clinical Guidelines in Bronchiectasis, and will help to further highlight future research priorities towards improving our understanding of this disease and quality of care for patients with bronchiectasis.

Appendices

Outputs arising from this work

Publications directly relevant to PhD

Peer-reviewed publications

1. McDonnell MJ, Hunt E, Ward C, Pearson J, Murphy D, O'Toole D, Laffey JG, Rutherford RM. Gastro-oesophageal reflux in chronic lung disease: current understandings of mechanisms and management. *Eur Respir J Open Res*, 2020 (in press).
2. McDonnell MJ, O'Toole D, Ward C, Pearson J, Lordan JL, De Soyza A, Loebinger MR, Chalmers JD, Laffey JG, Rutherford RM. A systematic review of gastro-oesophageal reflux in bronchiectasis: Current understanding and future risk. *Resp Med* 2018; 141: 132-143.
3. McDonnell MJ, Aliberti S, Goeminne PC, Restrepo MI, Finch S, Pesci A, Dupont LJ, Fardon TC, Wilson R, Loebinger MR, Skrbic D, Obradovic D, De Soyza A, Ward C, Laffey JG, Rutherford RM, Chalmers JD. Comorbidities and the risk of mortality in patients with bronchiectasis: an international multicentre cohort study. *Lancet Respir Med*. 2016; 4(12):969-979.
4. McDonnell MJ, Aliberti S, Goeminne PC, Dimakou K, Zucchetti SC, Davidson J, Ward C, Laffey JG, Finch S, Pesci A, Dupont LJ, Cowman S, Fardon TC, Skrbic D, Obradovic D, Loebinger MR, Rutherford RM, De Soyza A*, Chalmers JD*. Multidimensional severity assessment in bronchiectasis - An analysis of 7 European cohorts. *Thorax* 2016; 71:1110-1118.
5. McDonnell MJ, Ahmed A, Wall D, Bruzzi J, O'Mahoney M, Breen D, O'Regan A, Gilmartin JJ, Rutherford RM. Prevalence of hiatal hernias in non-cystic fibrosis bronchiectasis and associations with disease severity. *Respirology* 2015; 20(5):749-57.

6. Chalmers JD, Goeminne P, Aliberti S, McDonnell MJ, Lonni S, Davidson J, Poppelwell L, Salih W, Pesci A, Dupont LJ, Fardon TC, De Soyza A, Hill AT. The Bronchiectasis Severity Index: An International Derivation and Validation Study. *Am J Respir Crit Care Med.* 2014; 189(5): 576-585.
7. McDonnell MJ, Ward C, Lordan JL, Rutherford RM. Non-cystic fibrosis bronchiectasis. *QJM*. 2013; 106(8):709-715.

Peer-reviewed publications in submission

1. McDonnell MJ, O'Toole D, Das J, Aldhahrani A, Verdon B, Pearson JP, Lordan JL, De Soyza A, Bruzzi J, Huang J, Soussi N, Gominne P, Aliberti S, Polverino E, Ringhausen F, Loebinger MR, Chalmers JD, Laffey JG, Rutherford RM, Ward C. Macrolides attenuate gastro-oesophageal reflux-associated airway inflammation, remodelling and disease severity in bronchiectasis. In submission 2020.
2. McDonnell MJ, Haq I, Pearson J, Aldhahrani A, Verdon B, Lordan JL, De Soyza A, O'Toole D, Rutherford RM, Laffey JG, Ward C. Bronchiectasis primary bronchial epithelial cell culture models: an international collaboration. In submission 2020.
3. McDonnell MJ, Rutherford RM, Ward C, De Soyza A, Laffey JG, O'Toole D, Goeminne PC, Hill AT, Aliberti S, Obradovic D, Chalmers JD; on behalf of FRIENDS investigators. Impact of gastro-oesophageal reflux disease on mortality and exacerbations in bronchiectasis: data from the FRIENDS cohort. In submission 2020.
4. McDonnell MJ, Mokoka MC, Ward C, De Soyza A, O'Toole D, Laffey JG, Rutherford RM. The "New Age" of Bronchiectasis – State of the art review. In submission 2020.

