THE UNIVERSITY
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The lectin pathway of complement and bacterial colonisation in bronchiectasis

James Chalmers

A thesis submitted for the degree of Doctor of Philosophy

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Dedicated to my parents, Scott and Janet Chalmers.

Declaration

I declare that this thesis has been composed solely by myself and has not been previously submitted for a higher degree. All work presented in this thesis was performed by myself. Where others have contributed to the work, this has been appropriately acknowledged.

Abstract

Bronchiectasis is a chronic inflammatory lung disease associated with failure of the normal mucociliary escalator, chronic bacterial colonisation of the airways, neutrophil mediated inflammation and a resulting clinical syndrome of respiratory infections, lung damage and symptoms such as cough, sputum production and shortness of breath. These are few effective treatments and the cause of bronchiectasis is unknown in the majority of patients. It is hypothesised that unrecognised immune defects may predispose to bronchiectasis or affect the severity of lung disease.

Ficolin-2 is a circulating innate immune protein able to activate the lectin pathway of complement through interaction with mannose binding lectin associated serine protease-2. Through its structural and functional similarity to complement component C1q and mannose binding lectin, it is hypothesised that ficolin-2 may be involved in opsonophagocytosis of pathogens. A number of single nucleotide polymorphisms in the ficolin-2 gene have been described causing considerable variation in human ficolin-2 serum concentrations in healthy individuals.

In this thesis, the role of the key lectin pathway components ficolin-2 and mannose binding lectin, are investigated in patients with bronchiectasis.

We demonstrate a significant association between single nucleotide polymorphisms in the *FCN2* gene and disease severity in bronchiectasis. Specifically, patients with low expressing *FCN2* haplotypes have a higher frequency of chronic colonisation, colonisation with *P. aeruginosa*, more frequent exacerbations and worse health related quality of life. An association between MBL deficient genotypes and disease

severity is also demonstrated suggesting an important role for the lectin pathway of complement in modifying disease severity in bronchiectasis.

In-vitro studies identify that ficolin-2 is the major lectin pathway component responsible for complement activation on *P. aeruginosa* and that ficolin-2 binds to a wide range of clinically relevant pathogens.

Neutrophils isolated from the sputum of patients with bronchiectasis showed significant alterations in surface receptor expression and function compared to peripheral blood neutrophils, with a novel effect of neutrophil elastase cleavage of CD88 contributing to reduced phagocytosis by airway neutrophils. Despite loss of phagocytic receptors from sputum neutrophils, opsonisation by ficolin-2/MASP-2 complexes still enhanced phagocytosis of *P. aeruginosa* by sputum neutrophils, suggesting that ficolin-2 may be relevant in the clearance of *P. aeruginosa* in the airway.

In summary, ficolin-2 was found to be an important modifier of disease severity in bronchiectasis.

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Chapter 5 of the thesis includes analysis on samples from a randomised controlled trial performed by Dr Maeve Smith. This study was supported by the hard work of the bronchiectasis nurses, Kim Turnbull and Shelly MacQuarrie.

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Abbreviations

ABPA: allergic bronchopulmonary aspergillosis
ANOVA: analysis of variance
AOR: adjusted odds ratio
APC: Allophycocyanin
ASL: airway surface liquid
ATCC: American Type Culture Collection
BAL: bronchoalveolar lavage
BMI: body mass index
BSA: bovine serum albumin
CF: cystic fibrosis
CFU: colony forming units
CFTR- Cystic fibrosis transmembrane regulator
CHO: Chinese hamster ovary
CI- confidence interval
COPD: chronic obstructive pulmonary disease
CR1: complement receptor 1
CR3: complement receptor 3
CRP: C-reactive protein
CT: computed tomography
CVID: common variable immunodeficiency
CysNAc: N-acetylcysteine
DHR: dihydrorhodamine
DLCO: Diffusing capacity of carbon monoxide.
DMEM: Dulbecco's modified eagle medium
DMSO: Dimethyl sulfoxide
DTT: dithiothreitol
ECL: enhanced chemiluminescence reagent
EDTA: ethylenediaminetetraacetic acid
EGTA: ethylene glycol tetraacetic acid
ESR: erythrocyte sedimentation rate
ELISA: enzyme linked immunosorbant assay
ENaC: amiloride-sensitive epithelial sodium channe
FACS: fluorescence activated cell sorting
FCS: fetal calf serum
FEF: forced expiratory flow
FEV_1: forced expiratory volume in 1 second
FITC: Fluorescein isothiocyanate
fMLP: N-formyl-methionine-leucine-phenylalanine
FVC: forced vital capacity
GH: growth hormone
GlcNAc: N-acetylglucosamine
GM-CSF: granulocyte colony stimulating factor
HNP: human neutrophil peptides
HRCT: High resolution computed tomography
HRP: horseradish peroxidase

HSA: human serum albumin
ICAM-1: intercellular adhesion molecule-1
ICAM-2: intercellular adhesion molecule-2
Ig: immunoglobulin
IgA: immunoglobulin A
IPTG: isopropyl ß-D-thiogalactoside
IQR: interquartile range
IV- inverse variance
LB: Luria-Bertani
LCQ: Leicester cough questionnaire
LTB4: leukotriene B4
MASP-1: MBL associated serine protease-1
MASP-2: MBL associated serine protease-2
MBL: mannose binding lectin
MeoSAAPvN: substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide
MeOSu-AAPV-CMK: methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone
MH- Mantel-Haenszel
MMP: matrix metalloproteinase
MPO: myeloperoxidase
NETS: neutrophil extracellular traps
OR- odds ratio
ORF: open reading frame
PAF: platelet activating factor
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PE: Phycoerythrin
PI: propidium iodide
PPM: potentially pathogenic microorganisms
RNA: ribonucleic acid
RORγt: RAR related orphan receptor gamma
ROS: reactive oxygen species
RT-PCR: reverse transcription polymerase chain reaction
RV: residual volume
SCV: small colony variant
SD: standard deviation
SDS-PAGE: Sodium dodececyl-sulphate polyacrylamide gel electrophoresis
SE: standard error
SGRQ: St Georges Respiratory Questionnare
SLE: systemic lupus erythematosus
SLPI: secretory leukocyte protease inhibitor
SNP: single nucleotide polymorphisms
SPS: sodium polyanethole sulfonate
TAP: transporter antigen peptide
TBS: tris-buffered saline
TEMED: Tetramethylethylenediamine
TGF-β: transforming growth factor β
TLC: total lung capacity
TMB: 3,3',5,5'-Tetramethylbenzidine

TNF-α: tumour necrosis factor alpha
QoL: quality of life
VCAM-1: Vascular cell adhesion molecule
VDBP: vitamin-D binding protein
VEGF: Vascular endothelial growth factor

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Chapter 1

Introduction

1.1 Bronchiectasis

1.1.1 Background

Bronchiectasis is a chronic lung disorder characterised by recurrent cough, sputum production and recurrent respiratory infections (Murray, 2009). Pathologically, there is permanent dilatation of one or more bronchi with failure of the normal mucociliary escalator, leading to chronic airway inflammation and, in turn, chronic bacterial colonisation (Laennec, 1834).

Estimates of the prevalence of bronchiectasis vary, data from the United States reported a frequency of 52/100,000, from which it has been estimated there are a minimum of 30,000 patients with the disease in the UK (Weycker et al, 2005, DeSoyza et al, 2012). The prevalence increases with age (Weycker et al, 2005) and this is likely to be a significant underestimate of the true burden of the disease. For example 170 patients are followed up in secondary care for bronchiectasis at Ninewells Hospital, Dundee, which has a catchment population of 149,000 (General Register Office, Scotland) and approximately 800 patients are followed up in secondary care at the Royal Infirmary of Edinburgh with the population of NHS Lothian being 826,231 (General Register Office, Scotland). Given that only the most severe patients with bronchiectasis are typically followed-up in secondary care (Pasteur 2010) this gives an estimated prevalence of 97-114/100,000 for significant bronchiectasis followed up in secondary care, with the prevalence of less severe bronchiectasis likely to be much higher. Bronchiectasis may be underrecognised and underdiagnosed. O'Brien et al studied a cohort of 110 patients with a primary care

diagnosis of chronic obstructive pulmonary disease, performing high resolution computed tomography scanning and lung function testing. They identified bronchiectasis in 29% of patients, suggesting a high prevalence of bronchiectasis among COPD patients but also suggesting a degree of misdiagnosis as 19% of patients in this study had never smoked (O'Brien et al, 2000). Increasing awareness of the disease and improved diagnosis due to advances in computed tomography technology are likely to increase the reported prevalence. Recent data from the United States Medicare database found an annual increase in claims for bronchiectasis of 8.7% between 2000 and 2007 (Seitz et al, 2012). The rate of hospitalisations for bronchiectasis also increased significantly between 1993 and 2006, emphasising the growing recognition of this disease (Sietz et al, 2010).

Although in severe cases the appearances may be detected by chest x-ray (figure 1.1) radiologically, bronchiectasis is now defined by a characteristic appearance of bronchial dilatation on high-resolution chest computed tomography. In health, the overall diameter of a bronchus is approximately equal at any given level to its adjacent pulmonary artery, and a ratio of bronchus diameter : vascular diameter > 1 denotes abnormal dilatation. Cylindrical/tubular bronchiectasis is the most common subtype, but varicose and cystic bronchiectasis are also seen. Examples of cyclindrical, varicose and cystic bronchiectasis are shown in figures 1.2-1.4.

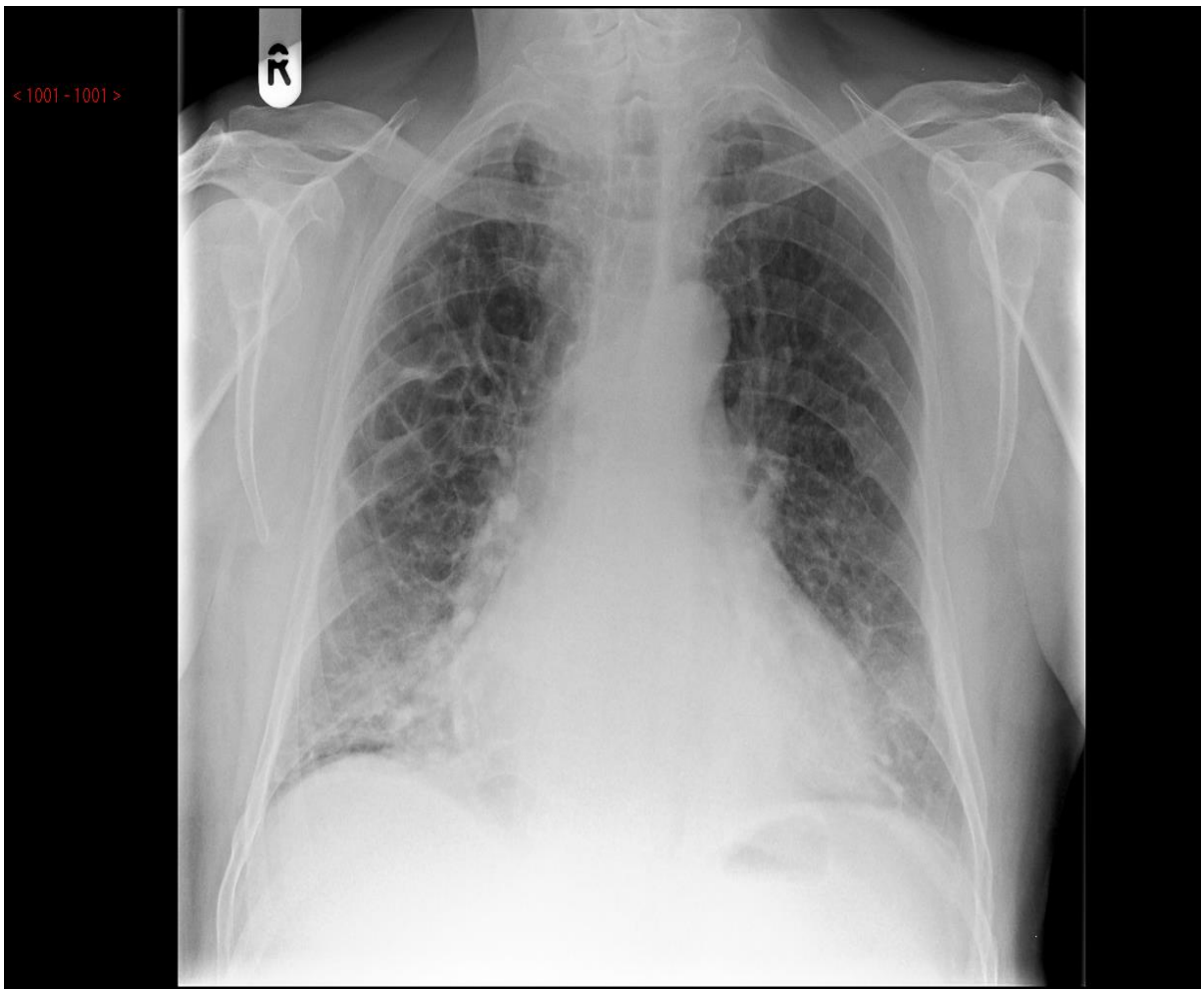

Figure 1.1 Chest radiograph from a 70 year old gentleman with severe bronchiectasis. The chest x-ray shows multiple thin walled cysts in the right upper lobe some of which contain fluid. There is peribronchial thickening in the right and left lower lobes. Incidentally there is slight cardiomegaly and old left sided rib fractures. The appearances are consistent with very severe cystic bronchiectasis.

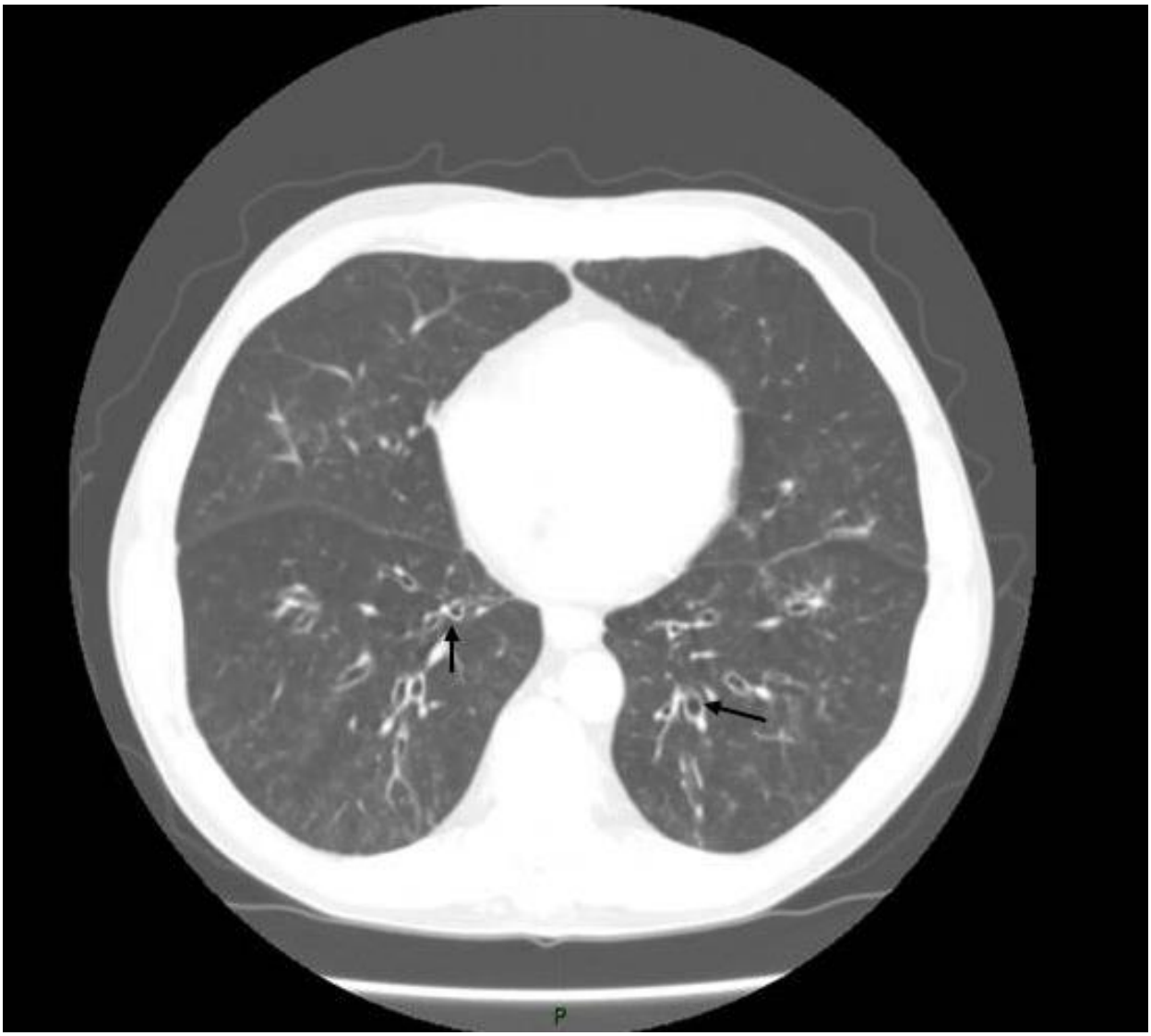

Figure 1.2. Chest Computed Tomography (CT) scan showing mild tubular bronchiectasis in both lower lobes (arrows show two examples where the diameter of the bronchus is larger than the adjacent vessel).

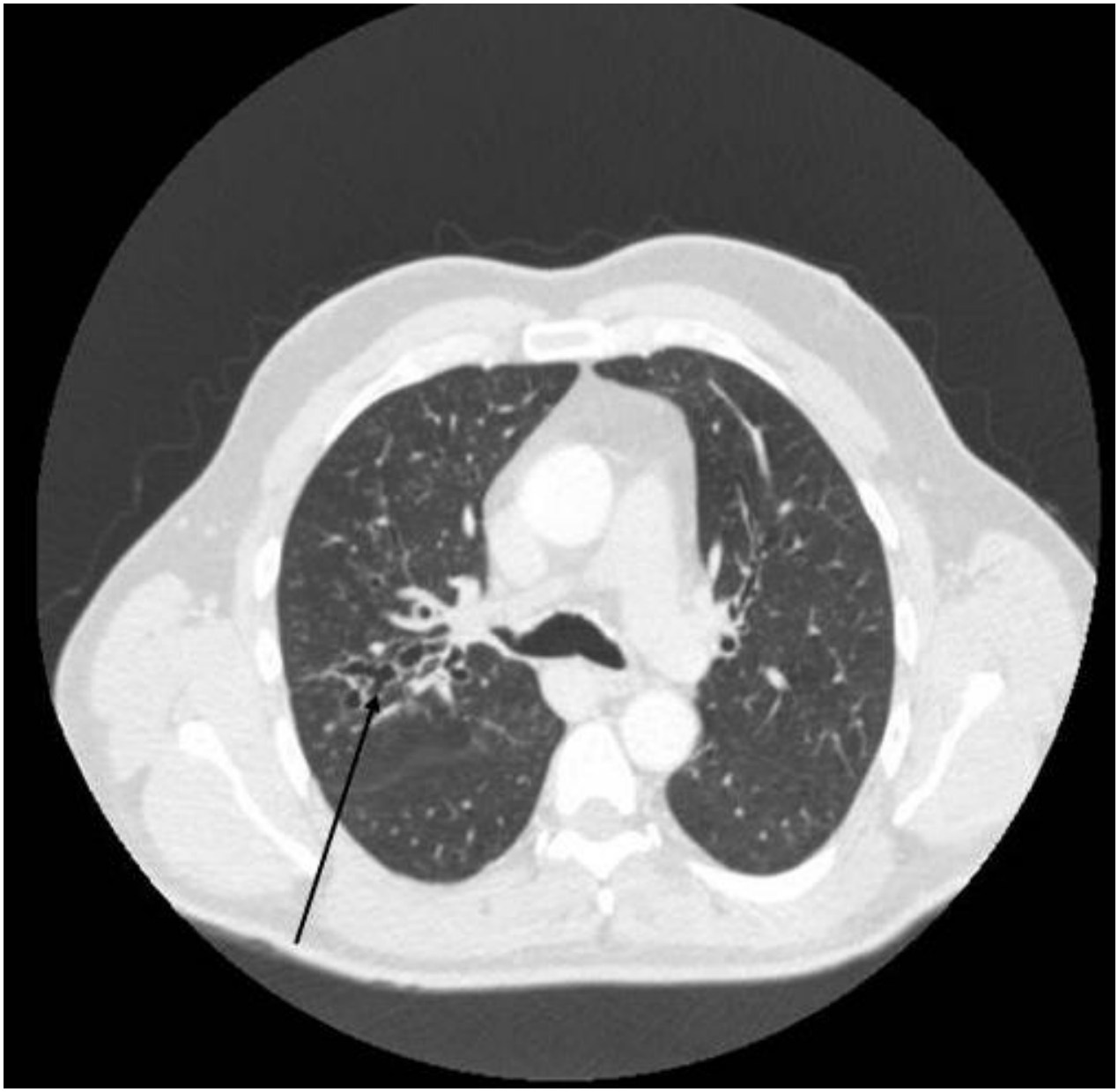

Figure 1.3. Chest CT scan showing varicose bronchiectasis in the right upper lobe- see arrow- the bronchus is enlarged and saccular.

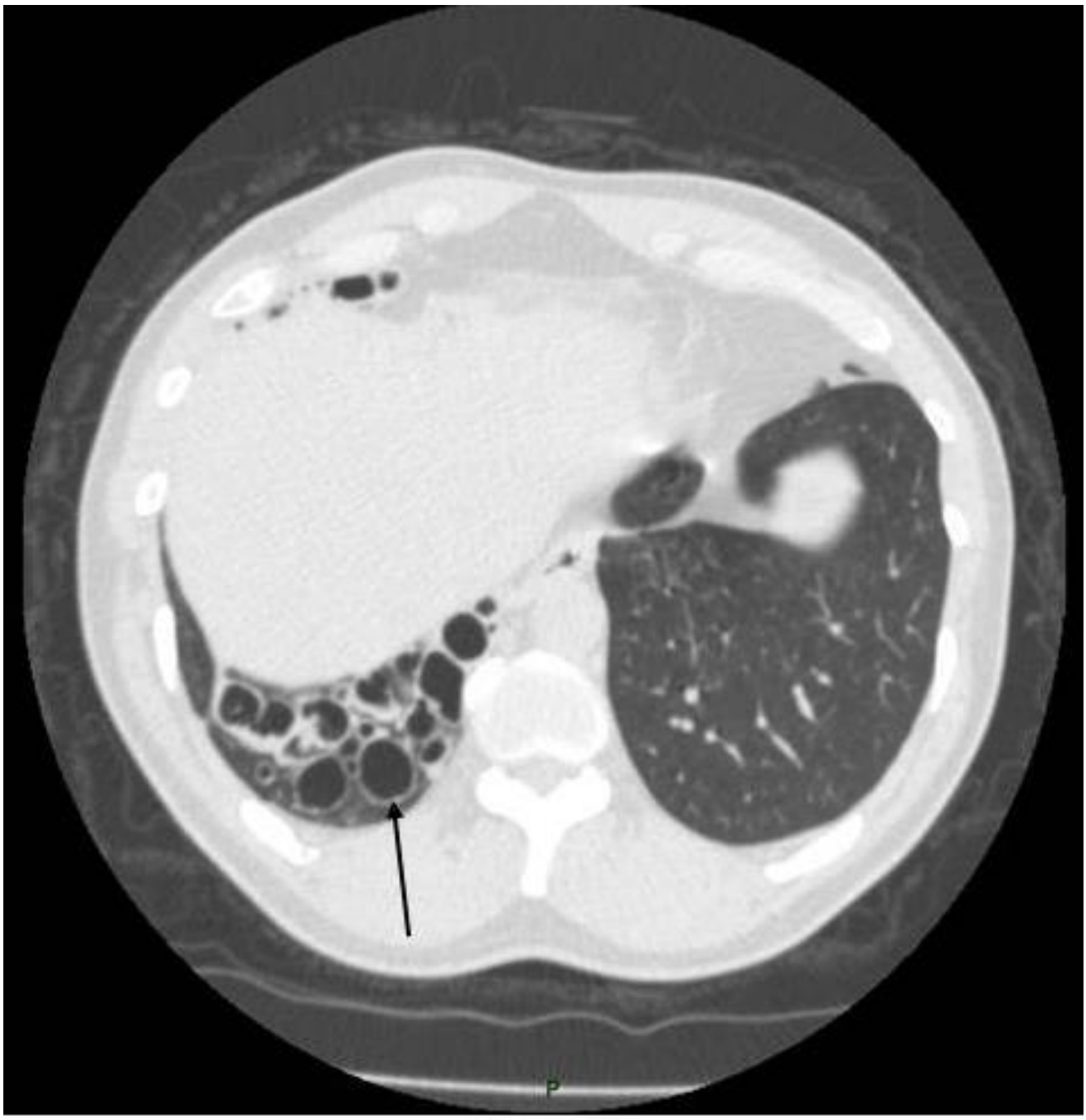

Figure 1.4. Chest CT scan showing severe cystic bronchiectasis in the right lower lobe. The arrow highlights one of the cysts.

Other radiological features associated with bronchiectasis include bronchial wall thickening, mucous plugging, mosaic attenuation , atelectasis, subsegmental, segmental or lobar collapse and emphysema (Kang et al 1995, Reiff et al 1995, McGuiness et al 2002). There is a relationship between the severity of bronchiectasis on CT scanning and the severity of clinical disease, although analysis of this is limited due to different scanning techniques and scoring systems (Lynch et al, 1999). There is no established scoring system for use in bronchiectasis and most studies have used scores validated in cystic fibrosis (Bhalla et al, 1991).

1.1.2 The clinical syndrome of bronchiectasis

The characteristic symptoms of bronchiectasis are cough and sputum production (Nicotra, 1995). Sputum may be mucoid, mucopurulent or purulent and the degree of purulence correlates with clinical markers of disease severity such as lung function, HRCT scanning and quality of life (Murray et al, 2009). Patients may also report dyspnoea (Smith et al, 1996), haemoptysis (Nicotra et al, 1995), chest pain (Munro et al, 1989, King et al 2012), fever, fatigue (Hester et al, 2012) and malaise. Patients may suffer recurrent respiratory tract infections, and this is a common reason for referral to secondary care. Bronchiectasis has a significant effect on health related quality of life, with mean total scores for the St.Georges Respiratory Questionnaire ranging from 36 (Chan et al, 2002), 41.1 (Murray et al, 2009), 44.4 (Wilson et al, 1997) to 45.5 (Martinez-Garcia et al, 2005) in clinically stable patients. These values are similar to those observed in other serious respiratory diseases such as moderate to

severe COPD (Tashkin et al, 2008) or idiopathic pulmonary fibrosis (Raghu et al, 2010).

Lung function impairment is variable in adults with bronchiectasis. Patients may have airflow obstruction, restriction or may have normal spirometry values. (Bahous et al,1984). Airflow obstruction is the most common pattern and is associated with more severe disease (Pande et al 1971, Bahous et al 1984, Roberts et al 2000). A lower forced expiratory volume in one second is associated with the severity of breathlessness measured by the Medical Research Council dyspnoea scale and is also associated with extent of disease on HRCT (Roberts et al, 2000). Forced vital capacity may be normal or reduced. Forced expiratory flow$_{25-75}$ (FEF_{25-75}) may be reduced and the RV/TLC ratio maybe increased suggestive of small airways disease and air trapping (Bahous et al 1984, Stockley et al 1984,Koulouris et al 2003). The response to bronchodilators is variable (Hill and Stockley 1986, Hassan et al 1999) between 1/3 and ¾ of patients with bronchiectasis may have a response to bronchial challenge testing with methacholine or histamine. (Bahous et al 1984, Pang et al 1989)

An exacerbation of bronchiectasis is defined as deterioration in a patient with known bronchiectasis from their stable condition, associated with increased cough, sputum production, increased sputum purulence or dyspnoea/wheeze (Pasteur et al, 2010). In addition to these subjective symptoms, patients may have objective evidence of reduction in forced expired volume in 1 second (FEV_1) or a reduction in exercise capacity. The frequency of exacerbation varies substantially in the literature depending on the severity of the cohort studied, from a mean of 2 to 5 exacerbations per year (Tsang et al 1999, Wilson et al 1998). Severe exacerbations may require

hospital admission and the British Thoracic Society have proposed guidelines recommending inpatient management of exacerbations for patients with cyanosis or confusion, breathlessness with respiratory rate $\geq$ 25/min, circulatory failure, respiratory failure, temperature $\geq 38^{O}C$, inability to take oral therapy, inability to cope at home and failure of oral antibiotic therapy (Pasteur et al, 2010).

1.1.3 The vicious cycle hypothesis

Originally proposed by Cole, the vicious cycle hypothesis is central to our current understanding of bronchiectasis (Cole, 1986). Airway structural damage, regardless of the initial insult or immunodeficiency that causes it, permits increasing bacterial colonisation of the lower airway (Chmiel et al, 2003, Charlston et al, 2011). A dysregulated immune response then occurs that fails to clear the initial infection but causes further airway structural damage through multiple mechanisms (Smith, 2011). This cycle repeats itself leading to disease progression. The concept is summarised in figure 1.5

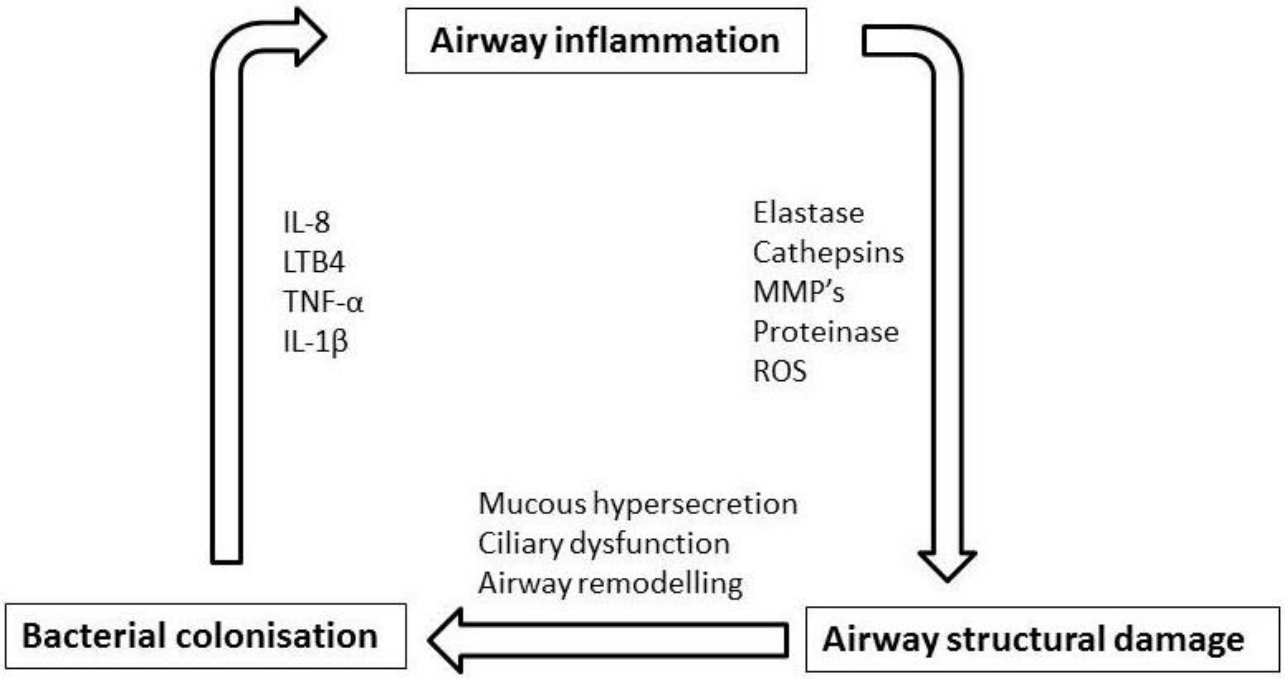

Figure 1.5. The vicious cycle hypothesis of bronchiectasis. Abbreviations IL-8=interleukin-8, LTB4= leukotriene B4, TNF-α= tumour necrosis factor α, IL-1β= interleukin-1β, MMP=matrix metalloproteinases, ROS= reactive oxygen species.

Although a large body of data supports the vicious cycle hypothesis, there remain areas that are poorly understood. It appears that significant inflammation and disease progression may occur in some patients in the absence of bacterial infection (Angrill et al, 2001). In addition, implicit in the vicious cycle hypothesis is the idea that clearance of bacterial infection or an anti-inflammatory strategy will be effective by 'breaking the cycle'. The relatively disappointing results to date with anti-inflammatory agents such as inhaled corticosteroids (Kapur et al, 2009) and with long term antibiotics (Evans et al, 2007, Tsang et al, 1999), leave this final question unresolved.

If incorrect, the vicious cycle hypothesis may hamper our understanding of bronchiectasis by trapping researchers in the idea that neutrophilic inflammation is intrinsically harmful and that suppression of inflammatory responses is the primary goal of therapy. Neither of these statements has been convincingly proven and indeed there is some data to suggest they may be incorrect. For example in cystic fibrosis, if the vicious cycle hypothesis were true, a 24 week placebo controlled phase II trial of a Leukotriene B4 receptor antagonist (BIIL284) would have been expected to produce benefit by reducing neutrophil recruitment. In reality, the trial resulted in increase exacerbations and hospital admissions in cytic fibrosis (Konstan et al, 2005), an effective that subsequent mechanistic work demonstrated may be due to a critical role of neutrophils in preventing bacterial proliferation and bacteraemia (Doring et al 2013). Therefore "breaking the cycle" is not inherently beneficial and indeed there is even evidence that future treatment of chronic lung disease may involve augmenting neutrophil responses. In a recent study, CFTR null and wild-type mice were chronically infected with multi-drug resistant *Pseudomonas aeruginosa*

and subsequently treated with recombinant pentraxin-3 (PTX3), a molecule that promotes neutrophil inflammation and phagocytosis. After treatment with PTX3, a significant reduction in colony forming units was seen to a level even below that of the normal mice. Furthermore, in whole blood assay, preopsonisation of *P. aeruginosa* with recombinant PTX3 significantly increased internalization by CF neutrophils and decreased the number of surviving bacteria inside the neutrophils (Paroni et al 2013).

1.1.4 Disorders associated with bronchiectasis

The most frequent disorders associated with bronchiectasis identified by studies in specialist centres are shown in Table 1.1. In a significant proportion of cases in adults, a single underlying cause cannot be identified. The disease is said to be post-infective where the patient gives a history of a previous severe infection such as pneumonia or pertussis in childhood (Nicotra et al, 1995, Pasteur et al, 2000, Kelly et al, 2003). Viral infections such as adenoviral pneumonia (Wynn-Williams 1953, Lanning et al 1980), measles (Kaschula, 1983), influenza (Laraya Cuasay et al, 1977) and respiratory syncytial virus (Massie and Armstrong, 1999) have also been associated with the development of bronchiectasis, often many years after the initial insult (Lanning et al, 1980). Determining whether a previous infection is directly related to the diagnosis of bronchiectasis is challenging, particularly when there is a long time interval between the infection and the onset of symptoms. Mycobacterial disease is an important infectious cause of bronchiectasis. Pulmonary *Mycobacterium tuberculosis* infection is implicated in around 15% of cases in

secondary care in the UK (Kelly, 2003) with the frequency likely to be much higher in areas where *M. tuberculosis* is endemic. Atypical *Mycobacteria* are also associated with bronchiectasis. Like all chronic lung diseases, bronchiectasis may be complicated by atypical mycobacterial infection. This has not been reported frequently in the UK, but appears to be very common in the United States, with rates of 23% isolated in sputum in one study (Nicotra et al, 1995) and a reported prevalence of 35% in the multicentre bronchiectasis research register in the United States (O'Connell et al, 2010). Why a small proportion of patients developing bacteria or viral infections develop bronchiectasis as a complication is unclear.

Bronchiectasis is associated with a number of inherited, acquired, infectious, allergic and autoimmune disorders (Table 1.1) (Nicotra et al, 1995, Pasteur et al, 2000, Kelly et al, 2003).

Underlying disorder	Frequency	Associations and clinical features
Idiopathic	30-53%	
History of severe infection	33-42%	Tuberculosis Pneumonia Pertussis Atypical mycobacteria
Connective tissue diseases	3-6%	Rheumatoid arthritis Systemic sclerosis SLE Ankylosing spondylitis
Allergic bronchopulmonary aspergillosis	1-7%	Associated with asthma
Immunodeficiency	1-8%	Common variable immunodeficiency X-linked agammaglobulinaemia IgA deficiency Specific antibody deficiencies
Cystic fibrosis	2-4%	Atypical cases may be diagnosed in adulthood.
Recurrent aspiration or inhaled foreign body	2-4%	A history of this may be absent
Primary ciliary dyskinesia	1-2%	Often associated with upper respiratory tract symptoms
Inflammatory bowel disease	1-2%	Ulcerative colitis Crohn's disease
Congenital airway structural abnormality	<1%	e.g Mounier-Kuhn syndrome

Table 1.1 Underlying causes of bronchiectasis in adult cohorts **(**Nicotra et al 1995, Pasteur et al, 2000, Kelly et al, 2003, Pasteur et al, 2010**).**

Almost all immunological deficiencies can be associated with bronchiectasis but common variable immunodeficiency, X-linked agammaglobulinaemia and IgA deficiency are the most frequently recognised of these disorders (Notarangelo et al, 2007). Although immunological defects may be diagnosed in childhood, they are increasingly recognised in adult patients with bronchiectasis (Pasteur et al, 2010). Such defects are reported to account for only 1-8% of cases of adult bronchiectasis, but even in specialist units, investigations are often limited to serum

immunoglobulins, protein electrophoresis and IgG subclass measurements (Pasteur et al, 2010, Pasteur et al, 2012). Although most research has focussed on primary immunodeficiencies, it is well recognised that bronchiectasis can complicate secondary immunodeficiency such as in leukaemia, lymphomas and human immunodeficiency virus infection (Kearney et al 1977, Knowles et al 1980, McGuinness et al 1993). It is presumed that a proportion of patients with "idiopathic" bronchiectasis have unrecognised immunodeficiency.

Specific treatments are recommended for patients with certain underlying disorders such as antibody deficiencies (immunoglobulin replacement) and allergic bronchopulmonary aspergillosis (corticosteroids and anti-fungals). Although not listed currently as a recognised underlying cause (Pasteur, 2010), bronchiectasis appears to be very common in chronic obstructive pulmonary disease. Martinez-Garcia et al studied 92 consecutive patients with moderate to severe COPD with HRCT scans and found a prevalence of bronchiectasis of 57.6%. Bronchiectasis in COPD was associated with more severe airflow obstruction, more positive sputum bacteriology and more frequent exacerbations (Martinez-Garcia et al, 2011). In the London COPD cohort, a study of 54 patients found a prevalence of bronchiectasis of 50%, with bronchiectasis associated with bacterial colonisation and airway inflammation. There was an associated with exacerbation severity but not exacerbation frequency (Patel et al, 2004). Not all studies have found such a high prevalence however, in the multinational Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) cohort, including 2,164 subjects, the prevalence of bronchiectasis was only 4% (Agusti et al, 2010). It seems likely that bronchiectasis associated with COPD will be recognised as a distinct subgroup.

Chronic asthma may also be a cause of bronchiectasis, although no studies have comprehensive excluded other possible causes in these patients (Paganin et al, 1996). The British Thoracic Society recommend that asthma should be considered as an underlying cause where no other cause can be found (Pasteur et al, 2010).

The vicious cycle concept can be adapted to illustrate the possible underlying causes in terms of their likely pathophysiology, figure 1.6. This is likely to be a gross over-simplification.

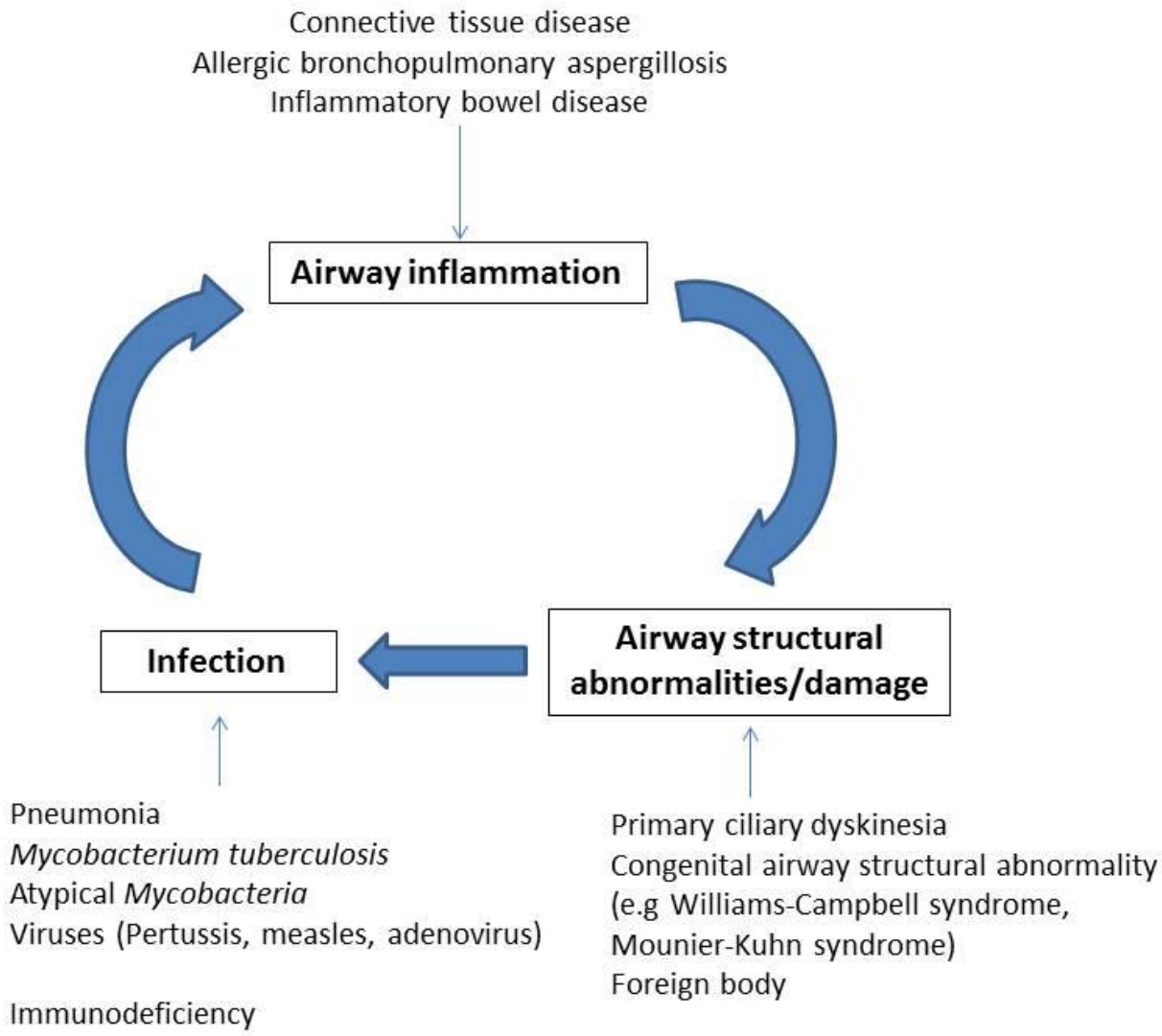

Figure 1.6. Underlying causes of bronchiectasis illustrated in terms of the "vicious cycle" concept.

1.1.5 Bacteria as drivers of airway inflammation and remodelling

Bacterial infection drives the vicious cycle of bronchiectasis. The most frequently isolated organisms in patients with non-CF bronchiectasis are *Haemophilus influenzae, P. aeruginosa, Moraxella catarrhalis, Streptococcus pneumoniae* and *S. aureus* (Pasteur 2010, Angrill, 2001, Angrill, 2002). Less frequently isolated pathogens include *Aspergillus* species, enteric gram negative organsisms, methicillin resistant *S. aureus* and atypical mycobacteria as has been discussed previously. The frequency of pathogens isolated in selected studies of adults with bronchiectasis shown in Table 1.2.

Author	Year	Country	N	Age	Method	Hi	PA	Sa	Sp	Mc	Asp	Myco	NP
Nicotra	1995	USA	123	57	Sputum	30	31	7	11	2	5	23	23
Evans	1996	UK	135	-	Sputum	ND	12	ND	ND	ND	ND	ND	ND
Cabello	1997	Spain	17	57	BAL	35	5	-	-	-	-	ND	60
Wilson	1997	UK	87	54	Sputum	20	25	-	-	-	-	-	38
Pasteur	2000	UK	150	53	Sputum	35	31	14	13	20	2	ND	5
Angrill	2001	Spain	49	57	BAL	26	20	-	2	-	-	ND	28
Palwatwichai	2002	Thailand	50	58	Sputum	14	20	-	6	4	-	6	36
Angrill	2002	Spain	42	58	Sputum	26	9	-	14	5	2	0	60
Angrill	2002	Spain	59	58	BAL	32	10	3	7	-	-	0	32
Kelly	2003	UK	100	57	Sputum	54	21	8	16	20	ND	ND	-
Tsang	2005	Hong Kong	86	58	Sputum	11	27	-	-	-	-	-	60
King	2007	Australia	89	57	Sputum	47	12	4	7	8	2	2	21
Garcia	2007	Spain	76	70	Sputum	18	20	-	-	-	-	-	-
O'Connell	2010	USA	230	-	Sputum	-	31	9	-	-	-	35	87
Wong	2012	N.Zealand	141	60	Sputum	28	12	3	3	4	-	-	-
Goeminne	2012	Belgium	479	67	Sputum	31	30	23	20	15	20	-	-
King	2012	Australia	178	58	Sputum	35	23	-	-	-	-	-	28
Chalmers	2013	UK	470	65	Sputum	30	15	9	6	12	ND	ND	23

Table 1.2. The frequency of bacterial isolates in adult patients with non-CF bronchiectasis. Adapated from Pasteur 2010. ND= not done. Dashes indicate data not reported. Some percentages may add up to more than 100% due to patients culturing more than one organism.

1.1.6 *Haemophilus influenzae*

H.influenzae is a Gram-negative rod shaped bacterium typically found as a commensal of the upper respiratory tract. *H. influenzae* is the most frequent pathogen causing chronic infection in non-CF bronchiectasis and chronic obstructive pulmonary disease. In cohorts of bronchiectasis patients, up to 50% will grow *H. influenzae* while clinically stable (Kelly et al, 2003), although the average across these studies is closer to 35% (Table 1.2). In contrast to *Pseudomonas aeruginosa*, there is little data about the impact of *H. influenzae* colonisation on clinical outcomes in bronchiectasis.

The mechanisms of chronic infection by *H. influenzae* are also less well studied than for other organism, but *H.influenzae* can survive intracellularly within macrophages and invade bronchial epithelial cells (Forsgren et al, 1994). *H.influenzae* therefore has both an intracellular and extracellular niche within the respiratory tract. King et al found evidence of an abnormal Th2 cytokine response to *H.influenzae* infection with reduce expression of CD40 ligand in bronchiectasis (King, 2003). The mechanism of this abnormal cytokine response has not been established. *H.influenzae* are also able to form biofilms to evade host immune responses.

1.1.7 *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous Gram-negative, aerobic coccobacillus found in soil, water, skin flora and many man made surfaces. *P. aeruginosa* is an opportunistic

human pathogen able to undergo diverse adapatations to permit chronic infection of the lower respiratory tract (Davies et al, 2009).

The phenotypic changes from acute to chronic *P.aeruginosa* infections have been extensively reviewed and include a reduction in invasive virulence factor expression such as flagellae, pili and toxins (Klausen et al, 2003). *P. aeruginosa* persistence is associated with transformation to a mucoid phenotype. Following a challenge, such as hypoxia, *Pseudomonas* strains expressing mucoid exopolysaccharide (alginate) are selected for and establish themselves within a protective environment (Li et al, 2005). The ability to form biofilms is central to survival of these organisms within the lung. Biofilms protect bacteria against clearance by the host immune system and also increase resistance to antibiotics (Bragonzi et al, 2009). Neutrophils have been shown to promote biofilm formation and once established within biofilms *Pseudomonas* becomes highly resistant to neutrophil phagocytosis (Parks et al, 2009). *Pseudomonas* within biofilms becomes non-motile, further protecting them from phagocytosis (Singh et al, 2000). Initiation of biofilm formation is dependent on the process of quorum sensing. As the number of bacteria within the lung increase, the concentrations of signalling molecules such as homoserine lactones increase. These diffuse freely into bacterial communities allowing organisms to sense the local population density. Once a critical mass of organisms is reached, quorum-sensing molecules induce gene expression that promotes biofilm formation (Amiel et al, 2010). The ability of *Pseudomonas* to adapt so rapidly and extensively to the lung is due to the hypermutable nature of these organisms. *Pseudomonas* can quickly express or inactivate genes, but also increases the frequency of mutation events within its genome to ensure survival in the face of multiple environmental (hypoxia,

ionic strength), immunological (neutrophils, complement), and antimicrobial challenges (Oliver et al, 2000).

In cystic fibrosis bronchiectasis, it is well established that *P.aeruginosa* colonisation leads to a more rapid deterioration in lung function and earlier mortality (Li et al, 2005). Consequently, eradication of *P. aeruginosa* is standard care in European Cystic fibrosis centres (Doring et al 2000, Ratjen et al 2010).

In non-cystic fibrosis bronchiectasis, the role of *P.aeruginosa* is less clearly established, with studies to date including relatively few patients. A recent study of 91 patients with 27 deaths suggested that patients culturing *P.aeruginosa* (n=20) were at an increased risk of long term mortality.(Loebinger et al, 2009) The same researchers previously reported in a cohort of 22 patients with *P.aeruginosa* colonisation that colonisation was associated with worse lung function, greater severity of lung disease and poorer quality of life (Davies et al, 2006). In this cohort, an association between *P.aeruginosa* colonisation and decline in FEV_1 and FVC over time was not found in contrast to the findings of Evans et al, who showed in 14 *P.aeruginosa* colonised patients that lung function deteriorated more rapidly (Evans et al, 1996). There are no controlled trials of *P. aeruginosa* eradication in non-CF bronchiectasis. The authors of each of these reports conclude that it is difficult to know if *P.aeruginosa* colonisation simply reflects a more severe disease, or whether *P.aeruginosa* really does impact prognosis in non-cystic fibrosis bronchiectasis directly.

1.1.8 *Staphylococcus aureus*

S.aureus is a major clinical problem in cystic fibrosis, particularly in children, and is also associated with ABPA or atypical CF in adults with bronchiectasis (Starner et al, 2006). Similar to *Pseudomonas*, *S.aureus* downregulates virulence factors during conversion to chronic infection and can form biofilms (Shah et al, 1999). The ability to form small colony variants (SCV's) is an important feature of *S.aureus* virulence. These variants are able to invade and persist within epithelial cells and are remarkably resistance to antibiotics (Shah et al, 1999, Kahl et al, 1998, Sandowska et al, 2002). Cystic fibrosis cells are more susceptible to invasion by SCVs and these variants are frequently isolated in CF (Mitchell et al, 2011). Their role in non-CF bronchiectasis is not known. Under anaerobic conditions, which can occur in poorly ventilated areas of the lung, *S.aureus* forms a polysaccharide intercellular adhesion that protects cells from neutrophil phagocytosis (Cramton et al, 2001). *S. aureus* is able to readily acquire resistance to antibiotics and the isolation of methicillin resistant *S. aureus*, while not common in non-CF bronchiectasis, is regarded as an indication for secondary care follow-up by the British Thoracic Society guidelines as it is difficult to treat and may be associated with a worse prognosis (Pasteur et al, 2010).

1.1.9. The relationship between bacterial infection and airway inflammation

In a study of patients with COPD, α-1-antitrypsin and bronchiectasis, Hill et al demonstrated a direct relationship between spontaneous sputum airway bacterial load and multiple markers of airway inflammation including myeloperoxidase, neutrophil

elastase, interleukin-8 and an inverse relationship with secretory leukocyte peptidase inhibitor (Hill et al, 2000). Similar results were shown by Angrill in a bronchoscopic study showing a direct relationship between bronchial colonisation, neutrophil count and airway inflammation (Angrill et al, 2001, Angrill et al, 2002). After initially disappointing data for long-term antibiotic therapies in bronchiectasis (Tsang et al, 2000), recent data with nebulised gentamicin among others suggests that bacterial clearance can reduce markers of inflammation and improve clinical outcome (Murray et al, 2010). This study randomised 27 patients to 12 months of nebulised gentamicin and 30 patients to nebulised 0.9% saline. 30.8% of patients infected with *P. aeruginosa* achieved eradication with gentamicin (3.7% with 0.9% nebulised saline). In patients that did not achieve eradication the study demonstrated a reduction in sputum bacterial load in the gentamicin group from 8.06 $\log_{10}$ cfu/ml at baseline to 6.17 $\log_{10}$ cfu/ml at the end of treatment in the gentamicin group. These changes in bacterial infection were associated with a significant reduction in sputum myeloperoxidase and neutrophil elastase activity (Murray et al, 2010). Improvements in clinical outcomes including the St.Georges respiratory questionnaire and Leicester cough questionnaire scores and a significant reduction in the frequency of exacerbations were also observed.

1.1.10 Other organisms

The frequencies of *S. pneumoniae, M. catarrhalis* and enteric Gram-negative organisms are variable but generally they account for a small minority of isolates (table 1.2). Bacterial persistence in bronchiectasis is the result of multiple inherited

and acquired immunological defects along with bacterial adaptations to promote chronic infection. Much of our understanding of the pathophysiology bronchiectasis is incomplete or is extrapolated from cystic fibrosis. A greater understanding of the mechanisms of immunological dysfunction and bacterial colonisation in bronchiectasis is essential for the development of new therapies.

1.1.11 Studies of immune function in bronchiectasis

1.1.12 The role of neutrophils

Bronchiectasis is believed to be primarily a neutrophil-driven disorder. Consistently neutrophils are found to be the most abundant cell type in bronchiectasis airway fluids (sputum and bronchoalveolar lavage) (Eller et al, 1994, Angrill et al, 2002). The number of neutrophils migrating to the airway is increased in stable patients and the numbers increase further with bacterial infection and exacerbation (Watt et al, 2004). This recruitment to the airway requires a number of co-ordinated processes that are highly active in patients with bronchiectasis.

1.1.13 Neutrophil recruitment and migration in bronchiectasis

Recruitment is driven by chemoattractants, with interleukin-8 (CXCL-8), Leukotriene B4, Interleukin-1 beta and tumour necrosis factor alpha believed to be of major importance (Mikami et al, 1998, Schleimer et al, 1991). Elevated levels of these pro-inflammatory cytokines have all been demonstrated in bronchiectasis airway secretions (Tsang et al, 1998, Stockley et al, 2000). Complement component

C5a is also a potent neutrophil chemoattractant but there is limited data to support a role for C5a in non-CF bronchiectasis (Fick et al, 1986).

Neutrophil detection of chemotactic stimuli leads to a co-ordinated process of cell signalling, cytoskeletal rearrangement and changes in surface receptor expression to facilitate migration.

Key to this process of transendothelial migration are the expression of the integrins CD11/CD18 on neutrophils and the expression of adhesion molecules on endothelial cells, principally intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the selectins (Downey et al, 2009, Cowburn et al, 2008). During the first stage of recruitment, rolling adhesion, upregulation of E-selectin and P-selectin on endothelial cells and an increase in the binding capacity of the leukocyte receptor L-selectin allow the neutrophil to adhere to and roll along the endothelial surface. During the second stage of this process, strong adhesion to the endothelial cell is achieved through binding of CD11/CD18 integrins present on the neutrophil to ICAM-1 and ICAM-2 (Bevilacqua et al, 1993). ICAM-2 is constitutively expressed on endothelial cells, but ICAM-1 expression is rapidly increased at sites of inflammation by the action of pro-inflammatory cytokines, principally tumour necrosis factor alpha but also Interleukin-1 beta (Yang et al, 2005). TNF-alpha and IL-1β are both elevated in the airway in patients with bronchiectasis (Fushillo rt al, 2008). Increased peripheral blood concentrations of ICAM-1, along with E-selectin and VCAM-1, have also been demonstrated in bronchiectasis patients (Zheng et al, 2000). During the process of neutrophil activation there is shedding of L-selectin and upregulation of CD11. Lower levels of CD11 have been shown on neutrophils from patients with cystic fibrosis during

exacerbations (Downey et al, 2007), but Pasteur et al found no abnormal values of CD11b in stable patients with bronchiectasis (Pasteur et al, 2000). Following strong adhesion, neutrophils migrate through the endothelial barrier, through the interstitium and finally through gaps between type I and type II pneumocytes to enter the alveolar lumen.

Up to 70% of transmigrating neutrophils cross the endothelial barrier by active engulfment by endothelial cells in a process that is dependent on the small GTPase RhoG (van Buul et al, 2007). Neutrophils are then able to migrate through the pericyte sheath and basement membrane using areas of low basement membrane density that appear to provide low resistance "tracks" for leukocyte recruitment (Wang et al, 2006).

Once neutrophils have migrated to the site of inflammation, they move towards a chemotactic stimulus along a concentration gradient until the rising concentration of chemoattractant serves to inhibit neutrophil migration and instead triggers its bacteriocidal functions (phagocytosis and degranulation). The chemotactic responses of bronchiectasis neutrophils appear to be intrinsically normal but the high levels of neutrophil chemoattractants in bronchiectasis sputum serve to enhance this process (Stockley et al, 1988). The process of neutrophil adhesion and migration to the lung is summarised in figure 1.7.

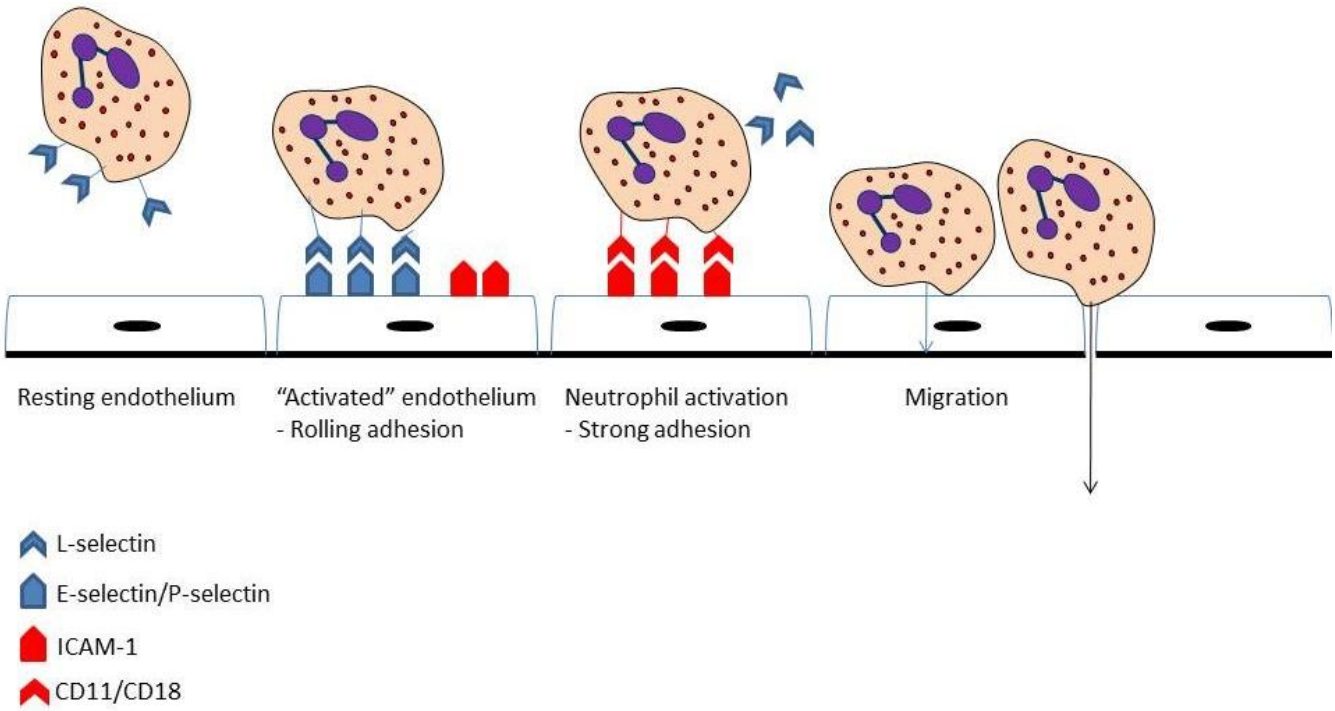

Figure 1.7. Neutrophil migration. The process begins with expression of E/P selectin on the surface of endothelial cells, followed by neutrophil activation with shedding of L-selectin and upregulation of CD11/CD18. CD11/CD18 binds to ICAM-1 leading to strong adhesion between neutrophils and endothelial cells. Neutrophils then migrate either by active engulfment by endothelial cells or at endothelial cell junctions. Abbreviations ICAM-1=intercellular adhesion molecule-1.

Increasing knowledge of the mechanisms of neutrophil migration may lead to targeted therapies in bronchiectasis. A phase II study of an anti-Interleukin-8 monoclonal antibody in COPD has been shown to be safe and was associated with symptomatic improvement (Mahler et al, 2004). Although monoclonal antibodies such as this are promising new treatments, anti-TNF-α therapy was not found to be effective in COPD (Rennard et al, 2007) or asthma (Wenzel et al, 2009), and was associated with a trend towards increased malignancy and pneumonia in the COPD trial. Similarly, as previously mentioned ,although LTB_4 is thought to be important in neutrophil recruitment in many chronic lung disorders, an LTB_4 receptor antagonist (BIIL284) result paradoxically increased respiratory exacerbations in phase II trial in cystic fibrosis (Konstan et al, 2005).

1.1.14 Neutrophil phagocytosis and killing

Phagocytosis is dependent on FcY and complement receptors, specifically FcγRIIIB (CD16), FcγRIIA (CD32), complement receptor 1 (CD35). and complement receptor 3 (CD11b/CD18). Fcγ receptors recognise phagocytic targets opsonised with IgG, while complement receptor 1 recognises the complement components C3b/C4b deposited on microorganisms and complement receptor 3 recognises complement component iC3b.

Ligation of phagocytic receptors initiates the process by which bacteria are engulfed by the neutrophil phagosome. Neutrophil granules then fuse with the phagosome releasing their toxic contents, resulting in bacterial killing.

Degranulating neutrophils release a range of mediators that have important effects in bronchiectasis. Neutrophil elastase is the most extensively studied, but cathepsin G, proteinase-3 and the matrix metalloproteinases are also increased in the airways of patients with bronchiectasis and have important effects (Tsang et al, 2000)

It is clear that neutrophils present in the bronchiectasis lung fail to effectively phagocytose and kill microorganisms, but the mechanism for this is complex and poorly understood. The mechanisms that bacteria use to evade neutrophil killing will be discussed in subsequent sections. King et al found no evidence of reduced phagocytosis by peripheral blood neutrophils in patients with bronchiectasis, suggesting that neutrophils from bronchiectasis may be relatively normal before they enter the inflamed airway (King et al, 2006). The inflammatory milieu in the airway is therefore strongly implicated in phagocytic dysfunction.

Large concentrations of neutrophil elastase are released into the bronchiectasis airway which overwhelm natural antiprotease defences such as α-1-antitrypsin and secretory leukocyte peptidase inhibitor. Elastase has multiple damaging effects in-vitro include directly damaging epithelial cells, slowing ciliary beat frequency and promoting mucous hypersecretion through effects on goblet cells (Voynow et al, 1999, Amitani et al, 1991). Elastase is directly pro-inflammatory. In clinical studies, elastase correlates with markers of disease severity such as lung function, radiological severity and 24-hour sputum volume (Tsang et al, 2000, Lloberes et al, 1992). Destruction of elastin, basement membrane collagen and proteoglycans by neutrophil proteases may contribute directly to progression of the disease (Shun et al, 2000, Stockley et al, 1984).

Importantly, however, there is strong evidence that elastase also impairs neutrophil phagocytosis by multiple mechanisms. Elastase cleaves FcγRIIIb, and has also been shown to cleave complement receptor 1 in patients with cystic fibrosis (Voglis et al, 2009, Berger et al, 1989). Elastase also cleaves iC3b from the surface of pathogens, leading to an important opsonin/receptor mismatch (Tosi et al, 1990). This evidence makes elastase a leading candidate for therapeutic manipulation in bronchiectasis and a number of pharmaceutical companies are developing oral elastase inhibitors for use in bronchiectasis and other chronic respiratory disorders (Stevens et al, 2011). Short and long term antibiotic therapy is also effective in suppressing levels of sputum neutrophil elastase activity (Chalmers et al, 2012).

Human neutrophil peptides (HNPs) – also known as α defensins – are the most abundant proteins stored in azurophilic granules by neutrophils. These peptides are found in high concentrations in the lungs of bronchiectasis patients and are essential for neutrophil killing of micro-organisms. Voglis et al, however, demonstrated that high concentrations of HNPs inhibit neutrophil function via a negative feedback loop resulting in reduced phagocytosis (Vogilis et al, 2009). Voglis et al also demonstrated reduced expression of Fcγ receptors and complement receptor 1 in the airway neutrophils of patients with bronchiectasis (Voglis et al, 2009).

Some of the mechanisms of neutrophil phagocytic dysfunction in bronchiectasis are summarised in figure 1.8.

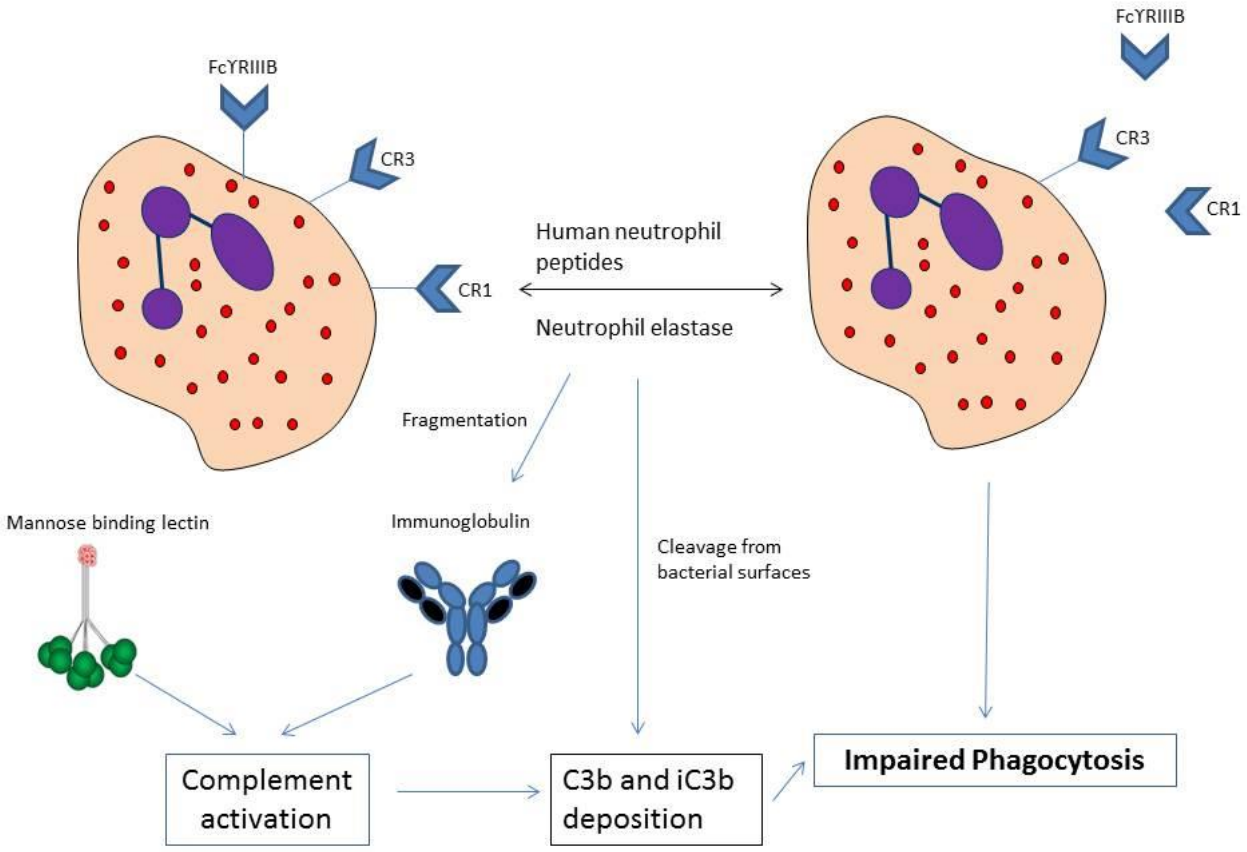

Figure 1.8. Neutrophil phagocytic impairment in bronchiectasis. Neutrophil elastase and neutrophil peptides released by activated neutrophils impair neutrophil functions through cleavage or downregulation of FcϒR receptors and complement receptor 1. Opsonisation of bacteria occurs through activation of complement via the classical or mannose binding lectin pathways. Elastase is also involved in the fragmentation of immunoglobulin which impairs the classical complement pathway, and in cleaving iC3b from bacterial surfaces thereby further reducing opsonic phagocytosis. Abbreviations CR1=complement receptor 1, CR3=complement receptor 3.

1.1.15 Macrophages

Less is known about the role of macrophages in bronchiectasis, although along with other inflammatory cells increased numbers of macrophages are demonstrable in endobronchial biopsies from patients with bronchiectasis (Zheng et al, 2001). Macrophages co-ordinate inflammatory responses by secreting inflammatory mediators including those mentioned previously (tumour necrosis factor alpha, interleukin-8 and leukotriene B4).

A crucial role of macrophages is the process of efferocytosis (Walker et al, 2005). The clearance of apoptotic cells by macrophages is a critical mechanism for the resolution of inflammation (Walker et al, 2005). Failure to clear apoptotic neutrophils from the airway is associated with increased inflammation and airway damage through secondary necrosis and unregulated release of granule products (Haslett et al, 1999). Large numbers of apoptotic and secondary necrosis neutrophils have been demonstrated in the airway of patients with bronchiectasis (Watt et al, 2004). Vandivier demonstrated that neutrophil elastase cleavage of phosphatidylserine on the surface of apoptotic cells prevents macrophage phagocytosis in patients with bronchiectasis (Vandivier et al, 2002). Vitamin D binding protein (VDBP) also appears to play a role in efferocytosis by activating alveolar macrophages, and Wood et al found an association between the GC2 variant of the VDBP gene and bronchiectasis in patients with α-1-antitrypsin deficiency (Wood et al, 2011). Macrophage clearance of apoptotic cells is a target for future therapeutic manipulation. The macrolide azithromycin, which has recently been shown to reduce exacerbations of bronchiectasis in a placebo controlled randomised

trial (Wong et al, 2012), has been shown to enhance phagocytosis of apoptotic cells by increasing expression of mannose receptor (Hodge et al, 2008).

1.1.16 T cells

T cell infiltration has been demonstrated in multiple studies of bronchiectasis patients (Silva et al, 1989, Sepper et al, 1995). The proportions of CD4+ and CD8+ cells are variable in biopsy studies and no consistent pattern of infiltration has been demonstrated. Th17 cells are controlled by the transcription factor RORγt and secrete interleukin-17, a potent pro-inflammatory cytokine and neutrophil chemoattractant (Dublin et al, 2008). They have an important role in the clearance of extracellular bacteria and fungi but may also be deleterious by exacerbating neutrophilic airway inflammation. A study of endobronchial biopsies from 17 patients with non-CF bronchiectasis found increased Th17 cells compared to healthy controls, with equivalent numbers of Th17 cells in non-CF bronchiectasis compared to CF patients. Similarly, increased levels of IL-17 were identified in bronchoalveolar lavage in non-CF bronchiectasis compared to controls (Tan et al, 2011). A crucial role for CD8+ T cells is demonstrated in patients with Transporter antigen peptide (TAP) deficiency. These patients are unable to make CD8+ T lymphocytes and suffer from severe recurrent infections often leading to bronchiectasis (Zimmer et al, 2005). Human immunodeficiency syndrome (HIV) patients also suffer recurrent infections and may develop bronchiectasis (Bard et al, 1998). A key role for CD4+ T cells was suggested by a study by Boyton et al who demonstrated an increased prevalence of the HLA-

DR1,DQ5 haplotype in bronchiectasis patients compared to control subjects (Boyton et al, 2006). The same group also identified a role for natural killer cells in susceptibility to bronchiectasis (Boyton et al, 2008).

1.1.17 Eosinophils

Little is known about the role of eosinophils in bronchiectasis, although some patients have been found to have high levels of eosinophils in sputum (Ip et al, 2003). Ip et al found high levels of sputum eosinophils to be associated with a lower FEV_1 (Ip et al, 1993). There is an apparent association between asthma and bronchiectasis that requires further study (Takemura et al, 2004). Increased serum levels of eosinophil cationic protein have been demonstrated in the serum of patients with bronchiectasis, although the clinical significance of this is unclear (Kroegel et al, 1998).

1.1.18 Epithelial cells

Bronchial epithelial cells serve multiple functions in innate immunity, providing a physical barrier to invasion, clearing micro-organisms and particulates through mucociliary function and participating in neutrophil recruitment through secretion of pro-inflammatory cytokines and adhesion of leukocytes (Devalia et al, 1993). The critical importance of the mucociliary escalator in airway defence is demonstrated in ciliary disorders such as primary ciliary dyskinesia which are associated with the early development of bronchiectasis (Noone et al, 2004). Nuclear medicine studies

have shown markedly delayed and disordered clearance of a radioactive aerosol in bronchiectatic regions of lung with normal clearance from the contralateral healthy regions of lung even in patients with bronchiectasis that is not due to a primary ciliary disorder (Isawa et al, 1990). Airway epithelium is covered by a thin film of liquid referred to as the airway surface liquid (ASL) (Matsui et al, 1998). This is separated into two compartments, the mucous layer and the periciliary layer. The periciliary layer is designed to facilitate ciliary function while the mucous layer traps particulates and microorganisms for clearance. Chloride and Sodium ion transport is tightly regulated to maintain ASL hydration and efficient mucociliary clearance (Sermet-Gaudelus et al, 2011). ASL homeostasis is maintained through the action of the amiloride-sensitive epithelial sodium channel (ENaC) and the cystic fibrosis transmembrane conductance regulator (CFTR) (Stutts, 1995). Dysfunction of the CFTR due to autosomal recessive mutations in the CFTR gene cause the clinical disorder of cystic fibrosis (Riordan et al, 1989). The mechanism by which CFTR dysfunction disrupts ASL homeostasis has been the subject of intense debate but is thought to be the result of ASL dehydration (Matsui et al, 1998). Active apical sodium reabsorption through ENaC occurs excessively due to a failure of tonic inhibition by CFTR (Knowles et al, 2002). This causes ASL dehydration which would normal be corrected by a switch from net NaCl absorption to secretion by chloride secretion through apical chloride channels (Mall et al, 2010). CF airway epithelia are unable to enhance chloride transport and therefore are unable to rehydrate depleted ASL (Caldwell et al, 2002). Loss of ASL effects both the periciliary layer and mucus layer leading to both ciliary dysfunction and vicious mucus plugs which are difficult to clear. This impairment of mucociliary clearance

leading to the clinical syndrome of CF. The detailed mechanisms of this process relevant to CF have been previously reviewed (Boucher et al, 2007).

Although the above focusses on cystic fibrosis, where the majority of this research has taken place, this is of great relevance to non-CF bronchiectasis. It has been shown that 30-50% of patients with bronchiectasis are heterozygous for mutations in the CFTR gene (Dumur et al, 1990, Bienvenu et al 2010), much more frequent than would be expected in the general population. The presence of CFTR mutations was associated with an increased frequency of *P. aeruginosa* colonisation in one study, as was evidence of epithelial dysfunction measured by nasal potential difference (Bienvenu et al, 2010). In addition, channelopathies such as variants in ENaC alone or in combination with CFTR mutations have been reported to be associated with diffuse bronchiectasis (Sermet-Gaudelus et al, 2011).

As mentioned above, epithelial cells play an important role in the recruitment of neutrophils to sites of inflammation. Airway epithelium expresses ICAM-1 in response to bacterial challenge, leading to increased adherence of leukocytes. This enhances phagocytic functions and promotes increased inflammation (Humlicek et al, 2004). Airway secretions in bronchiectasis enhance the expression of ICAM-1 by bronchial epithelial cells, an effect that will promote neutrophil recruitment (Chan et al, 2008). ICAM-1 is also the receptor for cellular entry of rhinovirus infections in humans (Staunton et al, 1989). It has been hypothesised that upregulation of epithelial ICAM-1 in chronic lung diseases will increase susceptibility to viral exacerbations. Rhinovirus is an important cause of COPD exacerbations (Malia et al, 2011). The role of viruses in bronchiectasis exacerbations has not been established. Evidence suggests that pro-inflammatory responses are excessive in airway epithelial

cells from patients with bronchiectasis when exposed to a bacterial trigger (Hill et al, 1998). Bronchial epithelial cells produce endothelin-1 which has been shown to promote neutrophil adhesion to endothelial cells, neutrophil recruitment to the airway and release of elatase (Zheng et al, 2000). There is limited additional clinical data regarding the role of epithelial cells in bronchiectasis and further studies are needed. HMG co-A reductase inhibitors (statins) have multiple pleiotropic properties beyond their cholesterol lowering effects (Chalmers et al, 2010), and two randomised controlled trials of statins in bronchiectasis are currently recruiting. Among their potential effects, statins inhibit release from bronchial epithelial cells of IL-8, IL-6, granulocyte colony stimulating factor (GM-CSF) and VEGF in response to IL-17, and suppress matrix metalloproteinase, IL-6 and GM-CSF release in response to TGF-beta (Murphy et al, 2008).

1.1.19 Anti-inflammatory mechanisms in the airways

While much of the above has focussed on the pro-inflammatory responses of inflammatory cells and epithelial cells in the bronchiectasis airway, there is also a simultaneous couter-regulatory anti-inflammatory response which limits inflammation induced damage and attempts to restore homeostasis. The active process of resolving inflammation is termed catabasis and is driven by the action of anti-inflammatory cytokines, pro-resolution lipid mediators and by apoptosis and clearance of inflammatory cells.

There has been a great deal of attention around the role of the pro-resolution lipid mediators resolvins, protectins and lipoxins all of which have been shown to have

pro-resolution effects in models of acute lung inflammation. Among the specialised pro-resolution effects of these mediators they can promote cessation of neutrophil infiltration, reduced chemokine and cytokine production and stimulation of efferocytosis and clearance of bacteria (Serhan and Chiang 2013).

Macrophages are critical to the suppression of lung inflammation and are the main orchestrators of this response. Interleukin-10 and TGF-β represent anti-inflammatory cytokines that can suppress the production of pro-inflammatory mediators (Raychaudhuri et al. 2000, Pittet et al 2001). Release of IL-1 receptor antagonist by macrophages prevents the pro-inflammatory effects of IL-1β and also downregulates neutrophil chemoattractants and the epithelial adhesion molecular ICAM-1 (Herold et al 2011). Macrophage derived matrix metalloproteinase 12 cleaves CXC chemokines impairing their function and reducing neutrophil recruitment (McQuibban et al 2002). Macrophages produce a range of mediators that promote neutrophil apoptosis including TNF-related apoptosis inducing ligand (TRAIL) (Herold et al 2008). Phagocytosis of apoptotic cells by macrophages induces an anti-inflammatory phenotype in macrophages in contrast to phagocytosis of necrotic cells which provokes pro-inflammatory cytokine release (Voll et al 1997).

Therefore the resolution of established inflammation is an active process co-ordinated by macrophages and a range of mediators. Unfortunately, there is an absence of data on resolution mechanisms in the context of non-CF bronchiectasis.

1.1.20 Immunoglobulin, complement and antimicrobial peptides

All primary antibody deficiency syndromes have been associated with bronchiectasis, due to reduced or absent levels of one of more immunoglobulin class or subset. Most of these disorders, such as common variable immunodeficiency (CVID) and X-linked agammaglobulinaemia, are diagnosed in childhood but rare cases are identified in adulthood (Pasteur et al, 2010). Acquired immunoglobulin deficiencies associated with haematological malignancy can cause bronchiectasis. Cohort studies have found a limited role of IgG subclass deficiencies or antibody deficiencies in general among unselected adult cohorts with bronchiectasis (Hill et al, 1998). Failure to produce specific antibody responses to organisms such as *H.influenzae* and *S.pneumoniae* can be demonstrated after vaccination and may occur in patients with normal total IgG levels (Stead et al, 2002).

An unresolved paradox in cystic fibrosis and bronchiectasis is the failure to effectively clear bacteria such as *P.aeruginosa* from the airway despite high levels of detectable antibodies against these pathogens. This occurs in part due to the ability of organisms to resist antibody binding and subsequent complement activation through biofilm formation and virulence factors. Immunoglobulin is also directly fragmented in the airway by neutrophil proteases leading failed opsonisation (Fick et al, 1984).

Additional innate defence mechanisms in the lung include lactoferrin, lysozyme, defensins, complement components, cathelicidins, surfactant proteins and other collectins. It has been suggested that abnormal salt concentrations in the airway of patients with cystic fibrosis impair the function of defensins, cathelicidin and others (Goldman et al, 1997, Smith et al, 1996), although this hypothesis is controversial

and the ionic conditions in the non-CF bronchiectasis have not been described. Mannose binding lectin deficiency has been described as a predisposing factor for bronchiectasis in patients with CVID (Gregersen et al, 2010, Fevang et al, 2005), and predispose to earlier infection with *Pseudomonas aeruginosa*, worse lung function and earlier death in adults with cystic fibrosis (Garred et al 1999, Chalmers et al, 2011). A small study of a related molecule, L-ficolin (ficolin-2), found a higher incidence of relative L-ficolin deficiency in bronchiectasis patients compared to controls (Kilpatrick et al, 2009). These pattern recognition molecules of the lectin pathway of complement are the major focus of this thesis and are described in detail in the following chapters.

1.2 The lectin pathway of complement and mannose binding lectin

Mannose binding lectin (also called mannan binding lectin, MBL) is a serum protein produced by the liver and encoded by the *MBL2* gene on chromosome 10 (Kilpatrick, 2003). It was the first molecule described as being able to activate the lectin pathway of complement and is also the most extensively studied. This section of the introduction therefore focusses on mannose binding lectin and specifically its relationship to cystic fibrosis.

1.2.1 Mannose binding lectin

Disease association studies (Kilpatrick, 2002) and experimental work in murine models (Shi et al, 2004; Molle-Kristensen et al, 2006) in vivo suggest a crucial role for MBL in innate immune responses to micro-organisms (Neth et al, 2000; Molle-Kristensen et al, 2006). MBL is a pattern recognition receptor that distinguishes self from non-self by binding glycoconjugates containing mannose, fucose or N-acetylglucosamine that are present on a wide variety of bacteria, viruses and fungi (Molle-Kristensen et al, 2006). Upon binding, MBL may activate the lectin pathway of complement (figure 1.9) via MBL associated serine protease 2 (MASP-2) leading to complement activation, with subsequent opsonisation or direct lysis of the target (Wallis et al, 2010). MBL also interacts independently of MASPs with receptors on phagocytes, and stimulates phagocytosis by "bridging" between phagocytes and micro-organisms or apoptotic cells (Shiratsuchi et al, 2008; Hodge et al, 2008)

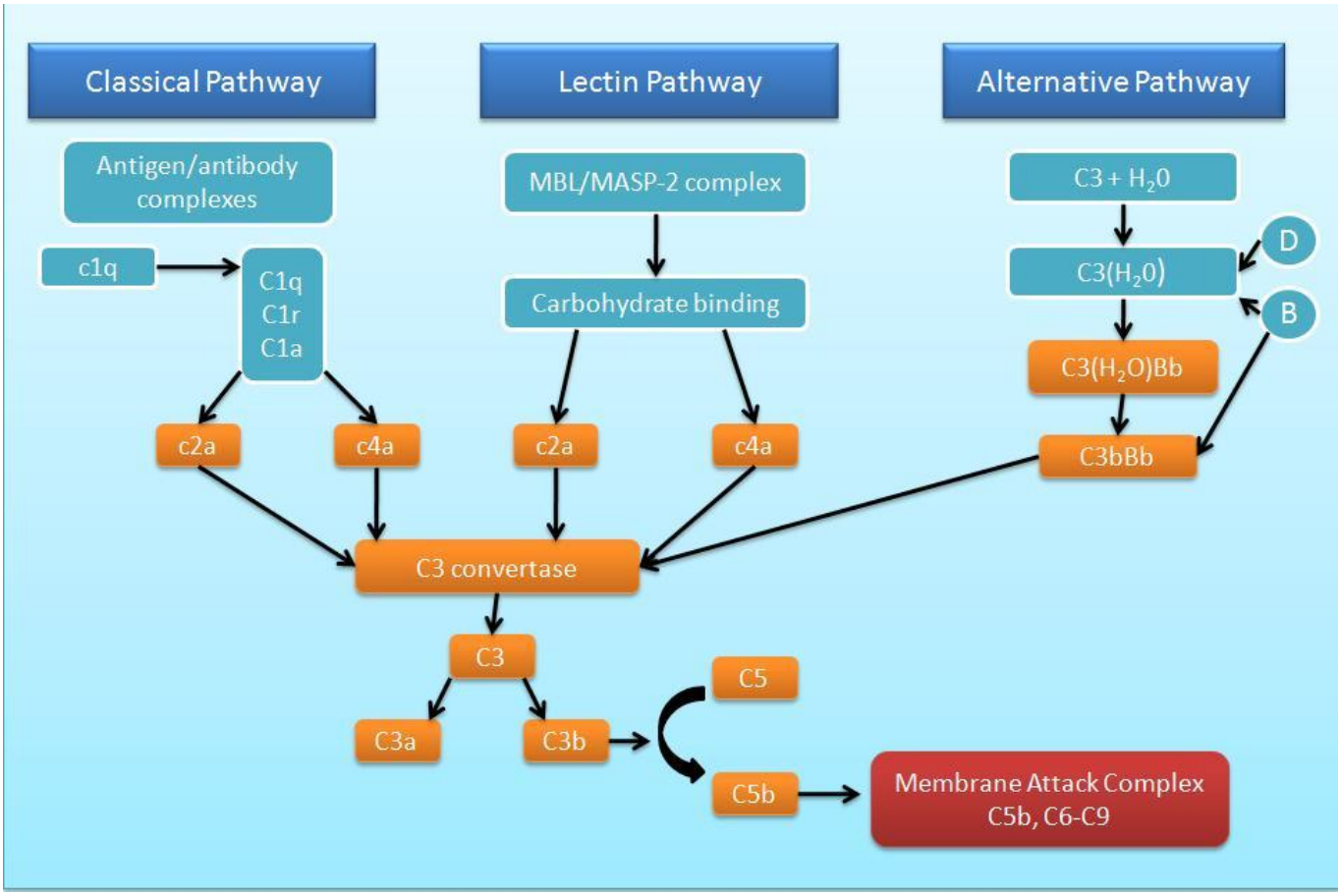

Figure 1.9 Complement activation pathways. The classical, lectin and alternative pathways are shown. The classical pathway is triggered by activation of the C1-complex on binding to immunoglobulin/antigen complexes. The lectin pathway is antibody independent and is triggered by binding of mannose binding lectin (MBL) or the ficolins to carbohydrates on the surface of microorganisms. The alternative pathway is triggered by spontaneous C3 hydrolysis independent of pathogen binding.

C3 is subsequently bound to complement factor B (B) in the presence of complement factor D (D) to form the alternative pathway C3 convertase. Each pathway progresses through the same route from this point to the formation of the membrane attack complex.

The concentration and activity of serum MBL is genetically determined to a major extent. Three single nucleotide polymorphisms in exon 1 of the *MBL2* structural gene (at codons 52, 54 and 57, referred to as the D, B and C alleles, and collectively designated "O") have an additive effect on serum MBL levels. The normal or wild type codons at those loci are termed "A". In addition, three variants in the promoter region of *MBL2* can influence serum levels, with the X/Y dimorphism (low and high respectively) having a marked effect. The X polymorphism at position -221 has a dominant negative effect on serum MBL concentrations, leading to reduced levels similar to the structural polymorphisms. As a consequence an individual carrying the X promoter polymorphism and the wild type structural allele has similar MBL serum levels to individuals carrying the structural variant alleles.

There is strong linkage disequilibrium between the promoter and structural gene variants. Consequently, only seven haplotypes (out of a possible 64) are commonly found combining to form twenty-eight genotypes (Garred et al, 2009). In disease association studies, these genotypes are usually grouped into assumed low (YO/YO and YO/XA), medium (YA/YO and XA/XA) and high (YA/YA and YA/XA) conferring categories (Wallis and Lynch, 2007). Most, but not all, individuals with A/A genotypes have serum MBL > 600 ng/ml and those with O/O genotypes generally have serum MBL < 200 ng/ml (Swierzko et al, 2009). The A/O groups, however, are highly heterogeneous with respect to serum MBL values, despite

average values being reported at approximately 400 ng/ml and perhaps a majority having concentrations < 600 ng/ml.

MBL deficiency or insufficiency is an imprecise concept with cut-off values used in the literature ranging from <100 to <1000 ng/ml serum MBL. Similarly, the A/A genotypes are sometimes compared with O/O or (O/O + O/XA), but at times both the homozygous and heterozygous (O/O + A/O) genotypes are regarded as insufficient. Some studies use both genotype grouping and serum MBL values to compare patients with healthy controls, but usually either one or the other is analysed on its own.

The *MBL2* gene products form a basic subunit consisting of a collagen-like triple helix with 3 globular heads. The basic subunit is capable of multimer formation up to hexamers. Only the higher multimers (trimers and above) bind to glycoconjugates with high affinity, or activate the lectin pathway of complement (figure 1.10).

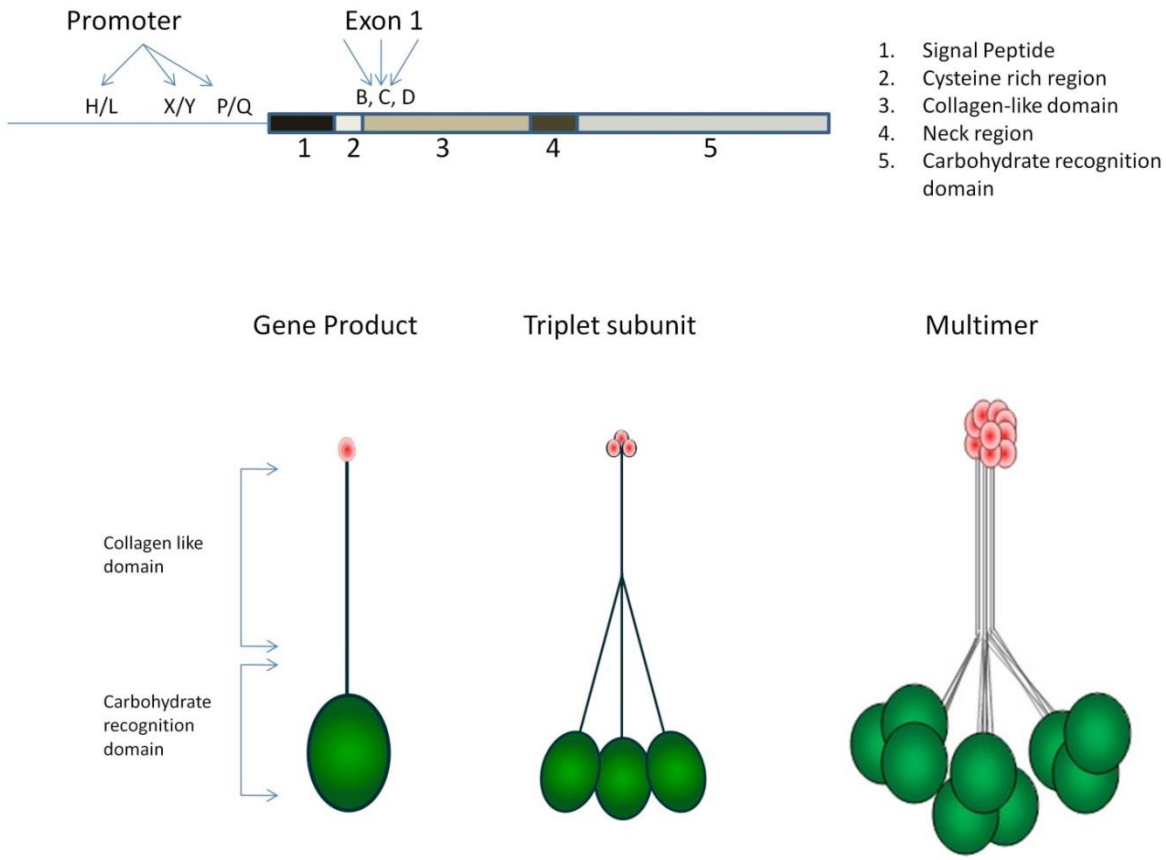

Figure 1.10. Structure of MBL. A representation of the single gene product, triplet subunit and MBL multimer is shown. Only the higher multimers are able to bind to glycoconjugates and activate the lectin pathway of complement. The variant alleles (B, C and D) result in fewer high order multimers and therefore functional MBL insufficiency (see text for further discussion).

The gravimetric values used for serum MBL are based on the multimeric protein from A/A homozygotes; the effect of variant alleles of the *MBL2* gene is to impair multimer formation from the basic subunits. The exon 1 variants B and C lead to disruption of the Gly-X-Y repeats, in turn leading to altered interchain disulfide bonding in the N-terminal cross-linking region. The D variant leads to aberrant disulphide bond formation resulting from a substitution of a cysteine for an arginine (Wallis and Cheng, 1999).

1.2.2 Cystic fibrosis and MBL

Cystic fibrosis (CF) is a chronic, multisystem disease arising from mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR). Over 1000 mutations in the CFTR gene are recognised but the ∂F508 mutation accounts for approximately 70% of cases in Caucasian populations (Andersen et al, 1938; Riordan et al, 1989).

The phenotypic defect in CF is in epithelial chloride transport and is responsible for abnormally thick mucus production manifesting itself as pancreatic and pulmonary insufficiency. Progressive lung disease is the major cause of morbidity and mortality. Airway damage leads to severe bronchiectasis (permanent dilation of the airways leading to fluid filled "cysts") and chronic colonisation of the airway with bacteria. Colonisation with *Pseudomonas aeruginosa*, *S. aureus*, *Burkholderia cepecia* complex and other opportunistic pathogens leads to recurrent pulmonary exacerbations, airway inflammation and progressive loss of lung function (Zemanick et al, 2010). This vicious cycle leads ultimately to death from respiratory failure.

There are, however, large variations in the progression of pulmonary disease in CF that are not accounted for by CFTR genotype. Consequently, it has been postulated that other "modifier genes" may account for the variable phenotype observed in CF (Collaco and Cutting, 2008).

A potential role for MBL as a modifier of severity in cystic fibrosis is suggested by the finding that MBL binds *P. aeruginosa*, *S. aureus* and *Burkholderia cepecia* complex along with other clinically relevant bacteria (Neth et al, 2000; Molle-Kristensen et al, 2006). MBL deficient mice are susceptible to more severe infections with *P. aeruginosa* (Molle-Kristensen et al, 2006) and *S. aureus* (Shi et al, 2004) than wild-type animals. Furthermore, although MBL is a serum protein, it accumulates in the lung during acute inflammation in cystic fibrosis in quantities sufficient to promote phagocytosis and cause complement activation (Fidler et al, 2009; Reading et al, 1997). MBL replacement therapy has been developed and has been suggested as a new therapeutic avenue for a disease with few effective treatments (Garred et al, 2002).

Studies of MBL as a disease modifier in cystic fibrosis have given inconsistent results. The first two studies to examine this topic (Garred et al, 1999; Gabolde et al, 1999) were in agreement that impaired lung function was associated with homozygosity for variant alleles (O/O). Further, Garred et al (1999) found a relationship between MBL insufficient alleles and earlier age of acquisition of *P. aeruginosa*, more frequent colonisation with *B. cepacia* and earlier age at death or lung transplantation. Although the relationship with reduced lung function was confirmed and extended to A/O heterozygotes in some studies (Yarden et al, 2004;

Trevisiol et al, 2005), it was not confirmed in others (Carlsson et al, 2005; Drumm et al, 2005).

Davies et al (2004) investigated age non-overlapping adult and paediatric groups and found that adults homozygous for variant alleles had worse lung function than patients with wild-type alleles. This relationship was not evident in adult heterozygotes or in children. This apparent relationship with age was given some support from Muhlebach et al (2006) who found that low (<200 ng/ml) serum MBL was linked to better lung function in patients under 15 years of age but poorer lung function in adult patients. Muhlebach et al suggested that low circulating MBL concentrations accelerate the age-related decline in lung function and that future studies should address that aspect carefully. This was convincingly done by Dorfmann et al (2008) in a large series, showing that MBL insufficiency (YO/YO and YO/XA genotypes) was associated with a more rapid decline in pulmonary function, especially in patients who also had high-producing genotypes of transforming growth factor β1.

Several other recent MBL genotyping studies are consistent with some aspects of previous studies. In agreement with Muhlebach et al (2006) and Davies et al (2004), Olesen et al (2006) found that lung function was actually better in patients (mostly children) with insufficient *MBL2* genotypes. Choi et al (2006) reported that variant alleles were associated with poor lung function in adult patients.

Although lung function impairment is the outcome most frequently studied with regard to MBL and disease severity, microbiology outcomes have also been extensively studied. Following the study by Garred et al (1999), the relationship with

earlier acquisition of *P. aeruginosa* was confirmed by Trevisiol et al (2005). Subsequently a number of studies examined the frequency of colonisation with *P.aeruginosa* in MBL insufficient vs MBL sufficient groups- the majority of these studies found no association with MBL insufficiency (including Buanawuti et al, (2007), Davies et al, (2004), Olesen et al (2006) and Trevisiol et al (2005)). Carlsson et al (2005) even found that *P.aeruginosa* was significantly less frequent in MBL insufficient individuals. *P.aeruginosa* colonisation is highly age dependent and the majority of cystic fibrosis patients become colonised over time. Age at acquisition is a potentially more useful outcome as earlier age at acquisition correlates with poor outcome. This was subsequently investigated in two large series by Dorfman et al (2008) and McDougal et al (2010), both of which confirmed the previous observation of earlier acquisition of *P.aeruginosa* in MBL, insufficient individuals.