Book chapters

1. McDonnell MJ, Ward C, Rutherford RM. Comorbidities and their impact on bronchiectasis. European Respiratory Monograph. Editors: James Chalmers, Eva Polverino, Stefano Aliberti. European Respiratory Society 2018; pp 45-61. ISBN 978-1-904097-92-1.

2. McDonnell MJ, Rutherford RM. Predisposing factors for the development of bronchiectasis. Bronchiectasis: The EMBARC manual. Editors: James Chalmers, Eva Polverino, Stefano Aliberti. Springerlink 2016; pp 129-145. ISBN 978-3-319-61452-6.
3. Ward C, Jones R, McDonnell MJ, Hunt E, Murphy D. Pathophysiology in the lung. Reflux Aspiration and Lung Disease. Editors: Alyn H Morice and Peter W. Dettmar. Springer UK Book Commission 2016; pp 55-69. ISBN 978-3-319-90525-9.

Conference presentations relevant to PhD

1. McDonnell MJ, Das J, O'Toole D, Ward C, Pearson J, Aldhahrani A, Verdon B, Lordan JL, De Soyza A, Bruzzi J, Soussi N, Loebinger MR, Chalmers JD, Laffey JG, Rutherford RM. Effects of gastro-oesophageal reflux and duodeno-gastro-oeophageal microaspiration in bronchiectasis. *Eur Respir J* 2018; 52 (62): 4950 (Oral presentation)
2. McDonnell MJ, Haq I, Verdon B, Pearson JP, Aldhahrani A, Lordan JL, De Soyza A, Brodlie M, O Toole D, Rutherford RM, Laffey JG, Ward C. Primary Bronchial Epithelial Cell Culture from Bronchial Brushings of Bronchiectasis Patients: An International Collaboration. *Am J Resp Crit Care Med.* 2018; 197: 1972 (Thematic poster) - *awarded Best Abstract Prize for Pulmonary Infections and Tuberculosis.
3. McDonnell MJ, Ward C, Rutherford RM, Verdon B, Pearson JP, Aldhahrani A, Lordan JL, De Soyza A, O Toole D, Laffey JG. Azithromycin attenuates release of bile acid-mediated neutrophilic and remodelling factors in bronchiectasis airway epithelial cells. *Am J Resp Crit Care Med.* 2018; 197: 2841 (Poster discussion)
4. McDonnell MJ, Rutherford RM, De Soyza A, Loebinger MR, Goeminne PC, Hill AT, Aliberti S, Polverino E, Chalmers JD; on behalf of the EMBARC investigators. Associations between gastro-oesophageal reflux, its management and exacerbations of bronchiectasis. *Eur Respir J* 2017; 50 (61) : 1968 (Oral presentation)
5. McDonnell MJ, Aliberti S, Goeminne PC, Dimakou K, Zucchetti SC, Davidson J, Ward C, Laffey JG, Finch S, Pesci A, Dupont LJ, Fardon TC, Skrbic D, Obradovic D, Cowman S, Loebinger MR, Rutherford RM, De Soyza A*, Chalmers JD*. Multidimensional severity assessment in bronchiectasis - An analysis of 7 European cohorts. *Eur Respir J* 2016; 48 (60): 274 (Oral presentation) and *Irish J Med Sc* 2016; 185 (9): 439 (Oral presentation)

6. McDonnell MJ, Fardon TC, Rutherford RM, Chalmers JD. Impact of comorbidities on disease severity and risk of mortality in non-cystic fibrosis bronchiectasis. *Eur Respir J* 2015; 46 (59): 467 (Oral presentation) and *Irish J Med Sc* 2015; 184 (11): 542. (Oral presentation)
7. McDonnell MJ, O'Mahony M, Breen D, Gilmartin JJ, O'Regan A, Rutherford RM. Increased disease severity and mortality associated with the Bronchiectasis-GORD phenotype. *Eur Respir J* 2015; 46 (59): 366 (Poster discussion)
8. McDonnell MJ, O'Toole D, Rutherford RM, De Soyza A, Lordan J, Pearson J, Ward C, Laffey JG. Azithromycin attenuates release of bile acid-mediated neutrophilic and remodelling factors from human bronchial epithelial cells. *Am J Resp Crit Care Med*, 2015: 190: 1291 (Poster discussion)
9. McDonnell MJ, O'Toole D, Rutherford RM, De Soyza A, Lordan J, Pearson J, Ward C, Laffey JG. Bile acids cause direct inflammation and injury and worsen acid induced injury in the pulmonary epithelium. *Am J Resp Crit Care Med*. 2015; 190: 1291 (Poster discussion) and Irish J Med Sc 2015; 184 (11): 521 (Oral presentation)
10. McDonnell MJ, Finch SC, Fardon TC, Rutherford RM, Chalmers JD. A comprehensive analysis of the impact of Pseudomonas aeruginosa colonisation in adult bronchiectasis. Irish J Med Sc 2015; 184 (11): 525 (Oral presentation)
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12. McDonnell MJ, Jones R, Crossfield G, Bourke SC, Forrest I, Simpson J, Rutherford RM, Griffin SM, Pearson JP, Ward C. Using questionnaires to measure the impact of gastro-esophageal reflux in chronic lung disease. *Am J Respir Crit Care Med*. 2014: 189: 1914 (Thematic poster)

13. McDonnell MJ, Chalmers JD, Goeminne P, Aliberti S, Lonni S, Davidson J, Poppelwell L, Salih W, Pesci A, Dupont LJ, Fardon TC, Hill AT, De Soyza A. International Validation of a Disease Specific Severity Index for Non-Cystic Fibrosis Bronchiectasis: The BSI Index. Chest. 2014; 145; 428 (Oral presentation) – *awarded Best Abstract Prize for Original Research, Chest World Congress)

Invited speaker at national and international conferences

- COPD and Beyond International Meeting, Barcelona, Spain. Bronchiectasis: Consigning it to History, April 2018.
- European Respiratory Society Postgraduate Course in Bronchiectasis, Milan, Italy. Immunology of Bronchiectasis, Sept 2017.
- Bronchiectasis World Congress, Milan, Italy. Age and Comorbidities in Bronchiectasis, July 2017.
- Irish Thoracic Society Annual Spring Meeting, Galway, Ireland. Phenotyping of Bronchiectasis, Mar 2017.
- British Thoracic Society Winter Meeting, London, UK, Highlights for Thorax Symposium - Multimodal Assessment of Severity in Bronchiectasis, Dec, 2016.
- Lung Forum, Danish International Respiratory Meeting, Copenhagen, Denmark. Microbiology of Bronchiectasis, Oct 2016.
- British Thoracic Society Bronchiectasis Short Course. London, UK. Gastroesophageal reflux in Bronchiectasis: Diagnosis and Management, Oct 2016.
- European Respiratory Society Clinical Highlights Meeting, Dublin, Ireland. Bronchiectasis: A Clinical Update, Nov 2014.
- British Lung Foundation, North East Bronchiectasis Research Interest Network Group (NEBRING), Lumley Castle, Durham, UK, Oct 2014.