Colonisation with *B. cepacia* complex also correlates with poor outcome in CF, but is less frequent. Following the study by Garred et al (2009), five other studies reported the frequency of *B.cepacia* colonisation (Davies et al (2004), Olesen et al (2006), McDougal et al (2010), Buranawuti et al (2007) and Carlsson et al (2005)) but none found a statistically significant association.

Studies of survival have also given conflicting results. In support of the observation by Garred et al (2009), Buranawuti et al (2007) found O/O genotypes (compared with A/A or A/O) to be associated with non-survival in adults. However, Carlsson et al (2005), Drumm et al (2005) and, most recently, McDougal et al (2010) found no significant association with survival or lung transplantation.

The discussion above focuses entirely on the pulmonary manifestations of cystic fibrosis. Three studies have also investigated the role of MBL in CF liver disease. The study by Gabolde et al (2001) found a strong association between MBL insufficient genotypes and liver disease, but this was not confirmed by subsequent studies by Tomaiuolo et al (2009) and Bartlett et al (2009).

Therefore, the literature on MBL as a disease modifier in cystic fibrosis has given conflicting results.

Consequently, we investigated the role of MBL insufficiency on several clinically important markers of disease severity by performing a systematic review and meta-analysis of the gene association studies.

The methodology of this systematic review has been described in the associated publication (Chalmers et al, 2011). The systematic review yielded 42 titles from which 19 were immediately excluded as irrelevant following title and abstract review. There were a further 7 exclusions from the 23 studies examined in depth. The remaining 16 studies satisfied the inclusion criteria and are shown in Table 1.3.

First author (year)	Location of study	Age of cohort	Definition of CF	N	Alleles investigated	Definition of deficiency	Outcomes
Carlsson (2005)	Lund, Sweden	Median 20.5 (range 4-54)	"genetically verified cystic fibrosis"	112	Exon 1 Promotor -550 (H/L) Promotor -221 (X/Y)	O/O or LXA/O	Spirometry Sputum microbiology - *P. aeruginosa* - *B. cepacia* - *S. aureus* Lung transplantation
Yarden (2004)	Combined data from cohorts in Belgium and Czech republic	Mean 13.4 (range 12-15)	∂F508 homozygous	179	Exon 1 Promotor -221 (X/Y)	O/O, XA/O, XA/XA	Spirometry Sputum microbiology - *P. aeruginosa* Age at first isolation of *Pseudomonas aeruginosa*
Gabolde (1999)	France	Mean 19.0 (SD=11.1)	∂F508 homozygous	22	Not stated but presumed to be Exon 1 and Promotor -221 (X/Y)	0/0 or compound heterozygous (presumed to mean XA/0	Spirometry Sputum microbiology - *P. aeruginosa*
Dorfman (2008)	National study, Canada	Mean 10.5 (limited to age <18 years)	Mutations associated with cystic fibrosis	1,393	Exon 1 and promoter -221 (X/Y)	O/O or XA/O	Spirometry Age of acquisition of *P. aeruginosa*
Drumm (2005)	Multicentre, USA and Canada	Mean 16.2 (4.1)- severe group, mean 28.6 (+/- 9.7)- mild group	Initial study- ∂F508 homozygous	808	Exon 1 and promoter -221 (X/Y)	O/O or XA/O	Spirometry Survival to death or lung transplantation
Garred (1999)	Copenhagen, Denmark	Median 16.2 (range 7.2-40.7)	Mutations associated with CF	149	Exon 1 Promotor -221 (X/Y)	O/O or XA/O	Death or lung transplantation after 10 years follow-up Sputum microbiology - *P. aeruginosa* - *B. cepacia* Age at acquisition of *P. aeruginosa*
Davies (2004)	London, UK	Adult cohort: mean 29.7(SE ± 0.5) Paediatric cohort 8.5±0.3	Genetic mutation associated with CF	298 adults 260 children	Exon 1 and Promotor -221 (X/Y)	O/O or XA/O	Spirometry Annual decline in FEV_1 Oxygen saturations Sputum microbiology - *P. aeruginosa* - *B. cepacia* Serum C-reactive protein Leucocyte count Number of IV antibiotics

Gabolde (2001)	France	Mean 14.5 (SD 8.7)- wild type group 17.9 (8.4)- deficient group.	∂F508 homozygous	216	Exon 1	O/O	The presence of liver cirrhosis.
Trevisiol (2005)	Trieste, Italy	Mean 17.0 (females) and 19.9 (males)	CFTR genotyping	47	Exon 1	O/O or A/O	Age at *P. aeruginosa* colonisation Spirometry
Olesen (2006)	Denmark	Median 14 (range 2-40)	Class I, II or III CFTR mutations	109	Exon 1 Promotor -550 (H/L) Promotor -221 (X/Y) Promotor +4 (P/Q)	O/O or XA/O	Spirometry Sputum microbiology - *P. aeruginosa* - *B. cepacia*
Buranawuti (2007)	USA	Children- mean 9.4 (range 0-16), adults 30.8 (17-66)	CFTR mutations	153 adults, 101 children	Exon 1	O/O	Spirometry Sputum microbiology - *P. aeruginosa* - *B. cepacia* Survival
Muhlebach (2006)	North Carolina, USA	Mean 12.8, range (0-45)	Positive sweat test and at least 1 CFTR mutation	148	Serum MBL levels	Serum MBL <200ng/ml	Spirometry Sputum microbiology - *P. aeruginosa* - *S. aureus*
Tomaiuolo (2009)	Naples, Italy	Not stated	∂F508 homozygous or compound heterozygous for ∂F508 and another severe CF mutation.	108	Exon 1 Promotor -550 (H/L) Promotor -221 (X/Y) Promotor +4 (P/Q)	O/O or XA/O	Liver disease
Bartlett (2009)	United States and Canada	Initial study- No liver disease Mean 26.7 (9.6). Liver disease 19.8 (7.3).	CFTR mutations	967	Exon 1 and Promotor -221 (X/Y)	O/O or XA/O	Liver disease
Choi (2006)	Bethesda, Maryland, USA	29.1 (+/- 4.9) (A/A group) and 34.3 (+/- 9.6) (A/O and O/O	∂F508 homozygous	51	Exon 1	O/O or A/O	FEV_1 DLCO

		group).					*P. aeruginosa colonisation* Disease severity scoring
McDougal (2010)	Bethesda, Maryland, USA	16 +/- 7.8	Generally verified cystic fibrosis	848	Exon 1 Promotor -221 (X/Y)	O/O or XA/O	FEV_1 Age at acquisition of *P. aeruginosa*

Table 1.3 Studies of Mannose binding lectin in cystic fibrosis. Abbreviations: MBL=mannose binding lectin, SD=standard devation, SE= standard error, IV=intravenous' FEV_1= forced expiratory volume in 1 second. DLCO=Diffusing capacity of carbon monoxide.

1.2.3 Mannose binding lectin and bacterial colonisation in cystic fibrosis

P. aeruginosa and *B. cepacia* are opportunistic pathogens. The presence of these organisms in the sputum of patients with cystic fibrosis is associated with poor outcome and deterioration in lung function (Ledson et al, 2002).

Pseudomonas aeruginosa

Seven studies reported the frequency of *P. aeruginosa* between MBL insufficient and sufficient groups. Reporting of data was inconsistent, with 2 studies combining the A/O and O/O groups into a single cohort and one study combining the A/A and A/O groups into a single cohort. This limited our ability to perform a pooled analysis. The acquisition of *P. aeruginosa* is age dependent and insufficient data was available to account for this in the analysis. The limited analysis did not suggest that the O/O genotype was associated with *P. aeruginosa* colonisation (OR 1.42; 95% CI 0.43 to 4.72, p=0.6).

Age at first acquisition of pseudomonas aeruginosa

Age of first acquisition is potentially a more useful outcome since earlier acquisition of *P. aeruginosa* is associated with poor outcome (Emerson et al, 2002). Data reporting for this outcome was inconsistent. Garred et al (1999) and Trevisiol et al (2005) combined the O/O and A/O groups into a single cohort while Dorfman et al (2008) and McDougal et al (2010) considered the O/O or XA/O group separately. Defining MBL insufficiency as reported in the papers (O/O or A/O for Garred et al (1999) and Trevisiol et al (2005), O/O or XA/O for Dorfman et al (2008) and McDougal et al (2010)) showed that all 4 studies indicated that MBL insufficiency was associated with earlier acquisition of *P. aeruginosa*: mean difference, 2.83 years; 95% CI 1.63 to 4.03 years, $p<0.0001$ (figure 1.6). However the results show significant heterogeneity ($I^2=73\%$) that may be due to the different definitions of insufficiency used in each study.

Chronic colonisation with Burkholderia cepacia

Seven studies reported the frequency of colonisation with *B. cepacia* complex. The analysis includes six separate cohorts as Davies et al (2004) reported paediatric and adult cohorts separately. Only one individual study found a statistically significant association with *B.cepacea* colonisation and MBL insufficiency (Garred et al, 1999). When comparing MBL insufficient (O/O) patients with MBL sufficient patients, there was a significant association with *B.cepacia* colonisation: OR 3.46; 95% CI,

1.14-10.52,p=0.03 (Figure 1.11). There were, however, only nine patients with *B.cepacea* colonisation across the seven studies in the O/O group. Combining the O/O and A/O group into a single cohort, we found no significant association between variant MBL alleles and *B.cepacea* colonisation (OR 1.26 95% CI 0.79 to 2.01,p=0.3).

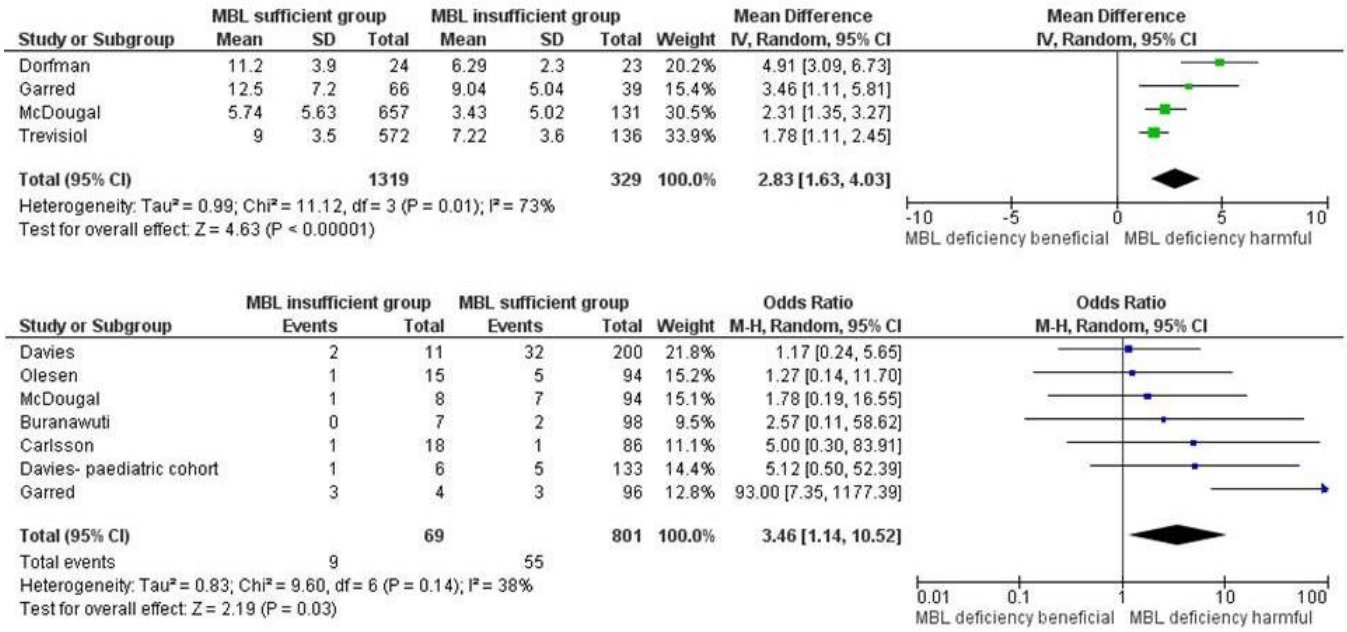

Figure 1.11. Age at acquisition of *Pseudomonas aeruginosa* and chronic colonisation with *Burkholderia cepacia.* Abbreviations, IV= inverse variance, M-H=Mantel-Haenszel. MBL= mannose binding lectin. SD= standard deviation.

1.2.4 MBL insufficiency and lung function in cystic fibrosis

Forced Expiratory Volume in 1 second

FEV_1 is an important marker of disease severity in cystic fibrosis. In total, nine studies contained valid data for FEV_1 as an outcome. Seven studies presented data for the exon 1 mutations (A/O) while one study presented a combination of exon 1 and the promoter -221 X/Y polymorphisms. One study used serum levels <200 ng/ml. In two studies, the adult and paediatric cohorts were presented separately.

The initial pooled analysis (figure 4A) suggested no significant effect of MBL insufficiency (defined as O/O or XA/O or a serum level <200 ng/ml) on reduced FEV_1 % predicted (mean difference -7.10% 95% CI -15.59 to 1.38, p=0.1). There was significant heterogeneity in the analysis, however. Visual inspection of the forest plots revealed the majority of the heterogeneity was due to three studies of exclusively paediatric cohorts (Dorfman et al (2008), Davies et al (2004) and Muhlebach et al (2006)) and the study by Olesen et al (2006), in which the median age was 14 years. The MBL insufficient group in the study by McDougal et al (2010) was significantly younger than the control group and hence was excluded.

FEV_1: subanalyses in predominantly adult cohorts

Excluding the above studies left six cohorts containing a majority of adult patients.

This sub-analysis revealed a correlation between MBL insufficiency and reduced FEV1 % predicted: mean difference, -19.65; 95% CI -10.83 to -28.48, p<0.0001, with non-significant heterogeneity (p=0.10) (figure 1.7).

The intermediate expressing MBL genotypes (A/O or XA/XA) were next compared to the wild-type or "high" expressing MBL genotypes. Seven studies contained sufficient data for this analysis. In the pooled analysis there was no statistically significant relationship between intermediate expressing genotypes and FEV_1: mean difference, -4.31; -9.25 to +0.64, p=0.09, with no significant heterogeneity (p=0.5).

Only three "adult" studies contained sufficient data to make a meaningful comparison between the O/O group and the intermediate expressing A/O group. These 3 studies, however, suggested a significantly lower FEV_1 % predicted in O/O individuals than A/O individuals: mean difference, -13.83 (-6.69 to -20.97, p=0.0001, I^2=0%).

FEV_1 subanalysis in predominantly paediatric cohorts

The five predominantly paediatric cohorts pooled together showed no significant effect of *MBL2* genotype on FEV_1: mean difference, 4.17; 95% CI, -1.10 to 9.43, p=0.1.

Forced vital capacity

Four studies contained satisfactory data for analysis of forced vital capacity. As before, there was a marked difference between the predominantly paediatric and the predominantly adult cohorts (figure 1.12). With the paediatric cohorts excluded, there was a significant association between MBL insufficiency (as defined above) with reduced FVC: mean difference, -13.31 (-2.97 to -23.66, p=0.01).

FEV1 % predicted and MBL deficiency

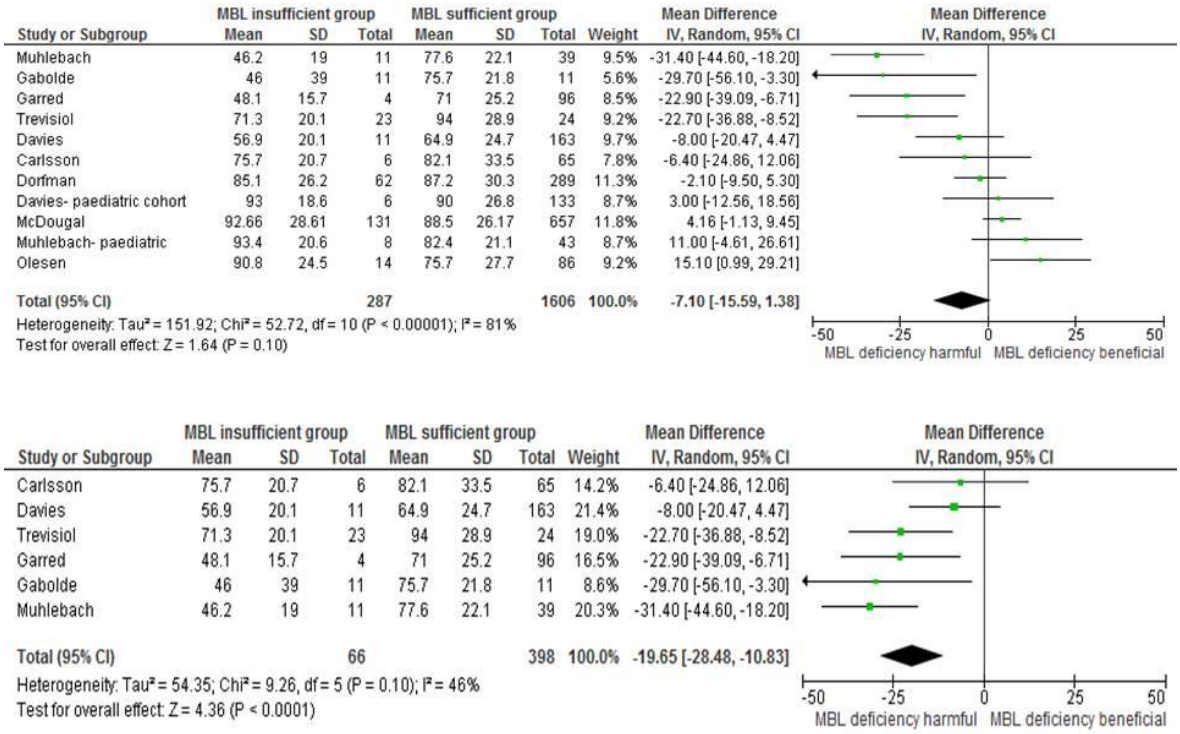

Figure 1.12. MBL insufficiency and lung function in cystic fibrosis. The top graph shows the effect of MBL deficiency on lung function across all studies that reported FEV_1 data. The bottom panel shows the sub-analysis in adult cohorts. Abbreviations IV= inverse variance, MBL= mannose binding lectin, SD= standard deviation.

Considering the impact of age on lung function, it is known that lung function declines with time in both children and adults with cystic fibrosis. This is potentially a major confounder if there are significant differences in age between MBL sufficient and insufficient groups. The studies included in this analysis variously attempted to adjust for the effects of age. Muhlebach et al (2006) and Davies et al (2004) divided their studies into adult and paediatric cohorts and there were no significant differences in age between the insufficient and sufficient adult cohorts. Garred et al (1999) similarly showed no significant differences in age between groups. The patients in the study by Gabolde et al (1999) were age matched. We excluded data from the study by McDougal et al (2010) as the MBL insufficient group was significantly younger than the control group, and therefore would be expected to have better lung function. It therefore seems unlikely that the differences observed in this analysis are due to imbalances in age between the insufficient and sufficient cohorts.

1.2.5 Death or end stage lung disease requiring transplantation

In cystic fibrosis studies, the end-point of death or transplantation is frequently combined as "end-stage cystic fibrosis". Seven studies reported data for these end-points. The methodology for assessing survival/end stage lung disease varied in each study. Garred et al (1999) followed their cohort for 10 years from 1989 to 1999. Similarly the study by McDougal et al (2010) was derived from the US cystic fibrosis twin and sibling study and provided follow-up data over several years. Buranawuti et al (2007) selected 38 samples from non-surviving adults patients for

analysis. Carlson et al (2005) and Muhlebach et al (2006) included patients attending a CF clinic who had undergone lung transplantation. Olesen et al (2006) followed up patients for 2-3 years following CF genotyping and recorded death or requirement for lung transplantation. Follow-up periods were unclear in the other studies.

In the analysis, the low expressing MBL group (O/O or XA/O) was compared to the wild type (A/A or high expressing groups). This analysis consisted of four studies and just failed to reach statistical significance (OR 3.96; 95% CI, 0.97-16.24, p=0.06). Combining the intermediate and high expressing MBL genotypes into a single group, as was done in seven studies, revealed a significant association of low expressing MBL genotypes with mortality or lung transplantation: OR 2.36 (1.06-5.22, p=0.03) without significant heterogeneity (p=0.14) (figure 1.13). This conclusion is, however, based on only 22 events in the MBL insufficient group across seven studies.

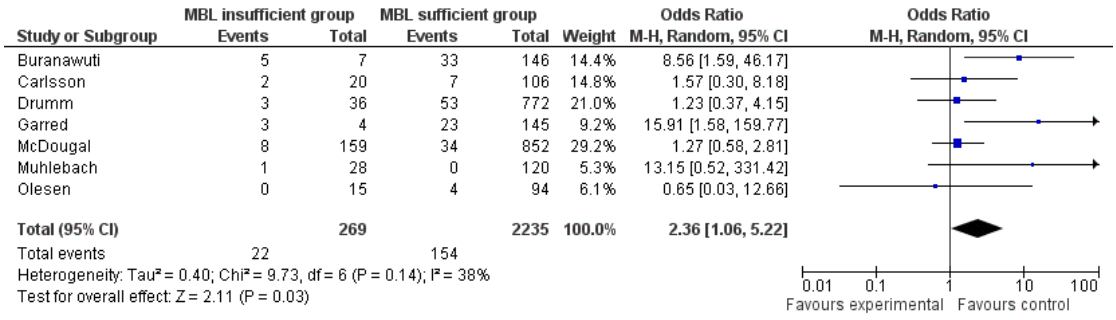

Figure 1.13. MBL insufficiency and end-stage cystic fibrosis.

1.2.6 MBL insufficient genotypes and chronic liver disease

Data were available from four studies. Only two studies considered the X/Y promoter polymorphisms. In the pooled analysis, O/O was not associated with presence of liver disease when compared to all other alleles (OR 2.65; 95% CI, 0.60 to 11.78, p=0.2), or to the A/A genotype only (OR 2.29; 95% CI, 0.62 to 8.44, p=0.21) but these three studies included only 51 patients with the O/O genotype. Similarly, the XA/O or O/O genotypes combined did not increase the risk of liver disease (OR 1.20; 95% CI, 0.73 to 1.97, p=0.5). There was no significant heterogeneity in this analysis.

Similarly, in the pooled analysis, intermediate expressing MBL genotypes were not associated with liver disease compared to wild-type: OR 0.95 (0.68 to 1.35), p=0.8, with no significant heterogeneity (I^2=0%). Pooling the intermediate and low expressing MBL genotypes also produced no significant effect: OR 1.00 (0.72 to 1.39), p=1.00, with no heterogeneity (I^2=0%).

Other outcomes

There were insufficient data regarding other outcomes identified in the cystic fibrosis literature, including decline in FEV_1 over time; pulmonary exacerbation rates; health related quality of life; measures of airway inflammation; radiographic severity by high resolution computed tomography; or growth during childhood. Investigators may wish to consider these in future studies.

1.2.7 Limitations of the analysis

Meta-analyses are entirely dependent on the quality of the source studies. Although we contacted each author to request additional (including unpublished) data, and assessed statistically for publication bias, it is possible, particularly in the smaller studies, that authors selectively report positive findings and do not report negative associations. We encountered heterogeneity in the alleles genotyped, as some studies only examined the exon 1 mutations while others also studied the promoter variants. We encountered heterogeneity in methodology, such as the duration of follow-up for survival studies that significantly limit these analyses. In some cases, lung function data were not fully reported and had to be obtained from authors or converted to allow meta-analysis. This introduces a degree of error and bias into the assessment of lung function. Some studies were not limited to patients with the ∂F508 mutation. Other gene modifiers appear to influence the CF phenotype. Along with the CFTR genotype, *TGFβ* and *MASP2* have been identified as severity modifiers and appear to interact with *MBL2* in CF patients. This cannot be adjusted for in this analysis and requires further study.

1.2.8 MBL replacement therapy

If mannose binding lectin insufficiency is associated with a poorer prognosis in patients with cystic fibrosis, can replacement of mannose binding lectin by intravenous infusion be used to improve outcomes in these patients?

The safety and tolerability of intravenous MBL therapy has been assessed by Valdimarsson et al (2004) and Petersen et al (2006), using plasma-derived and recombinant MBL respectively. Both studies showed that administration of MBL could restore the ability to activate the lectin pathway of complement, and was well tolerated by patients. Frakking et al (2009) and Brouwer et al (2009) extended these observations to children with chemotherapy-induced neutropenia, a group of patients at higher risk of opportunistic infections. Twice weekly infusions of MBL in this patient group effectively restored the activity of the lectin pathway of complement. In addition, MBL replacement increased neutrophil phagocytosis of zymosan *ex vivo*. High doses of MBL were required to restore MBL pathway activity to normal levels in this patient cohort, but the treatments were well tolerated with no evidence of systemic complement activation.

Although MBL replacement appears to be effective in restoring the MBL pathway in adults and children with MBL insufficiency, there is as yet no evidence from randomised clinical trials to show a beneficial effect in treatment or prevention of infectious disease. The only clinical evidence in support of mannose binding lectin replacement in cystic fibrosis comes from a case report (Garred et al, 2002). In this case, the authors administered purified mannose binding lectin to a patient with severe cystic fibrosis and *P. aeruginosa* infection. The infusions were well tolerated. Assessing the influence of MBL is impossible in an uncontrolled study but the authors report that the patient's condition stabilised during treatment. On the basis of the available studies a controlled trial of MBL replacement therapy is cystic fibrosis may be justified.

Several factors limit enthusiasm for the potential of MBL replacement therapy in CF. First, the results of the present analysis suggest any benefit may be limited to the small proportion (approximately 10-15%) of patients with most severe (O/O, XA/O) insufficiency. Second, all studies so far have used intravenous infusions of MBL. In the Garred report, the patient received twice weekly infusions. Given that MBL replacement may be required long term, there are practical and economic considerations to treating patients indefinitely with intravenous infusions. The potential cost of MBL replacement therapy would require careful consideration relative to the potential benefits.

On the other hand, a few patients seem to experience long-term benefits from short courses of MBL therapy (Valdimarsson, 2003).

1.2.9 Conclusions

The available evidence suggests that the *MBL2* gene is a major modifier of lung disease in cystic fibrosis. MBL deficiency is associated with reduced lung function, earlier infection with *P. aeruginosa*, colonisation with *B. cepacia* and a higher rate of end-stage CF. This justifies an in-depth study of the lectin pathway of complement in non-CF bronchiectasis to determine if MBL or other lectin pathway components are modifiers of disease.

1.3 Ficolins and Ficolin-2

1.3.1 Discovery of ficolins

Ficolins were initially described as transforming growth factor β-binding proteins present in porcine uterus, characterised by the possession of both fibrinogen-related and collagen-like domains (Ichijo et al, 1991). Subsequently, a molecule designated P35 was described capable of binding to N-acetylglucosamine and function as an opsonin for phagocytosis of *Salmonella* by neutrophils (Matsushita et al 1996). This initial study was the first indication that ficolins may function as part of the innate immune response to pathogens. A very similar molecule named L (for liver)-ficolin was independently purified from human plasma on GlcNAc-Sepharose (Le et al, 1997). Both resembled two other previously discovered plasma proteins: EBP-37, which bound elastin (Harumiya et al, 1995), and hucolin, which bound a corticosteroid derivative (Edgar, 1996).

It soon became clear that authors were identifying the same molecule, a 35kDa innate immune protein composed of a fibrinogen-like domain combined with a collagen-like domain that is now designated ficolin-2 (or L-ficolin). Ficolin-2 is structurally and functionally related to mannose binding lectin and complement component C1q, but the presence of the fibrinogen-like domain distinguishes ficolins from the collectins, which by definition are composed of a collagen-like domain with a C-type lectin domain.

1.3.2 Human ficolins

Three human ficolins have been identified, all of which circulate in serum: Ficolin-1 (Monocyte ficolin or M-ficolin); Ficolin-2 (Liver ficolin or L-ficolin) and Ficolin-3 (Hakata antigen or H-ficolin). Ficolins 1 and 2 have 80% homology at amino-acid level while ficolin-3 has only approximately 50% homology with the other ficolins (Matsushita, 2012). Ficolin-1 is predominantly found in monocytes and granulocytes, while ficolin-2 is only expressed in the liver and circulates in serum (Honore et al, 2008). Ficolin-3 was originally identified and defined by autoantibodies present in a small group of patients with systemic lupus erythematosus (Sugimoto et al, 1998). Like ficolin-2, ficolin-3 is synthesised in the liver (secreted into bile as well as blood) but it is also synthesised in the lung where it is secreted into the alveolus and bronchi (Akaiwa et al, 1999). Ficolin-3 is present at the highest concentration of all of the lectin pathway components in serum and is also reported to be the most potent at activating complement (Hummelshoj et al, 2008).

A comparison of the properties of the ficolins is summarised in Table 1. All three have the ability to activate the lectin pathway of complement, an activity known to be shared with just two collectins, mannan-binding lectin (MBL) and CL-L1 (Keshi et al, 2006, Hansen et al, 2010, Ma et al, 2004).

All three ficolins share the ability to bind acetylated sugars like GlcNAc. Ficolin-2 (like MBL) appears to be a major pattern recognition molecule in human plasma (Ma, 2004). It has a uniquely complex set of binding sites, potentially conferring the ability to recognise and interact with a wide range of microorganisms (Garlatti et al, 2007).

	Ficolin-1	**Ficolin-2**	**Ficolin-3**
Molecular size (subunit)	35K	35K	34K
Molecular size (native)	900K	420K	610K
Location	Monocytes; lung; spleen	liver; serum	liver; bile; lung; serum
Chemical specificity	acetylated sugars; GlcNAc; GalNAc; Sialic acid	acetylated sugars; LPS; 1,3–β-glucans, lipoteichoic acids; elastin; steroids; fibrinogen; fibrin	acetylated sugars; D-fucose>GlcNAc; polysaccharide from *A.viridans;* LPS from *Hafnia alvei*
Microbial specificity	*E. coli; S. aureus; S. agalactiae*	*S. aureus; S. pneumoniae;* *S .typhimurium;* *E. coli;* *P. aeruginosa;* *M. bovis; G. lamblia; T. cruzi;* *A. fumigatus; Influenza A*	*A.viridans; T.cruzi; Influenza A*
Complement activation	Yes	Yes	Yes
Opsonic activity	Presumed	Yes	?
Collagenase sensitivity	Yes	Yes	No

Table 1.4. The Human Ficolins.

1.3.3 Ficolins in other species.

Mice possess two ficolins. Ficolin A is present in liver and serum and is said to be analogous to ficolin-2 in humans, while ficolin B is expressed in bone marrow and spleen and is associated with macrophages similar to human ficolin-1 (Fujimori et al, 1998, Ohashi and Erickson, 1998). The two murine ficolins have approximately 60% homology at amino-acid level. There is no homologue of ficolin-3 in mice and FCN3 exists as a pseudogene containing several stop codons. (Endo, 2004)

In pigs, ficolins also consist of two homologous molecules, designated ficolin-α and ficolin-β. Although first discovered in uterine tissue, porcine ficolin-α is more abundant in liver and blood where two isoforms, "little ficolin" (Mr~400 000) and "big ficolin" (Mr~800 000) were described (Ohashi and Erickson, 1997). Ficolin-β, with around 80% identity to ficolin-α, was found to be expressed mainly in neutrophils (Ohashi and Erickson, 1998, Fujimori et al, 1998).

These findings have prompted the generalisation that ficolins can be classified into soluble serum ficolins and cell-bound ficolins whatever the species. This view is supported by a similar dichotomy in the toad, *Xenopus laevis* (Kakinuma et al, 2003).

1.3.4. Structure of ficolins

The primary structure of ficolin-2 is composed of 288 amino acids forming a gene product of apparent Mr 35 000 after glycosylation (Matsushita, 1996). A short N-terminal region implicated in multimer formation is followed by a series of 19 (Gly-X-Y) repeats forming the collagen-like region or domain. This is attached via a short

linking sequence to a large globular domain with a distinctive fold, homologous to the C-terminal domains found in fibrinogen chains. This fibrinogen-like domain occurs in several apparently unrelated proteins, including tenascins, the acetylated sugar-binding tachylectins from a horseshoe crab (*Tachypleus tridentatus*), and the sialic acid-binding lectin from the slug, *Limax flavus* (MacDonald and Kilpatrick, 2007).

The combination of fibrinogen-like domain and collagen-like region in ficolin-2 (and other ficolins) permits the gene product to form a basic subunit consisting of a triple helical tail and a trio of globular heads. This 3-dimensional structure is often likened to a bowl of tulips and resembles the shape of the complement component C1q and the collectin family, despite those other molecules not having primary sequence homology with ficolins or with each other. The triplet subunits can then associate to form higher multimers. The major form in plasma is believed to be a tetramer of subunits (12-mer) with an apparent Mr of approximately 400 000 (Ohashi and Erickson, 1998, MacDonald and Kilpatrick, 2007).

Ficolin-2 uniquely possesses a complex set of binding sites constituting a recognition surface that can detect various acetylated structures and neutral sugars in the context of extended polysaccharides. This conclusion is based on studies of its trimeric recombinant recognition domains solved by X-ray crystallography (Garlatti et al, 2007, Garlatti et al, 2010). There is an outer binding site (S1) close to the only calcium binding site. This could be considered the ancestral binding site, as it is homologous to that of the horseshoe crab tachylectin 5A as well as that found in human ficolin-1 and ficolin-3. Surprisingly, S1 is not responsible for recognition of

acetylated sugars, unlike its counterpart in tachylectin 5A. Instead, ficolin-2 possesses three inner binding sites (S2, S3, S4) that are located on both sides of a cleft between the upper parts of the protomers. It is S3 that is mainly responsible for binding acetylated structures. It also binds 1,3-β-glucans with assistance from the minor site, S4. The innermost S2 has affinity for galactose. The ficolin-2 recognition groove with its contiguous subsites (S2-S4) is reminiscent of the peptidoglycan binding proteins of invertebrates (Garlatti, 2007).

The ficolins, collectins and complement component C1q, all have a similar 3-dimensional shape suited to function as multivalent recognition molecules with increased affinity for ligands achieved by multiple protein–oligosaccharide interactions. It is noteworthy that ficolin-2 possesses a semi-open structure intermediate between the compact assembly of C1q and the wide open arrangement of MBL, the latter of which has little interaction between the lectin domains and a buried surface 8% the size of that of C1q (Garlatti, 2007).

The collagen-like region is responsible for the formation of the helical tails of the molecule and also for functional signalling. In particular, the lysine-57 residue is a key component of the binding site for MBL-associated serine proteases and also for calreticulin, a putative complement/collectin/ficolin receptor on phagocytes (Lacriox et al, 2009).

1.3.5 Biochemical specificity

The complex arrangement of binding sites described allows ficolin-2 to bind a wide range of ligands. Ficolin-2 binds acetylated sugars and also to non-saccharide acetylated compounds (Krarup et al, 2004). Nevertheless, glycan array studies have established that ficolin-2 does not bind to most acetylated oligosaccharides found on mammalian glyco-conjugates (Krarup et al, 2008). Rather, ficolin-2 has complex recognition requirements, and binding probably requires the presence of two or more acetylated sugar groups presented in an appropriate conformation. In another glycan array study, ficolin-2 preferentially recognised disulphated N-acetyllactosamine and tri- and tetrasaccharides containing terminal galactose or N-acetylglucosamine, and binding was sensitive to the orientation of the bond between N-acetyllactosamine and the adjacent saccharide (Gout et al, 2010).

Ficolin-2 is the major 1,3-β-glucan binding protein in human plasma (Ma et al, 2004) and can bind to lipoteichoic acid a major component of the Gram-positive bacterial cell wall (Lynch et al, 2004). Potentially, therefore, ficolin-2 could bind to a wide variety of fungi and Gram-positive bacteria. Ficolin-2 recognises and binds to the viral envelope glycoproteins (E1 and E2) of the hepatitis C virus and triggers the lectin pathway of complement by binding to a virally-infected human hepatoma cell line (Lui et al, 2009).

Ficolin-2 also appears to bind human DNA, suggesting a mechanism for attaching to apoptotic or necrotic autologous cells. Clearance of apoptotic cells is a critical mechanism for the resolution of inflammation. Two reports support the view that ficolin-2 can opsonise autologous dead or dying cells and cellular debris (Jensen et

al, 2007, Kuraya et al, 2005) . Kuraya et al concluded that ficolin-2 binds to apoptotic cells and activates complement via the lectin pathway. Jensen et al, however, observed binding to necrotic but not apoptotic cells, and only at supra-physiological concentrations of ≥20 μg/ml. High concentrations of ficolin-2 also promoted the uptake of necrotic Jurkat cells by monocyte-derived macrophages in a phagocytosis assay (Jensen et al, 2007) The importance of ficolin-2 to this clearance of apoptotic cells has not been established. It has already been mentioned that ficolin-2 can bind to a protein, elastin (Hariymiya, 1995) and a lipid (Edgar, 1996). Significantly, it can bind to the pentraxins, C-reactive protein (Zhang et al, 2009) and pentraxin 3 (PTX3) (Ma et al, 2009). Under inflammatory conditions, pentraxins appear to enhance complement activation via ficolin-2.

1.3.6 Binding to micro-organisms

Ficolin-2 has been found to bind to the Gram-negative bacteria, *Salmonella typhimurium* (Ra strain) (Matsushita, 1995), *Escherichia coli* and Gram-positive capsuled bacteria such as *S. aureus* and *S. pneumoniae* (Krarup et al, 2004, Krarup et al, 2005, Aoyagi et al, 2008). All interactions were partially sensitive to GlcNAc. Ficolin-2 binding to the intracellular bacterium *Mycobacterium bovis* has also been reported (Carroll, 2009). It also binds the opportunistic fungal pathogen, *Aspergillus fumigatus* (Ma et al, 2009). This last interaction can be partially inhibited by GlcNAc or Curdlan (a β-1,3-glucose polymer). The interaction with *Aspergillus fumigatus* is enhanced in the presence of pentraxin-3. Similar synergy was observed between ficolin-2 and C-reactive protein in response to *P. aeruginosa* (Zhang et al, 2009).

Binding of ficolin-2 to the protozoan causing Chagas' disease, *Trypanosoma cruzi*, (Cestari et al, 2009), as well as to the intestinal protozoan, *Giardia lamblia* (Evans-Osses et al, 2010), has been demonstrated.

1.3.7 Complement activation

As previously mentioned, ficolin-2 is one of the few molecules known to activate the lectin pathway of complement, along with mannose binding lectin, ficolin-1, ficolin-3 and the collectin CL-11/CL-K1. (Matsushita et al, 2000) This arises after forming a complex with MBL-associated serine proteases (MASP)-1, -2 and -3, of which MASP-2 is crucial for complement activation. MASP-2 binding takes place at a site on the collagen-like region (Lacroix, 2009). Ficolin-2-MASP-2 interaction leads to activation of the latter, enabling it to cleave complement components C2 and C4 in a manner similar to the C1q,r,s complex of the classical pathway initiated by antigen-antibody formation. The roles of MASP-1 and its alternatively spliced gene product MASP-3 are less clear, but evidence is accumulating that MASP-1 may link complement to the coagulation system (Presanis et al, 2004, Krarup et al, 2007, Krarup et al, 2008, Gulla et al, 2009) as well as collaborating with MASP-2 in the generation of the C3 convertase (Kocsis, 2010). MASP-3 is primarily found complexed to ficolin-3 and appears to regulate ficolin-3 mediated complement activation (Skjoedt et al, 2010).

Unsurprisingly, ficolin-2 has been shown to possess opsonic activity by enhancing phagocytosis of Salmonella by human neutrophils (Matsushita, 1995). Although such opsonisation may be mediated by complement activation, it has also been suggested

that ficolin-2 can opsonise bacteria by binding calreticulin on phagocytes via its collagen-like domain (Lacroix, 2009). Ficolin-2 can co-operate with pentraxins to opsonise bacteria and initiate the lectin pathway, as mentioned earlier.

1.3.8. Genetics

The human ficolin-2 gene (*FCN2*) has been localised to chromosome 9 (9q34) (Endo, 1996). The ficolin-1 gene (*FCN1*) is similarly located on chromosome 9, while *FCN3* is found on chromosome 1. The ficolin-2 gene has eight exons (Figure 1.9). The first exon encodes a signal sequence and the first nine N-terminal residues. Exons 2 and 3 encode a collagen-like region similar to that found in collectins. The fourth exon encodes a link or connecting region. Exons five to eight encode a domain similar in structure to that of the C-terminal portion of the human fibrinogen β and γ chains that is characterized by the conservation of 24 mainly hydrophobic amino acid residues.

A large number of functionally important single nucleotide polymorphisms in the *FCN2* gene have been described (Hummelshoj et al, 2005). Hummmelelshoj et al first described five polymorphisms in the promoter region and nine in the structural gene from a Danish population. Compatible results were reported by Herpers et al (Herpers et al, 2006), describing ten single nucleotide polymorphisms in 1888 Dutch blood donors. A later study compared five different ethnic groups; some ethnic-specific polymorphisms were noted, but most were found in all populations (Hummelshoj, 2008).

The most intensively studied single nucleotide polymorphisms are the promoter polymorphisms rs3124952 (-986 G>A), rs3124953 (-602G>A), rs28969369 (-64A>C) rs17514136 (-4A>G), and the exon 8 polymorphisms rs7851696 (Ala258Ser) and rs17549193 (Thr236Met). A frame shift mutation at position +6442 in exon 8 is theoretically capable of causing complete ficolin-2 deficiency, but homozygosity for this mutation has not been described. Indeed no example of complete ficolin-2 deficiency has yet been described. The single nucleotide polymorphisms implicated in influencing protein concentration in one or more studies are listed in Table 2. Although individual SNPs have a statistically significant impact on ficolin-2 serum levels at population level, they are not particularly useful for predicting individual ficolin-2 serum levels (Munthe-Fog et al, 2007). As yet, little data exists on whether non-genetic factors such as acute-phase responses influence circulating ficolin-2 levels. It has also been reported that the two common SNPs in exon 8 cause changes in the fibrinogen binding domain, leading to altered binding to GlcNAc. The T236M SNP is associated with significantly reduce GlcNAc binding while the Ala258Ser SNP is associated with increased lectin binding.

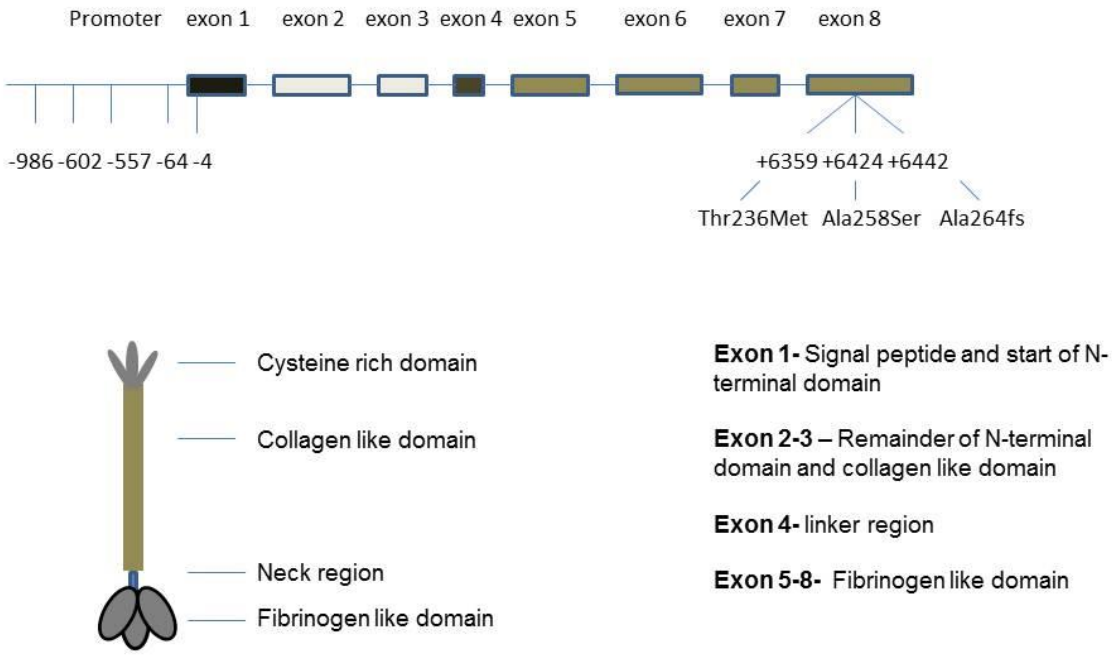

Fig.1.14. The human *FCN2* gene. The position of the major single nucleotide polymorphisms is shown. The mutation at +6442 leads to a deletion (fs=frameshift mutation).

SNP No	region & position	base substitution	amino acid substitution
rs3124952	promoter –986	A>G	-
rs3124953	promoter –602	G>A	-
rs3811140	promoter –557	A>G	-
rs28969369	promoter –64	A>C	-
rs17514136	promoter –4	A>G	-
rs17549193	exon 8 +6359	C>T	Thr236Met
rs7851696	exon 8 +6424	G>T	Ala258Ser

Table 1.5. Selected single nucleotide polymorphisms in *FCN2*.

Considerable linkage disequilibrium exists between pairs of promoter and structural gene dimorphisms, complicating the investigation of the relationship between allele expression and protein concentration. The majority of SNP's can be classified into 6 functional haplotypes which strongly influence serum levels. Authors (Munthe-Fog, 2007) have stratified serum ficolin-2 concentration according to those haplotypes as shown below.

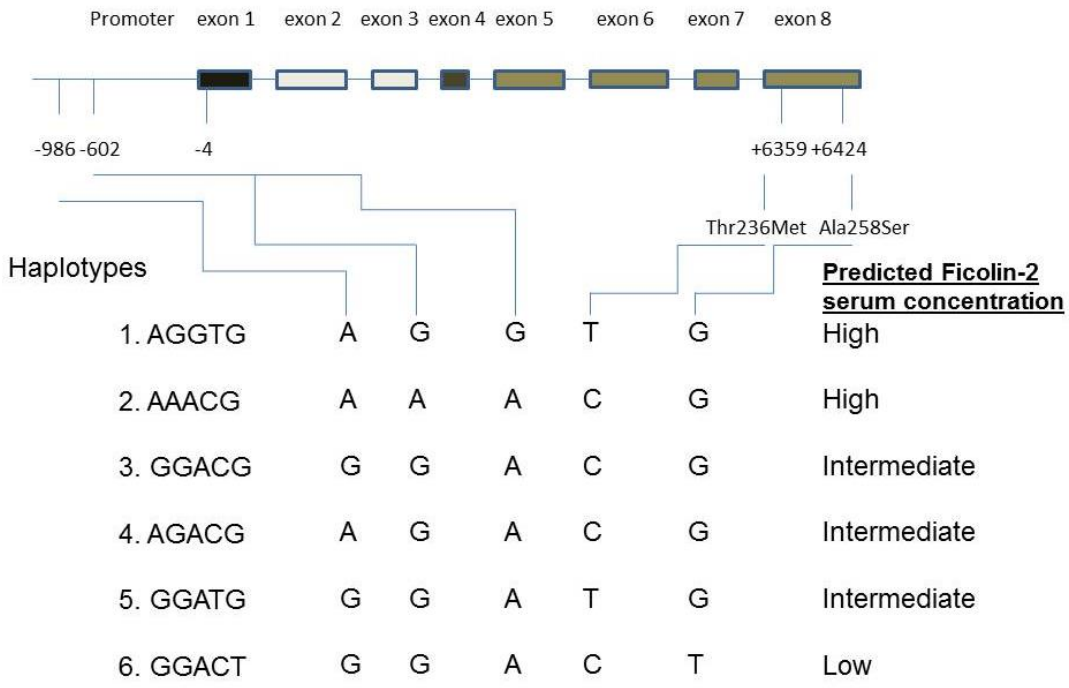

Figure 1.15. *FCN2* haplotypes having impact on ficolin-2 serum levels.

1.4 Ficolin-2 in health and disease

1.4.1 Ficolin-2 in healthy subjects

In healthy adult individuals, the distribution of serum ficolin-2 is Gaussian, so the mean and median are exactly the same. That value has generally been reported to be between 3 and 4 μg/ml (Kilpatrick, 2007) although more recently it has been determined at 5.4μg/ml (Munthe-Fog, 2007). Most normal values fall within the range 1000 to 6000 ng/ml, although occasionally much higher values are detected.

One study has investigated serum ficolin-2 throughout life (Sallenbach, 2011). The highest values were found between 1 and 4 years of life (median 11300 ng/ml), then dropped during later childhood (4-16 years, median 8660 ng/ml) before reaching a stable adult level (median 3370) after 16 years of age.

Although no absolute deficiency of ficolin-2 has yet been discovered, it seems possible that relative deficiency ("insufficiency") defined by low serum ficolin-2 ± immunogenetics could contribute to disease susceptibility.

1.4.2 Ficolin-2 in neonatal and perinatal infections

Ficolin-2 insufficiency was associated with perinatal infections in preterm Polish babies (St Swierzko et al, 2009). In a cohort of over 1800 consecutive deliveries, the rate of perinatal infections in babies with cord blood ficolin-2 < 1000 ng/ml (the lowest 9%) was twice that of babies with higher concentrations (13.7% v. 7.7%; $p<0.01$). This relationship was not independent of gestational age and birthweight,

but suggests that ficolin-2 insufficiency could be one of several factors that contribute to the adverse consequences of prematurity and low birthweight.

A further study, however, found no significant relationship between low cord blood ficolin-2 and sepsis in 47 premature infants (Schlapback et al, 2010). This was despite finding significant relationships between low ficolin-3 and Gram-positive sepsis and between low MBL and Gram-negative sepsis.

1.4.3 Infections in adults

Uraemic patients have an increased susceptibility to infection, and peritonitis is a common complication in patients on continuous ambulatory peritoneal dialysis (CAPD). The +6359 C>T variant in the *FCN2* gene, causing a Thr ►Met alteration and a concomitant decrease in lectin activity, was found to be more frequent in CAPD patients with a history of staphylococcal peritonitis, compared with CAPD patients without such a history (Meijvis et al, 2011). In the former, exit site *S. aureus* was also more prevalent (Meijvis et al, 2011).

A study of patients with neutropenic sepsis following haematological malignancy found no evidence of an effect from ficolin-2 (Kilpatrick et al, 2003).

However, the situation appears to be very different in the context of liver transplantation, where the recipient assumes the phenotype of the donor. The variant *FCN2* +6359 allele was associated with a doubling of the bacterial infection risk within the first year post-transplant and the risk was enhanced by co-inheritance of *MBL2* variant alleles (de Rooij et al, 2010). Patients with one or more lectin pathway

genetic variants and infection had an increased mortality rate which was statistically significant. As well as those bacterial infections causing sepsis and pneumonia, the normal (high lectin activity) *FCN2* +6359 was associated with protection from cytomegalovirus after liver transplantation (de Rooij, 2011). Again, the combination of *FCN2* and *MBL2* risk alleles conferred a particularly high degree of susceptibility (De Rooij et al, 2011).

1.4.4 Respiratory infections

There is a strong theoretical basis to believe ficolin-2 may be important in respiratory infections. As discussed previously, ficolin-2 binds to and opsonises a wide variety of important respiratory pathogens (including capsulated *S.pneumoniae* and *S.aureus*).

In perhaps the first disease association study involving ficolin-2, an association between ficolin-2 insufficiency and recurrent respiratory infections in children was reported. This relationship was particularly marked for patients with co-existing allergic disorders (mostly rhinitis and/or asthma with high IgE) (Atkinson et al, 2004). This preliminary observation prompted a fresh, prospective study on children aged 1 to 16 years (mean 8.9) to confirm or refute the previous retrospective findings, and to distinguish between infection and allergy (Cedzynski et al, 2009). Ficolin-2 insufficiency was indeed significantly associated with asthma and/or allergic rhinitis in the context of recurrent respiratory infections, but not with those allergic disorders in the absence of infection or with recurrent respiratory infections in the absence of allergy (Cedzynski, 2009). The reason for this relationship is not

clear, but it is possible that ficolin-2 confers some protection from microorganisms that exacerbate allergic inflammation in the lung.

Studies of ficolin-2 genetics in respiratory infections are in their infancy but a study of FCN2 genotypes and invasive pneumococcal disease found no significant association (Chapman et al, 2007).

Our pilot study demonstrated a clear association between low serum ficolin-2 and idiopathic bronchiectasis (Kilpatrick et al, 2009). Children with cystic fibrosis develop bronchiectasis and chronic bacterial colonisation, particularly with the opportunistic pathogen *P. aeruginosa*. A study of 96 children with cystic fibrosis followed up longitudinally found a significant relationship between polymorphism in the *FCN1* (rs2989727 and rs1071583) and *FCN2* genes (rs7865453 and rs7851696), and earlier age of onset of *P.aeruginosa* colonisation (Haerynck et al, 2012). Rs7865453 is particularly interesting as this is the Ala258Ser mutation in exon 8 that is most strongly associated with low serum ficolin-2 levels (Haerynck et al, 2012)

1.4.5 Other infectious diseases and disorders

The distribution of *FCN2* haplotypes in leprosy patients differed significantly from healthy controls (de Messias-Reason, 2009). The authors interpreted their findings as an indication that normal (relatively high) ficolin-2 concentrations protect against *M. leprae* infections, but serum ficolin-2 was not actually measured.

A comparison of mild and severe *Plasmodium falciparum* malaria revealed that serum ficolin-2 concentration is highest during acute severe disease, but this

difference was not reflected in the distribution of the *FCN2* haplotypes (Faik et al, 2011).

The distribution of *FCN2* variants was found to be altered in post-streptococcal disease (Messias-Reason, 2009). A haplotype associated with low levels of ficolin-2 was slightly but significantly more frequent in patients with chronic rheumatic heart disease (CRHD) compared with healthy controls. Conversely, another haplotype was more common in controls than in either CRHD or rheumatic fever patients. Since ficolin-2 can readily bind to *Streptococcus pyogenes* and thereby activate complement (Aoyagi, 2008), it is conceivable that these immunogenetic differences are related to an altered innate response.

A similar *FCN2* investigation in Behcet's disease was essentially negative (Chen et al, 2006) as was the outcome of serum ficolin-2 measurements in sarcoidosis (Svenden et al, 2008). Nevertheless there was a trend towards lower circulating ficolin-2 in sarcoid patients, in contrast to an increase noted for MBL.

1.4.6. Animal studies

The differences between human and animal ficolins have previously been discussed. It is difficult to extrapolate the results of animal studies directly from mice to humans because of these differences. In addition to the absence of ficolin-3 in mice, although ficolin-A in mice is analogous to ficolin-2 in humans, there are significant differences in binding specificity (Hummelshoj et al, 2012). Mannose binding lectin is represented by two proteins (MBL-A and MBL-C) in mice compared to a single

molecule in humans (Liu et al, 2001) In addition, given the emerging evidence of a role for pentraxins in amplifying the lectin pathway, it is important to note essential differences between human and mice pentraxins. In humans, C-reactive protein is the major acute phase reactant in human plasma, in contrast to mice where serum amyloid P is the major acute phase reactant and CRP is virtually absent (Yuste et al, 2007).

Having noted these differences, studies of animal ficolins are informative. Porcine ficolin-α was found to neutralise porcine reproductive and respiratory virus (a major pathogen of swine) in-vitro in a GlcNAc-dependent manner (Keirstead et al, 2011). However, variant alleles of ficolin-α were not associated with common infectious diseases (pneumonia, enteritis, serositis, septicemia) at necropsy, despite significant associations with MBL-A, MBL-C and surfactant protein A (Keirstead et al, 2011).

The lectin pathway of complement has been described as important in mice for host defence against *S. pneumoniae* pneumonia (Ali et al, 2012). MASP-2 knockout mice, with no functioning lectin pathway activity, had an increased severity of infection in a model of *S. pneumoniae* pneumonia. In this study, ficolin-A was the major lectin pathway component opsonising *S. pneumoniae*, and ficolin A null mice also had increased susceptibility to infection. Using MASP-2 knockout mice, it has also been reported that the lectin pathway of complement is not critical to defence against *P. aeruginosa* pneumonia (Kenawy et al, 2012) but is a major contributor to ischaemia reperfusion injury (Schwaeble et al, 2011). The role of ficolins in these particular contexts has not been fully explored. Ficolin-A knockout mice were found to be more susceptible to influenza A infection (Pan et al, 2012). The authors found that

human ficolin-2 could bind to influenza A virus and activate the lectin pathway of complement, preventing viral entry into cells.

Human ficolin-2 cDNA has been cloned into an expression plasmid and used in a murine model of *Salmonella typhimurium* infection (Ma et al, 2011). Administration of ficolin-2 in that form protected mice from a potentially lethal challenge with *Salmonella*, with bacterial counts dramatically reduced a week after infection, compared with empty vector-treated controls. The recombinant ficolin-2 enhanced monocyte phagocytosis of *Salmonella* in a dose-dependent manner (Ma et al, 2011).

1.4.8 Conclusions

Ficolin-2 is a major pattern recognition molecule in human serum, capable of binding to a wide range of micro-organisms and activating the lectin pathway of complement. Emerging data links ficolin-2 to defence against several important human pathogens, as well as diverse processes including apoptotic cell clearance. There are large variations in ficolin-2 serum levels due to genetic polymorphisms in humans. Disease association studies have thus far been limited. Studies in humans and animals suggest that variation in ficolin-2 may be associated with susceptibility to disease.

1.5 Aims and hypothesis

The lectin pathway of complement is involved in pattern recognition against a range of micro-organisms that are relevant to human respiratory infections. Work in humans and animals suggest that ficolin-2 and related molecules modify immune responses relevant to non-CF bronchiectasis, including apoptotic cell clearance, collaboration with the pentraxins and opsonophagocytosis.

Our review of data from studies of mannose binding lectin and cystic fibrosis, support the hypothesis that MBL is a modifier of disease severity.

We hypothesised that single nucleotide polymorphisms in the ficolin-2 and MBL genes would be important disease modifiers in bronchiectasis. Further we hypothesise that ficolin-2 would be important for opsonophagocytosis of *Pseudomonas aeruginosa* and other important bacteria.

Based on previous data, primarily from cystic fibrosis, we hypothesised that bronchiectasis would be associated with impaired neutrophil phagocytosis. We hypothesised that in-vitro this inflammation and neutrophil dysfunction could be improved by opsonisation with ficolin-2.

The aims of this thesis are therefore:

1) To define disease severity in non-CF bronchiectasis by investigating the association between airway bacterial load, airway inflammation and clinical markers such as exacerbations, health related quality of life and lung function. These investigations will be used to inform the studies of genetic modifiers of disease severity reported in subsequent chapters.

2) To determine if single nucleotide polymorphisms in the ficolin-2 and mannose binding lectin genes or serum levels of these proteins are associated with disease severity and chronic colonisation in bronchiectasis.

3) To investigate the role of ficolin-2 and MBL in opsonophagocytosis of *Pseudomonas aeruginosa.*

4) To investigate the mechanisms of inflammation and neutrophil dysfunction in the airway of patients with bronchiectasis.

Chapter 2

Materials and Methods

The study and all procedures in human subjects were approved by the Lothian Research Ethics committee, approvals, 08/S1102/58, 08/S1103/38, 08/S1103/61 and 10/S1103/27. All participants gave written informed consent to participate.

2.1 Patients with bronchiectasis and controls

Patients with bronchiectasis were recruited from a regional specialist bronchiectasis clinic at the Royal Infirmary of Edinburgh, Edinburgh, UK. The diagnosis of bronchiectasis was based on the presence of bronchial dilatation on high resolution CT scanning and a compatible clinical history of cough with sputum production and recurrent respiratory infections (Pasteur et al 2010). Patients aged <18 years, patients with active allergic bronchopulmonary aspergillosis, active mycobacterial disease, cystic fibrosis, asthma, common variable immunodeficiency and current smokers (within the previous 2 years) were excluded. We also excluded patients with long term use of oral or nebulised antibiotic therapy or long term oral corticosteroid therapy at any dose. Full medication histories, including over the counter medication use, were obtained at study entry. At the time of inclusion all patients were clinically stable with no antibiotic use in the preceding 4 weeks.

Clinical assessments and management

All patients underwent clinical assessments including spirometry (forced expiratory volume in one second, forced vital capacity with the highest of three technically satisfactory measurements recorded) high resolution CT scanning and chest radiography (Miller et al 2005). Patients were reviewed at each clinic visit by a consultant respiratory physician, specialist registrar or clinical research fellow. Patients were managed according to a standardised protocol based on the British Thoracic Society guidelines for the management of bronchiectasis (Pasteur et al 2010).

Symptoms and quality of life

Patients completed the St Georges Respiratory questionnaire (Wilson et al 1997) and the Leicester cough questionnaire (Murray et al 2009) as validated measures of quality of life and cough severity in bronchiectasis respectively. The LCQ has a score from 3-21 (a score of 3 is most severe cough) and the SGRQ has a score from 0-100 (100 indicates maximum impairment). The minimal important clinical difference is 1.3 Units and 4 Units respectively for these questionnaires (Raj 2009 and Jones 1991).

Exacerbations and hospitalisation

Unscheduled hospitalisations in the previous year for severe exacerbations were recorded and verified using an administrative database that records all regional

hospital admissions. Outpatient antibiotic use for exacerbations of bronchiectasis was quantified and verified against prescription records. All patients included were followed up every 6 months from 2008-2012. Exacerbations were defined as in the British Thoracic Society bronchiectasis guidelines as an acute deterioration with worsening of cough, increased sputum production or change in viscosity, increased sputum purulence with or without increasing wheeze, breathlessness or haemoptysis (Pasteur et al 2010). Hospitalisation was recommended for patients meeting the British Thoracic Society criteria for inpatient management of exacerbations (development of cyanosis or confusion, breathlessness with respiratory rate $\geq$ 25/min, circulatory failure, respiratory failure, temperature $\geq 38^{\circ}C$, inability to take oral therapy, inability to cope at home and failure of oral antibiotic therapy) (Pasteur et al 2010).

Chronic colonisation

Patients were classified as chronically colonised according to an accepted definition, if they grew a potentially pathogenic microorganism on 2 occasions at least 3 months apart in 1 year while clinically stable (Pasteur et al 2000). Patients had early morning sputum samples for qualitative and quantitative bacteriology at each clinic appointment at least twice per year and were provided with sputum pots to provide specimens at other times.

Radiological severity

Radiological severity of bronchiectasis was defined according to a modified Reiff score (Reiff et al 1995, Pasteur et al 2000). This takes into account the number of lobes involved (with the lingula scored as a seperate lobe) and the severity of dilatation (tubular-1 point, varicose-2 points, cystic- 3 points). The maximum score is 18.

Control subjects

Controls matched for age and sex were recruited from the healthy spouses of patients attending the outpatient clinics at the Royal Infirmary of Edinburgh. Controls completed a short health questionnaire to establish that they did not have chronic respiratory disease. Medication histories were also taken. Controls were excluded if they were aged <18 years bronchiectasis, asthma, COPD, were taking inhaled corticosteroids, long term oral corticosteroids or other immunosuppressive medications. We also excluded controls with chronic liver disease or active malignancy.

2.2 DNA extraction and genotyping

Genomic DNA was isolated from EDTA-anticoagulated whole blood samples using the Nuccleon BACC-3 kit (Gen-Probe Inc). 10mls of blood was added to 50ml

polypropylene tubes (Falcon) with 40ml of reagent A. This was mixed for 4 minutes at room temperature then centrifuged at 1300g for 4 minutes. Pellets were dissolved in 2ml of lysis reagent and incubated at 37°C for 10 minutes. Deproteinisation was performed by adding 500µl sodium perchlorate and mixing by inverting manually. 2ml of chloroform was added and inverted 7 times. 300µl of nucleon resin was added and the mixture centrifuged at 1300g for 3 minutes. The upper phase containing DNA was transferred to a fresh centrifuge tube and centrifuged at 1300g to pellet any carried over resin. Two volumes of 100% ethanol (cooled to 4°C) was added and inverted manually to precipitate the DNA. DNA was pelleted at 4000g for 5 minutes and the supernatant discarded. 70% ethanol was used to wash DNA and the pellet air dried for 10 minutes. DNA was resuspended in 1ml of sterile water for 2-4 hours and stored at -80°C until use.

Isolated DNA was quality-tested and genotyping performed at the Wellcome Trust Clinical Research Facility Genetics Core, Edinburgh. Validated Taqman allele specific PCR primers were purchased from Applied biosystems and PCR performed on the Applied Biosystems 7900HT according the manufacturer's instructions. Analysis was conducted with Sequence detection system (SDS) software version 2.4. A typical allelic discrimination plot is shown in figure 2.1.

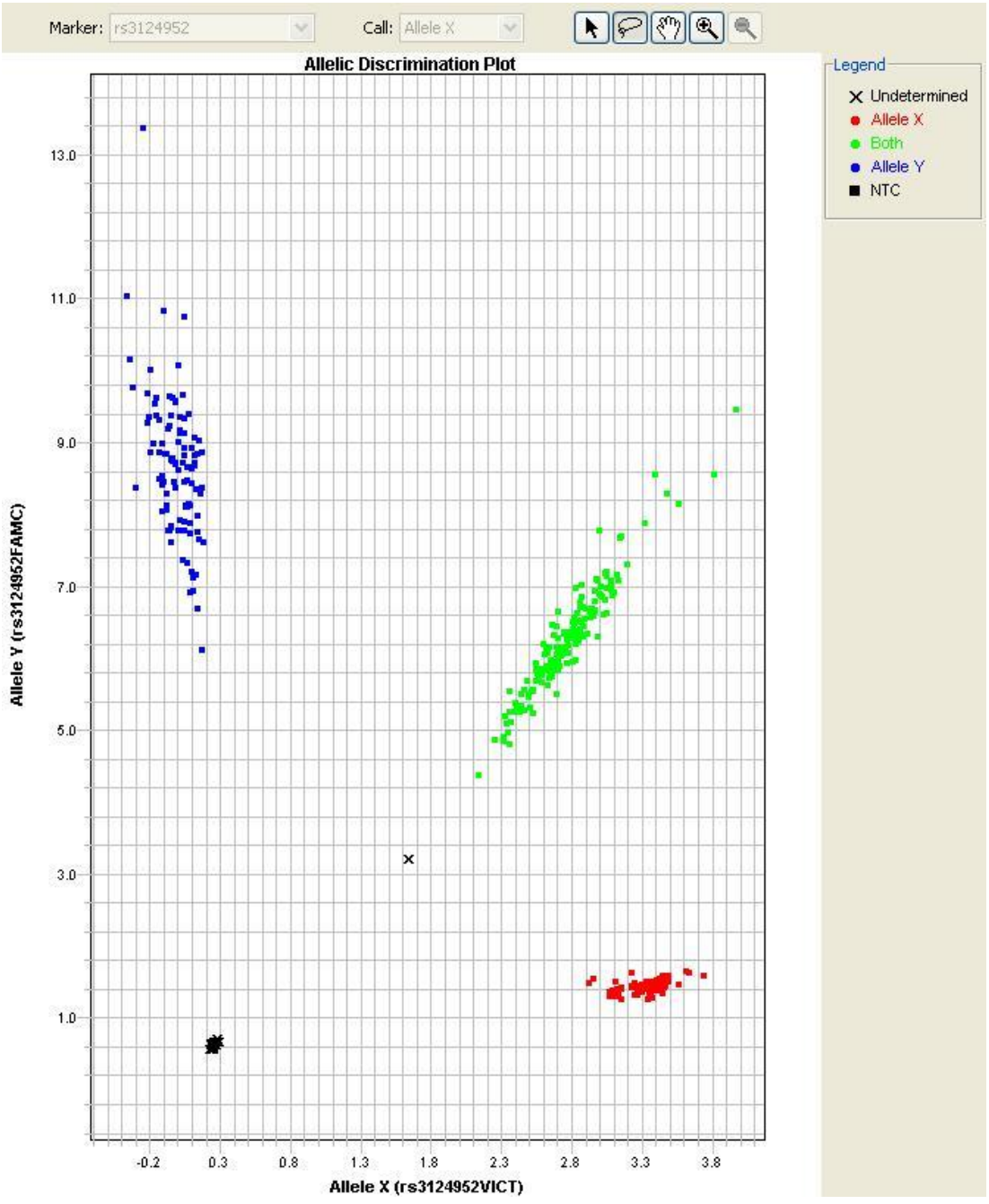
Marker: rs3124952
Call: Allele X
Allelic Discrimination Plot
Legend
Undetermined
Allele X
Both
Allele Y
NTC
Allele Y (rs3124952FAMC)
13.0
11.0
9.0
7.0
5.0
3.0
1.0
-0.2
0.3
0.8
1.3
1.8
2.3
2.8
3.3
3.8
Allele X (rs3124952VICT)

Figure 2.1. A typical allelic discrimination plot (In this case the plot shown is for the *FCN2* promoter polymorphism at position -986, rs3124952). A small number of samples as shown with (X) could not be determined. Such samples were re-extracted and retested.

Ficolin-2 SNP's

The authors studied 6 SNPs known to have the greatest effect on Ficolin-2 serum levels: the promoter polymorphisms rs3124952 (-986 G>A), rs3124953 (-602G>A), rs28969369 (-64A>C), rs17514136 (-4A>G) and the exon 8 polymorphisms rs7851696 (Ala258Ser) and rs17549193 (Thr236Met) (Hummelshoj et al 2005). Strong linkage disequilibrium between ficolin-2 SNP's produce 6 well characterised haplotypes which are associated with varying serum ficolin-2 levels. These can be classified into high, intermediate and low expressing haplotypes as previously described (Munthe-Fog et al 2007) . SNP's in MASP-2 rs72550870 (D120G) (Stengaard-Pedersen et al 2003, Thiel et al 2007), FCN3 rs28357092 (1637delC) were also determined by the same methods.

MBL genotyping

The authors studied 6 SNPs known to have the greatest effect on MBL serum levels, these are the exon-1 polymorphisms B- rs1800450, C rs1800451, D rs5030737, the promoter polymorphisms H/L, rs11003125, X/Y, rs7096206 and the 5' UTR SNP P/Q, rs7095891. These SNP's comprise 7 well-characterised "secretor haplotypes" (HYPA, LYPA, LYQA, LXPA, HYPD, LYPB and LYQC) which strongly influence circulating MBL levels (Garred et al 2009).

2.3 Definition of low ficolin-2 serum levels

The optimal way of defining abnormally low Ficolin-2 levels or ficolin-2 "deficiency" is not known. Based on a previous pilot study (Kilpatrick et al 2009), a priori, low serum Ficolin-2 levels was defined as a serum level <1600ng/ml with a sensitivity analysis performed using the lower cut-off of <1200ng/ml.

There is no genetic definition of Ficolin-2 deficient or insufficiency although it is well recognised that ficolin-2 SNP's form 6 haplotypes (Munthe-Fog et al 2007). These can be grouped into high, intermediate and low expressing haplotypes based on the associated serum ficolin-2 levels as described and shown in Table 2.1.

Haplotype	**Predicted Ficolin-2 serum level**
AGGTG	High
AAACG	High
GGACG	Intermediate
AGACG	Intermediate
GGATG	Intermediate
GGACT	Low

Table 2.1. Described Ficolin-2 haplotypes and the associated predicted effect on ficolin-2 serum level.

To determine the optimal genetic definition of low ficolin-2 serum levels, the authors conducted an analysis to determine the accuracy of individual SNP's, haplotypes and haplotype combinations to predict low Ficolin-2 serum concentrations (defined as <1600ng/ml). Discrimination was determined using the area under the receiver operator characteristic curve and curves were compared using the method described by Hanley and McNeil (Hanley and McNeil 1983). This data is presented in chapter 4.

2.4 Definition of MBL deficiency

There is no universally agreed definition of MBL deficiency. MBL function may be assessed by genotype, serum levels or functional activity in complement activation assays (Eisen 2010). Although these are strongly correlated, they do not give identical results. To account for this, we present data using 3 definitions of MBL deficiency determined a priori-

1) Genotypes associated with MBL deficiency
2) Serum levels <500ng/ml
3) Serum levels <200ng/ml

Haplotypes	Abbreviated genotype	Abbreviated combined genotypes	MBL expression
HYPD/LYQC/LYPB	O	O/O	Low
LXPA	XA	XA/O	Low
LYPA/LYQA	YA	YA/YO	Intermediate
HYPA	YA	XA/XA	Intermediate
		XA/YA	High
		YA/YA	High

Table 2.2. Haplotypes and abbreviated genotypes according to serum MBL expression.

Repeat measurements of serum MBL

To determine the impact of changes in MBL on disease severity over time, serum MBL was measured at study baseline, mid-point and at the end of the study in all patients. In addition, to determine the effect of exacerbations on serum MBL, 68 patients attending for treatment of exacerbations at the Royal Infirmary of Edinburgh bronchiectasis service were recruited.[23] Serum was taken at day 1 (start of exacerbation) and day 14 (end of exacerbation). Patients were treated with

intravenous antibiotic therapy based on their previous sputum microbiology for 14 days. Repeat measurements were then made at least 3 months post exacerbation to determine return of MBL levels to baseline levels.

2.5 Serum measurement of lectin pathway components

Ficolin-2 ELISA

Ficolin-2 ELISA was performed as described (Kilpatrick et al 1999). Microtitre plates (Immunlon 4, Dynatech) were coated with (GN4) monoclonal antibody against Ficolin-2 (100µl/well) in 0.25M sodium phosphate, pH 7.5 overnight at 4ºC. After washing three times (250 µl/well) with 0.1% Tween-20 in PBS, the wells were blocked with 1% bovine serum albumin (BSA) in 0.1M sodium phosphate, pH 7.5, for 2 h at 37°C. A further 3 washes with 0.1% Tween-20 in PBS were performed. Serum samples were diluted 30 fold in 0.1M sodium phosphate, pH 7.5, containing 0.1% BSA and incubated at 37°C for 2 h. The wells were washed again as above, and then incubated overnight at 4°C with biotinylated anti-ficolin-2 (GN5) at 5µg/ml in 0.1 M sodium phosphate, pH 7.5, containing 0.1% BSA. After a further wash step, avidin–biotin–peroxidase complex (Vectastain ABC kit, Vector Laboratories) was added and a further incubation at 37°C for 2 h performed. The wells were then washed twice with Tween-PBS followed by a further three washes with just PBS. Finally, substrate solution (0.1 mg:ml ABTS in 50 mM citrate phosphate buffer, pH 5.3, containing 0.015% H_2O_2) was added and the plates left in the dark at room temperature for 20 min. The A_{405} was measured using an ELISA reader and the concentration of ficolin-2 in serum samples determined against a standard curve

constructed with doubling dilutions of a serum previously calibrated against purified Ficolin-2. For the results presented, the mean intraassay coefficient of variation was 4.1% and the mean interassay co-efficient of variation was 9.4%.All low values were confirmed by reassay at 1 in 10 dilution.

Mannose binding lectin ELISA

Serum MBL measurement was performed as described (Kilpatrick et al 2009).

To prepare Mannan coated plates, Immulon-4 (Dynex) plates were coated with 100µl/well of 50µg/ml poly-D-lysine in carbonate buffer ((15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) at 4°C overnight. The plates were washed twice with distilled water and then once with phosphate buffered saline. Mannan solution (Sigma) at 0.1mg/ml was added (100µl/well) followed by 100µl/well of 0.1% glutaraldehyde in PBS. After incubation at room temperature for 10 minutes plates were washed three times with PBS and stored prior to use.

Plates were blocked with 3% BSA in PBS for 1 hour at room temperature followed by washing with calcium buffer (20mM Tris/0.5M NaCl, 10mM $CaCl_2$, 1% BSA and 0.05% Triton X-100, pH 9.0). Serum samples were diluted at 1 in 20 in calcium buffer and incubated for 1 hour at room temperature. Plates were washed three times with calcium buffer followed by incubation with mouse anti-MBL (HYB131-01) at 1µg/ml followed by goat anti-mouse immunoglobulin conjugated to alkaline phosphatase diluted 1 in 4000 in calcium buffer (Sigma) followed by p-nitrophenyl phosphate as a substrate (1mg/ml of p-nitrophenyl phosphate in 0.1M glycine buffer,

pH 10.3 containing 1mM zinc and magnesium). The reaction was termination with 3M NaOH (50µl/well) and the A_{410} was measured. This assay demonstrated intra-assay coefficient of variation of 3.5% and inter-assay co-efficient of variation of 7%.

MASP-2 and Ficolin-3 ELISA

MBL associated serine protease-2 and Ficolin-3 serum levels were determined using commercially available ELISA assay kits (Hycult Biotechnology, Uden, Netherlands).

2.6 Measurement of Systemic inflammation

It has been reported that bronchiectasis and other inflammatory lung diseases are associated with systemic elevation of adhesion molecules that are involved in leucocyte recruitment to the inflamed lung (Grosso et al 1998, Riise et al 1998, Zheng et al 2000). To determine if bacterial load correlates with these markers, Soluble Intercellular adhesion molecule-1 (ICAM-1), soluble E-selectin and soluble vascular cell adhesion molecule-1 (VCAM-1) were measured in serum using commercially available ELISA kits (R+D systems, Abingdon, UK). C-reactive protein and Erythrocyte sedimentation rate were also measured as systemic inflammatory markers. Measurements of these markers were performed by the National Health Service clinical biochemistry laboratory.

2.7 Sputum processing

Patients were taught to provide appropriate early morning sputum samples under the direction of a bronchiectasis specialist nurse and respiratory physiotherapist. Spontaneous early morning sputum was used for all bacteriology and airway inflammation studies. Spontaneous early morning sputum samples containing less than 10 squamous cells and more than 25 leukocytes per low power microscope field were acceptable (White et al 2003). All sputum processing was performed within 2 hours of expectoration. Sputum was homogenized and liquified using an equal volume of 0.1% dithiothreitol and serially diluted using sterile 0.9% saline to achieve dilutional factors of 10^{-1} to 10^{-4}. Cetrimide (Difco) *Pseudomonas* isolation agar, chocolate with bacitracin agar and sheep blood agar plates were inoculated with 100µl of sample. Colony forming units for each predominant pathogen were then identified by standard procedures and counted after 48hrs aerobic incubation (5%CO_2 for growth on chocolate with bacitracin agar) at 37°C to determine the sputum bacterial density, expressed as $\log_{10}$ colony forming units/ml (cfu.ml^{-1}) (Murray et al 2010).

For measurement of markers of airway inflammation, sputum, not treated with DTT, was ultracentrifuged at 50,000×g for 90 minutes at 4ºC. The sol phase was removed and immediately frozen at -80ºC.

Myeloperoxidase activity was measured as a marker of neutrophi mediated airway inflammation. 25µl of patient sputum sol diluted in PBS or purified myeloperoxidase (Sigma) were added to 96 well microplates (Serotec). 25µl of the colorimetric substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was added for approximately 5

minutes and the reaction then terminated with 50µl of sulfuric acid. Plates were read in a microplate reader at 450 and 560nm.

Neutrophil elastase activity was measured by chromogenic assay. Human neutrophil elastase (Sigma) was diluted in 50mM sodium acetate, 200mM NaCl, pH 5.56 and stored at -80ºC until use. Elastase or patient sputum sol samples were diluted in 50mM HEPES, 150mM NaCl + 0.05% Igepal CA630, pH 8. Sputum samples were diluted 1 in 50. Standard or samples were then added to 96 well microplates, 40µl per well. The synthetic substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MeoSAAPvN) was dissolved in DMSO at a stock concentration of 50mM. 200µl of the stock substrate was diluted in 4.8mls of 50mM HEPES, 150mM NaCl + 0.05% Igepal CA630, pH 8 and 40µl added to each well. Cleavage of the substrate was monitored by kinetic assay in a microplate at 37°C for 30 minutes. Reads were performed every 2 mins. Values were calculated against a standard curve of doubling dilutions of purified neutrophil elastase.

Interleukin 8, tumour necrosis factor-α and interleukin-1ß were measured by commercially available enzyme linked immunosorbant assay (ELISA, R+D systems, Abingdon, UK) using kits previously validated for sputum use according to established methodology (Stockley and Bayley 2000).

Validation of sputum ELISA's

All assays for sputum were validated as described (Stockley and Bayley 2000). Standard curves for each assay were obtained using pure mediator provided by the manufacturer following the assay protocol. Three pools of sputum sol were prepared

from 4 patients each with mucoid (grade 1), muco-purulent (grade 2) and purulent sputum(grade 3 or 4) based on a previously published sputum colour chart (Murray et al 2009). Three parameters were assessed in validating assays: Reliability, recovery of spiked mediators and the effect of sample dilution.

Reliability: The quantity of mediator in each sputum pool was determined by interpolation against the standard curve. Individual samples were assayed 5 times on a single plate to obtain the intraassay co-efficient of variation. Each sample was also assayed 5 times on different plates to obtain the inter-assay co-efficient of variation.

Spike and recovery: a known quantity of each mediator was spiked into the 3 pools of sputum. These "spiked" samples were then assayed and compared to the values obtained for the original pool. The obtained value was divided by the predicted value to calculate the % recovery.

Dilution effect: The 3 sputum pools were assayed at dilutions ranging from 1 in 1000, to 1 in 5. Low serum dilutions were used to exclude the prozone effect (Hook effect) on ELISA at high concentrations of antigen.

An assay was deemed to be valid if it had a reliable standard curve, an intra-assay and inter-assay coefficient of variation <10%, recovery of spiked samples in the range 80-120% of predicted and a linear dilution effect (Stockley and Bayley 2000). The results of the assays used in this study, for Interleukin-8, tumour necrosis factor-α and interleukin-1ß are shown below, figure 2.2. These assays passed each validation step and were used in the study. Table 2.3, shows the validation data for all sputum assays tested. Assays for Interleukin-6 (R+D systems), Interleukin-10

(R+D systems), Complement C5a (Hycult biotechnology and R+D systems) were not used in the study as they failed validation.

The figures below show the validation data for the IL-8, TNF-α and IL-1ß. The first figure (2.2) shows the linearity of dilution effect. In view of these results, dilution of 1 in 20 was used for each assay with re-assay at higher or lower dilution in cases where results were outside the dynamic range of the assay.

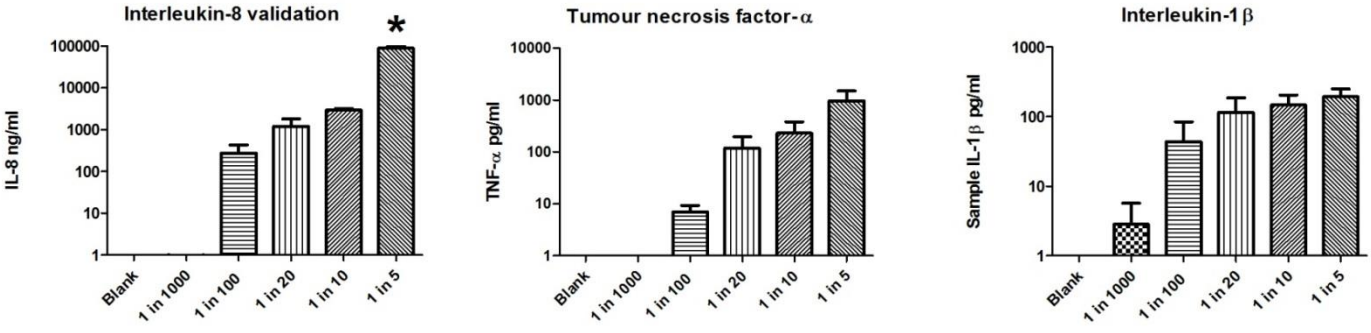

Figure 2.2. Validation of assays for sputum cytokine measurements: linearity of dilution effects for sputum measurement of interleukin-8, tumour necrosis factor-α and interleukin-1ß. * indicates outside the dynamic range of the assay.

Each assay gave a linear, reliable standard curve and representative standard curves are shown below (figure 2.3).

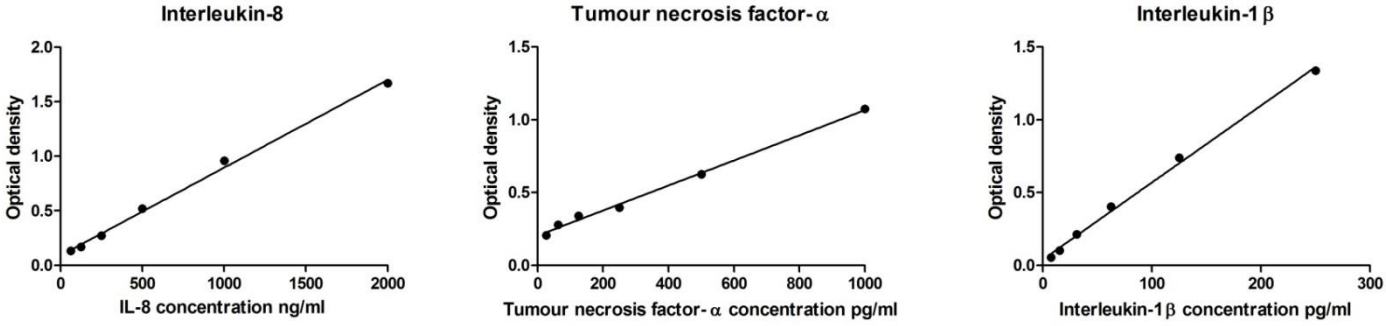

Figure 2.3. Validation of sputum assays for inflammatory mediators: representative standard curves for sputum measurement of interleukin-8, tumour necrosis factor-α and interleukin-1ß.

Finally, spike a recovery experiments demonstrated a majority of values between 80% and 120% of expected recovery with a median recovery and range within acceptable levels of variability. The results of the individual spike and recovery experiments are shown below (figure 2.4) and the overall assessment of each assay tested is shown in table 2.3. Table 2.3 also contains data for assays that were evaluated but were ultimately not used due to failed assay validation in sputum.

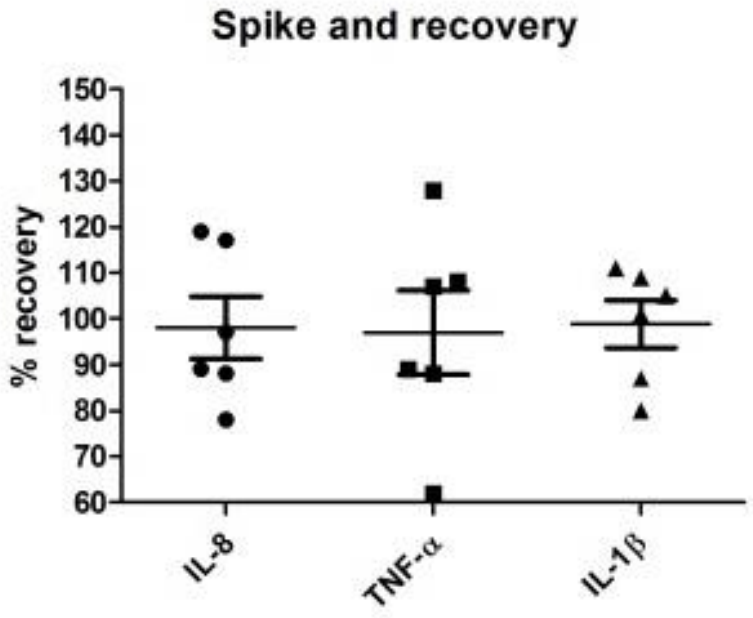

Figure 2.4. Results of spike and recovery experiments for the 3 assays validated for use in sputum. The bars show median with interquartile range with each icon representing the results of a single spiking experiment. Results are expressed as % recovery compared to the quantitiy of spiked standard.

Mediator	Spike-recovery (median %-IQR)	Intra-assay co-efficient	Inter-assay co-efficient	Linear dilution effect
Myeloperoxidase	102% (94-105)	4.2%	3.7%	yes
Elastase	102 (83-115)	7.1%	7.7%	yes
Interleukin-8	93% (86-118)	8.5%	8.1%	yes
TNF-α	98% (81-113)	4.1%	9.8%	yes
IL-1ß	103% (85-110)	2.7%	6.7%	yes
IL-6	71% (48-89)	9.6%	19.1%	no
IL-10	90% (57-162)	5.8%	34.2%	no
C5a (R+D systems)	48% (31-68)	8.8%	65.2%	no
C5a (Hycult Biotechnology)	67% (41-90)	11.7%	28.0%	no
Pentraxin-3	106% (82-114)	6.7%	11.1%	yes
Ficolin-2	77% (56-91)	6.2%	15.8%	no

Table 2.3. Validation of commercial ELISA kits for use in sputum

2.8 Bacteria and bacterial strains

Pseudomonas strain PA01, a fully sequenced *Pseudomonas* strain (Stover et al 2000), and all other bacterial strains unless otherwise indicated were kindly provided by Professor John Govan's laboratory at the University of Edinburgh.

Binding and phagocytosis assays were performed using *E.coli* strain 25922 (ATCC) , *Streptococcus pneumoniae* serotype 3 (ATCC), *S. aureus* (R6390 a methicillin sensitive strain, ATCC), *M. catarrhalis* (clinical isolate from a patient with non-CF bronchiectasis), *Haemophilus influenzae* (clinical isolate) *and Burkholderia cenocepacia.*

Environmental strains of *Pseudomonas aeruginosa* were obtained from hospital and non hospital environments such as sinks, drains, vegetables and ponds. Identification was based on colony morphology, blue-green pigmentation and positive oxidase test or API 20NE kits and specifies specifc PCR as described (Macdonald et al 2010).

Clinical isolates of *P. aeruginosa* from patients with *Cystic fibrosis* were provided by Professor John Govan's laboratory at the University of Edinburgh. These were first isolates of patients with CF aged from 2 months to 40 years All isolates were non-mucoid. Further details of these strains are described previously (Macdonald et al 2010).

Bacterial culture

All *Pseudomonas* strains were grown overnight in Luria Bertani (LB) broth at 37ºC in an orbital shaker at 250rpm overnight (12-16 hours) to achieve a stationary-phase

suspension. Before use, bacterial suspensions were diluted 1:20 in fresh LB broth and incubated at 37°C for a further 90 minutes to reach log phase. Bacterial suspensions were standardised via dilution to an optical density of 0.1 at 595nm using a spectrophotemeter (WPA UV 1101, Biotechn Photometer, Biochrom Ltd, Cambridge, UK). Bacteria were centrifuged at 1500g for 15 minutes and resuspended in the required buffer prior to experimental use.

2.9 Sodium dodececyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

Prior to western blotting, cell samples were washed with PBS and lysed at 4°C in lysis buffer (25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.3M NaCl, 1.5mM $MgCl_2$, 0.2mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100 (v/v), 1mM sodium orthovanadate and 0.5mM dithiothreitol (DTT). For non-reducing conditions buffer containing no DTT was used. Lysates were shaken for 20 minutes at 4°C and cleared by centrifugation at 13,000rpm for 10 minutes at 4°C.

Lysates or protein samples were solubilised in 4x SDS-PAGE sample buffer (50mM Tris-HCl, 10% glycerol (v/v), 2% SDS (v/v), 0.1% bromophenol blue (v/v), 10% β-mercaptoethanol (v/v), pH 6.8). For non-reducing conditions the buffer contains no β-mercaptoethanol. Samples were incubated at 95°C for 5 minutes and stored until analysis.

10% or 12% SDS-PAGE gels were used for western blotting as appropriate. Separating gels consisted of 0.375M Tris base (pH 8.8), 0.1% SDS (v/v), 10-12% acrylamide (v/v), 0.1% ammonium persulphate (v/v) and 0.02% Tetramethylethylenediamine (TEMED) (v/v). Stacking gels consisted of 0.13M Tris base (pH 6.8), 0.1% SDS (v/v), 4.5% acrylamide (v/v), 0.1% ammonium persulphate (v/v) and 0.02% TEMED (v/v).

.

Western blotting was performed under reducing and non-reducing conditions depending on the experiment, using a vertical electrophoresis tank Biorad Mini Protean II system. Samples were electrophoresed at 100–150 volts using electrophoresis buffer (50mM Tris base, 250mM glycine, 0.1% SDS (v/v) for 1–2 hours. Proteins were transferred onto Hybond C nitrocellulose membranes (Amersham Pharmacia Biotech) in transfer buffer (210mM glycine, 24.7mM Tris base, 20% methanol (v/v)) at 100 volts for 60 minutes, in a Mini Protean II blotting tank or at 4°C overnight. Equal protein loading was confirmed by staining with 1% Ponceau S (AMS Biotechnology) for 5 minutes to visualise protein bands. Non-specific binding sites were blocked by incubation with Tris-buffered saline (TBS)-Tween 20 (150mM NaCl, 20mM Tris HCl (pH 7.4), 0.1% Tween 20 (v/v)) containing 5% non-fat dried milk powder for 1 hour at room temperature. Membranes were probed with appropriate antibodies diluted in TBS-Tween 20 containing 5% non-fat dried milk powder for 1 hour or overnight at 4ºC. Membranes were then washed in TBS-Tween 20 (3x 10 minutes washes), before exposure to appropriate species-specific HRP-conjugated secondary antibodies (DAKO 1:1000 dilution unless otherwise stated) for 1 hour at room temperature, diluted in TBS-

Tween 20 containing 5% non-fat dried milk powder. Membranes were then finally washed in TBS-Tween 20 (3x 10 minute washes). Bands were identified using enhanced chemiluminescence reagent (ECL) (Amersham Pharmacia Biotech) according to the manufacturer's instructions and developed in a Konica SRX-101A film processor. Pre-stained molecular weight markers (Invitrogen) were used to estimate the size of chemoreactive bands. For western blotting of ficolin-2, anti-ficolin-2 clone GN5 was used followed by anti-mouse-HRP secondary antibody (DAKO).

2.10 Recombinant proteins

Bacterial expression of ficolin-2

A full length human ficolin-2 (FCN2) cDNA clone was purchased from Geneservice(USA). Polymerase chain reaction was performed using the following primers

FCN2 f: 5'-TAAGGATCCGATGGAGCTGGACAGAGCTGTG-3'

FCN2 rev 5'-TTCGAATTCCTAGGCAGGTCGCACCTTCAT-3'

BamH1 and EcoR1 restriction sites (underlined above) were incorporated for subsequent cloning. The Ficolin-2 open reading frame (ORF) PCR product was gel purified using a Qiagen DNA purification kit. The purified Ficolin-2 ORF was subsequently ligated into the pRSET-B expression vector (6-His tagged) with DNA ligase- Figure 2.5.

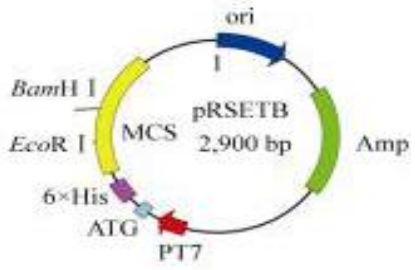

Figure 2.5- pRSETB plasmid used for cloning and expression of human ficolin-2.

Transformation of vector into BL-21 strain *Eschericia coli* bacteria was performed as follows. 0.5µg of pcDNA 3.1 vector, with a His-tagged FCN2 insert was added to 100µl of bacteria, mixed and placed on ice for 30 minutes. Bacteria were heat shocked for 50 seconds at 42°C and immediately transferred to ice for 2 minutes. 900µl of media was added and tubes were incubated for 45 minutes at 37°C shaking at 700rpm. Bacteria were pelleted for 5 minutes at 5000rpm and resuspended in 200µl of media. Serial dilutions were plated onto Luria-Bertani (LB) (Sigma-Aldrich) agar plates containing 50µg/ml ampicillin (Sigma-Aldrich) and incubated overnight at 37°C. The next day colonies were picked and inoculated into 10ml of LB broth containing 50µg/ml ampicillin and grown overnight. 2.5ml of overnight culture was inoculated into 500ml of LB broth containing 50µg/ml ampicillin. Bacteria were grown to an optical density (OD)600 of 0.7 to 0.8 and induced with 1mM isopropyl ß-D-thiogalactoside (IPTG) (Sigma-Aldrich) and then grown overnight at 37°C. Cultures were subsequently centrifuged at 8,000rpm for 20 minutes. Pellets were resuspended in 5ml of BugBuster Protein Extraction Reagent (Novagen) per gram of wet cell paste followed by the addition of 1µl/ml Benzonase

(Novagen). This was separated into 50ml conical tubes, placed on a rotary shaker for 20 minutes and centrifuged at 11,500rpm for 20 minutes.

Expressed ficolin-2 was purified from the cleared lysate using Ni-NTA His·Bind® Resin. 1ml of Ni-NTA His·Bind® Resin (Novagen), per purification, was washed with 4ml 1X Ni-NTA Bind Buffer (300mM NaCl, 50mM sodium phosphate buffer, 10mM imidazole, pH 8.0), the beads were centrifuged at 1000rpm for 5 minutes, and the top bind buffer layer was removed. 4ml of cleared lysate was loaded onto the prepared Ni-NTA His·Bind slurry and mixed gently by shaking at 4°C for 60 minutes. This was then loaded onto a disposable polypropylene column (1-5ml bed volume) (Pierce Biotechnology), flow through collected and 3 washes were carried out using Ni-NTA Wash Buffer (300mM NaCl, 50mM sodium phosphate buffer, 20mM imidazole, pH 8.0), 4x 500ìl elutions were performed with Ni-NTA Elution Buffer (300mM NaCl, 250mM imidazole, 50mM sodium phosphate buffer, pH 8.0) and elutions were collected in 4x 1.5ml eppendorf tubes. Fractions were analysed using SDS-PAGE and elutions dialysed against 1L cold 1X PBS overnight (dialysis tubing size – 14.3mm, molecular weight cut off 12-14KDa). The presence of ficolin-2 in the elutions was confirmed by ELISA and Western blot.