Awards relating to PhD

- Newcastle University Travel Award, European Respiratory Society Meeting, Paris, Sept 2018
- American Thoracic Society Best Abstract Award for Pulmonary Infections and Tuberculosis, San Diego, May 2018
- William Stokes Trainee Research Award Finalist, Royal College of Physicians of Ireland, Dublin, Ireland, Oct 2016
- Research Medal for Recognition of Outstanding Research, Galway University Hospitals Medical Board, Galway, Ireland, June 2016
- Irish Thoracic Society Travel Award, European Respiratory Society Meeting, London, Sept 2016
- Original Research Oral Presentation Prize, Chest World Congress, Madrid, Spain, Mar 2014

Publications arising from other projects during timescale of PhD

1. Gardner A, Mavin E, Vernon B, McDonnell MJ, Ward C, Brodlie M. Recombinant acid ceramidase reduces inflammation and infection in cystic fibrosis. *Am J Respir Crit Care Med*, accepted May 2020.
2. Chalmers JD, Laska IF, Franssen FME, Janssens W, Pavord I, Rigau D, McDonnell MJ, Roche N, Sin DD, Stolz D, Suissa S, Wedzicha J, Miravitlles M. Withdrawal of Inhaled Corticosteroids in Chronic Obstructive Pulmonary Disease: A European Respiratory Society Guideline. *Eur Respir J*, accepted May 2020.
3. Brennan M, McDonnell MJ, Ward C, Alamer A, Duignan N, Rutherford RM. Bronchiectasis in the elderly—a disease that has not gone away. *Curr Geri Rep*. 2020.
4. Kefala AM, Fortescue R, Alimani GS, Kanavidis P, McDonnell MJ, Magiorkinis E, Megremis S, Paraskevis D, Voyiatzaki C, Mathioudakis GA, Papageorgiou E, Papadopoulos NG, Vestbo J, Beloukas A, Mathioudakis AG. Prevalence and clinical implications of respiratory viruses in stable chronic obstructive pulmonary disease (COPD) and exacerbations: A systematic review and meta-analysis protocol. *BMJ Open* 2020; 10:e035640.
5. Morris-Rosendahl DJ, Edwards M, McDonnell MJ, John S, Alton EW, Davies JC, Simmonds NJ. Whole Gene Sequencing of CFTR Reveals a High Prevalence of the Intronic Variant c.3874-4522A>G in Cystic Fibrosis. *Am J Respir Crit Care Med.* 2020; 201 (11): 1438-1441.
6. Lahousse L, Bahmer T, Cuevas-Ocaña S, Flajolet P, Mathioudakis AG, McDonnell MJ, Uller L, Schleich F, Dortas Junior S, Idzko M, Singh D, Ricciardolo FLM, Adcock IM, Usmani O, Spanevello A, Bonvini SJ. ERS International Congress, Madrid, 2019: highlights from the Airway Diseases, Asthma and COPD Assembly. *ERJ Open Res.* 2020; 6 (1):00341-2019.

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10. Mokoka MC, McDonnell MJ, MacHale E, Cushen B, Boland F, Cormican S, Doherty C, Doyle F, Costello RW. Inadequate assessment of adherence to maintenance medication leads to loss of power and increased costs in trials of severe asthma therapy. Results from a systematic literature review and modelling study. *Eur Respir J* 2019; 18; 53(5).
11. Spencer S, Donovan T, Chalmers JD, Mathioudakis AG, McDonnell MJ, Tsang A, Pilkington G. Intermittent prophylactic antibiotics for bronchiectasis protocol. *Cochrane Database of Systematic Reviews* 2019, CD01324.
12. Schleich F, Bikov A, McDonnell MJ, Mathioudakis AG, Andersson C, Bonini M, Uller L, Idzko M, Singh D, Lopez-Campos JL, Bossios A, Adcock IM, Usmani O, Spanevello A, Bonvini SJ. Airway Disease: Research highlights from the 2018 ERS International Congress. *ERJ Open Res*. 2019 (5): 00225-2018.
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23. Duignan N, McDonnell MJ, Mokoka MC, Rutherford RM. High prevalence of stress urinary incontinence in adult patients with bronchiectasis. *Ir Med J.* 2016 Aug 8;109(7):440.
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