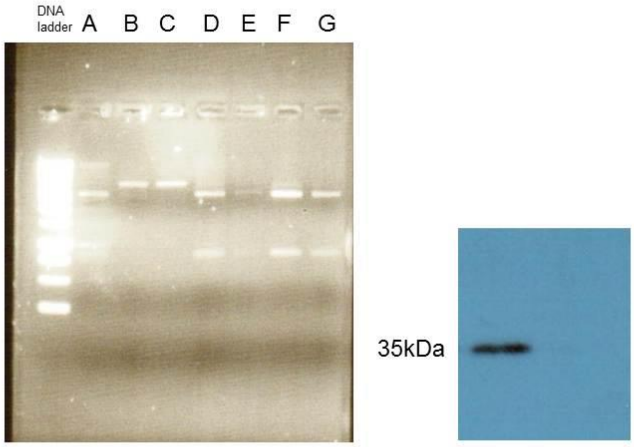

Figure 2.6. The first panel shows results of cloning of the FCN2 gene into the pRSETB plasmid. DNA samples were run on a 1% agarose gel. Lanes B and C show successful incorporation of the insert into the plasmid while empty vector are shown in lanes A and D-G. Following expression and purification of ficolin-2 the presence of recombinant protein was confirmed by western blotting (second panel). A band at 35kDa is shown under reducing conditions corresponding to ficolin-2 monomers.

Mammalian expression of ficolin-2

Stable CHO K1 cell lines expressing recombinant ficolin-2 were a kind gift from Dr Nicole Thielens (Lacroix et al 2009). Ficolin-2 was cloned into the pcDNA 3.1(+) mammalian expression plasmid (Invitrogen) containing a neomycin resistance gene. Ficolin-2-CHO stable lines were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% heat inactivated FCS and G418 (Invitrogen). 6 days after

confluence the supernatants were pooled and Ficolin-2 purified. For purification of Ficolin-2, N-acetylglucosamine (GlcNAc) and N-acetylcysteine (CysNAc) beads were generated. Sepharose 4CL beads (Sigma) were activated by incubation with 10% divinyl sulfone. After washing, the beads were derivatized in 0.25 M Na_2CO_3 containing 10% (w/v) GlcNAc or CysNAc at pH 11 overnight. The beads were washed with dH_2O, and residual active groups were blocked by incubation with 0.1 M ethanolamine, pH 9.0.

The cell culture supernatant was dialysed against 145 mM NaCl, 5 mM $CaCl_2$, and 20 mM Tris-HCl (pH 7.4). This was then applied to the CysNAc column equilibrated in 145 mM NaCl, 5 mM $CaCl_2$, and 20 mM Tris-HCl (pH 7.4) as above. Bound ficolin-2 was then eluted using the same binding buffer containing 0.3M GlcNAc. The protein containing fractions were dialysed into binding buffer and then concentrated by ultrafiltration (Krarup et al 2004). As above, the presence of ficolin-2 in the elutions was confirmed by ELISA and western blot. Function of the recombinant ficolin-2 expressed in both bacterial and mammalian systems was tested using binding to acetylated bovine serum albumin in a solid phase binding assay as described below.

MBL

Recombinant mannose binding lectin was purchased from R+D systems (Abingdon, UK) and functionality tested using a mannan binding assay as above.

Ficolin-3

Recombinant Ficolin-3 was was purchased from R+D systems (Abingdon, UK) and functionality tested by adding increasing concentrations to acetylated BSA coated microplates in a functional ELISA as described below.

Pentraxins

Recombinant Pentraxin-3 and serum amyloid P were purchased from R+D systems. Recombinant C-reactive protein was purchased from Sigma.

Complement component C5a

Recombinant complement component C5a was purchased from R+D systems.

2.11 Isolation of peripheral blood neutrophils and monocytes

Peripheral blood neutrophils were isolated from patients with bronchiectasis and healthy volunteers by Percoll-gradient density centrifugation as previously described (Haslett et al 1985).

Human peripheral blood was obtained from the antecubital vein of healthy volunteers Blood was collected into tubes containing sodium citrate (1% final concentration) to prevent coagulation. Erythrocytes were sedimented with 0.6% (w/v) dextran T500 followed by fractionation of leukocytes on a discontinuous Percoll™ gradient

(prepared in Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS) with final concentrations of Percoll of 55, 70, and 81%) at 720 g for 20 min. Monocytes were aspirated from the 55/70 interface, and PMN from the 70/81% interface, and washed three times in PBS (without Ca^{2+}/Mg^{2+}) before culture. Monocytes were enriched by anti-CD14 microbead magnetic cell sorting (Miltenyi Biotec). Cells from the 55/70 interface we centrifuged at 300g for 10 minutes and resuspended in 16µl of MACS buffer (PBS with 2% fetal calf serum and 2mM EDTA) per 10^7 cells counted with a haemocytometer. 4µl of CD14 microbeads (Miltenyi Biotec) were added per 10^7 cells and incubated for 20 minutes at 4ºC. The MACS column was equilibrated with 3mls of MACS buffer and placed in a magnet (MACS separator, Miltenyi Biotec). Cells were washed with 2mls of MACS buffer per 10^7 cells and centrifuged at 300g for 10 minutes, with the supernatant removed. Cells were resuspended in 1ml of cold MACS buffer and applied to the column. Unlabelled cells passing through the column were collected and the column washed with 3 x 3ml of MCS buffer. The column was then removed from the magnet and flushed with 2 x 5ml of MACS buffer. Cells were resuspended in 5ml of RPMI and then resuspended at the indicated concentration in RPMI or Iscove's media (IMDM). Isolated monocytes with typical purity of >95% as assessed by flow cytometry with CD14 antibody (DAKO) were matured into macrophages by incubation culture for 5 days in IMDM containing 10% autologous serum.

2.12 Isolation of sputum neutrophils

For sputum neutrophil studies patients underwent sputum induction as described with 3% hypertonic saline. Sputum neutrophils were isolated by a modification of previous described methodology (Hartl et al 2007, Voglis et al 2009). Sputum was processed immediately upon expectoration. Sputum plugs were selected and sputum diluted 8 fold in phosphate buffered saline (PBS). After centrifugation at 800g for 10 minutes, sputum was mixed with 4 volume of 0.1% dithiothreitol(DTT). Sputum was then incubated with DTT for 15 minutes at 4ºC and the homogenised sputum was then passed through a 48µm filter. The filtrate was then washed with PBS to remove DTT. The cell suspension after filtration consisted of a majority of neutrophils with a few alveolar macrophages in bronchiectasis patients. The cell suspension in PBS was then applied to a percoll gradient (40% and 50% percoll) and centrifuged for 20 minutes at 400g. Cell viability was assessed by trypan blue exclusion and cell purity assessed by Diff-Quick stained cytospins. Neutrophils were typically 95% pure using this method.

9 healthy volunteers underwent sputum induction with sputum processing as described above. Insufficient neutrophils were obtained to perform assessment of airway neutrophil receptor expression or to perform phagocytosis assays. Cytospins suggested that in healthy non-smokers, induced sputum contained small numbers of macrophages with few neutrophils. Consequently sputum neutrophil receptor expression in bronchiectasis patients was compared to the patient's own peripheral blood neutrophils stimulated with the synthetic chemoattractant fMLP (0.1µM), an approach that has been used previously (Berger et al 1989).

Initial experiments in which peripheral blood neutrophils were processed according to the sputum neutrophil protocol established that treatment with dithiothreitol and the filtration process did not affect surface expression of any of the markers under study, or significantly affect phagocytosis or oxidative burst by peripheral blood neutrophils (these results are presented in the relevant chapter). Sputum neutrophils were examined by flow cytometry on a BD FACSCALIBUR flow cytometer.

2.13 Definitions of severe bronchiectasis for sputum neutrophil and peripheral blood neutrophil studies

Severity of bronchiectasis was determined according to the criteria described by Smith (Smith 2011). Patients with severe bronchiectasis have severe radiological disease as defined by Reiff criteria (Reiff et al, 1995), are colonised with *P. aeruginosa*, expectorate purulent sputum when stable and have frequent exacerbations (typically defined as $\geq$ 3 per year). Patients with mild bronchiectasis have mild radiological disease, are not chronically colonised, expectorate mucoid sputum and do not have frequent exacerbations. A priori, patients matched for age and sex in these subgroups were identified and recruited.

2.14 Binding assays

Solid phase binding assay

The solid phase binding assay was performed as described (Lynch et al 2004). Nunc maxisorb microtitre plates were coated with acetylated BSA, Mannan, *P. aeruginosa*

(PA01) or *E.coli* in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6). After overnight incubation, wells were blocked with 300µl 1% human serum albumin (HSA) in TBS (10mM Tris-Cl, 140mM NaCl, pH 7.4) for 1 hour and subsequently washed with TBS/0.05% Tween 20 containing 5mM $CaCl_2$. Serum samples or recombinant Ficolin-2 was diluted in 100µl of 10mM Tris-Cl, 140mM NaCl, 2mM $CaCl_2$, 0.1% HAS, pH 7.4. Diluted samples were added to the wells and incubated for 2 hours at 37^{o}C. After washing 4 times with wash buffer, bound ficolin-2 was detected with biotinylated anti-ficolin-2 GN5 followed by streptavidin-HRP. After repeating the wash steps as above, 200µl of 3,3',5,5'-Tetramethylbenzidine was added to each well for 30 minutes. The reaction was stopped with 100µl of HCL and absorbance measured at 450nm with subtraction of absorbance at 650nm using a microplate reader.

Depletion assay

P. aeruginosa (1 x 10^8) were incubated with 25µl of serum or recombinant protein in TBS (10 mM Tris-HCl (pH 7.4), 140 mM NaCl), 0.05% (v/v) Tween 20, 5 mM $CaCl_2$. GlcNAc-sepharose beads or mannan-agarose beads (Sigma) were used as a positive control. After incubation at 4ºC for 1 hour, samples were centrifuged at 7000g for 10 minutes. Supernatants were collected and unbound proteins quantified by ELISA. A result of 100% indicates no binding to microorganisms whereas 0% indicates complete binding. Values between 100% and 75% were not considered as significant binding as previous described (Hummelshoj et al 2012). <75% was regarded as significant binding (Hummelshoj et al 2012).

Flow cytometry assay

Bacteria (1 x 10^8) were blocked for 1 hour at room temperature with 3% BSA in PBS. The samples were centrifuged at 3000×g for 15 minutes and the supernatant aspirated before re-suspension in the indicated concentration of Ficolin-2, Ficolin-3 or mannose binding lectin in calcium buffer (0.5M sodium chloride, 20mM Tris-Hcl, 10mM $CaCl_2$, 1% BSA, pH 7.4. These were incubated at 37°C for 1 hour with the bacteria before centrifugation at 3000g for 15 minutes, aspiration of supernatant and incubation at 4°C for 30 minutes with anti-ficolin-2 (5µg/ml), anti-ficolin-3 (5µg/ml) or anti-MBL (1µg/ml). Samples were then centrifuged and re-suspended in PBS for flow cytometry on a BD FACS Calibur flow cytometer.

2.15 Treatment of neutrophils with sputum sol or reagents

Healthy donor neutrophils (0.5 x 10^6 per experiment) were incubated for 30 minutes with either purified neutrophil elastase, cathepsin G (Sigma), or patient sputum sol (at a final concentration ranging from 10% sputum sol to 0.6% in PBS) or PBS control at 37°C. Following incubation, cells were washed extensively with PBS and subject to neutrophil studies as shown below.

2.16 FACS analysis of neutrophil surface markers

Samples of freshly prepared neutrophils were centrifuged at 300g for 5 minutes and resuspended in PBS containing the following primary antibodies or appropriate isotype control antibody. The antibodies used were as follows:

Mouse monoclonal anti-CD88 labelled with Allophycocyanin, Mouse monoclonal anti-complement receptor 1 (CD35) Phycoerythrin (clone 594708), mouse monoclonal anti-CD16b (clone 245536) labelled with Allophycocyanin (all R+D systems, Abingdon, UK), mouse monoclonal anti-complement receptor 3 (CD11b) labelled with Allophycocyanin (clone ICRF44) and mouse monoclonal CD62L (clone DREG-56) labelled with Phycoerythrin both from BD Bioscience, Oxford, UK. Neutrophil viability was assessed by staining with Annexin-V Fluorescein isothiocyanate and propidium iodine (Apoptosis detection kit, Sigma Aldrich).

Antibodies were incubated at $4^{o}C$ for 30 minutes, then centrifuged at 300g for 5 minutes, resuspended in PBS and immediately subject to flow cytometry on a FACSCalibur flow cytometer. A minimum of 10,000 events were counted in all analyses.

In the case of peripheral blood neutrophils, receptor expression was studied with and without maximal activation induced by treatment for 15 minutes with 1µM platelet activating factor and 0.1µM fMLP (Farnworth et al. 2008).

2.17 FITC-labelling of bacteria

P. aeruginosa strain PA01 and *E.coli* strain (ATCC 25922) were grown on Pseudomonas isolation agar and nutrient agar plates respectively. Freshly isolated colonies were inoculated into 10mls of Luria-Bertani broth at cultured overnight at 37^{o}C with gentle shaking. Cultures were then subculture 1 in 10 and grown for 3 hours to enter logarithmic growth phase. Cultures (OD_{600}=0.1) were then labelled with Fluorescein isothiocyanate (FITC) as described below.

Bacterial suspensions were serially diluted and plated out on agar plates to determine colony counts. Overnight cultures of bacteria were heat inactivated 60°C for 1 hour before centrifugation at 3000×g for 15 minutes and OD brought to 1 with cold PBS. Samples were washed with 1%BSA-Hanks' balanced salt solution (HBSS). 100µL of FITC solution (0.5mg/ml in PBS) was added and placed on a rotary mixer at 4^{o}C for 30 minutes. 900µl of ice cold 1%BSA-HBSS was added and samples were centrifuged at 10000rpm for 2 minutes, and re-suspended in 1mL 1%BSA-HBSS. Labelling was confirmed by FACS analysis and microscopy. Labelled bacteria were stored at -80ºC until use. Results of the FACS analysis after labelling are shown below in figure 2.7.

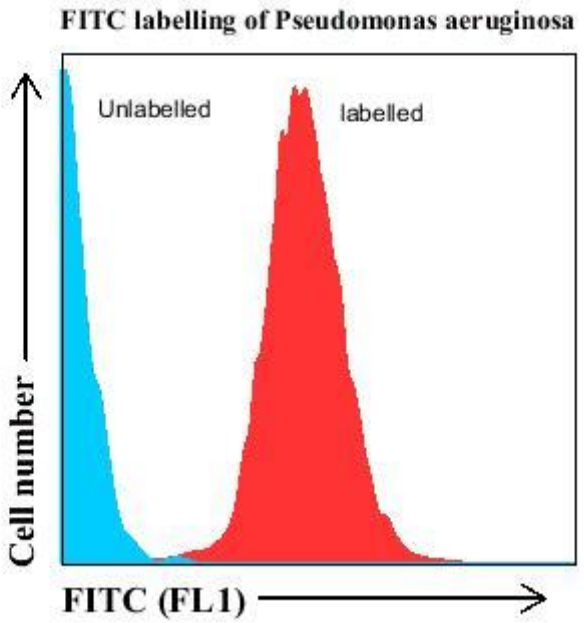

Figure 2.7. Flow cytometry analysis confirming labelling of *Pseudomonas aeruginosa* strain PA01 with FITC.

2.18 Generation of depleted sera

Lectin pathway depletion

To generate serum depleted of all lectin pathway components, pooled serum samples from healthy donors were incubated with GlcNAc beads equilibrated in binding buffer (50mM Tris-HCl, 200mM NaCL, 10mM $CaCl_2$, pH 7.8) overnight at 4°C (Krarup et al 2004). Following incubation the concentration of Ficolin-2, MBL and Ficolin-3 in the serum was determined by ELISA. This method resulted in complete depletion of these components from serum (no detectable proteins by ELISA).

Specific Ficolin-2 depletion

Serum samples were incubated with anti-Ficolin-2 (10µg/ml) and depleted with magnetic pan-mouse IgG dynabeads (Invitrogen) were applied according to the manufacturer's instructions. Ficolin-2 depletion was confirmed by ELISA prior to use.

Specific MBL depletion

Serum samples were applied to a mannan-agarose column (Sigma) equilibrated in binding buffer 20mM Tris-HCl, 150mM NaCl, 2mM $CaCl_2$, 1% BSA, pH 7.4, for 2 hours at 4°C. MBL depletion was confirmed by ELISA prior to use.

C1q and MASP-2 *negative serum*

C1q depleted serum was purchased from Merck. MASP-2 negative serum was obtained from a patient with a homozygous D120G mutation, a condition that results in complete MASP-2 deficiency (Stengaard-Pedersen et al 2003, Thiel et al 2007). This serum contained normal levels of MBL, Ficolin-2 and Ficolin-3 but no detectable levels of MASP-2 by ELISA.

2.19 Complement activation assays and Lectin pathway isolation

Complement can be activated by the classical, alternative and lectin pathways. To isolate the pathways responsible for opsonisation of *P. aeruginosa* and other bacteria, and to determine the contribution of the lectin pathway to overall complement activation, several inhibitors of complement activation were used.

Incubation of sera with 10 mM EDTA blocks all 3 complement activating pathways, as does heat inactivation at 57ºC for 1 hour. Incubation with 10 mM EGTA + 10 mM Mg^{2+} selectively blocks the classical and lectin pathways (Fine et al 1973) allowing study of the alternative pathway in isolation. Incubation with 100mM mannose primarily inhibits mannose binding lectin while incubation with 100mM N-acetylglucosamine blocks all lectin pathway components (MBL, ficolin-1, ficolin-2 and ficolin-3). C1q depleted serum was used to selectively block the classical pathway. Purified C1q (Sigma) was added to C1q depleted serum to recapitulate the blocked classical pathway. C3 depleted serum was obtained from Sigma and was used as a negative control for C3 deposition.

A literature search identified 3 methods to specifically examine the lectin pathway of complement activation. C1q deficient serum was used to exclude classical pathway activation. Alternative pathway inhibition was achieved by high ionic strength using high NaCl concentration buffer (Petersen et al 2001) and the anticoagulant Sodium polyanethole sulfonate (SPS) (Palarasah et al 2011, Heja et al 2012). Low serum dilution (0.5%) also results in reduced activity of the alternative pathway (Harboe et al 2004, Ferguson et al 2006) which most accurately reflects the low levels of complement components present in the lung. Previously studies suggest the

alternative pathway is relatively inactive in the lung (Watford et al 2000). Each of these experimental conditions was tested to determine the impact of lectin pathway activation on opsonisation of PA01.

C4 cleavage assay

Lectin pathway activation was quantified as described (Petersen et al 2001). Microplate wells were coated with 100µl of *P.aeruginosa* in coating buffer as described above (Lynch et al 2004). After overnight incubation, wells were blocked with 300µl 1% human serum albumin (HAS) in TBS (10mM Tris-Cl, 140mM NaCl, pH 7.4) for 1 hour and subsequently washed with TBS/0.05% Tween 20 containing 5mM $CaCl_2$ (wash buffer). Serum samples diluted with or without supplementary recombinant ficolin-2 were diluted (typically 1 in 10) in 20mM Tris-Cl, 1M NaCl, 10mM $CaCl_2$, 0.1% HAS, pH 7.4 which prevents activation of endogenous C4 and dissociates the C1 complex (composed of C1q, C1r and C1s). 100µl of diluted samples were added to the plate for 2 hours at 37^{o}C. Plates were washed with wash buffer, followed by addition of 0.1µg of purified human C4. After 90 minutes at 37°C, the plates were washed again and C4b deposition detected with anti-C4 (Hycult biotechnology).

SPS assay

Initial experiments identified a dominant role for the alternative pathway in complement activation against *P. aeruginosa* (PA01). As described above, Microtitre

plates were coated overnight with 1 x 10^8 cfu/ml of PA01 in coating buffer. Plates were washed 3 times in 10mM Tris-HCl, 140mM NaCl, pH 7.4. 50% pooled human serum was diluted in 10mM Tris-HCl, 140mM NaCl, pH 7.4 containing 10mM EGTA and 10mM Mg^{2+} with increasing concentrations of sodium polyanethole sulfonate (0.1mg to 1mg/ml). Serum samples were incubated for 1 hour at 37°C followed by washing 3 times in wash buffer. Complement activation was detected by incubation with anti-complement C4 (HYB 162-04, Bioporto) or anti-complement c3 (HAV 003-05, Bioporto) followed by anti-mouse HRP detection antibody. Complete inhibition of alternative pathway activity was defined as an optical density of C4 and C3 activation equivalent to the negative control (heat inactivated serum).

Lectin pathway activity was measured using mannose binding lectin activation on mannan using a modification of a previously described protocol (Petersen et al 2001). Microtitre plates coated with mannan from *Saccharomyces cerevisiae* (Sigma) at 10µg/ml in coating buffer and incubated overnight at 4°C. Wells were blocked and subsequently doubling dilutions of pooled serum were incubated in 20mM Tris-HCl, 10mM $CaCl_2$, 140mM NaCl, 0.05% triton X-100, 0.1% human serum albumin, pH 7.4 containing increasing concentrations of sodium polyanethole sulfonate (0.1mg to 1mg/ml).These were incubated at 37°C for 1 hour. Well were washed and complement activation detected by incubation with anti-complement C4 (HYB 162-04, Bioporto) and anti-complement c3 (HAV 003-05, Bioporto) followed by HRP detection antibody. Complete inhibition of lectin pathway activity was defined as an optical density of C4 and C3 activation equivalent to the negative control (MBL depleted serum)

Low serum concentration

The alternative pathway is relatively inactive at low serum concentrations and is therefore thought to have a limited role in complement activation in the lung (Watford et al 2000, Ferguson et al 2006). Therefore low concentrations of C1q $^{-ve}$ serum should only be able to activate complement via the lectin pathway. Using the alternative pathway assay described above, very low C3 or C4 deposition was detected when incubating serum with 10mM EGTA and 10mM Mg^{2+} at a concentration of 2% serum. Lectin pathway activity was still detectable at 0.5% serum concentration and was used for subsequent experiments. These data are shown in chapter 4.

2.20 Phagocytosis assays

Neutrophil phagocytosis assay

A flow cytometry based phagocytosis assay was used (Farnworth et al 2008). Labelled *P. aeruginosa and E.coli* were opsonised as indicated at 37^{o}C for 1 hour. Opsonised bacteria were then added at a multiplicity of infection 10:1 to patient or control neutrophils (0.5 x 10^6 per experiment). Phagocytosis was permitted at 37^{o}C for up to 30 minutes and then terminated by placing the samples on ice. Excess bacteria were removed by washing in PBS and cells subsequently analysed by FACS. To differentiate phagocytosed (intracellular) bacteria from adherent (extracellular bacteria), cells were incubated with 0.1% trypan blue to quench extracellular fluorescence. A minimum of 10,000 events were counted.

Time point studies were performed to determine the optimum point to terminate phagocytosis. These data are shown below. Validation of the assay suggested a strong correlation between flow cytometry and microscopy results. Validation that the observed increase in fluorescence was the result of phagocytosis was achieved by pre-treatment of neutrophils with 100µg/ml cytochalasin-D prior to phagocytosis (Mimura and Asano, 1976). This inhibits actin polymerisation in neutrophils and therefore prevents bacterial ingestion.

Figure 2.8 shows a strong relationship between the concentration of serum used for opsonisation and the percentage of positive cells, indicating the proportion of neutrophils taking up FITC labelled bacteria. After opsonisation with 20% serum, neutrophils are saturated within 5 minutes with no further increases in % positive cells. Saturation was achieved between 10 and 20 minutes after opsonisation with 10% serum but 100% phagocytosis was not achieved at concentrations below 10% serum. After opsonisation with 1% serum, 98% phagocytosis was achieved after 30 minutes. As expected, cytochalasin-D completed inhibited neutrophil phagocytosis. Even small concentrations of opsonins increase phagocytosis with significant differences between 0.5% serum and no serum ($p=0.001$).

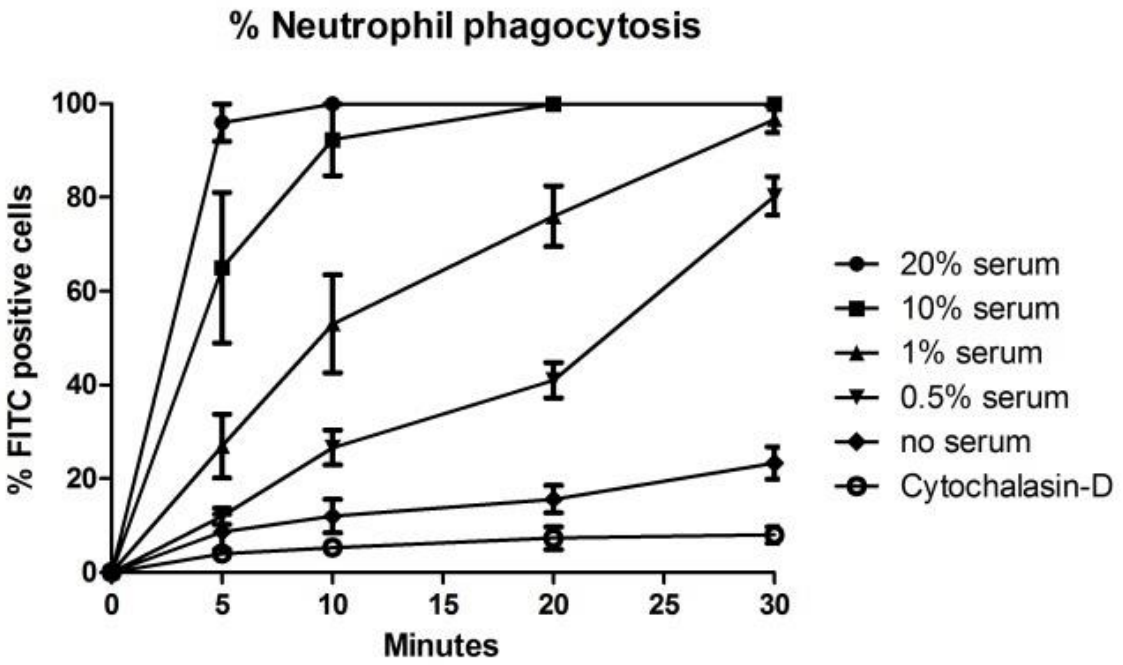

Figure 2.8. Flow cytometry phagocytosis assay. Bacteria were incubated with increasing concentrations of donor serum and incubated with FITC-labelled PA01 for the indicated times. Results are the mean of 3 independent experiments.

Examining mean fluorescence, which is thought to estimate the number of bacteria ingested per neutrophil, there were similar concentration serum concentration dependent increases in phagocytosis. Using two way ANOVA, all serum concentrations were significantly different compared to no serum ($p<0.0001$). The same inhibitory effect was observed with cytochalasin-D, these results are shown in figure 2.9.

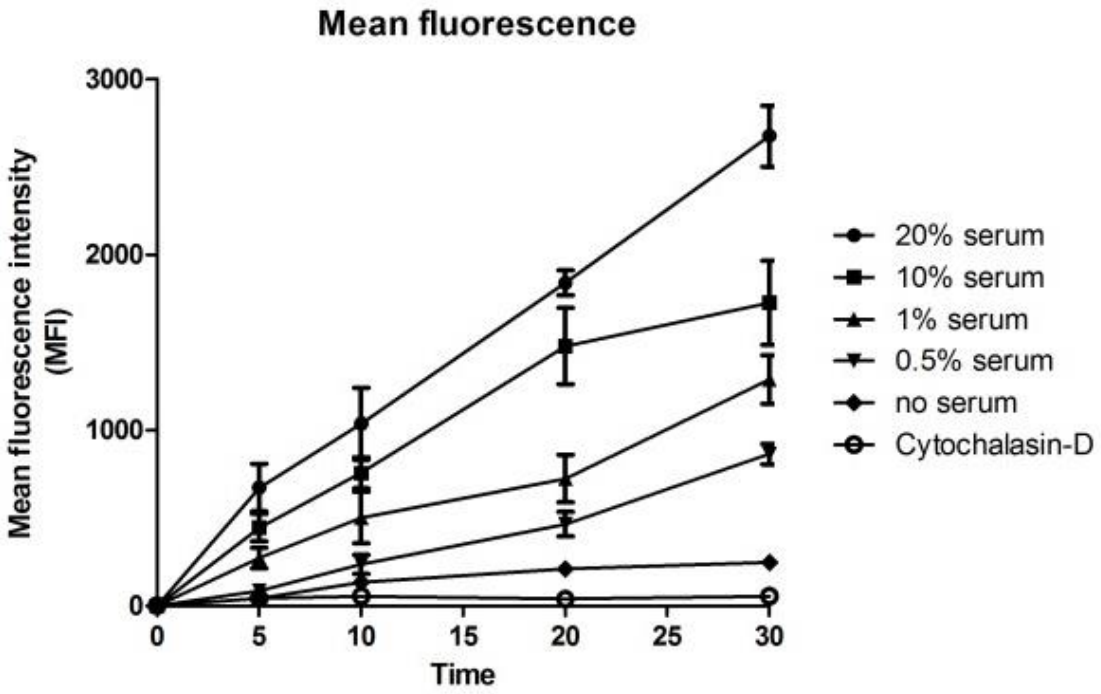

Figure 2.9. Flow cytometry phagocytosis assay. Bacteria were incubated with increasing concentrations of donor serum and incubated with FITC-labelled PA01 for the indicated times. Results are the mean of 3 independent experiments.

Again, even small amounts of serum opsonins increased phagocytosis. These results suggested that this assay is sensitive to changes in serum opsonins and would be suitable to study the effects of ficolin-2 and mannose binding lectin on phagocytosis.

To further validate the phagocytosis assay used in these experiments, the neutrophil phagocytosis assay was performed in the same way as described above, but instead of using flow cytometry, the proportion of positive cells was identified using microscopy. Phase contrast microscopy was used to identify neutrophils and fluorescence microscopy indicated the presence of intracellular FITC-labelled bacteria. The proportion of cells containing bacteria was determined by counting 100 neutrophils from randomly selected fields.

The figure below shows a representative microscopy image from an experiment in which PA01 was incubated with 20% serum for 1 hour following which phagocytosis was permitted for 30 minutes at 37ºC. Large numbers of FITC-labelled PA01 are seen within neutrophils.

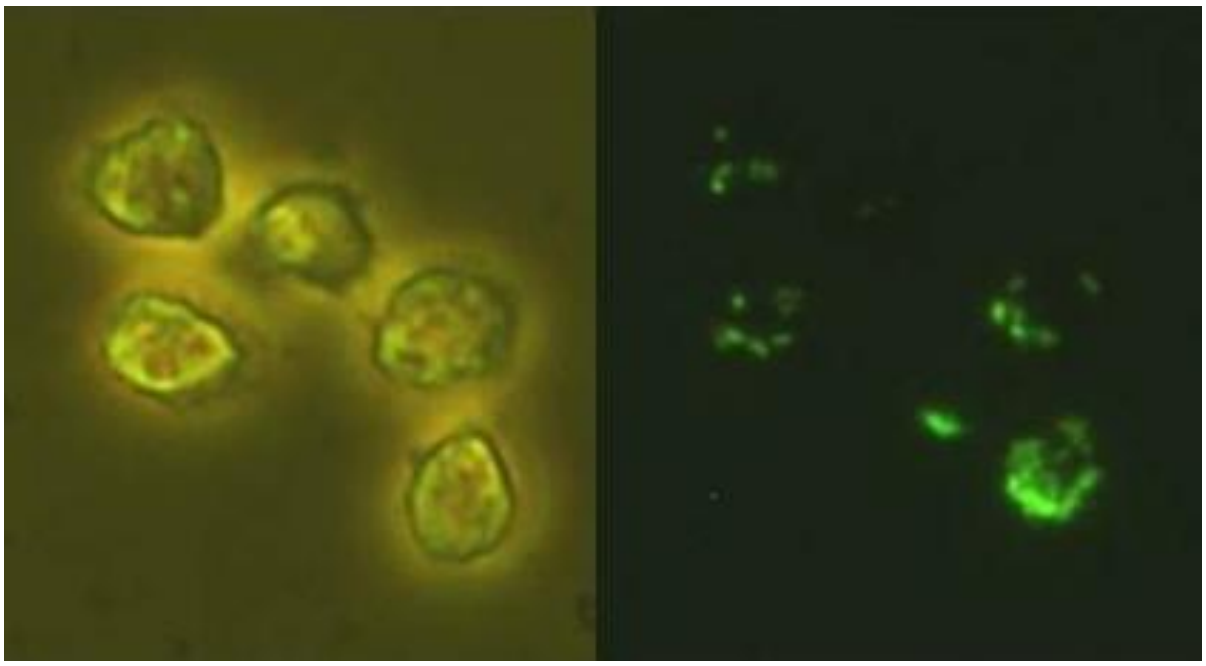

Figure 2.10. Phase contrast and fluorescence microscopy images of neutrophil phagocytosis. The panel on the left shows the phase contrast image. Green PA01 is clearly visible within the cells and this is confirmed on the fluorescence image (right) showing large numbers of labelled bacteria within the cells.

The correlation between the observed % phagocytosis rate determined by both flow cytometry and microscopy methods is shown below and assessed using linear regression. There was a strong correlation between both methods, $r=0.97, p<0.001$, figure 2.11.

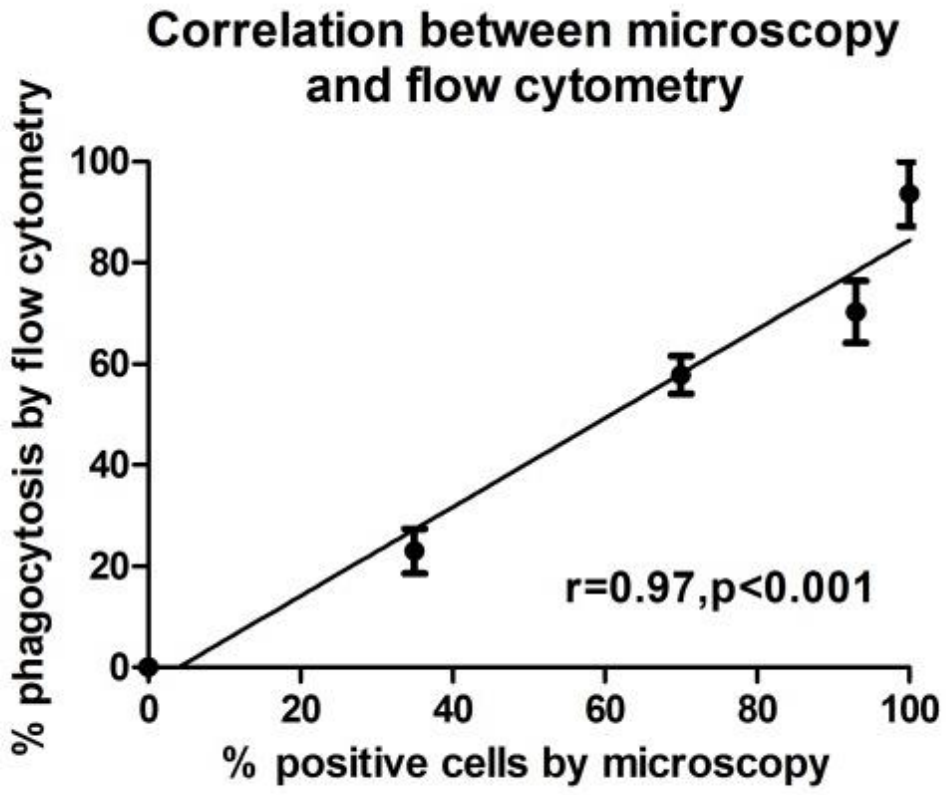

Figure 2.11. Validation of the flow cytometry phagocytosis assay against microscopy based methods.

This analysis demonstrated a strong linear correlation between phagocytosis determined by flow cytometry and microscopy. Flow cytometry based assays were therefore used for subsequent investigations of the effect of ficolin-2 and mannose binding lectin on neutrophil phagocytosis.

Macrophage phagocytosis assay

After 5 days incubation, adherent monocyte derived macrophages (0.5 x 10^6 per experiment) were washed with PBS and subsequently incubated for up to 1 hour at 37°C with FITC-labelled *P.aeruginosa* and *E.coli.* Bacteria were opsonised as indicated prior to incubation with macrophages After 1 hour, cells were washed 3 times with PBS to remove non-phagocytosed bacteria. Cells were incubated with 0.1% trypan blue to quench extracellular fluorescence. Macrophages were then removed from plates by incubation with PBS-EDTA and gentle agitation and transferred to FACS tubes for flow cytometry. Macrophages were identified by their forward and side scatter properties on a BD FACSCALIBUR flow cytometer. A minimum of 10,000 events were counted (Farnworth et al 2008).

2.21 Role of Pentraxins

Previous reports that C-reactive protein enhances complement activation and killing of *P. aeruginosa* via the lectin pathway (Zhang et al 2009) and that Ficolin-2 collaborates with pentraxin-3 to enhance complement activation and phagocytosis of *Aspergillus fumigatus* (Ma et al 2009) led us to investigate the role of pentraxins in complement activation and neutrophil phagocytosis against *P. aeruginosa*.

Pentraxin-3 ELISA

Serum and sputum pentraxin-3 was measured using a commercial sandwich ELISA purchased from R+D systems (Abingdon, UK).

*P. aeruginosa (*PA01) were prepared as described above. Bacteria (1 x 10^8) were opsonised for 1 hour at 37°C in PBS (control), 0.5% $C1q^{-ve}$/ficolin-2^{-ve} serum, 0.5% $C1q^{-ve}$/ficolin-2^{-ve} serum supplemented with 5µg/ml recombinant ficolin-2 or 0.5% $C1q^{-ve}$/ficolin-2^{-ve} serum supplemented with 5µg/ml recombinant ficolin-2 and 10µg/ml recombinant pentraxin-3, 10µg/ml Serum amyloid P or recombinant C-reactive protein (10µg/ml).

C3 deposition was determined by flow cytometry. Bacteria (1 x 10^8) were blocked for 1 hour at room temperature with 3% BSA in PBS. The samples were centrifuged at 3000×g for 15 minutes and the supernatant aspirated before re-suspension in PBS, depleted serum or depleted serum containing ficolin-2 and pentraxins as above. Sera were diluted in calcium buffer (0.5M sodium chloride, 20mM Tris-Hcl, 10mM $CaCl_2$, 1% BSA, pH 7.4). Samples were incubated at 37°C for 1 hour with the bacteria before centrifugation at 3000×g for 15 minutes, aspiration of supernatant and incubation at 4°C for 30 minutes with anti-complement C3-FITC (MP Biomedicals). Samples were centrifuged and re-suspended in PBS for flow cytometry.

For phagocytosis assays, bacteria were opsonised as described above and then exposed to fresh human neutrophils as described under phagocytosis assays.

2.22 Neutrophil oxidative burst assay

Neutrophil oxidative burst was measured using the Dihydrorhodamine fluorescence method as previously described (Walrand et al 2003, Farnworth et al 2008). Neutrophils with or without the indicated treatments were incubated with 0.1mmol/L dihydrorhodamine followed by a priming agent (1µM PAF, complement component c5a or control) for 15 minutes followed by an activating agent (0.1µM fMLP or control) for 15 minutes. DHR fluorescence was then measured as mean FL-1 fluroescence on a BD FACSCalibur flow cytometer. The mean fluorescence equates to the conversion of dihydrorhodamine to rhodamine through the generation of H_2O_2 metabolites by activated neutrophils.

2.23 Neutrophil killing assay

1 x 10^6 cfu/ml *P. aeruginosa* PA01 was opsonised for 1 hour at 37°C in complement component C5 deficient serum (Sigma). C5 deficient serum was used to prevent activation of the terminal complement components and cell lysis as PA01 is sensitive to serum killing. Initial experiments indicated that the C5 deficient sera were able to opsonise PA01 with C3 normally (see C3 activation assay above) with no bacterial killing. Opsonised bacteria were added to 1 x 10^7 neutrophils (a multiplicity of infection of 0.1) and incubated for 90 minutes at 37°C. Bacteria without neutrophils were used as a negative control and bacteria treated for 90 minutes with gentamicin 10µg/ml were used as a positive control (100% killing). Following incubation with neutrophils, samples were centrifuged at 1000g for 5 minutes. The supernatants were aspirated and the neutrophils lysed with 0.1% Triton x-100 (no effect on PA01

viability was observed at this concentration of Triton x-100). Serial dilutions were made and 100µl samples plated on LB agar overnight at 37°C in triplicate.

2.24. Regulation of Ficolin-2 secretion

Expression of ficolin-2 in human liver cell lines

Immortalised human hepatocyte cells lines HepG2 and HuH-7 were kind gifts from Professor Iredale's laboratory at the University of Edinburgh. Cells were maintained in DMEM media containing 10% fetal calf serum, 1% penicillin/streptomycin and 1% L-glutamine. Cells were grown in 5% CO_2 and 95% atmospheric air at 37ºC.

To investigate regulation of ficolin-2 secretion by hepatocytes, HuH-7 and HepG2 cells were seeded into 6 well plates (1 $x10^4$ cells per plate). After 48 hours, the media was changed to 1ml media containing one of the following agents (obtained from Sigma)

- Hormones (T3, human Growth Hormone)
- Proinflammatory cytokines (interleukin 1-beta, Interleukin-6, Tumour necrosis factor-α, transforming growth factor ß)
- Medications (Simvastatin, Dexamethasone)
- Lipopolysaccharide

Cells were cultured for 72 hours after which the culture supernatants were collected and stored at -70°C until analysed. Cells were counted using a haemocytometer and ficolin-2 and MBL synthesis were measured using ELISA. Results are expressed as ng/10^6 cells.

Real time polymerase chain reaction

FCN2 mRNA expression levels were quantified using real time PCR. Total RNA was extracted from cells using QIAshredder columns and the RNeasy mini kit (Qiagen) according to the manufacturers instructions followed by DNase treatment. For DNase treatment, 2µl RQ1 RNase-Free DNase (Promega) and 2µl 10x reaction buffer (Promega) was added to 2µg RNA, made up to a final volume of 20µl with nuclease-free H_2O and incubated for 30 minutes at 37°C. Immediately, 2µl RQ1 DNase stop solution (Promega) was added to each tube for 10 minutes at 65°C to terminate the reaction. 'Clean' RNA was stored at -80°C until further use. cDNA synthesis was performed using 1.5µg RNA in a 40µl reaction as follows (10x Taqman RT buffer-4µl, 25µM MgCl- 8.8µl, dNTP- 8µl, Random hexamers- 2µl, RNase inhibitor- 0.8µl, multiscribe reverse transcriptase- 1µl, RNA- 15.4µl).

PCR condition were: 25°C: 10 minutes, 48°C: 40 minutes, 95°C: 5 minutes.

Taqman primer probe mix for ficolin-2 was purchased from Applied Biosystems. Real-time PCR was performed in a 25µl volume per well, incorporating 12.5µl of 2x Gene expression master mix (Applied biosystems), 1.25µl 20x FCN2 primer/probe mix, 8µl dH_20 and 1.25µl 18s primer/probe mix (Applied biosystems). 18s was used as a house-keeping gene. The amount of mRNA expression is reported as the Ct (threshold cycle) value compared to untreated control cells.

Cardiac surgery study

We recruited 10 patients undergoing coronary artery bypass surgery, an invasive surgical procedure associated with a significant acute inflammatory response that has been previously used in the literature as a model for acute phase responses (Bruins et al 1997). We measured serum levels of Ficolin-2 and mannose binding lectin 24 hours pre-operatively and then at 24, 72, 120 and 168 hours after cardiac surgery. Acute phase responses of Ficolin-2 and MBL were compared to classical acute phase reactant C-reactive protein measured by the biochemistry department at the Royal Infirmary of Edinburgh.

2.25 Studying the relationship between airway and systemic inflammation and airway bacterial load

Several studies were performed to determine the relationship between airway bacterial infection and airway and systemic inflammation. The aim of these studies were to determine if bacterial load was associated with markers of airways and systemic inflammation in patients with bronchiectasis, and to determine if antibiotic therapy, both during stability and during acute exacerbations could reduce airway and systemic inflammation. The studies are summarised in figure 2.12. The methods for each individual component of the study are described below

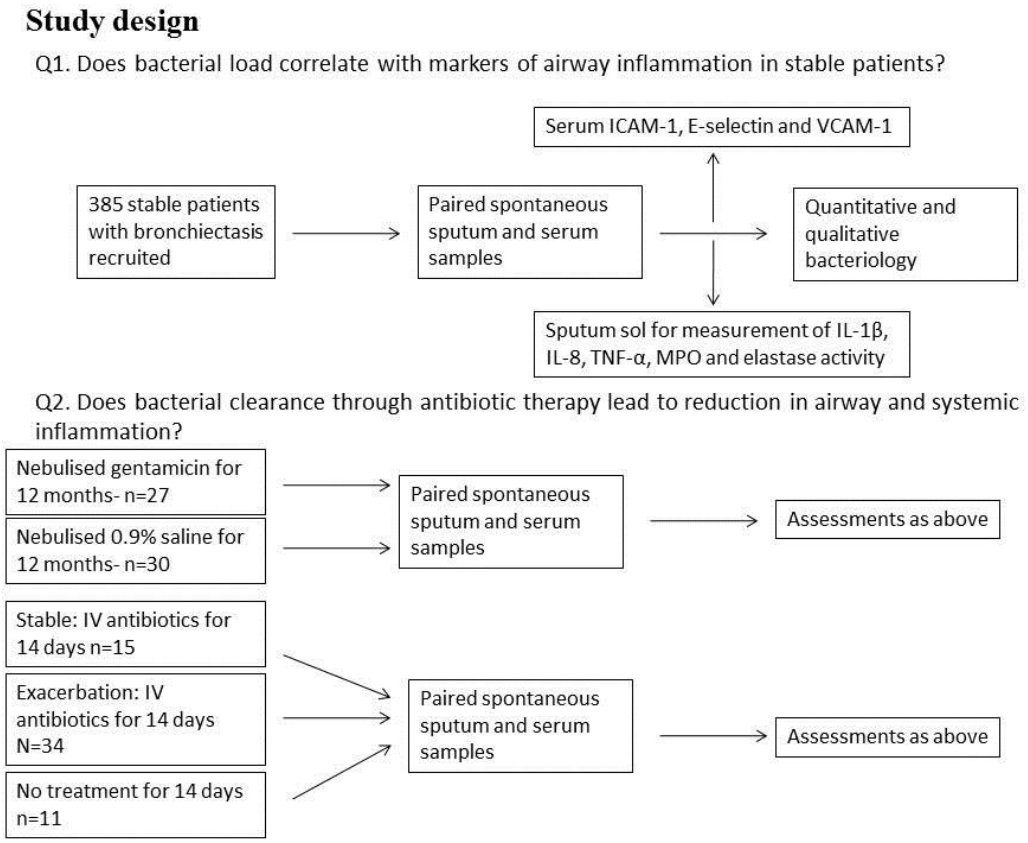

Figure 2.12. Summary of studies into the relationship between airway colonisation and airway and systemic inflammation.

In study 1, we investigated the relationship between bacterial load and airway and systemic inflammation during clinical stability

This study included stable serum and sputum samples for 385 patients with bronchiectasis recruited as part of the primary study. All patients were clinically stable at recruitment and free from antibiotic therapy for at least 4 weeks. The inclusion and exclusion criteria are as described above for the study of ficolin-2 in

bronchietasis. Data on the relationship between bacterial load and clinical outcomes are presented for the first year of follow-up after bacterial load measurement.

In study 2 the effect of long term antibiotic treatment using nebulised gentamicin on markers of airway and systemic inflammation was assessed. We used data from a randomised controlled trial of nebulised gentamicin. Details of this randomised controlled trial have been described previously (Murray et al, 2010). Patients were randomised to receive 80mg twice daily nebulised gentamicin or to receive nebulised 0.9% saline for 12 months. Randomisation was performed by the study pharmacist and the study was approved by the NHS Scotland multi-centre research ethics committee. The primary outcome of the study was the impact of nebulised gentamicin on quantitative bacterial load, and the results of the primary and secondary outcomes of the study have been published (Murray et al, 2010). The inclusion criteria for the study were culture of pathogenic organisms in at least 3 sputum samples when clinically stable in the preceding 12 months, at least two exacerbations in the past year, and successful toleration of a test dose of gentamicin 80mg without a fall in FEV_1 >15% or >200ml. Patients had to have an FEV_1 greater than 30% predicted and had to be ex-smkers of at least 1 year with less than 20 pack year history. Patients were also required to be clinically stable at study entry defined as above, as the absence of requirement for antibiotics in the preceding 4 weeks. Exclusion criteria were: current use of long term antibiotics, current smoking, cystic fibrosis, active pulmonary mycobacterial infection, active sarcoidosis, active allergic bronchopulmonary aspergillosis, chronic obstructive pulmonary disease, poorly controlled asthma, creatinine clearance <30ml per minute, vestibular instability and

previous documented intolerance to aminoglycosides (including ototoxicity and nephrotoxicity).

The study was not blinded and patients were aware of their treatment allocation.

The study was performed principally by Dr Maeve Murray.

To investigate the impact of long term antibiotic therapy on airway and systemic inflammation we used sputum and serum samples from the 27 bronchiectasis patients randomised to receive nebulised gentamicin 80mg twice daily along with samples from 30 patients randomised to receive nebulised 0.9% saline for 12 months. Microbiology and assessments of airway and systemic inflammation were performed as described above.

In study 3 the effect of bacterial clearance using 14 days intravenous antibiotic therapy on markers of airway and systemic inflammation in stable patients was assessed. 15 patients received 14 days of intravenous antibiotics based on their previous microbiology results. Patients were recruited from patients attending the specialist bronchiectasis service at the Royal Infirmary of Edinburgh and were free from exacerbations or oral antibiotic therapy for at least 4 weeks prior to study. None of the patients were receiving long term antibiotic or corticosteroid therapy. A sputum sample was obtained and processed immediately from all eligible patients. Patients growing $>1 \times 10^7$ cfu/ml were chosen for study. For comparison, 11 patients who attended for routine review and were found to have bacterial loads while clinically stable of $>1 \times 10^7$ cfu/ml had sputum samples taken 2 weeks apart without antibiotics therapy. This study was not placebo controlled.

In study 4 the effect of bacterial clearance using 14 days intravenous antibiotic therapy on markers of airway and systemic inflammation in patients with exacerbations of non-CF bronchiectasis was assessed. 34 patients with severe exacerbations of non-CF bronchiectasis requiring intravenous antibiotic therapy (defined according to BTS guideline definitions, Pasteur et al 2010) were recruited. After clinical assessment to confirm exacerbation patients were treated with intravenous antibiotic therapy based on their previous microbiology results. Sputum and serum samples were obtained taken at baseline and after 14 days of antibiotic therapy.

2.26. Statistical methods

Statistical software used for analyses in this thesis were SPSS version 21 for windows (SPSS inc., Chicago, IL)., Graphpad Prism version 5 (Graphpad software, San Diego, CA, USA) and Snpalyze version 8 (Dynacom, Japan).

Results are expressed as median with interquartile range or mean +/- standard deviation (SD) as appropriate. Each in-vitro experiment was performed in triplicate unless otherwise indicated. For clinical data, comparisons of categorical data were performed using chi-square test while continuous data were compared using the Mann-whitney U test (2 groups) or Kruskal-Wallis test (more than 2 groups). Pair-wise comparisons between in-vitro conditions are made using an unpaired students t test. Analysis of variance with the Bonferroni posthoc test was used to determine differences between multiple groups for in-vitro experiments.

The genotype frequencies were checked for consistency among cases and controls separately with those expected from the Hardy-Weinberg equilibrium.

The accuracy of genetic definitions of ficolin-2 insufficiency to predict low serum Ficolin-2 levels was assessed using the area under the receiver operator characteristic curve. Curves were compared using the method described by Hanley and McNeil (Hanley and McNeil 1983).

Cox-proportional hazards regression analysis was used to investigate the association of ficolin-2 serum levels and SNP's with mortality. These models utilised forced entry of all other co-variates to minimise residual confounding. For analysis of chronic colonisation or *P. aeruginosa* colonisation, multivariable analysis was performed using logistic regression. Models were constructed adjusted for all available, biologically plausible confounders. Model adequacy was assessed with the Hosner-Lemeshow goodness of fit test.

A p-value <0.05 was considered statistically significant for all analyses.

CHAPTER 3

The impact of short and long term antibiotic therapy on airway and systemic inflammation in bronchiectasis

3.1 Introduction

The vicious cycle hypothesis of bronchiectasis argues that bacterial colonisation of the normally sterile respiratory tract provokes and perpetuates airway inflammation (Cole, 1986). This neutrophil mediated inflammation leads to airway structural damage and further impairment of the mucociliary escalator, leading to increased bacterial load (Cole, 1986, Watt et al, 2004, Fuschillo et al, 2008). This cycle repeats continuously throughout the course of the disease, leading to the clinical syndrome of cough, sputum production and recurrent exacerbations that is characteristic of bronchiectasis (Pasteur et al, 2010).

According to this hypothesis, if bacteria are the primary drivers of airway inflammation, bacterial clearance through the use of short or long term antibiotic therapy would be expected to reduce airway inflammation, allow airway healing and modify the long term course of the disease.

There is strong evidence from cystic fibrosis bronchiectasis to support this view, with data suggesting that bacterial colonisation is associated with airway inflammation and that antibiotic treatment during stability and at exacerbation can reduce markers of inflammation (Sagel et al, 2011, Ordonez et al, 2003, Downey et al, 2007). Extrapolation from cystic fibrosis to non- cystic fibrosis bronchiectasis is difficult as the pathophysiology is different and the failure of recombinant DNase treatment to provide benefit in non-cystic fibrosis bronchiectasis demonstrates that responses to

treatment can be very different in cystic fibrosis compared to non-cystic fibrosis bronchiectasis (O'Donnell et al, 1998). The evidence base in non-cystic fibrosis bronchiectasis is limited, although a study of 49 patients with stable bronchiectasis using bronchoalveolar lavage demonstrated a link between bacterial load and airway inflammation, and a study of COPD patients included 43 with bronchiectasis (Angrill et al, 2001, Hill et al, 2000).

Raised systemic inflammation has been shown to be associated with disease severity and lung function impairment in 87 stable patients with bronchiectasis (Wilson et al, 1998).

There are limited studies evaluating the effect of bacterial clearance in non-cystic fibrosis bronchiectasis. A 2007 Cochrane review of randomised controlled trials of antibiotic therapy in non-cystic fibrosis bronchiectasis suggested no benefit in terms of lung function or exacerbation frequency (Evans et al, 2007). In addition, some studies suggest that antibiotic therapy in bronchiectasis had little or no effect on markers of airway inflammation, further questioning the hypothesis that antibiotics can "break the cycle" (Tsang et al, 1999) Conversely, three studies of long-term antibiotics have demonstrated reduced levels of myeloperoxidase and neutrophil elastase activity in sputum following prolonged antibiotic treatment (Murray et al, 2010, Lin et al, 1997, Hill et al, 1988).

The aim of the studies described in this chapter was to determine if bacterial load was associated with markers of airways and systemic inflammation in patients with bronchiectasis, and to determine if antibiotic therapy, both during stability and during acute exacerbations impacted on this. Finally, we aimed to determine the association

of bacterial load with key clinical markers of severity in bronchiectasis, including exacerbation frequency and health related quality of life.

3.2 Characteristics of the cohort

The stable cohort in this study included 385 patients. Median age was 67 years, interquartile range 56-74, 42.9% of the study cohort were male. 81.6% of patients had idiopathic/post-infective bronchiectasis in the stable cohort. The "other" causes of bronchiectasis were previous ABPA (n=36), rheumatoid arthritis (n=19), inflammatory bowel disease (n=13), and one patient each with alpha-1-antitrypsin deficiency, yellow-nail syndrome and Kartagener's syndrome. 40.3% of the population were using inhaled corticosteroids. The demographics of the study population for each of the studies described in this chapter are shown in table 3.1.

Cohort	N	Age	Gender % male	FEV1 % predicted	FVC % predicted	Radiological Severity Score	BMI kg/m^2	% idiopathic / post-infective bronchiectasis
Study 1 Stable cohort	385	67 (56-74)	42.9%	69.2 (51-91.1)	81.0 (67.4-101)	4 (2-5)	25.9 (22.4-29.6)	81.6%
Study 2 Nebulised gentamicin group	27	58 (53-67)	33.3%	72.9 (60-81.2)	88.9 (82.7-94)	9 (4-12)	25.4 (20.9-29.0)	71.3%
Nebulised 0.9% saline group	30	64 (56-69)	50%	63.4 (45.5-80.4)	85 (71.1-94.4)	6 (3-12)	27.1 (24.1-29.5)	66.7%
Study 3 Stable patients intravenous antibiotics (14 days)	15	66 (55-70)	53.3%	61.3 (36.0-77.1)	73.5 (60.0-97.0)	9 (4-12)	25.0 (20.8-26.9)	66.7%
Stable patients (no treatment for 14 days)	11	61 (48-70)	54.5%	62.3 (39.5-75.3)	81.3 (57.6-90.1)	8 (3-12)	25.3 (20.3-28.2)	63.6%
Study 4 Acute exacerbation of bronchiectasis- intravenous antibiotics (14 days)	34	66 (54-71)	52.9%	61.4 (39.5-81.8)	77.4 (60.3-95.9)	9 (3-12)	24.8 (22.1-27.7)	79.4%

Table 3.1. Cohorts included in the studies described in this chapter.

3.3 Relationship between airway bacterial load and airway inflammation

Pathogenic microorganisms were isolated in the baseline sputum cultures from 75.3% of patients. Of those with positive cultures, predominant organisms isolated were *Haemophilus Influenzae* 38.6%, *Pseudomonas aeruginosa* 21.0%, *S. aureus* 12.4%, *M. catarrhalis* 11.4%, *S. pneumoniae* 9.7% and others (primarily enteric gram negative organism) 9.3%. Total numbers add up to greater than the total number of patients as some patients grew more than one organism.

Bacterial load was strongly associated with each of the markers of airway inflammation measured in sputum (figure 3.1). Compared to the 22.1% of patients that grew no potentially pathogenic microorganisms (PPMs), statistically significantly higher levels of inflammatory markers were found at 10^5cfu/ml. The

median levels of each of the five markers increased progressively with increasingly bacterial load. Analyses adjusting for inhaled corticosteroid use found the same results (Chalmers, 2012, online supplementary material). The same results were observed when examining patients with idiopathic/post-infective bronchiectasis and other causes (Chalmers et al, 2012, online supplementary material).

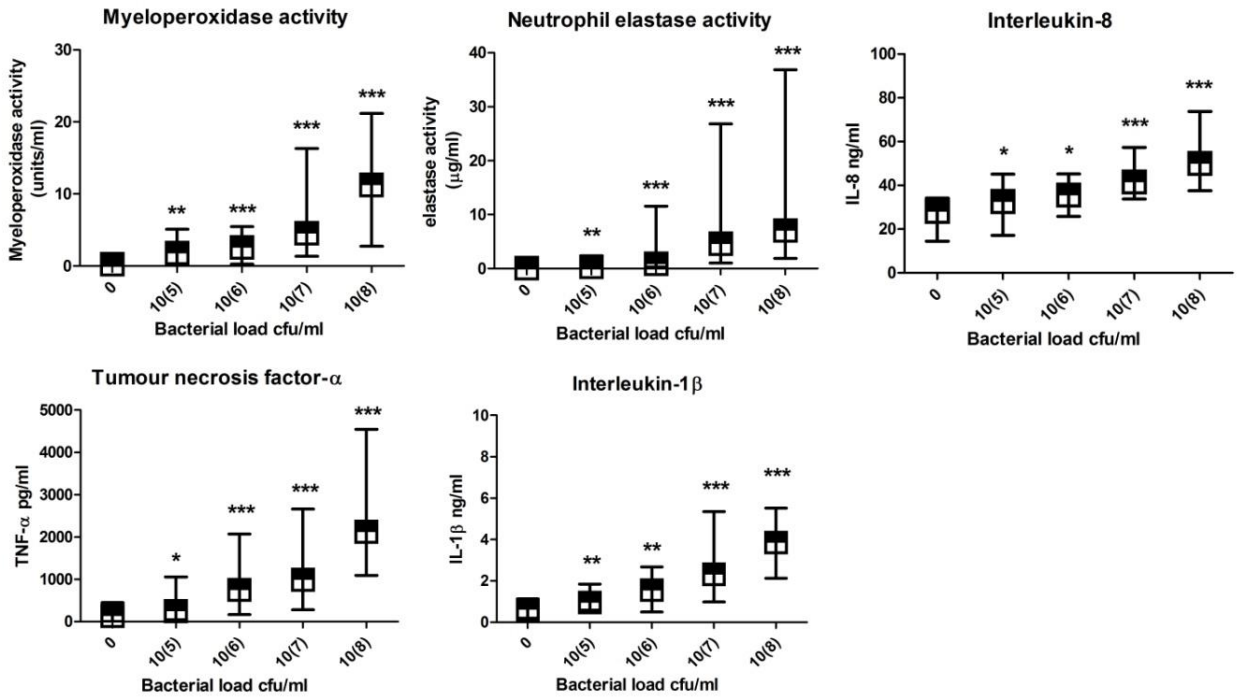

Figure 3.1. The relationship between bacterial load when stable and markers of airway inflammation in sputum. Data are presented as median (boxes) with interquartile range (bars). *p<0.05, **p<0.001 ***p<0.0001. All display comparisons are Mann-Whitney U test compared to patients growing no PPMs. Comparison across multiple groups (Kruskal-Wallis test) showed a direct relationship between airway bacterial load and airway inflammation (p<0.0001 for all markers).

3.4 Relationship between airway bacterial load and systemic inflammation

Analysis of soluble adhesion molecules in serum showed elevated levels of ICAM-1, E-selectin and VCAM-1 in patients growing 1 x 10^7cfu/ml or more compared to the group with no PPMs (Figure 3.2). Differences between bacterial loads of 1 x 10^5cfu/ml and 1 x 10^6cfu/ml with the no PPM group were not statistically significant ($p>0.05$).

When compared to the healthy control population, overall bronchiectasis patients had higher levels of ICAM-1 (171ng/ml (interquartile range 114.2–296.2) in controls vs. 270.2ng/ml (168.7-459.0) in bronchiectasis patients, $p<0.0001$)), while less striking differences in E-selectin (56.8ng/ml (36.4-80.9) in controls vs. 64.9ng/ml (43.7-94.6) in bronchiectasis patients, $p=0.02$) were observed and the levels of VCAM-1 (582ng/ml (456-924.1) in controls vs. 686.6 (527.8 to 921.4),$p=0.07$) were not significantly different between bronchiectasis patients and control subjects.

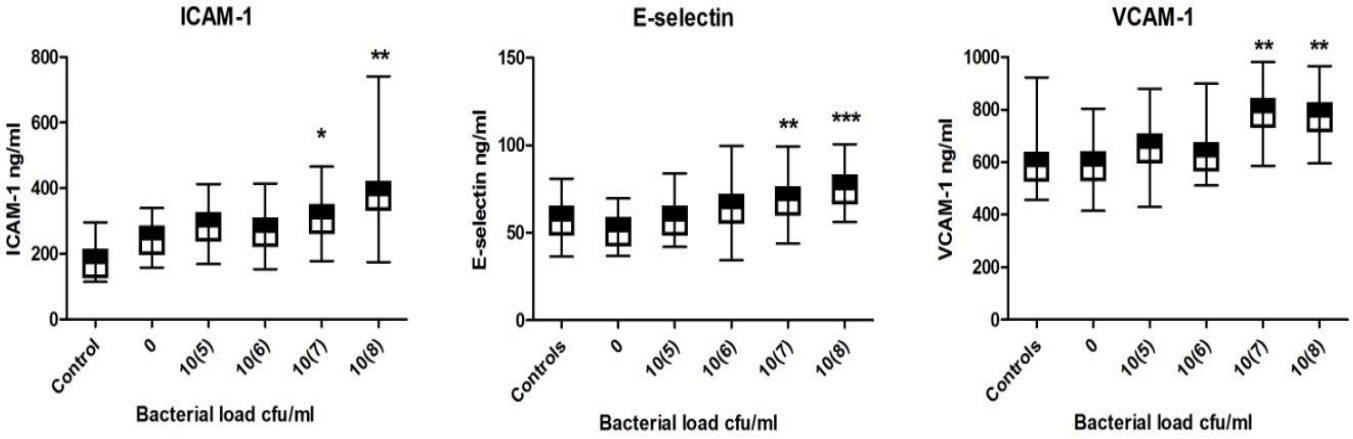

Figure 3.2. The relationship between bacterial load when stable and markers of systemic inflammation in serum. Data are presented as median (boxes) with interquartile range (bars). *p<0.05, **p<0.001 ***p<0.0001. All display comparisons are Mann-Whitney U test compared to patients growing no PPMs. Comparison across multiple groups (Kruskal-Wallis test) showed a direct relationship between airway bacterial load and systemic inflammation (p=0.003 for ICAM-1, p=0.001 for E-selectin and p=0.0007 for VCAM-1).

3.5 The impact of 14 day antibiotic therapy on markers of airway inflammation and systemic inflammation + exacerbations.

For stable patients, 15 were treated with IV antibiotics for 14 days and 11 received no therapy for 14 days.

Table 3.2 shows the characteristics and microbiology of patients included in the IV antibiotic study.

Sputum and serum markers were then compared between these two groups. At baseline, all patients had sputum cultures that grew PPMs at 1 x 10^7 cfu/ml or greater. Following 14 days of intravenous antibiotic therapy, all patients had no significant bacterial growth in sputum. In contrast, in the control group that received no antibiotic therapy, there was no reduction in the bacterial load (baseline mean 3.2 x 10^8 cfu/ml vs. 2.1 x 10^8 cfu/ml at day 14, p=0.4).

Patient No	Treatment allocation	Age	Gender	FEV_1 % predicted	Radiological severity score	Predominant organism at baseline	Organism at 14 days
1	IV antibiotics	55	Female	81.8%	10	*E.coli*	None
2	IV antibiotics	68	Female	113.2%	3	*S.pneumoniae*	None
3	IV antibiotics	53	Female	61.3%	4	*H.Influenzae*	None
4	IV antibiotics	67	Male	46.9%	15	*P.aeruginosa*	None
5	IV antibiotics	71	Male	61.4%	9	*P.aeruginosa*	None
6	IV antibiotics	43	Female	82.6%	9	*H.Influenzae*	None
7	IV antibiotics	62	Male	36.0%	18	*H.Influenzae +S.aureus*	None
8	IV antibiotics	68	Male	30.0%	12	*P.aeruginosa*	None
9	IV antibiotics	66	Male	59.4%	9	*S.maltophilia*	None
10	IV antibiotics	72	Female	74.9%	2	*M.Catarrhalis*	None
11	IV antibiotics	70	Male	19.9%	5	*S.aureus + H.influenzae*	None
12	IV antibiotics	61	Male	29.2%	18	*E.coli*	None
13	IV antibiotics	52	Female	77.1%	15	*P.aeruginosa + E.coli*	None
14	IV antibiotics	65	Female	57.9%	12	*H.Influenzae*	None
15	IV antibiotics	75	Male	70.8%	4	*S.pneumoniae*	None
1	No treatment	79	Female	56.5%	12	*P.aeruginosa*	*P.aeruginosa*
2	No treatment	61	Male	33.3%	18	*P.aeruginosa*	*P.aeruginosa*
3	No treatment	48	Female	101.6%	3	*H.Influenzae*	*H.influenzae*
4	No treatment	67	Female	39.5%	1	*S.pneumoniae + H.influenzae*	*H.Influenzae*
5	No treatment	33	Female	66.7%	6	*A.hydrophila*	*M.catarrhalis*
6	No treatment	56	Male	66.0%	4	*H.influenzae*	*H.Influenzae*
7	No treatment	79	Male	37.6%	11	*H.influenzae + S.pneumoniae*	*H.Influenzae*
8	No treatment	70	Male	46.5%	2	*P.mirabilis*	*P.mirabilis*
9	No treatment	48	Male	62.3%	9	*H.influenzae*	*H.influenzae +*
10	No treatment	61	Female	80.1%	8	*S.aureus*	*M.catarrhalis S.pneumoniae*
11	No treatment	66	Male	75.3%	13	*S.pneumoniae*	*+ S.aureus S.pneumoniae*

Table 3.2. Characteristics and Microbiology of patients included in the IV antibiotic study. All patients had a bacterial load of $>1 \times 10^7$ cfu/ml at baseline.

Bacterial clearance through 14 days of antibiotic therapy was associated with a significant reduction in all of the markers of airway inflammation measured (figure 3.3). In addition, antibiotic treatment was associated with a statistically significant reduction in ICAM-1 concentration in serum and E-selectin also decreased with treatment (76.7ng/ml, 61.7-91.8ng/ml pre-treatment vs. 53.3ng/ml, 44.6 – 68.8ng/ml post-treatment, p=0.04). Although VCAM-1 serum levels decreased with treatment (782ng/ml, 302.2-1001ng/ml pre-treatment vs. 387.1, 121.3 -919.7 post-treatment), this difference was not statistically significant, p=0.4. There were no significant differences in patients receiving no treatment for any markers between day 0 and 14.

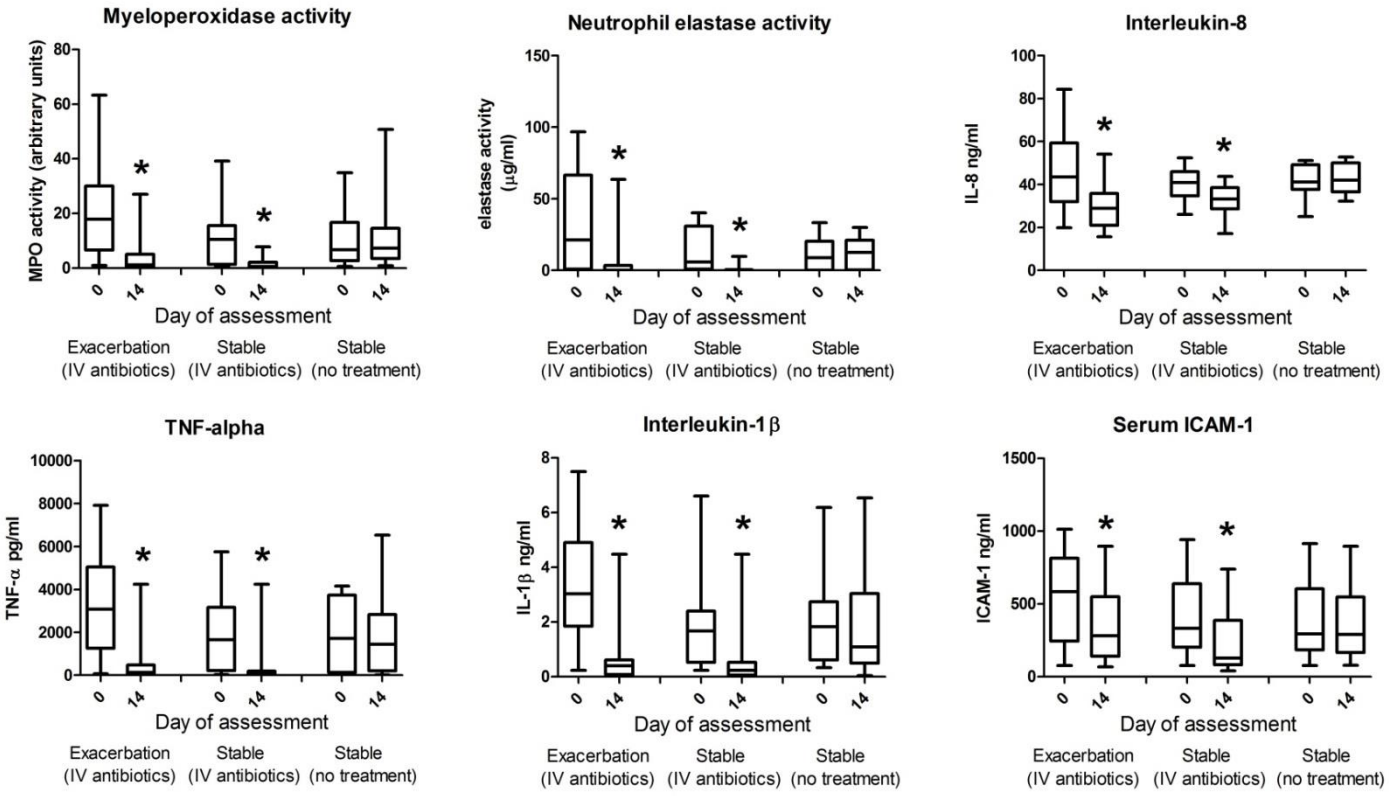

Figure 3.3. Response of markers of airway and systemic inflammation to 14 days of intravenous (IV) antibiotics. *$p<0.05$ when compared the day 0 values. All other comparisons $p>0.05$. Box plots show median with interquartile range with the whiskers representing the minimum and maximum values.

For patients with acute exacerbations of bronchiectasis (n=34), all patients had a significant growth of pathogens from baseline (the most frequent pathogens were *H.influenzae* in 11 patients, *S. pneumoniae 9* patients, *P. aeruginosa* 8 patients, *S. aureus 3* patients, *M. catarrhalis* 2 patients, gram-negative enterobacteriaceae 4 patients- numbers add up to more than 34 as 3 patients grew more than one pathogen). At day 14, only 4 patients still had a significant growth of bacteria (all *P. aeruginosa)*. The data demonstrated a significant reduction in all markers of airway inflammation ($p<0.0001$ for all comparisons, figure 3.3) and ICAM-1 ($p<0.05$) after 14 days of antibiotic therapy in patients with exacerbations of bronchiectasis, figure 3.3. Differences in serum E-selectin and VCAM-1 were not statistically significant ($p=0.1$ and $p=0.3$ respectively).

3.6 The impact of 12 months nebulised gentamicin on markers of airway and systemic inflammation

The organisms isolated in the patients treated with nebulised gentamicin and nebulised 0.9% saline in this study have been reported previously (Murray et al, 2010). In addition, it has been previously reported that nebulised gentamicin resulted in a statistically significant reduction in MPO activity and neutrophil elastase activity in the treatment group, which was not evident in the saline group (Murray et al, 2010). Results are therefore only presented for sputum IL-8, TNF-α, IL-1β and the soluble adhesion molecules in serum. Nebulised gentamicin resulted in significant reduction in sputum IL-8, TNF-α and IL-1β, compared to patients treated with 0.9% saline. For the soluble adhesion molecules, reductions were evidence in ICAM-1

compared to both baseline and the saline treated groups at 12 months. E-selectin was significantly different from baseline in the gentamicin group but did not differ compared to the saline group. No differences were evident in the levels of VCAM-1 at any stage in the study. These data are shown in Table 3.3.

	Gentamicin n=27			Saline n=30		
Time point (months)	0	12	15 (3m post treatment)	0	12	15 (3m post treatment)
IL-8 ng/ml	38.4 (34.8-44.1)	33.2 (25.0-37.5)*#	36.4 (33.4-42.8)#	39.1 (37.8-46.8)	42.9 (36.1-48.5)	42.5 (39.9-50.1)
TNF-α pg/ml	1346 (485.1-3581)	485.4 (115.1-1286)*#	1028 (600.1-3920)	1281 (374.9-2874)	1421 (290-3074)	1295 (409.6-4246)
IL-1β ng/ml	2.2 (0.96-4.0)	0.99 (0.46-2.2)*#	2.2 (0.90-3.7)	2.1 (0.59-3.4)	2.0 (0.68-3.0)	2.4 (1.1-3.4)
ICAM-1 ng/ml	304.7 (190.9-463.8)	245.3 (167.4-359.4)*#	299.2 (245.9-436.9)	278.8 (163.2-459.7)	318.7 (177-458.3)	299.4 (174-444.9)
E-selectin ng/ml	72.7 (50.7-91.7)	54.4 (36.5-77.1)*	67.4 (41.4-81.8)	65.6 (45.1-80.1)	63.1 (47.2-80.8)	61.5 (40.7-80.2)
VCAM-1 ng/ml	671.2 (473.4-869)	591.5 (362.7-836.6)	712 (378.3-891)	671.6 (399.1-878.7)	642 (447.1-862)	643.8 (391.6-894.1)
% positive microbiology	100%	33.3%*#	66.7%	100%	96.7%	96.7%

Table 3.3. The impact of nebulised gentamicin treatment on markers of airway and systemic inflammation. *$p<0.05$ when compared with baseline (time point 0). # $p<0.05$ when compared with the same time-point in the saline treated group.

3.7 *Pseudomonas aeruginosa* and radiological severity of bronchiectasis influence airway inflammation

Pseudomonas aeruginosa

P. aeruginosa was associated with increased airway inflammation independent of the bacterial load compared to isolation of other pathogens. Patients with *P.aeruginosa* were each matched by log bacterial load and radiological severity (+/- 1) to patients that had isolated other PPMs (n=81 and 162 respectively). The median bacterial load

was 4.4 x 10^7cfu/ml vs. 4.6 10^7cfu/ml,p=0.3. Median radiological severity was 8 (4-12) vs. 7 (4-12),p=0.8.

Patients isolating *P.aeruginosa* had higher levels of MPO activity- 13.5U/ml (4.0-38.6) vs. 2.6 (0.6-50.8),p<0.0001, elastase activity- 4.9µg/ml (1.2-68.0) vs. 1.2µg/ml (0.4-147.2),p<0.0001 , IL-8- 52.2ng/ml (34.0-64.5) vs. 39.5 (30.0-49.7), p=0.001 and TNF-alpha- 2722pg/ml (971.2-5098) vs. 1024pg/ml (277-2529),p<0.0001. The difference between the two groups in levels of Interleukin 1-beta- 2.8ng/ml (0.9-5.8) vs. 2.3ng/ml (1.3-4.9),p=0.8 was not statistically significant.

Radiological severity

There was a direct relationship between increasing radiological severity and severity of airway inflammation (p<0.0001 for sputum elastase, MPO, TNF-alpha, IL-1beta and p=0.001 for IL-8) (figure 3.4).

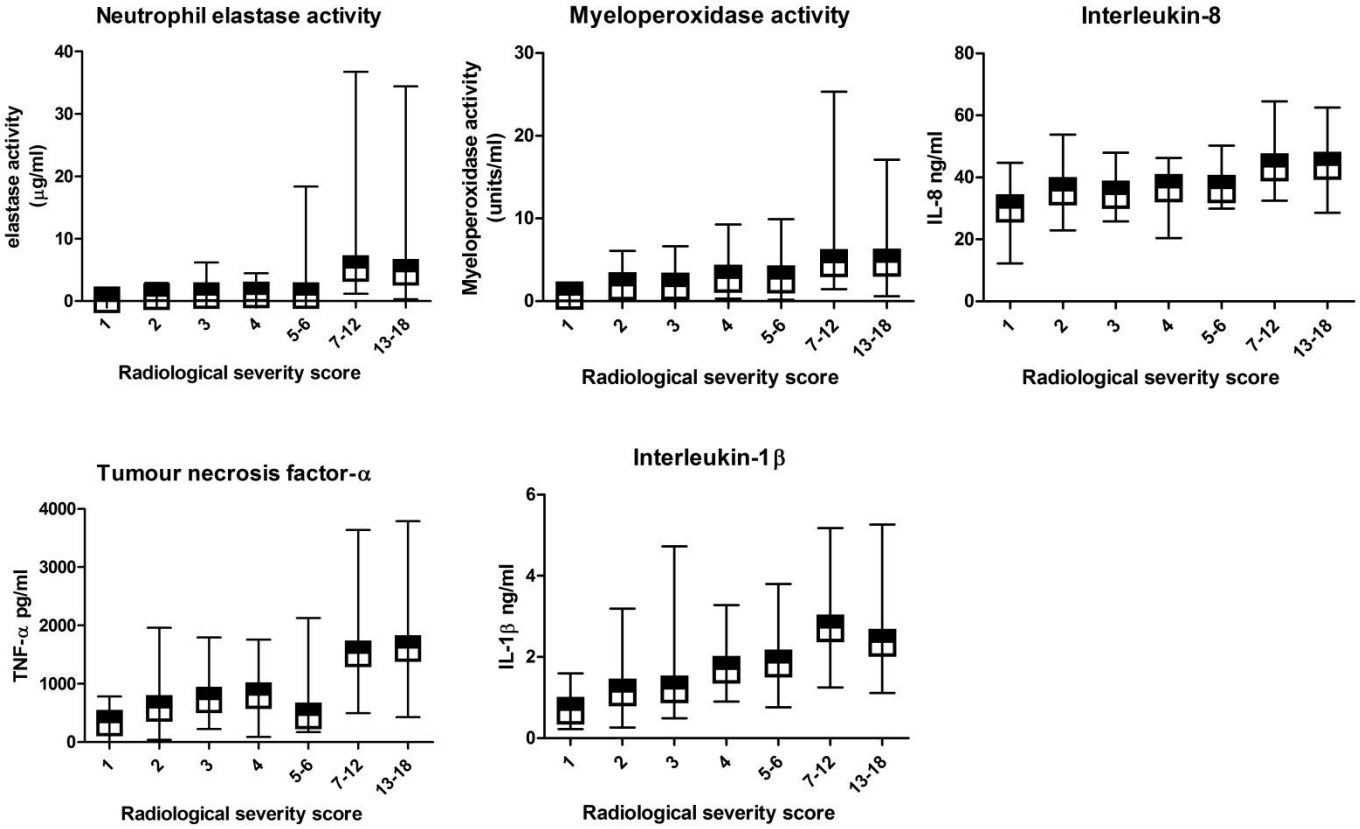

Figure 3.4. The relationship between airway inflammation and radiological severity (CT scan scoring). median (boxes) with interquartile range (bars) are shown.

3.8 Spirometry and airway inflammation

These data demonstrate a significant association between FEV_1% predicted and airway inflammatory markers. There was a significant association between FEV_1% predicted and elastase activity ($p<0.0001$), MPO activity ($p<0.0001$), Interleukin-8 ($p<0.0001$), tumour necrosis factor alpha ($p=0.005$) and IL-1beta ($p<0.0001$).

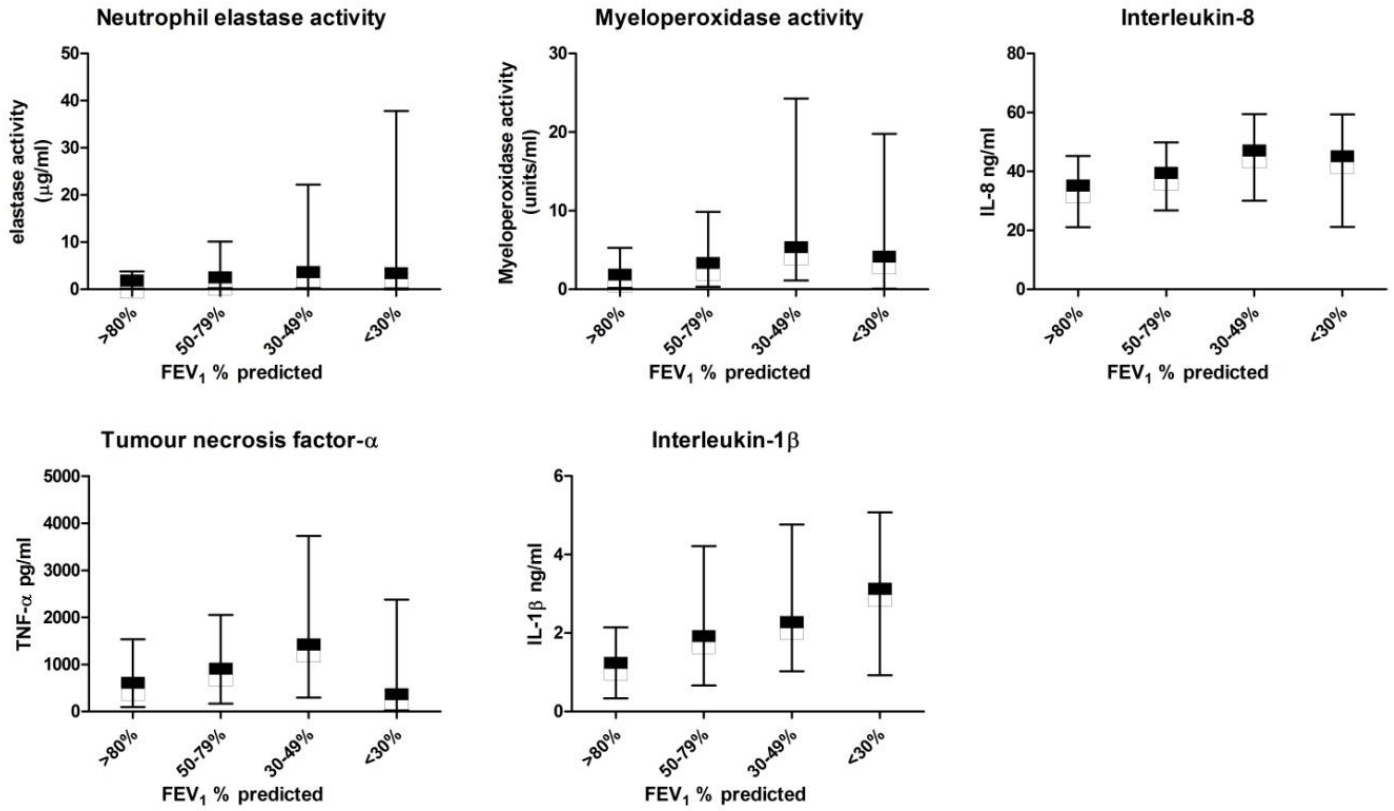

Figure 3.5. Relationship between airway inflammation and lung function. The relationship between airway inflammation and forced expiratory volume in 1 second. Data are shown as median (boxes) with interquartile range (bars) are shown.

3.9 Independent relationship between airway inflammation and bacterial colonisation

A multivariable logistic regression analysis was performed to determine if airway inflammation was associated with a positive bacterial culture, independent of radiological severity and FEV_1 % predicted. Neutrophil elastase (Adjusted Odds Ratio (AOR) 1.08, 95% CI 1.03-1.14,p=0.003), myeloperoxidase (AOR 1.18, 95% CI 1.09-1.28,p<0.0001), Interleukin-8 (AOR 1.03, 95% CI 1.02-1.05,p<0.0001), TNF-alpha (AOR 1.11, 95% CI 1.06-1.15,p<0.0001) and IL-1β AOR 1.61, 95% CI 1.34-1.94,p<0.0001) were each independently associated with bacterial colonisation. Adequate model fit was demonstrated for all analyses by the Hosner-Lemeshow goodness of fit test (p>0.05).

3.10 Airway bacterial load predicts exacerbation frequency, hospital admissions for severe exacerbations over the subsequent 12 months and patient symptom scores.

The prognostic importance of airway bacterial load was investigated by following patients for 1 year after their initial visit. The mean number of self-reported exacerbations during this year was 3.3/year (standard deviation 2.9, range 0-15/year).

After 1 year, there was a significant correlation between the number of exacerbations and the airway bacterial load (figure 3.6). Similarly, patients with higher bacterial loads had experienced more frequent unscheduled hospitalisations (figure 3.6). After adjusting for confounders, bacterial load remained a significant predictor of recurrent exacerbations: AOR 1.20, 1.11-1.29, p<0.0001 for each 1 log unit increase in bacterial load (for this analysis, recurrent exacerbations were defined as $\geq$3 per year).

For unscheduled hospitalisations, bacterial load was an independent predictor of hospitalisation AOR 1.11, 1.01-1.21, p=0.02 for each 1 log unit increase in bacterial load. Adequate model fit was demonstrated for all analyses ($p>0.05$). There was a significant association between bacterial load and subsequent severity of cough symptoms using the Leicester Cough Questionnaire ($p<0.0001$, Kruskal-Wallis test) and the St. George's Respiratory Questionnaire ($p<0.0001$, Kruskal-Wallis test). The differences were greater than the minimal clinically important difference at 10^5cfu/ml or greater for the LCQ and at 10^6cfu/ml or greater for the SGRQ.

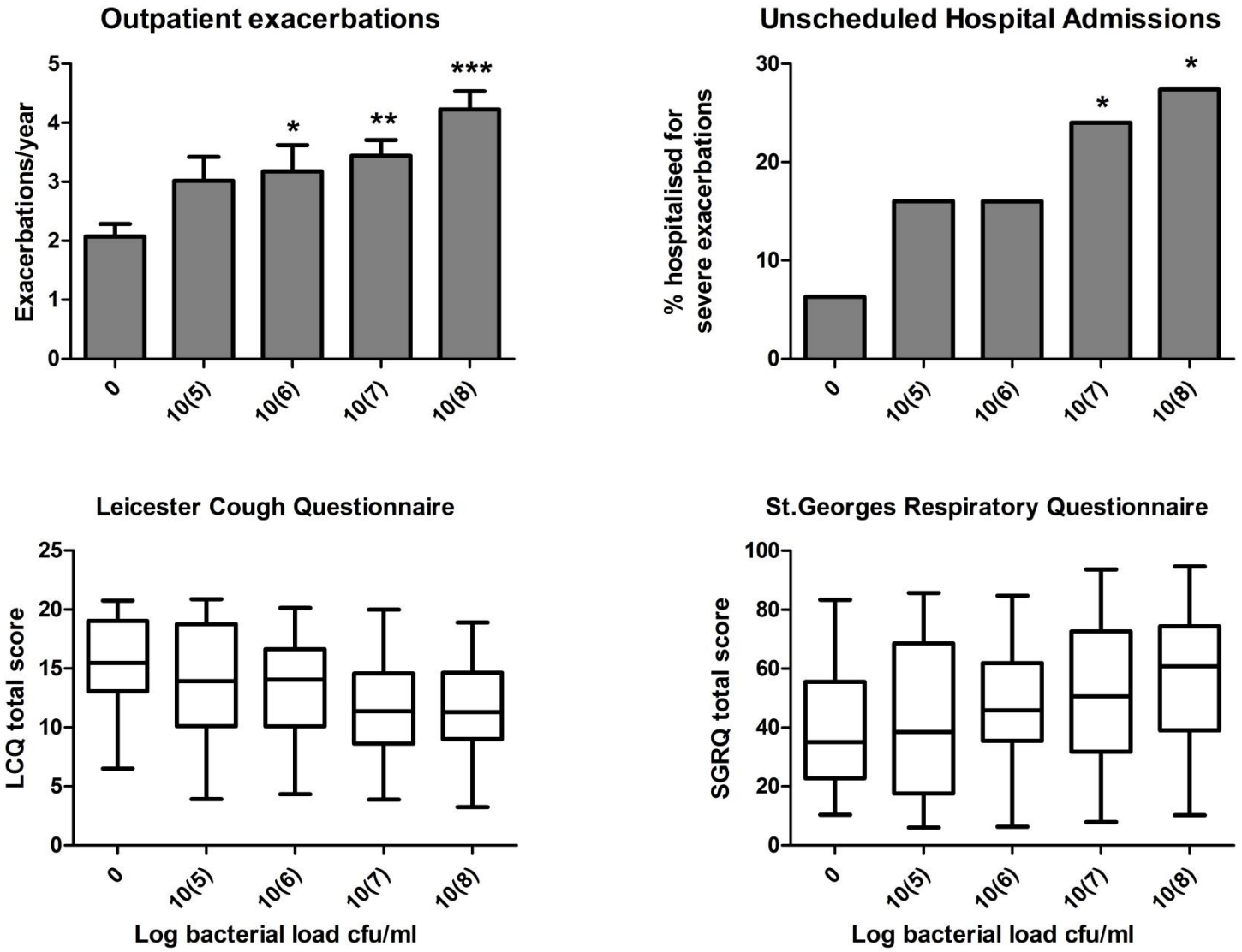

Figure 3.6. Outpatient exacerbations, unscheduled hospital admissions and symptom questionnaires after 1 year of follow-up. Data are presented as mean and standard error (outpatient exacerbations) and as median (IQR) for symptom questionnaires. For unscheduled hospital admissions, data are presented as % of patients hospitalised during follow-up. *p<0.05, **p<0.01 ***p<0.0001

3.11 Discussion

This chapter demonstrates a direct relationship between bacterial load and airway and systemic inflammation in a large population of patients with non-CF bronchiectasis. Over 1 year, patients with high bacterial loads had a higher frequency of exacerbations and were more likely to require hospitalisation with severe exacerbations. This relationship was independent of severity of bronchiectasis as assessed by CT scanning and spirometry. Higher bacterial loads were associated with more severe respiratory symptoms, as assessed by the St. George's Respiratory Questionnaire, and more severe cough, as assessed by the Leicester Cough Questionnaire.

These relationships demonstrate a central role for bacterial infection in provoking inflammation and affecting short-term prognosis in patients with bronchiectasis, providing strong evidence in support of the vicious cycle hypothesis (Cole, 1986).

The logical extension of the vicious cycle hypothesis is that bacterial clearance through antibiotic therapy should "break the cycle" and thereby have a positive impact on inflammation and prognosis.

This study found a significant improvement in markers of airway inflammation, both using short term (14 days) treatment with intravenous antibiotics, and using longer term treatment with 12 months of nebulised gentamicin. These data provided strong evidence that antibiotic treatment can alter the underlying airway inflammation in bronchiectasis, providing hope of improving clinical symptoms and the prognosis of the disease. This study provides the basis for future large randomised controlled trials

of long term antibiotics in non-CF bronchiectasis. A number of trials of inhaled and oral antibiotics in non-CF bronchiectasis are now underway. To date, results of trials of long term antibiotics in non-CF bronchiectasis have produced conflicting results, with many trials limited in terms of sample size and duration. A systematic review of clinical trials published in 2007 reported no benefit of prolonged antibiotics in bronchiectasis (9 trials including 378 patients) in terms of reducing exacerbations (OR 0.96 95% CI 0.27-3.46) (Evans et al, 2007).[12] The authors could find no evidence on benefits in terms of markers of airway inflammation. A previous study randomised patients with bronchiectasis in a double blind placebo controlled study to erythromycin or placebo and found no benefit of antibiotic treatment on airway inflammatory markers, including IL-1α, IL-8, TNF-α and LTB_4 (Tsang et al, 1999). Conversely Hill et al showed reduced elastase activity in patient sputum following treatment with amoxicillin (Hill et al, 1988). A study of nebulised gentamicin demonstrated reduced levels of myeloperoxidase with nebulised antibiotics, and most recently our group demonstrated reduced myeloperoxidase and elastase levels in patients treated with nebulised gentamicin (Lin et al, 1997, Murray et al, 2010). An important finding of the present study was that inflammatory markers returned to baseline in patients treated with nebulised gentamicin 3 months after cessation of treatment. This suggests that to maintain efficacy, treatment has to be continuous or to have off-drug intervals of less than 3 months. This is important for designing future antibiotic interventions.

One of the goals of this study was to demonstrate that bacterial clearance with antibiotics reduced markers of airway inflammation. This provides strong evidence firstly that bacterial colonisation directly influences airway inflammation, rather than

reflecting the underlying lung damage which should not be affected by antibiotics. Secondly it provides a basis for future large trials of long-term antibiotic therapy. If bacterial infection directly contributes to airway inflammation and independently predicts exacerbations and hospitalisation for severe exacerbations, long term antibiotic treatment should be expected to improve the long term prognosis of the disease and quality of life (Wilson et al, 1997, Loebinger et al, 2009). A recent study has demonstrated that chronic bacterial colonisation, severe exacerbations and systemic inflammation were all associated with more rapid lung function decline over 24 months (Martinez-Garcia et al, 2007). Our data suggest that these three important phenomena are closely linked.

Bronchiectasis has been described as an "orphan" disease. In contrast to cystic fibrosis where guidelines recommend the use of surveillance sputum cultures and the evidence base for suppressive antibiotic therapy is strong (Flume, 2007), there is no such standard of care in non-CF bronchiectasis. The British Thoracic Society has recently published guidelines for the management of non-CF bronchiectasis[4] that includes recommendations to monitor sputum cultures when patients are stable, and to consider long term antibiotic therapy in patients with frequent exacerbations. These recommendations are based on expert opinion and there is a clear need for studies specifically in non-CF bronchiectasis.

Our study strongly supports the use of surveillance bacteriology in stable patients with non-CF bronchiectasis, as bacterial colonisation predicts future exacerbation risk, hospitalisations and quality of life. Furthermore, our study provides evidence to support antibiotic treatment in stable patients, as both short and long term antibiotic treatment were associated with reduced airways and systemic inflammation.

The relationship between airway bacterial load and airway inflammation is recognised in COPD where several studies have demonstrated a progressive increase in MPO, elastase and pro-inflammatory cytokines with increasing bacterial load (Hill et al, 2000). The data reported in this study are supported by Angrill et al, who performed bronchoalveolar lavage (BAL) on 49 patients with non-CF bronchiectasis, and demonstrated an increase in neutrophil numbers, MPO, elastase and cytokines in patients with bronchiectasis compared to controls, along with an increase in these markers with increasing bacterial load in BAL fluid (Angrill, 2001).

The present study expands on this previous work by demonstrating the importance of bacterial colonisation in a large cohort of patients with exclusively non-CF bronchiectasis. The advantage of this large cohort was the ability to adjust for other potential modifiers of inflammation, such as the severity of radiological lung disease and airflow obstruction. Even after accounting for these variables, patients with high bacterial loads had higher levels of airway inflammation.

We expand on the data in stable patients by demonstrating in patients with exacerbations of bronchiectasis that antibiotic treatment for 14 days results in a significant reduction in airway and systemic inflammation.

Bronchiectasis is a neutrophil driven inflammatory disorder (Cole, 1986, Watt et al, 2004, Fuschillo et al, 2008). Release of pro-inflammatory cytokines – such as Interleukin-1β, IL-8, TNF-α and LTB4 – in the airway promote neutrophil recruitment from the peripheral circulation (Stockley et al, 1988). Pro-inflammatory cytokines, particularly TNF-α and IL-1β, increase the expression of ICAM-1 and E-selectin on vascular endothelial cells (Stockley et al. 1988). Activation of

CD11/CD18 on circulating neutrophils allows neutrophils to adhere to these ligands on the inflamed endothelium, leading to neutrophil migration (Downey et al, 2009).

In this study we measured ICAM-1, E-selectin and VCAM-1 as soluble vascular adhesion molecules. As bacterial load was associated with markers of neutrophil mediated airway inflammation, we hypothesised that bacterial colonisation would promote neutrophil recruitment through up-regulation of adhesion markers. Previous studies have shown that these markers are elevated in patients with bronchiectasis compared to controls, and that they are elevated in other inflammatory lung diseases including COPD and cystic fibrosis (Riise et al, 1994, De Rose et al, 1998, Zheng et al, 2000). Our data showed that ICAM-1 was elevated in bronchiectasis patients compared to matched controls and that E-selectin and VCAM-1 were also elevated at bacterial loads of 10^7 cfu/ml or greater. These data suggest that even during clinical stability there is an increased drive to recruit leukocytes to the airway, contributing to the cycle of inflammation. These findings may have relevance beyond airway inflammation as raised levels of endothelium derived inflammatory markers (particularly ICAM-1) have been linked to an increased frequency of myocardial infarction (Ridker et al, 1998), stroke (Fassbender et al, 1999) and diabetes (Song et al, 2007), which are increased in patients with chronic lung disease, in addition to predicting lung function decline (Walter et al, 2008). The finding that this marker decreases with antibiotic treatment is therefore an interesting area for further research.

It should be noted that although this study found a significant relationship between airway cytokines and neutrophil markers with bacterial load, the overlap between the groups was large and the role of these markers in clinical practice is unclear.

The relevance of this study to the following chapters regarding ficolin-2 and mannose binding lectin is clear. Bronchiectasis is a chronic inflammatory disorder and we hypothesise that ficolin-2 or mannose binding lectin deficiency may predispose to more severe bronchiectasis and greater airway inflammation through a failure to clear bacteria via opsonophagocytosis. These studies therefore link airway inflammation and airway infection, providing an explanation for why ficolin-2 "deficiency" may cause disease progression. Furthermore, if short or long term antimicrobial treatment is able to reverse airway inflammation in bronchiectasis, a logical conclusion is that poor prognosis in patients with ficolin-2 or MBL "deficient" SNP's could be reversed with long term antimicrobial treatment. This idea, that patients with a genetic susceptibility to bacterial infection and poor outcome may be managed with different treatments, known as pharmacogenetics, is the concept that underpins the following chapters.

Conclusion

Chronic colonisation with high bacterial loads in non-CF bronchiectasis is associated with airway and systemic inflammation, a greater risk of exacerbations and worse health-related quality of life. Both short and long term antibiotic therapy reduce markers of airway and systemic inflammation. This study highlights the importance of monitoring sputum bacteriology when clinically stable, and provides the evidence base for future intervention studies to reduce the bacterial burden in the airways.

CLINICAL TRIAL REGISTRATION

The randomised controlled trial described in this chapter was registered: Clinical Trial Registration Number NCT00749866

CHAPTER 4

Ficolin-2 in bronchiectasis

4.1 Introduction

Bronchiectasis is a chronic inflammatory lung disease characterised by permanent dilation of the bronchi leading to a clinical syndrome of cough, sputum production and recurrent respiratory infections (Smith 2011) Although bronchiectasis is associated with a number of immunodeficiencies, autoimmune disorders and severe infections, the cause is not identified in more than 50% of cases even in specialist centres (Pasteur et al 2000). It is speculated that a significant number of patients with "idiopathic" bronchiectasis have unrecognised immune defects.(Brown et al 2011).

Bronchial damage allows chronic colonisation of the airway with damaging bacterial pathogens, most frequently with nasopharyngeal commensals such as *Haemophilus influenzae* or *S. pneumoniae* but also with opportunistic bacteria such as *P. aeruginosa*. (Chalmers et al 2012). *P. aeruginosa* is of particular interest as it has been associated with an increased rate of disease progression in cystic fibrosis and non-cystic fibrosis bronchiectasis.(Evans et al 1996, Wilson et al 1997, Loebinger et al 2009). This pathogen is highly resistant to antibiotics and once initial infection is established, *P. aeruginosa* adapts to its niche by forming biofilms and downregulating invasive virulence factors to evade pulmonary innate immunity (Singh et al 2000, Parks et al 2009).

Why some patients with bronchiectasis are susceptible to opportunistic *P. aeruginosa* infection and more severe disease is not clear.

Complement is critical to pulmonary immunity against *P. aeruginosa* and other bacteria. (Lavoie et al 2011) Studies in mice and humans suggest that the alternative pathway is the dominant pathway for complement mediated defence against *P. aeruginosa* in serum with a small but significant contribution from the lectin pathway (Younger et al 2003). The relative importance of each pathway in the context of the bronchiectasis lung is less clear. Studies using human bronchoalveolar lavage suggest that alternative complement pathway components are present at very low levels in the lung, that alternative complement activation is negligible and that the majority of complement activation occurs via the classical and lectin pathways (Watford et al 2000).

Ficolin-2 is a component of the lectin pathway of complement and one of the major pattern recognition molecules in human serum. (Matsushita et al 1996) Like other ficolins it consists of a collagen-like tail and a fibrinogen-like binding domain that interacts with a wide range of carbohydrates expressed on bacterial, fungi, viruses and host cells.(Ma et al 2004, Krarup et al 2005, Garlatti et al 2007) It is one of a small number of molecules able to activate the lectin pathway of complement (along with the other ficolins, MBL and CL-L1) via MBL-associated serine protease-2 (Thiel et al 1997). Single nucleotide polymorphisms in the FCN2 gene lead to low ficolin-2 serum levels (Hummelshoj et al 2005) and preliminary data have linked low ficolin-2 serum levels with recurrent respiratory tract infections in childhood (Cedzynski et al 2009) and with bronchiectasis (Kilpatrick et al 2009).

The hypothesis of this study was that ficolin-2 may be an important component of complement mediated defence against *P. aeruginosa* and other bacteria and may

therefore be associated with bacterial colonisation and disease severity in bronchiectasis.

4.2 Bronchiectasis cohort

The study included 470 patients with non-cystic fibrosis bronchiectasis and 414 healthy control subjects. The characteristics of the study population are shown in Table 4.1. There were no significant differences in demographics or co-morbid illnesses between bronchiectasis patients and controls. The majority (80.4%) of the cohort had idiopathic/post-infective bronchiectasis. None of the control subjects had a history of chronic respiratory disease and none were prescribed inhaled corticosteroids (table 4.1)

	Bronchiectasis cohort	Control cohort	
N	470	414	
Age	67 (58-75)	66 (56-77)	0.2
Gender (% female)	259 (55.1%)	211 (51.0%)	0.2
BMI kg/m^2	25.4 (21.9-29.3)	Not recorded	n/a
Co-morbidities			
Chronic cardiac disease	108 (23.0%)	99 (23.9%)	0.7
Cerebrovascular disease	46 (9.8%)	42 (10.1%)	0.9
Chronic renal impairment	32 (6.8%)	33 (8.0%)	0.5
Diabetes mellitus	58 (12.3%)	41 (9.9%)	0.3
Causes of bronchiectasis			
Idiopathic/post infective	378 (80.4%)	Not applicable	
Previous ABPA	41 (8.7%)		
Connective tissue disease	28 (6.0%)		
Inflammatory bowel disease	14 (3.0%)		
Others	9 (1.9%)		
Medications			
Inhaled corticosteroid use	196 (41.7%)	0 (0%)	<0.0001

Table 4.1. Baseline characteristics of the study cohort and control subjects. Abbreviations, ABPA= allergic bronchopulmonary aspergillosis. BMI= body mass index.

4.3 Ficolin-2 SNP's and Haplotypes: defining Ficolin-2 Insufficiency

Genotyping success was >95% and all SNP's were in Hardy-Weinburg equilibrium ($p>0.05$). The expected relationships between *FCN2* SNP's and serum levels were observed. As shown in figure 4.1, The promoter polymorphisms had significant effects on ficolin-2 serum levels with the lowest serum levels associated with the exon-8 polymorphism (Ala258Ser). All of the relationships were statistically significant. At $p<0.0001$ with the exception of the promoter polymorphism at position -4 ($p=0.0004$).

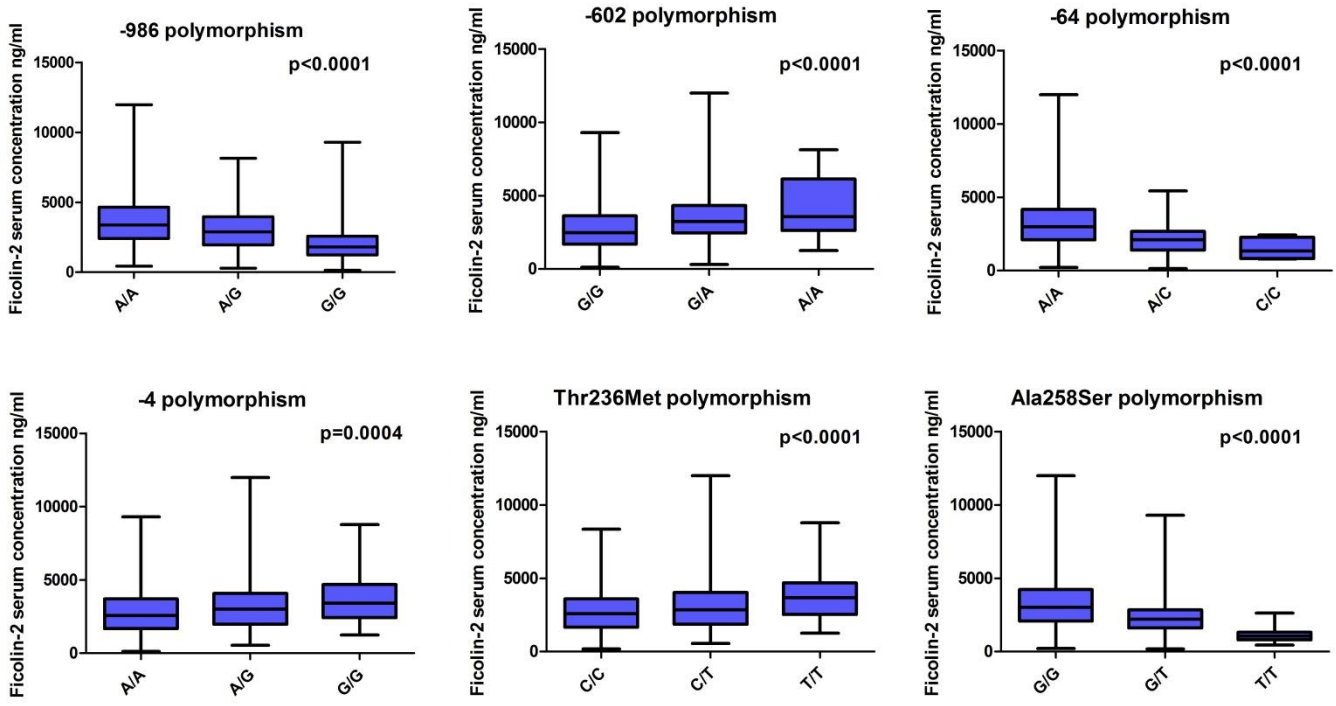

Figure 4.1. The effect of single nucleotide polymorphisms in the FCN2 gene on ficolin-2 serum levels. The graphs show median with interquartile range (boxes) and range (bars). N=884 (bronchiectasis patients and controls are shown together as the same relationships were observed when analysing each group seperately (data not shown).

Similarly, the inferred haplotypes had the expected impact on ficolin-2 serum concentrations, although with large and overlapping ranges. In view of this large variation within individual SNP and haplotype groups, an analysis was performed to identify the most accurate way of defining Ficolin-2 insufficiency using genetic data. Ficolin-2 insufficiency was defined a-priori as a ficolin-2 serum level <1600ng/ml, and receiver operator characteristic curves used to calculate the discriminatory ability of each individual SNP, each haplotype or pairs of haplotypes to identify low ficolin-2 serum levels. The results are shown in table 4.2

	Area under the curve
Individual SNP's	
FCN2 (pro-986G>A)	0.58 (0.55-0.62),p=0.01
FCN2 -602 G>A	0.55 (0.52-0.59),p=0.1
FCN2 -64 A>C	0.55 (0.52-0.59),p=0.1
FCN2 -4 A>G	0.54 (0.51-0.57),p=0.2
FCN2 (exon 8- T236M)	0.63 (0.60-0.67),p=0.0001
FCN2 (exon 8- A258S)	0.60 (0.57-0.63),p=0.006
Haplotypes	0.66 (0.63-0.68),p<0.0001
Haplotype pairs	0.76 (0.72-0.79), p<0.0001

Table 4.2 Comparison of analysis based on genotype, haplotype and haplotype pairs for identifying low serum Ficolin-2 levels. Analysis based on N=1768 chromosomes from bronchiectasis patients and controls.

This analysis demonstrated that each individual SNP was relatively poor at discriminating between deficient and non-deficient populations. Haplotypes improved this discrimination ($p<0.05$) comparing the area under the curve against

individual SNP's. Haplotype pairs however, significantly improved the discrimination between insufficient and sufficient groups. This method is similar to that used in studies of mannose binding lectin (see chapter 1.2 for further details). Of the 6 well described FCN2 haplotypes AGGTG and AAACG were classified and high expressing haplotypes, GGACG, AGACG and GGATG were classified and intermediate expressing haplotypes and GGACT was defined as a low expressing haplotype for the purposes of this analysis as previously described (Hummelshoj et al 2007). The distribution of ficolin-2 serum levels according to haplotype pairs are shown in figure 4.2. The differences between groups were statistically significant ($p<0.0001$).

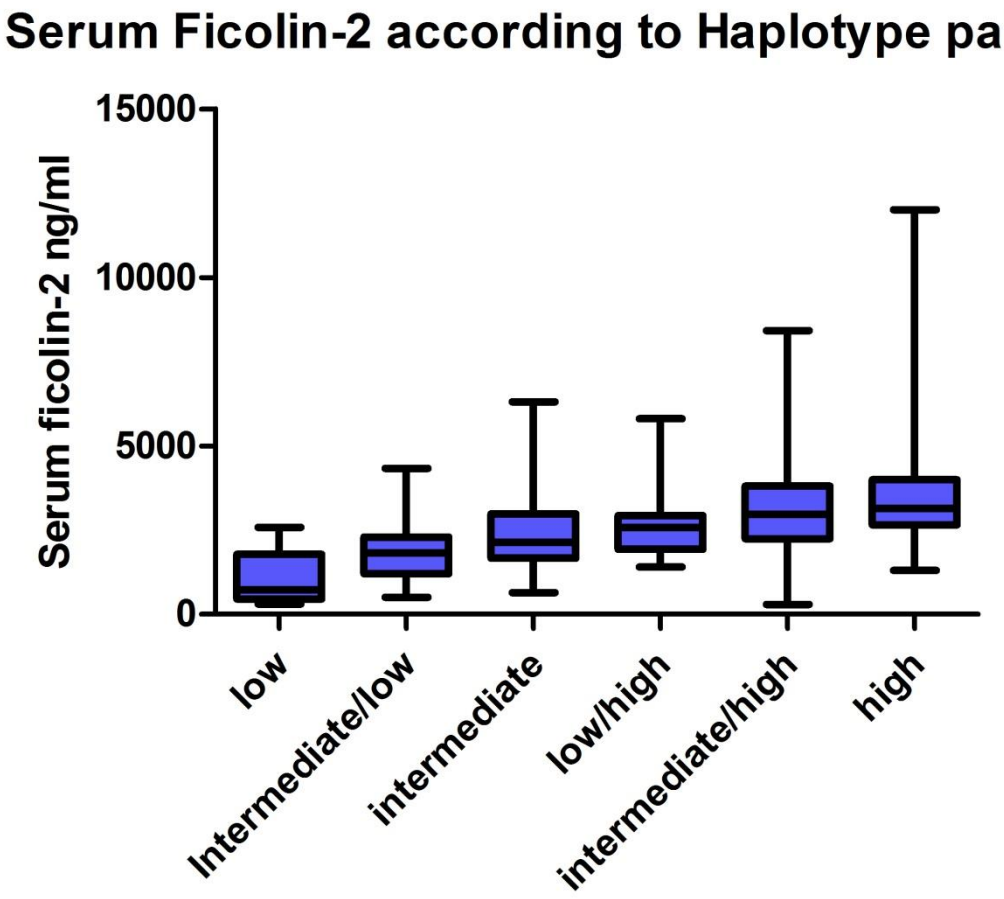

Figure 4.2. Pairs of ficolin-2 haplotypes more accurately stratify patients according to ficolin-2 serum levels. Low indicates patients with two low expressing haplotypes (GGACT/GGACT), Intermediate/low indicates patients with one intermediate expressing haplotype and one low expressing haplotype (GGACG/GGACT, AGACG/GGACT and GGATG/GGACT) and so on.

4.4 Single nucleotide polymorphisms in the FCN2 gene are more frequent in bronchiectasis patients than controls.

The high expressing FCN2 haploytype AGGTG was significantly less frequent in bronchiectasis patients compared to controls (OR 0.75 95% CI 0.60-0.93). Other differences were not statistically significant. This is shown in table 4.3.

Haplotype	Bronchiectasis patients		Controls		Odds ratio (95% CI)	p-value
	N	% patients	N	% patients		
AGGTG	193	20.5%	213	25.7%	0.75 (0.60-0.93)	0.01
AAACG	176	18.7%	157	19.0%	0.98 (0.78-1.25)	1.0
GGACG	364	38.7%	299	36.1%	1.12 (0.92-1.36)	0.3
AGACG	39	4.1%	34	4.1%	1.01 (0.63-1.62)	0.9
GGATG	40	4.3%	22	2.7%	1.63 (0.96-2.76)	0.09
GGACT	88	9.4%	89	10.7%	0.86 (0.63-1.17)	0.3

Table 4.3. The frequency of FCN2 haplotypes in bronchiectasis patients and controls. P-values derived from Chi-square test. 95.7% of bronchiectasis patients haplotypes and 98.3% of control haplotypes formed the 6 common haplotypes.

The analysis of individual SNP's are shown below. There were no significant differences in the frequency of FCN3 or MASP-2 SNP's between bronchiectasis and control subjects- Table 4.4. There was a significant relationship between the FCN2 promoter SNP 986 G>A with the AA genotype, which is associated with higher expression of serum ficolin-2, being less frequent in bronchiectasis patients compared to controls (p=0.01). The multiple chi square test indicated that the distribution of this SNP across the population was significantly different between bronchiectasis patients and controls (p=0.04). In addition, there was a significant associated between the AA genotype in the FCN2 SNP -4 A>G which also affects the promoter region of the gene (p=0.04). This allele is associated with slightly higher concentrations of serum ficolin-2 but has a lesser effect on ficolin-2 serum concentrations than other SNP's figure 4.1. No other statistically significant differences were identified.

SNP	Bronchiectasis patients	Controls	Odds ratio (95% CI)	Chi square test	Multiple chi square
N	470	414			
FCN2 (pro-986G>A)					
GG	152 (32.3%)	115 (27.8%)	1.24 (0.93-1.66)	0.2	
GA	226 (48.1%)	189 (45.7%)	1.10 (0.85-1.44)	0.5	
AA	92 (19.6%)	110 (26.6%)	0.67 (0.49-0.92)	**0.01**	**0.04**
FCN2 -602 G>A					
GG	295 (62.8%)	267 (64.5%)	0.93 (0.71-1.22)	0.6	
AG	156 (33.2%)	131 (31.6%)	1.07 (0.81-1.42)	0.7	
AA	19 (4.0%)	16 (3.9%)	1.05 (0.53-2.07)	1.0	0.9
FCN2 -64 A>C					
AA	376 (80.0%)	335 (80.9%)	0.94 (0.68-1.31)	0.8	
AC	85 (18.1%)	74 (17.9%)	1.01 (0.72-1.43)	1.0	
CC	9 (1.9%)	5 (1.2%)	1.60 (0.53-4.80)	0.6	0.7
FCN2 -4 A>G					
AA	292 (62.1%)	227 (54.8%)	1.33 (1.02-1.75)	**0.04**	
AG	150 (31.9%)	153 (37.0%)	0.80 (0.61-1.06)	0.1	
GG	28 (6.0%)	34 (8.2%)	0.71 (0.42-1.19)	0.2	0.07
FCN 2 (exon 8- T236M)					
CC	247 (52.6%)	215 (51.9%)	1.03 (0.79-1.34)	0.9	
CT	181(38.5%)	158 (38.2%)	1.01 (0.77-1.33)	1.0	
TT	42 (9.4%)	41 (9.9%)	0.89 (0.57-1.40)	0.7	0.9
FCN2 (exon 8- A258S)					
GG	382 (81.3%)	325 (78.5%)	1.19 (0.85-1.65)	0.3	
GT	83 (17.7%)	79 (19.1%)	0.91 (0.65-1.28)	0.6	
TT	5 (1.0%)	10 (2.4%)	0.43 (0.15-1.28)	0.2	0.2
FCN3 (+1637 delC L117fs)					
GG	461 (98.1%)	406 (98.2%)	0.99 (0.38-2.59)	0.8	
Gfs	9 (1.9%)	8 (1.8%)	N/A	N/A	
Fs	0 (0%)	0 (0%)	N/A	N/A	1.0
MASP-2 (CUB 1 D120G)					
AA	447 (95.1%)	404 (97.5%)	0.48 (0.23-1.02)	0.08	
AG	22 (4.7%)	10 (2.5%)	1.98 (0.93-4.24)	0.1	
GG	1 (0.2%)	0 (0%)	N/A	N/A	0.1

Table 4.4. The frequency of single nucleotide polymorphisms in FCN2, FCN3 and MASP-2 compared between bronchiectasis patients and matched healthy controls. fs=frameshift.

Serum ficolin-2 was significantly lower between bronchiectasis patients and control subjects. Using the previous definitions of low ficolin-2 levels. At <1600ng/ml 18.7% of bronchiectasis patients were "deficient" compared to 7.8% of controls,$p<0.0001$. Using <1200ng/ml, the figures were 8.1% for patients with bronchiectasis and 1.9% for controls,$p<0.0001$. No relationships between Ficolin-3 and MASP-2 serum levels with bronchiectasis were observed.

4.5 Impact of Ficolin-2 insufficiency on disease severity

Patients with serum ficolin-2 insufficiency had more severe disease, as demonstrated by a higher frequency of chronic bacterial colonisation, a higher frequency of *P. aeruginosa* colonisation, more frequent exacerbations, more frequent hospitalisations for severe exacerbations during follow-up and worse symptoms and health related quality of life using the Leicester cough questionnaire and SGRQ. The difference in radiological severity of bronchiectasis by CT scoring was not statistically significant ($p=0.052$). These results are shown in Table 4.5. There was no relationship between MASP-2 or Ficolin-3 serum levels or SNP's with disease severity.

Severity marker	**Low ficolin-2 levels ≤1600ng/ml**	**Ficolin-2 >1600ng/ml**	**p-value**
N	88	382	
Age	66 (56-74)	63 (52-72)	0.2
Gender	46 (52.3%)	165 (43.2%)	0.1
Bacteriology			
Chronic colonisation	72 (81.8%)	240 (62.8%)	0.0007
P.aeruginosa	28 (31.8%)	40 (10.5%)	<0.0001
H.influenzae	20 (22.7%)	121 (31.7%)	0.1
S. aureus	12 (13.6%)	31 (8.1%)	0.1
S. pneumoniae	4 (4.5%)	26 (6.8%)	0.4
M. catarrhalis	9 (10.2%)	45 (11.8%)	0.7
GNE	10 (11.4%)	36 (9.4%)	0.6
Pulmonary function			
FEV_1 % predicted	70.8% (46.7-85.4%)	73.6% (55.2-92.3%)	0.2
FVC % predicted	80.6% (66.9-96.7%)	84.6% (70.6-99.7%)	0.2
Exacerbations and QOL			
Annual exacerbation frequency*	3 (1-4)	2 (1-4)	0.009
Hospitalisations*	40 (45.5%)	71 (18.6%)	<0.0001
SGRQ	51.7 (34.8-69.7)	44.3 (27.2-61.4)	0.008
LCQ	12.5 (9.5-16.9)	14.4 (11.0-17.8)	0.01
HRCT (Reiff) score	4 (2-12)	3 (2-6)	0.05
BMI kg/m^2	25.3 (22.3-29.4)	25.4 (21.8-29.2)	0.8

Table 4.5 Bronchiectasis patients with low serum levels of Ficolin-2 have a higher frequency of bacterial colonisation and a greater severity of disease. *Abbreviations*: GNE= gram negative enterobacteriaceae, FEV_1= forced expiratory volume in 1 second, FVC= forced vital capacity, SGRQ= St.Georges respiratory questionnaire, LCQ= Leicester cough questionnaire, HRCT= high resolution computed tomography, BMI= body mass index. *these data refer to the patients history at baseline. Longditudinal follow-up data during the study are presented later.

In the genetic analysis low and intermediate expressing haplotypes were associated with a greater disease severity (figure 4.3). This included a higher frequency of chronic bacterial colonisation (p=0.02 by chi square test).

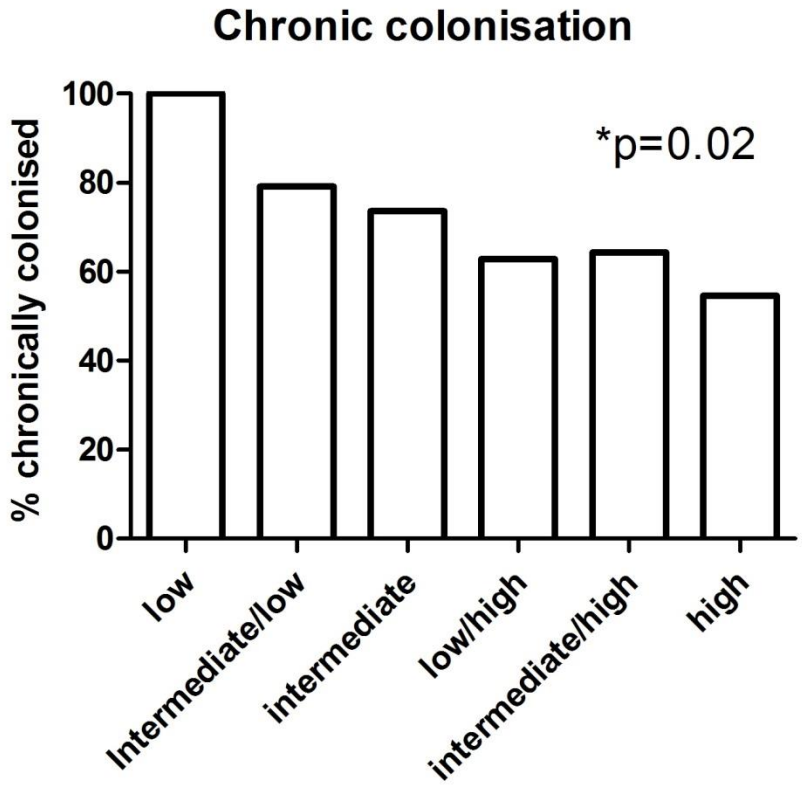

Figure 4.3. The frequency of chronic bacterial colonisation (defined as isolation of a potentially pathogenic bacterial species on at least 2 occasions 3 months apart while clinically stable) stratified by ficolin-2 haplotype.

In addition, there was a strong correlation between FCN2 haplotype and hospitalisation for severe exacerbations, with a significantly higher proportion of patients in the low, intermediate/low and intermediate expressing haplotype groups

being admitted to hospital for severe exacerbations over 4 years follow-up (p<0.0001 by log-rank test), figure 4.4.

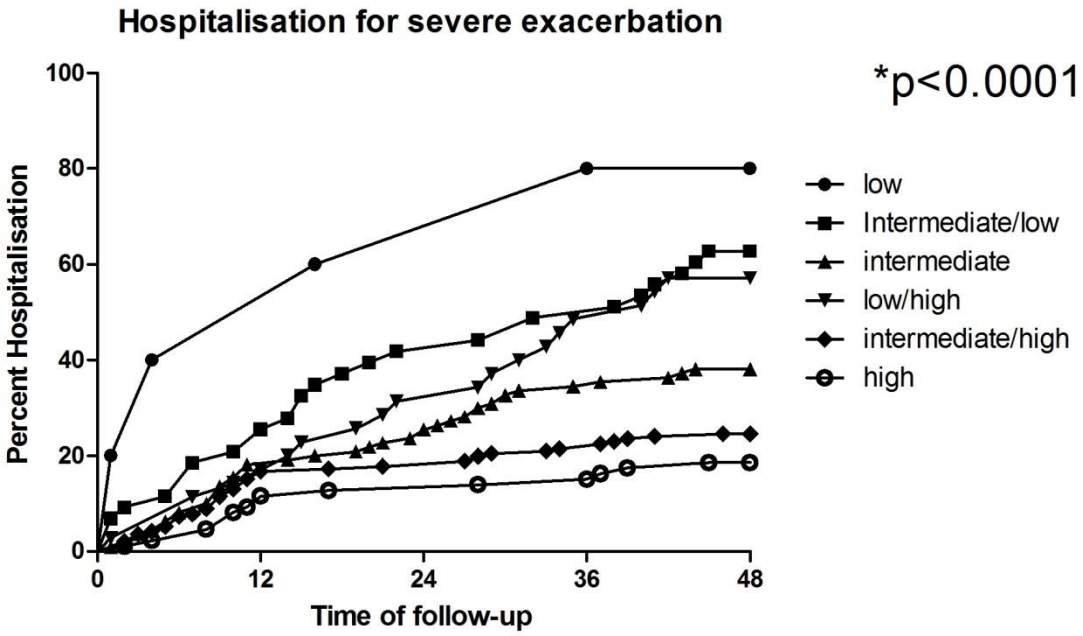

Figure 4.4. The frequency of hospitalisation during the study period. p<0.0001 refers to log-rank test comparing time to first hospitalisation stratified by ficolin-2 haplotype.

There were also significant relationships between *FCN2* haplotype status and quality of life, as measured by the St Georges respiratory questionnaire (p=0.003 by Kruskal-wallis test) and the Leicester cough questionnaire (p=0.009 by Kruskal-wallis test), this is shown in figure 4.5.

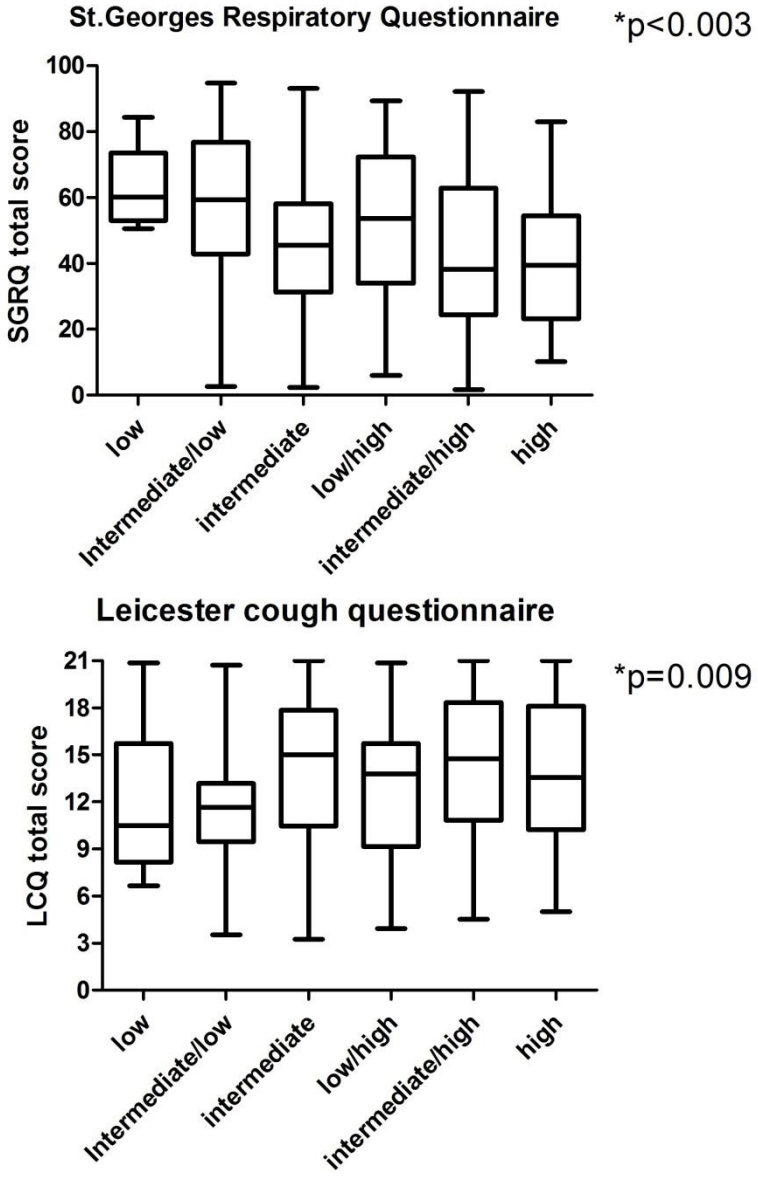

Figure 4.5. Quality of life using the St.Georges Respiratory Questionnaire and cough severity using the Leicester cough questionnaire assessed at the start of the study and displayed according to FCN2 haplotype. Data are presented as median (lines), interquartile range (boxes) and range (bars). Statistical comparisons refer to Kruskal-

wallis test. Abbreviations- SGRQ= St Georges Respiratory Questionnaire, LCQ= Leicester cough questionnaire.

Patients with low expressing haplotypes (median 5 IQR 1-8) and intermediate/low expressing haplotypes (median 4 IQR 1-5) had higher annual exacerbation frequency compared to the others groups (p=0.02), see figure 4.6. There were no significant differences in the scoring for radiological severity between FCN2 haplotype groups.

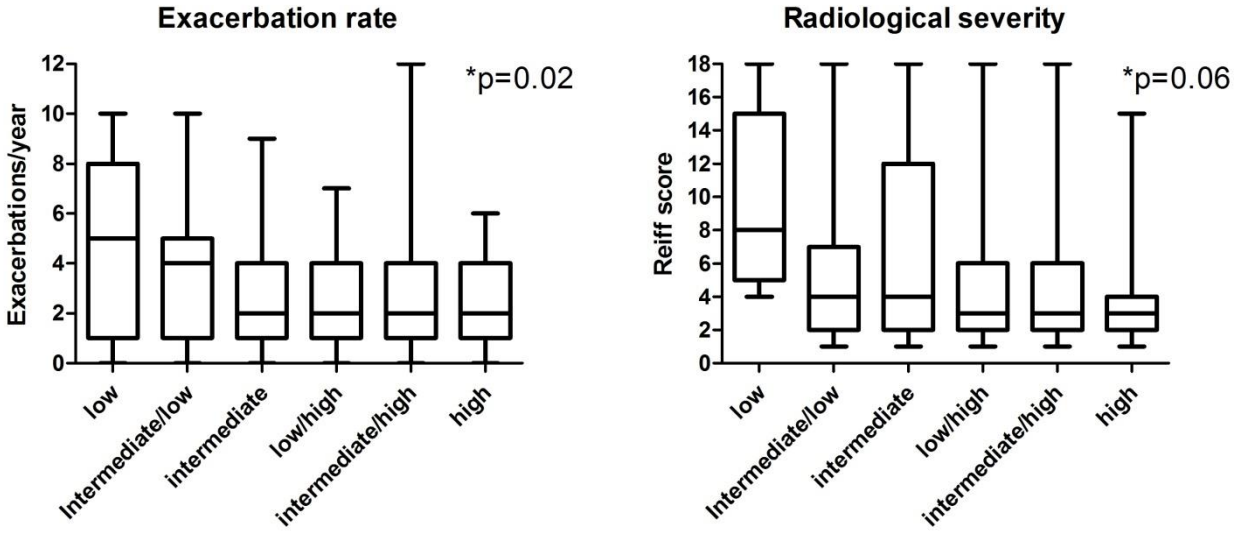

Figure 4.6. Exacerbation frequency and radiological severity in patients with bronchiectasis stratified according to FCN2 haplotype. Data are presented as median (lines), interquartile range (boxes) and range (bars). Statistical comparisons refer to Kruskal-wallis test.

Examing spirometry results at entry into the study, there were no significant differences in forced expiratory volume in 1 second or forced vital capacity between haplotype groups, figure 4.7.

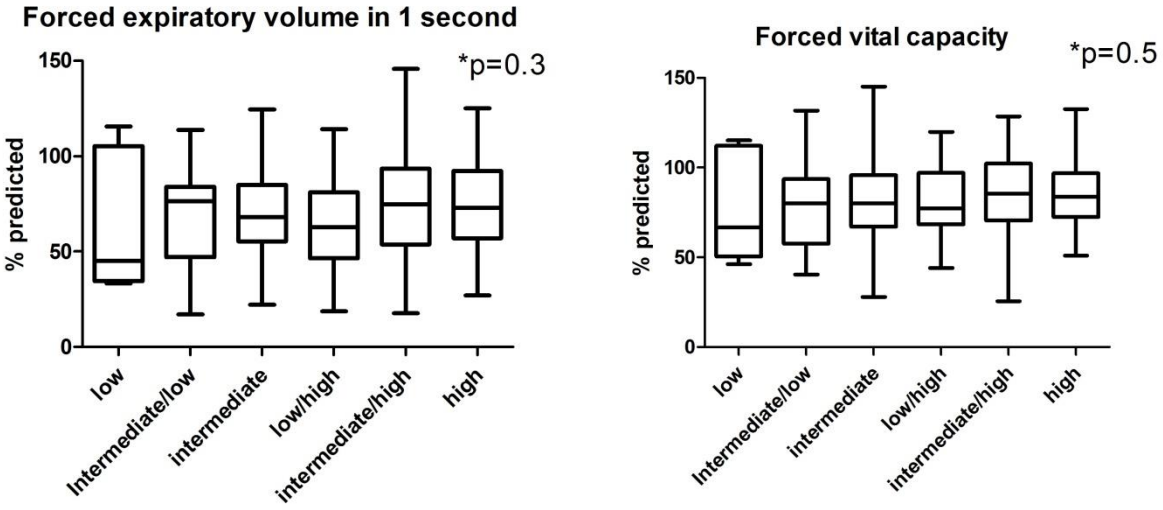

Figure 4.7. forced expiratory volume in 1 second and forced vital capacity at study entry stratified according to FCN2 haplotype. Data are presented as median (lines), interquartile range (boxes) and range (bars). Statistical comparisons refer to Kruskal-wallis test.

After 4 years follow-up, patients in the intermediate/high and high expressing groups had the lowest mortality (10.5% and 5.8%) respectively and patients in the low and intermediate/low haplotypes had the highest mortality rates, 20% and 16.3% respectively, but these differences were not statistically significant (p=0.2 by log rank test). Figure 4.8.

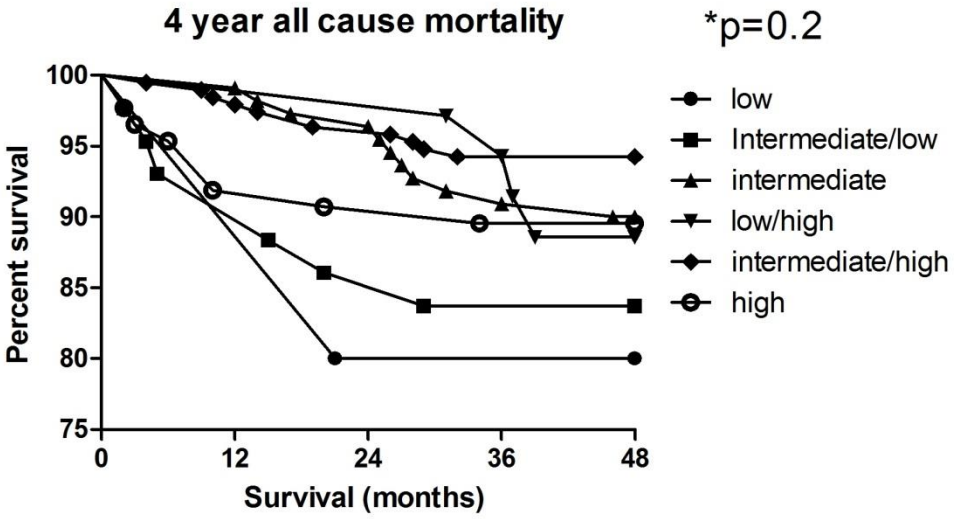

Figure 4.8. Impact of ficolin-2 haplotypes on survival in bronchiectasis. Statistical significant is shown in each figure. P-value refers to comparison of time to death using the log-rank test.

In addition to having worse severity of disease using clinical indices, there was a significant relationship between FCN2 haplotype, bacterial load and airways inflammation. The frequency of *P. aeruginosa* was significantly higher in the low, intermediate/low and intermediate group compared to patients with high expressing haplotypes. Other enteric Gram-negative organisms were also less frequent in patients with high expressing haplotypes, see figure. The frequency of *H. influenzae* and *S. aureus* were not significantly different between groups. This is shown in figure 4.9.

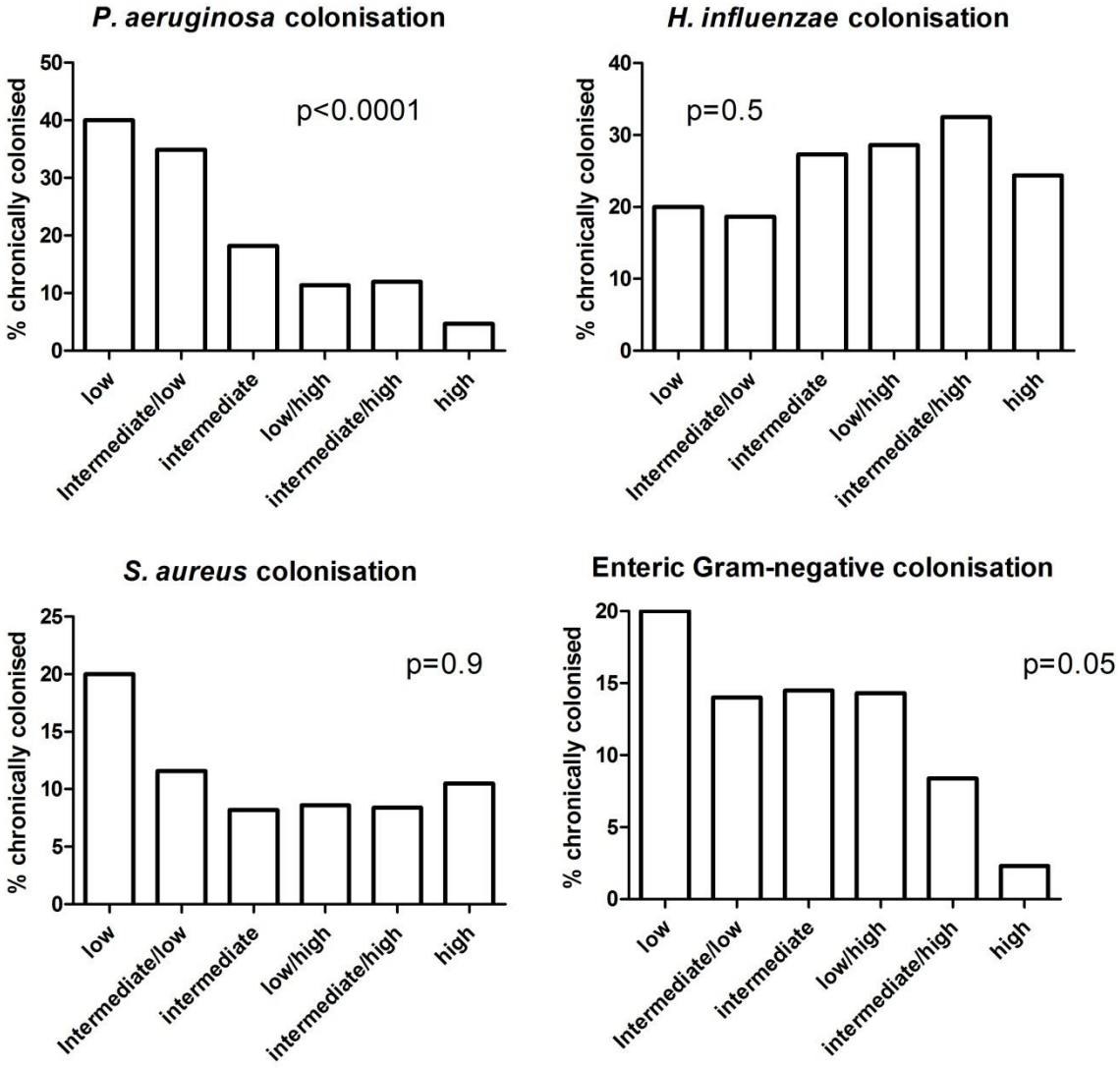

Figure 4.9. Relationship between FCN2 haplotype and bacterial colonisation. There was a statistically significant relationship between FCN2 haplotype and *P. aeruginosa* colonisation ($p<0.0001$ by chi square test) and colonisation with enteric Gram-negative organisms, $p=0.05$ by chi square test).

All the markers of airway inflammation measured were higher in patients with lower expressing FCN2 haplotypes with the exception of interleukin-8 where not significant relationship was observed (p=0.5). Figure 4.10.

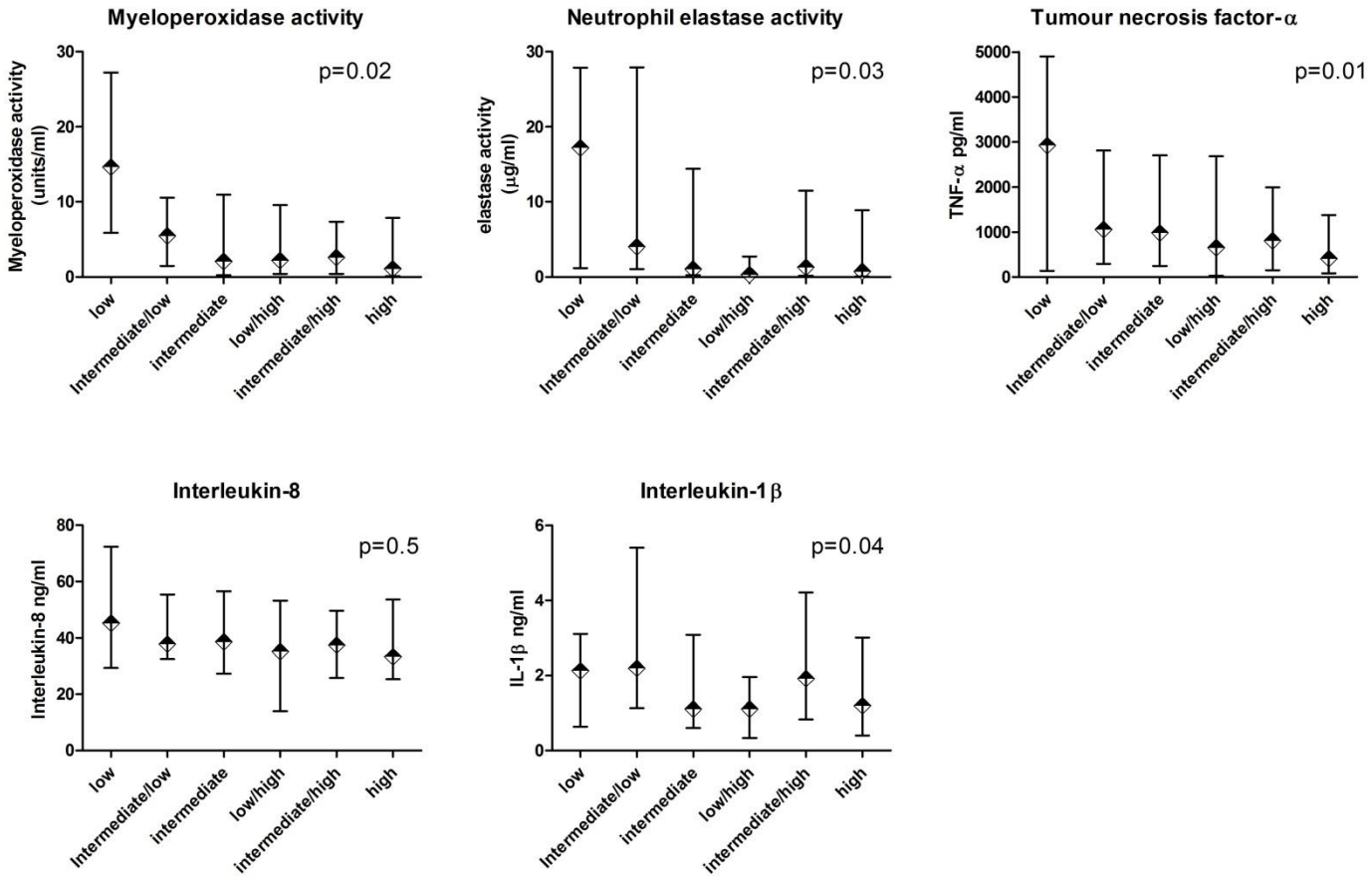

Figure 4.10. The relationship between FCN2 haplotype and inflammatory markers measured in sputum from patients with bronchiectasis. Comparisons refer to Kruskal-wallis test. Data are shown as median (diamonds) with interquartile range (bars).

As expected based on the results in chapter 3, airway inflammation was dependent on bacterial load and the higher levels of inflammatory markers present in patients with low expressing FCN2 haplotypes was associated with higher bacterial loads in sputum- figure 4.11.

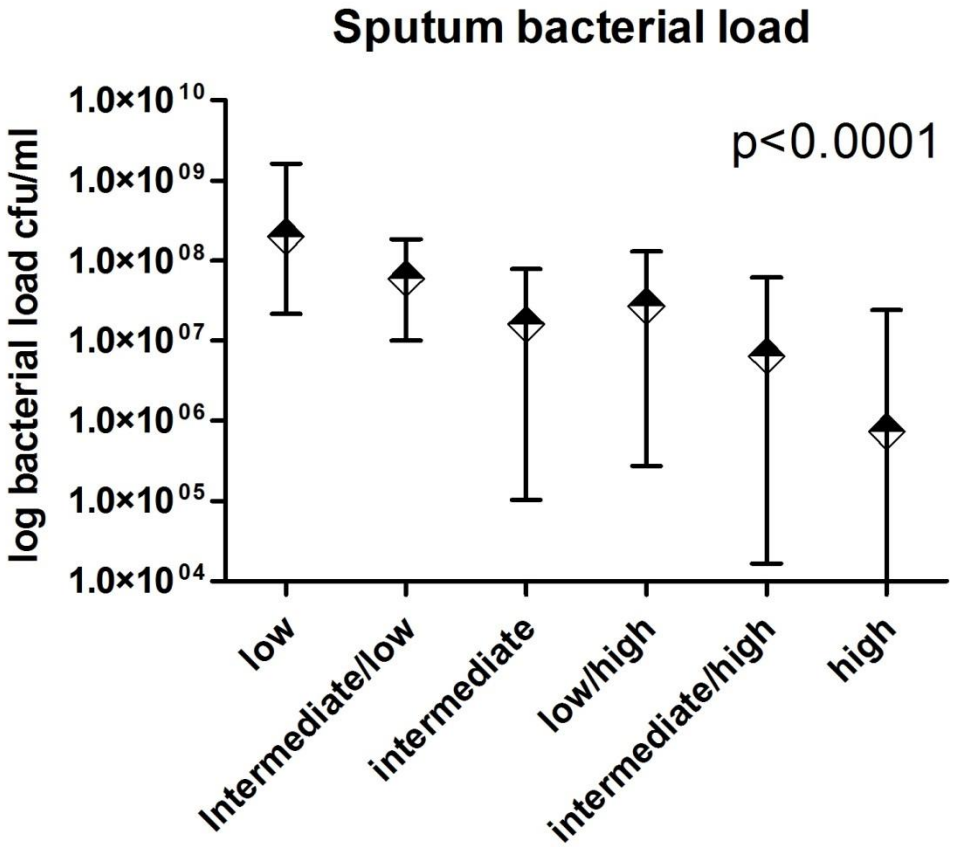

Figure 4.11. The relationship between FCN2 haplotype and sputum bacterial load in patients with bronchiectasis. Comparisons refer to Kruskal-wallis test. Data are shown as median (diamonds) with interquartile range (bars).

In multivariable analysis, adjusted for age, gender, smoking status, spirometry, radiological severity, BMI and inhaled corticosteroid use there was a significant relationship between *FCN2* haplotype status and chronic colonisation. Table 4.6 shows the results of the multivariable analyses. Survival was analysed by cox-proportional hazards regression while colonisation and *P. aeruginosa* colonisation was analysed by logistic regression. There was a significant relationship between the presence of low/intermediate haplotypes and chronic colonisation, and a strong relationship between these haplotypes, and a combination of intermediate expressing haplotypes with *P. aeruginosa* colonisation. None of the groups had a significantly higher mortality during follow-up.

Group	Colonisation	*Pseudomonas aeruginosa*	4 year mortality
High (reference)	1.0	1.0	1.0
Low	6.0 (0.2-25)	8.6 (0.8-92.3)	1.08 (0.11-10.1)
Low/intermediate	**4.4 (1.6-12)***	**11.3 (3.1-41.8)*****	1.44 (0.51-4.07)
Intermediate	1.8 (0.9-3.4)	**3.46 (1.04-11.5)#**	0.87 (0.35-2.17)
Low/high	1.0 (0.4-2.5)	2.38 (0.4-12.9)	1.07 (0.32-3.56)
Intermediate/high	1.4 (0.7-2.4)	2.56 (0.8-8.5)	0.48 (0.20-1.17)
Serum levels			
≥1600ng/ml (reference)	1.0	1.0	1.0
<1600ng/ml	**2.1 (1.2-3.9)****	**4.7 (2.4-9.2)*****	1.76 (0.90-3.45)

Table 4.6. Multivariate analysis of ficolin-2 haplotypes and serum levels with chronic colonisation, *P. aeruginosa* colonisation and survival. Statistically significant relationships are highlighted in bold: *p=0.004, **p=0.01, ***p<0.001, #p=0.04. All other comparisons p>0.05.

4.6 Regulation of Ficolin-2 expression in hepatocytes

In view of the large variation in serum concentrations of ficolin-2 in patients with bronchiectasis and controls within different haplotypes/genotypes, we hypothesised that ficolin-2 would be influenced by external factors. A leading hypothesis for the higher frequency of low ficolin-2 serum levels in bronchiectasis patients was that ficolin-2 may be consumed during the inflammatory response. It is known, for example, that complement components such as C3 ad C4 may be consumed in diseases involving immune complex deposition and widespread complement activation such as systemic lupus erythematosus (Birmingham et al, 2010). In addition, although many blood proteins are upregulated during acute inflammatory responses (including mannose binding lectin) some may be negatively regulated by the acute phase response, serum 25 hydryoxyvitamin-D being one example (Gama et al, 2012).

We therefore investigated whether hormones, pro-inflammatory mediators or the acute phase response may influence expression and secretion of ficolin-2.

In-vitro studes

Since ficolin-2 is exclusively expressed in hepatocytes, in-vitro studies to investigate possible hormonal factors affecting ficolin-2 expression and secretion were performed in hepatocyte cells lines. Two cell lines, HepG2 and HuH-7 (kind gifts from the laboratory of Professor John Iredale) were used. First, expression of ficolin-2 in these cell lines were confirmed by western blot (figure 4.12). Both cells lines secreted significant quantities of ficolin-2 into the media as shown. Western blotting

under reducing conditions is shown and found a band at 80kDa. The molecular weight of ficolin-2 is 35kDa. Subsequently this was found to be a dimer of ficolin-2 with MASP-2, which is also produced by the hepatocytes (Krarup et al, 2004).

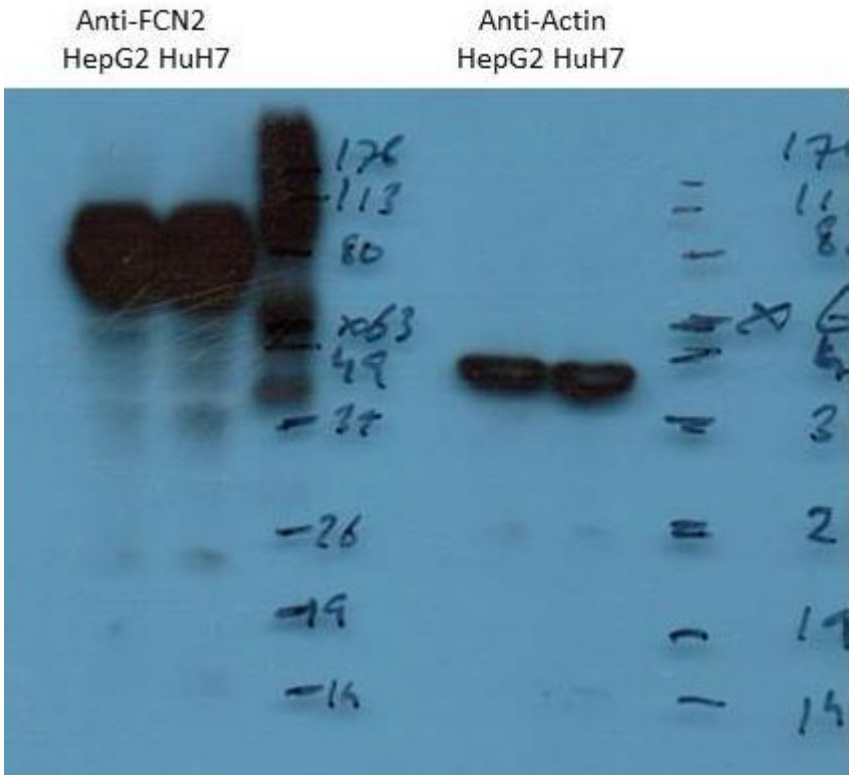

Figure 4.12. Detection of ficolin-2 secretion by HepG2 and HuH-7 hepatocyte cell lines. Actin loading controls are shown. The band was found to correspond to ficolin-2/MASP-2 dimers (approximately 80kDa).

We then proceeded to test possible modifiers of hepatocyte ficolin-2 expression by ELISA and real time PCR. HuH-7 cells were used for these experiments with the ELISA results verified by also performing in HepG2 cells. Possible modifiers were selected on the basis of previous data showing that mannose binding lectin expression in hepatocytes was increased by growth hormone, interleukin-6 and

thyroid hormones. mRNA expression was compared to expression for the housekeeping gene 18s. Cells were counted using a haemocytometer and ficolin-2 and MBL synthesis were measured using ELISA. Results are expressed as ng/10^6 cells. The results of the real time PCR are shown in figure 4.13 and figure 4.14. None of the agents testing caused a statistically significant increase in mRNA expression with the exception of high doses of lipopolysaccharide.

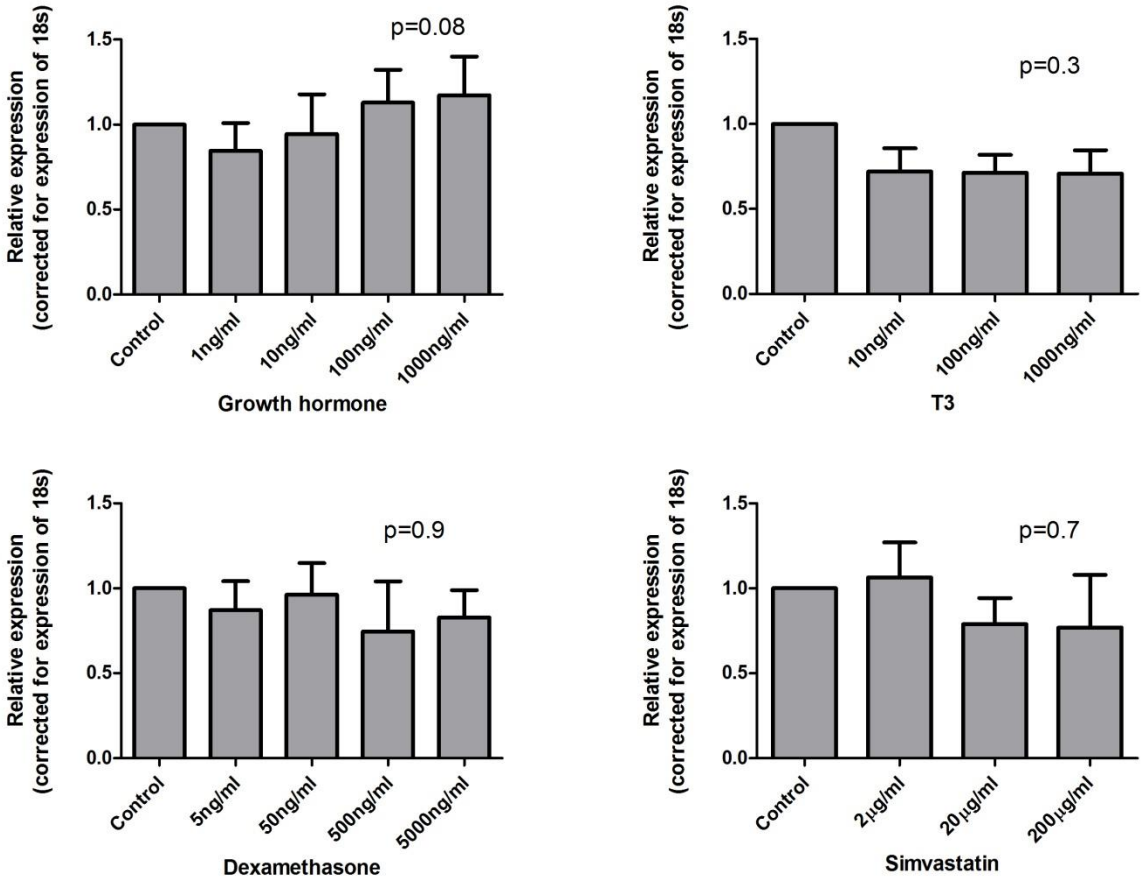

Figure 4.13. The effect of hormones and selected drugs on mRNA expression of FCN2. Data are presented as mean with standard error of the mean from 3 independent experiments.

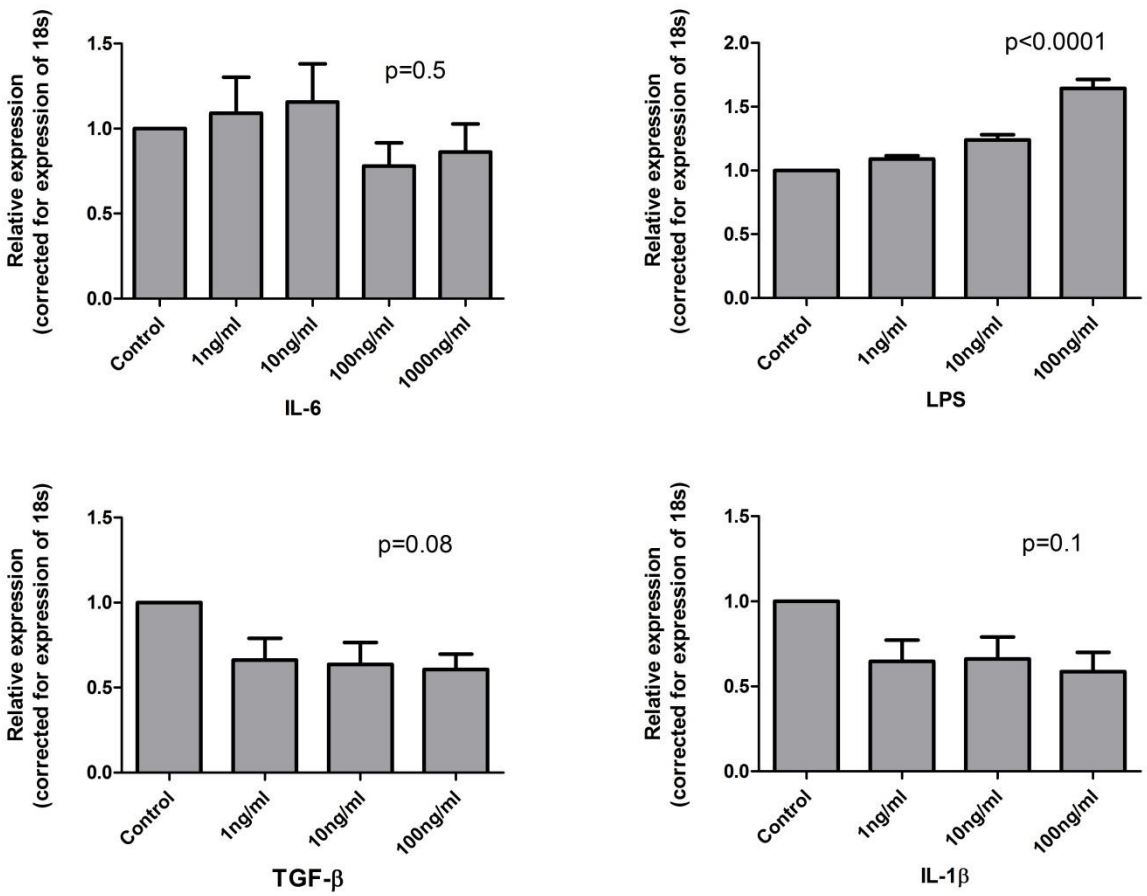

Figure 4.14. The effect of inflammatory mediators and lipopolysaccharide on mRNA expression of FCN2. Data are presented as mean with standard error of the mean from 3 independent experiments.

These results were further tested by measuring secreted protein in the culture media.

The results of this analysis are shown in figure 4.15 and figure 4.16. No significant differences were identified using this method, suggesting that hormones and pro-

inflammatory mediators did not have a large effect on FCN2 expression and secretion in hepatocytes.

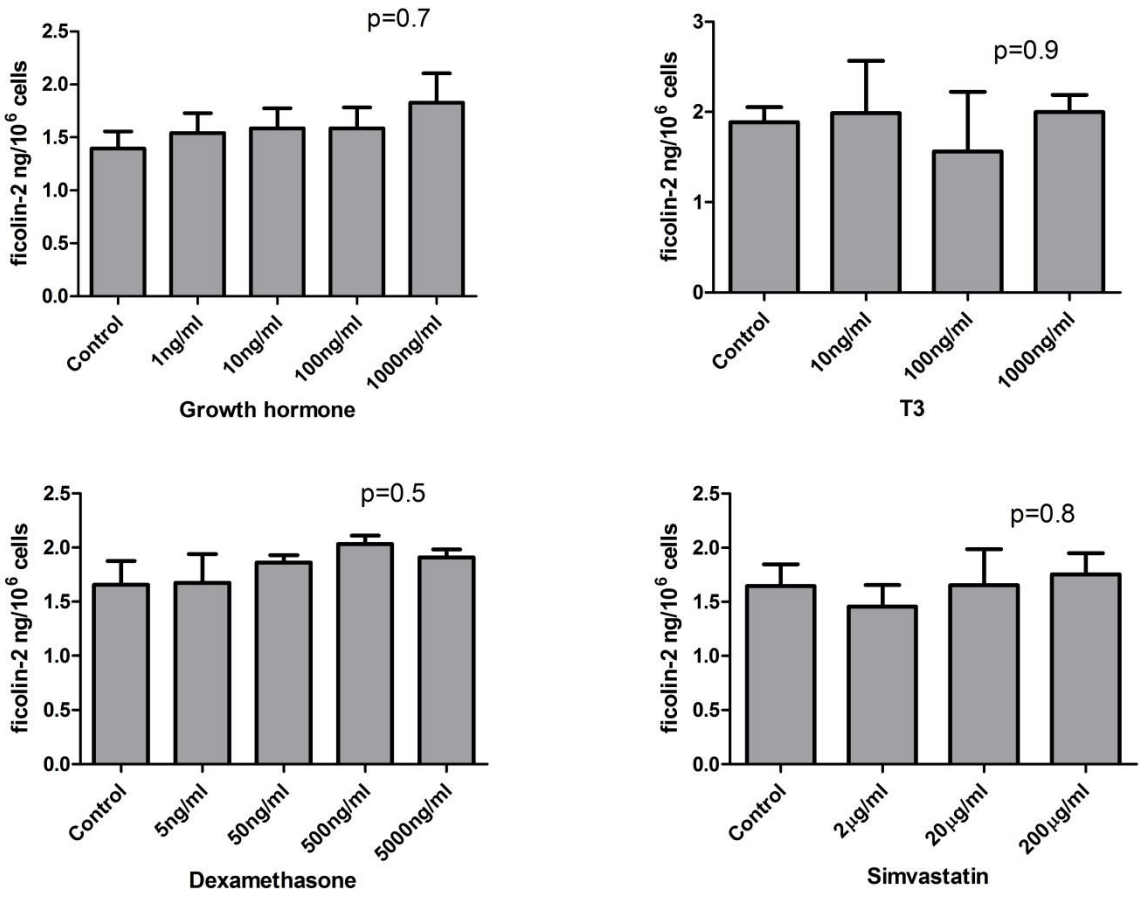

Figure 4.15. Ficolin-2 secretion by HuH-7 treated with hormones and selected medications. Data are presented as mean with standard error of the mean from 3 independent experiments.

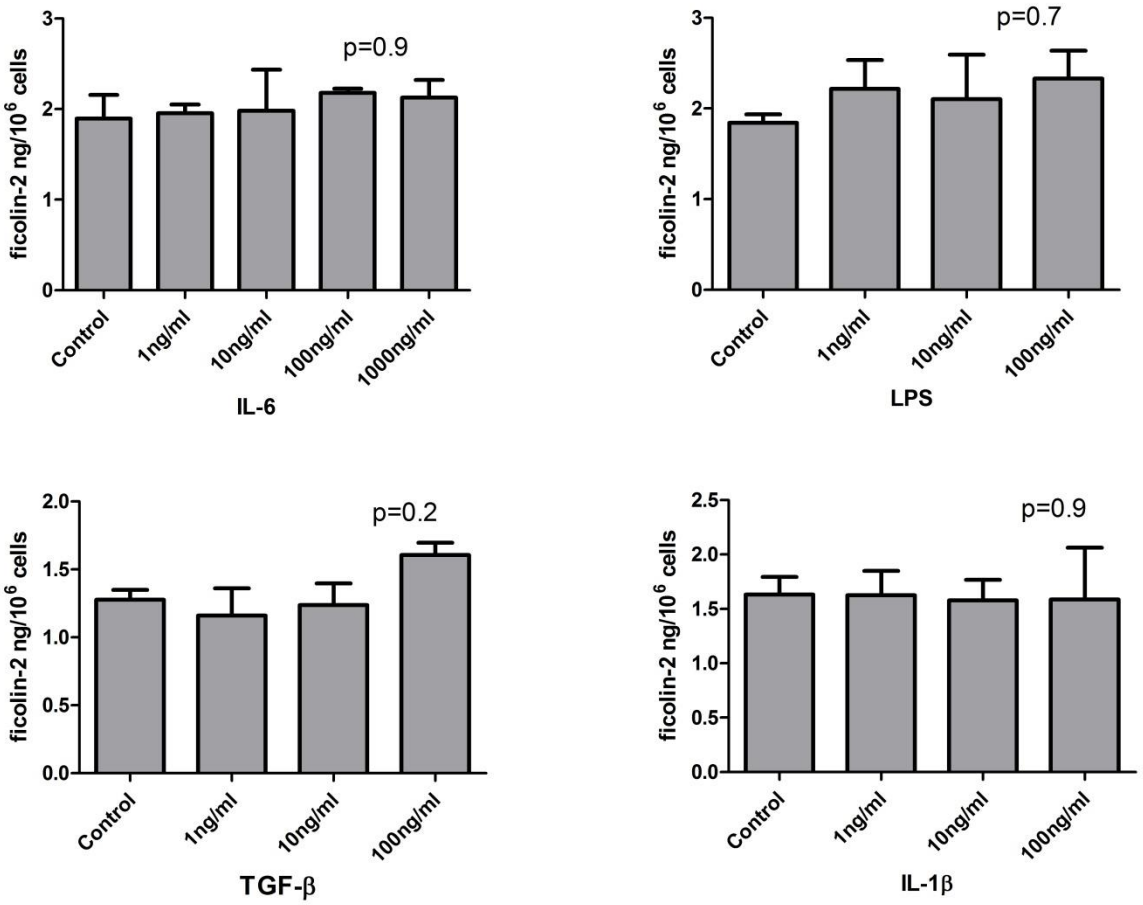

Figure 4.16. Ficolin-2 secretion by HuH-7 treated with pro-inflammatory mediators and lipopolysaccharide. Data are presented as mean with standard error of the mean from 3 independent experiments.

In-vivo studies of acute phase response

10 patients were recruited prior to cardiopulmonary bypass surgery, a procedure known to be associated with acute phase response and previously used in studies of acute phase responses (Eisen et al, 2007). Blood samples for serum measurement of ficolin-2 and mannose binding lectin were taken pre-operatively, pre-operatively and

then at 24, 72, 120 and 168 hours after cardiac surgery. Acute phase responses of Ficolin-2 and MBL were compared to classical acute phase reactant C-reactive protein measured by the biochemistry department at the Royal Infirmary of Edinburgh.

8 patients completed the study with blood samples taken at all 5 time-points and the data for those patients completing the study is shown in figure 4.17. All patients exhibited a typical post-operative acute phase response with significant elevation in C-reactive protein peaking at 72 hours and declining following this. Mannose binding lectin increased during the acute phase response (repeated measures ANOVA p=0.01) with a peak increase above baseline at 72 hours of 114%. No significant increase in serum levels of ficolin-2 was observed (p=0.9).

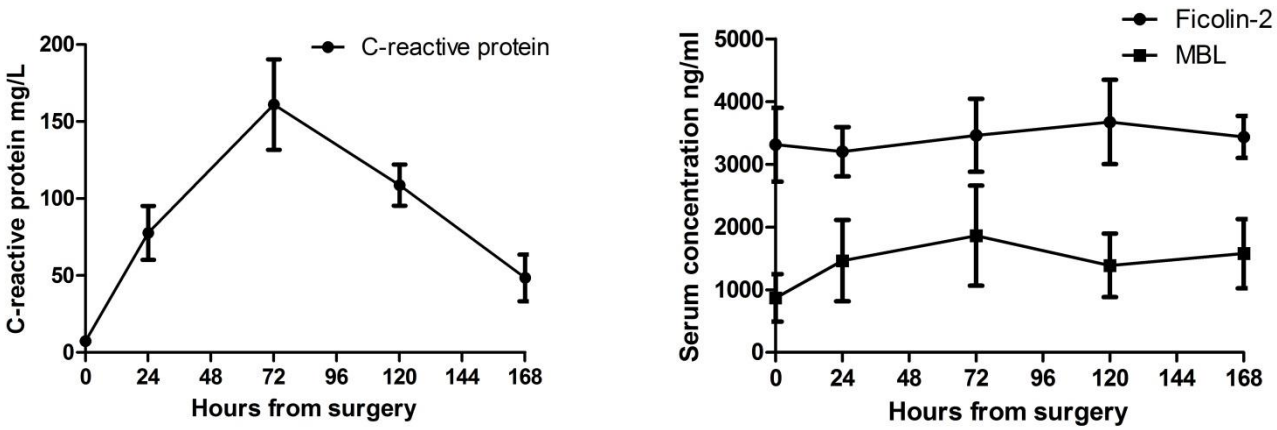

Figure 4.17. Acute phase response in 8 patients undergoing cardiac bypass surgery.

These data suggest that ficolin-2 is neither positively or negatively regulated during acute inflammation.

4.7 Ficolin-2 is present in sputum from bronchiectasis patients but not from healthy controls.

Sputum ficolin-2 was measured by ELISA in 72 patients with bronchiectasis using spontaneous sputum and 10 control subjects provided induced sputum. None of the control subjects had detectable levels of ficolin-2 in induced sputum.

Ficolin-2 was detectable by ELISA in 47 of the 80 bronchiectasis sputum samples. The median level in patients with detectable ficolin-2 was 383.1ng/ml (187.0-620.7ng/ml). The highest detectable level was 3.1ng/ml, equivalent to the levels found in serum.

In the 72 patients studied, there was a correlation between serum and sputum ficolin-2 levels, $p<0.0001$, figure 4.18. None of the patients with ficolin-2 levels <1600ng/ml had detectable sputum ficolin-2.

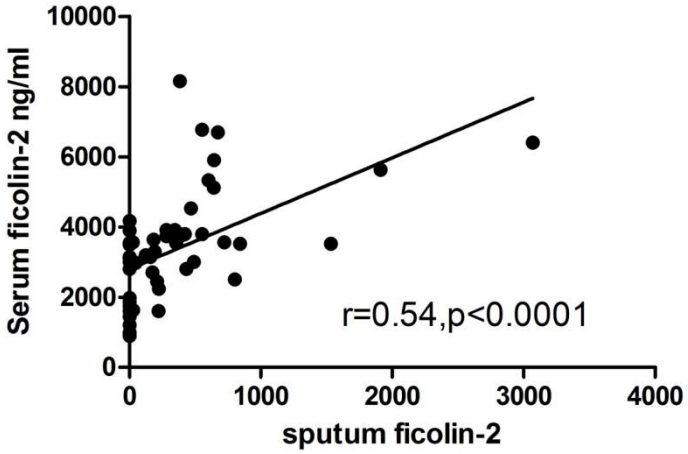

Figure 4.18. Correlation between sputum and serum measurement of ficolin-2.

These data would suggest that ficolin-2 is present in the bronchiectasis airway in concentrations high enough to cause complement activation and to enhance phagocytosis.

4.8 Discussion

Bronchiectasis is a chronic inflammatory lung disease characterised by chronic airway bacterial infection, exacerbations, hospitalisation and premature death in the most severe cases (Smith 2011, Chalmers et al 2011, Loebinger et al 2009). Despite significant advances in the understanding of chronic lung disease over past decades, the underlying cause of bronchiectasis in the majority of patients remains unknown (Pasteur et al 2000, Brown et al 2011). It is presumed that unrecognised immune defects are present in a proportion of patients with idiopathic bronchiectasis. The clinical course of the disease varies, with some patients having a relatively benign course with little impact on quality of life, and others having severe impairment of lung function and frequent exacerbations (Pasteur et al 2010, Chalmers et al 2011). Modifiers of this clinical course have not previously been identified in non-CF bronchiectasis, although it is well recognised in cystic fibrosis that genes other than CFTR modify outcome, including TGF-ß, SERPINA1 and mannose binding lectin (Collaco and Cutting 2008, Dorfman et al 2008, Chalmers et al 2011).

This study therefore sought to investigate whether the components of the lectin pathway of complement were implicated in susceptibility to bronchiectasis, with severity of bronchiectasis or susceptibility to *P. aeruginosa*. *P. aeruginosa* is of considerable interest in bronchiectasis because it is associated with worse lung function, worse impairment of quality of life and decreased survival in multiple studies .(Evans et al 1996, Wilson et al 1997, Loebinger et al 2009).

We report a significant association between low expressing FCN2 haplotypes and disease severity in bronchiectasis, and a strong associated with *P. aeruginosa*

colonisation. The findings of this study are supported by both in-vitro and in-vivo data in the literature. A study of 96 patients with cystic fibrosis found a strong and statistically significant effect of FCN2 polymorphisms (rs7865453 and rs7851696) and earlier onset of *P. aeruginosa* colonisation (Haerynck et al 2012). This polymorphisms (rs7865453- Ala258ser) results in low serum ficolin-2 concentrations and was also the variant most strongly associated with *P. aeruginosa* colonisation in the present study. Variants in the MBL2 and FCN1 genes were also significantly associated with earlier *P. aeruginosa* colonisation in the study by Haerynck et al suggesting a very important role for the lectin pathway in susceptibility to colonisation with *P. aeruginosa*. Susceptibility to *P. aeruginosa* in bronchiectasis and in cystic fibrosis remain poorly understood and our data are the first demonstration of genetic susceptibility to *P. aeruginosa* colonisation in non-CF bronchiectasis.

There are a number of recognised underlying causes for bronchiectasis, including severe infections such as pneumonia and tuberculosis, immunodeficiencies such as common variable immunodeficiency, allergic bronchopulmonary aspergillosis and connective tissue diseases (e.g rheumatoid arthritis) and inflammatory bowel disease (Pasteur et al, 2010). Circumstantial evidence links ficolin-2 to a number of these conditions. A study in MASP-2 knockout mice has identified an important role for the lectin pathway in defence against pneumococcal pneumonia, the most common cause of pneumonia worldwide. Ficolin-2 was found to be the major lectin pathway component responsible for defence against pneumococcal infection (Ali et al 2012). Most diseases that predispose to severe pneumonia have been associated with bronchiectasis as severe infections are reported as the most frequent cause of

bronchiectasis in adults (Pasteur et al, 2010). In addition, ficolin-2 binds to, and activates complement against *Mycobacteria* (Carroll et al 2009) and has been linked to recurrent respiratory tract infections in childhood (Cedzynski et al 2009). As discussed above, a role for ficolin-2 in defence against *Aspergillus fumigatus* has been demonstrated (Ma et al 2011). In the present study, the majority of patients had idiopathic bronchiectasis with smaller number of other causes. No interaction was found with underlying cause and either low ficolin-2 serum levels or *P. aeruginosa* colonisation. This is an area for future research in larger cohorts.

The method used to study Ficolin-2 "deficiency" has varied across the limited number of disease association studies to date (Chapman et al 2007, de Messias-Reason 2009, Faik et al 2011). Many use individual SNP's (Chapman et al 2007, Haernyck et al 2012, Meijvis 2011), while others have examined individual haplotypes (Chapman et al 2007, de Messias-Reason 2009, Faik et al 2011). We demonstrated that although on the population level individual SNP's have a significant effect on serum ficolin-2 levels, they are unreliable to predict individual patients serum ficolin-2. The strong linkage disequilibrium between FCN2 SNP's produce 6 common haplotypes (Hummelshoj et al 2007). Haplotypes also have strong effects on ficolin-2 levels,with patients carrying the GGACT having the lowest serum ficolin-2 levels. In this study we used a new approach combining low, intermediate and high expressing ficolin-2 haplotypes to produce haplotypes pairs that was significantly more discriminatory in identifying individuals with low serum ficolin-2. This is the same approach as is used in disease association studies for mannose binding lectin, where only individuals with 2 MBL deficient haplotypes have complete MBL deficiency (Kilpatrick 2003). This approach should be repeated

in independent cohorts to confirm if this should become the standard method of defining ficolin-2 “insufficiency”.

A leading hypothesis for why low serum ficolin-2 concentrations were more frequent in bronchiectasis patients was that chronic inflammation may cause consumption of complement components, or that ficolin-2 may be a negative acute phase reactant. Our in-vitro studies in hepatocytes and in-vivo studies in patients undergoing cardiac surgery suggest no significant acute phase response and no evidence that low ficolin-2 serum levels are due to consumption.

We demonstrate that ficolin-2 is presented in the infected bronchiectasis airway by measuring ficolin-2 in sputum. Induced sputum from healthy donors contained no ficolin-2, consistent with the hypothesis that ficolin-2 may diffuse into the airway under conditions of inflammation. This is already known to be the case for mannose binding lectin (Fidler et al, 2010). We found low levels of sputum ficolin-2 in patients with with low serum levels, again consistent with the hypothesis that under conditions of acute inflammation in patients with low ficolin-2 serum levels, ficolin-2 may fail to diffuse into the airway to enhance opsonophagocytosis.

This study is the largest genetic study of patients with non-CF bronchiectasis and the only study the authors of aware of to demonstrate a genetic modifier of disease severity. The largest previous study the authors could identify was a study of 96 patients with idiopathic bronchiectasis that identified an association between bronchiectasis and homozygous HLA-C group 1 (Boyton et al 2006). A key stage in any genetic study is the validation of a genetic association in an independent cohort. This is challenging in bronchiectasis as no large genetic studies have previously been

undertaken. Although our previous pilot data (Kilpatrick et al 2009), the data from cystic fibrosis (Chalmers et al 2011) and the in-vitro data (discussed in chapter 5) strongly support our findings, it is essential that future genetic studies of bronchiectasis should validate the role of ficolin-2. As large international collaborations begin to develop for this disease, it is hoped that more genetic studies will be conducted in bronchiectasis (Aksamit et al 2012).

Conclusions

Single nucleotide polymorphisms associated with low ficolin-2 serum levels are associated with disease severity and susceptibility to *P. aeruginosa* infection in bronchiectasis. Ficolin-2 should be further evaluated as an important disease modifier and therapeutic target in bronchiectasis.

CHAPTER 5

The role of Ficolin-2 in opsonophagocytosis of *Pseudomonas aeruginosa* in bronchiectasis

5.1 INTRODUCTION

In the previous chapter, we have demonstrated a significant association between single nucleotide polymorphisms in the ficolin-2 gene and susceptibility to chronic bacterial colonisation, particularly with *P. aeruginosa*. Up to 80% of patients with bronchiectasis grow pathogenic micro-organisms when clinically stable, suggesting that bronchiectasis is associated with a failure to remove bacteria from the airways by phagocytosis.

Clearance of inhaled particles, including microbes but also allergens, irritants and environmental particulates is a key function of the innate immune system in the lung. This is achieved through co-ordinated processes involving the mucociliary escalator, secretion of antimicrobial peptides and mucins by epithelial cells, and leucocytes both present in the airway, such as the alveolar macrophage, and recruited to the airway during inflammatory responses. (Hiemstra, 2006).

While macrophages are the dominant phagocyte in the healthy lung (Martin and Frevert, 2005), neutrophils are the dominant cell type identified in the airways of patients with bronchiectasis (Fuschillo et al, 2008) as well as other chronic lung diseases such as cystic fibrosis and chronic obstructive pulmonary disease. The primary function of neutrophils is to phagocytose and kill bacterial pathogens. Phagocytosis is dependent on two classes of neutrophil receptors, the Fcγ receptors (FcγRIIA (CD32) and FcγRIIIb (CD16) and the complement receptors CR1 (CD35)

and CD3 (CD11b/CD18 integrin). After engagement of phagocytic targets, primarily with FcγRIIIb and CD11b, pseudopods encircle and engulf bacteria, followed by fusion of phagocytic granules. Bacterial killing occurs through proteases, anti-microbial peptides and reactive oxygen species. It has also been reported that neutrophils are able to kill some pathogens extracellularly through neutrophil extracellular traps (Brinkmann et al, 2004). These NETS are principally composed of DNA with granule products such as elastase and myeloperoxidase. Their role in chronic lung disease is a subject of ongoing research.

Successful neutrophil phagocytosis requires opsonisation of pathogens with soluble particles that permit recognition by specific receptors. Complement is the primary system for opsonising pathogens (reviewed in Chapter 1) and complement activation results in deposition of C3b (recognised by complement receptor 1/CD35 on phagocytes) or C3bi (recognised by complement receptor 3/CD11b). Pathogens may be opsonised directly with immunoglobulin (recognised by FcγRIII (CD16) (Greenberrg and Grinstein, 2002). Without this mechanism for enhancing pathogen recognition by phagocytes, the process of pathogen clearance is significantly impaired. Nevertheless, non-opsonic phagocytosis does occur. The mechanisms of non-opsonic phagocytosis in neutrophils are poorly described compared to macrophages where a large number of scavenger receptors involved in non-opsonic clearance have been described (among them mannose receptor, CD136, CD163 and CD36) (Donnelly and Barnes, 2012).

Other receptors are involved in phagocytosis without being direct phagocytic receptors. For example, complement activation generates complement component C5a, which interacts with its receptor CD88 expressed on macrophages, epithelial

cells and particularly neutrophils. Among its effects, CD88 engagement primes neutrophils to enhance functional responses such as phagocytosis and the generation and release of granule enzymes and superoxide anions which are critical to bacterial killing (Mollnes 2002, Hopken et al 1996). C5a/CD88 interaction is reported to shows a paradoxical effect, enhancing phagocytic and killing functions at low concentrations but caused impaired leukocyte function at higher concentrations as occur in sepsis (Ward 2004).

The persistence of bacteria within the airway suggests a possible role for phagocytic failure in the bronchiectasis lung. When investigated the majority of patients with bronchiectasis do not have defects in the classical or alternative complement pathways by conventional screening.

Neutrophil elastase has emerged as an important mediator of phagocytic dysfunction in cystic fibrosis, where it has been shown to cleaves FcγRIIIb and complement receptor 1 in vivo (Berger et al, 1989). Elastase also cleaves iC3b from the surface of pathogens, leading to an important opsonin/receptor mismatch (Tosi et al, 1990).The role of elastase in non-CF bronchiectasis has not been extensively investigated. Voglis et al, showed phagocytic defects associated with human neutrophil peptides in non-CF bronchiectasis (Voglis et al, 2009).

Since ficolin-2 is involved in complement activation via the lectin pathway, it is reasonable to hypothesise that low expression of ficolin-2 due to single nucleotide polymorphisms may lead to phagocytic failure due to reduce opsonisation.

In the clinical studies described in chapters 3 and 4, we have shown first, high levels of neutrophil elastase in the airway of patients with non-CF bronchiectasis and

bacterial colonisation, and secondly an association between low expression of ficolin-2 and bacterial colonisation and disease severity in bronchiectasis. We now investigate the in-vitro relevance of these findings to opsonophagocytosis and neutrophil function.

Given the strikingly higher frequency of *P. aeruginosa* in patients with low expressing FCN2 haplotypes demonstrated in the preceding chapter, we focus on the in-vitro activity of ficolin-2 against *P. aeruginosa*.

5.2 Recombinant ficolin-2 and mannose binding lectin

Recombinant ficolin-2 was expressed in both a bacterial and mammalian system as described in chapter 2, materials and methods. The functional ability of these ficolins to bind to ligands and activate complement via MASP-2 was assessed as described (Lynch et al, 2004) using microplates coated with lipoteichoic acid from *S. aureus*, a known ligand of ficolin-2.

As shown in figure 5.1, bacterial ficolin-2 was non-functional and did not bind significantly to lipoteichoic acid.

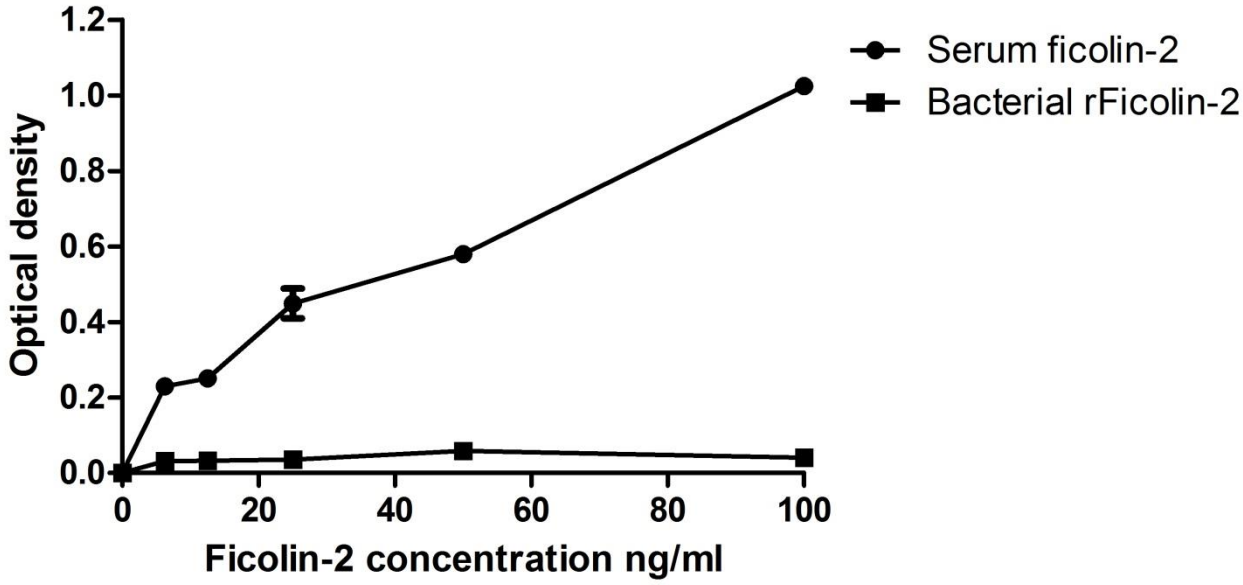

Figure 5.1. Binding of ficolin-2 in serum and ficolin-2 expressed in *E.coli* to lipoteichoic acid from *S. aureus*. Approximately 100ng/ml of recombinant protein and a pool of serum containing 2000ng/ml ficolin-2 measured by ELISA was diluted 1 in 20 and applied to the binding assay.

Subsequent discussions indicated that other researchers had established that ficolins undergo extensive post-translational modifications and that attempts to express functional ficolin in both bacteria and insect cells had been unsuccessful (Dr Nicole Thielens, personal communication). Ficolin-2 was therefore expressed in a stable CHO cell line provided by Dr Nicole Thielens. Ficolin-2 expressed by this method bound to lipoteichoic acid, as did the positive control in both experiments (ficolin-2 in serum samples from healthy donors), figure 5.2.

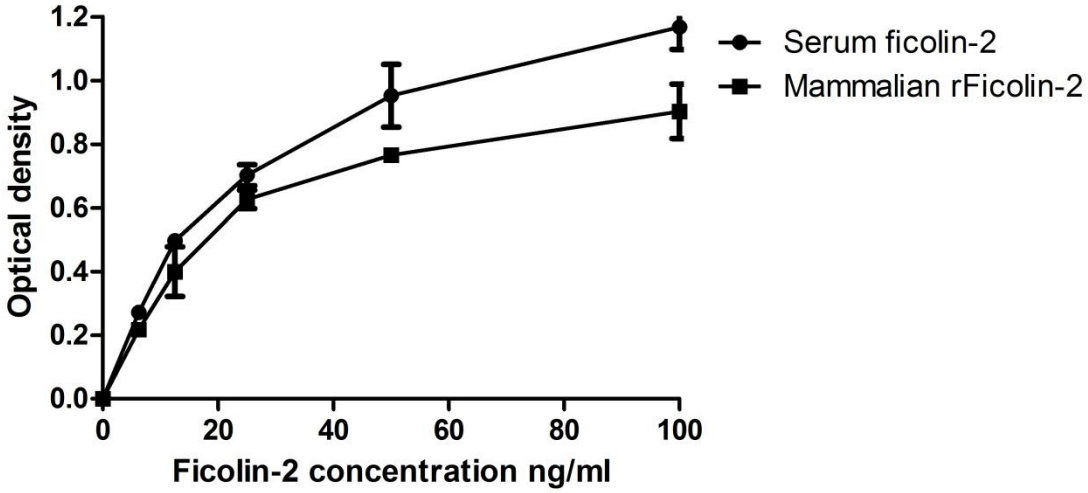

Figure 5.2. Binding of ficolin-2 to lipoteichoic acid from *S. aureus.* 100ng/ml of recombinant ficolin-2 expressed in CHO cells and diluted in TBS was applied to the binding assay and serially diluted. Binding of this recombinant ficolin-2 was compared to a serum pool containing 2000ng/ml ficolin-2 and diluted 1 in 20 in TBS.

Mannose binding lectin was purchased from R+D systems and functionally tested using binding to mannan by ELISA. Recombinant MBL was found to be functional and like rFicolin-2, was able to activate complement. (figure 5.3).

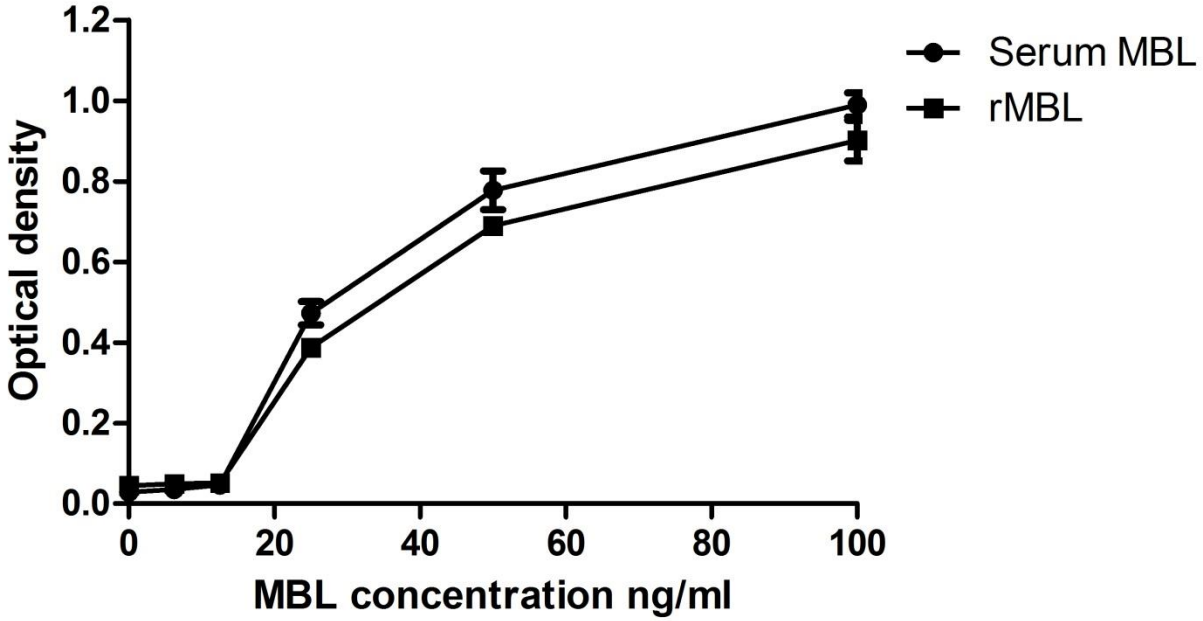

Figure 5.3. Binding of recombinant mannose binding lectin to mannan in a functional ELISA.

5.3 Ficolin-2 binding to *Pseudomonas aeruginosa* and other respiratory pathogens

Figure 5.4 shows the relative binding of the lectin pathway components Ficolin-2, mannose binding lectin and Ficolin-3 to respiratory pathogens. Binding is expressed as a percentage of the binding observed compared to a positive control (Acetylated BSA for Ficolin-2 and ficolin-3 and Mannan for MBL). Ficolin-2 bound strongly to *P. aeruginosa* PA01, *Haemophilus influenzae*, and *S. pneumoniae* serotype 3. Lesser

binding was observed to *M. catarrhalis, S. aureus and E. Coli.* No binding of ficolin-2 to *B. cepacia* or mannan was observed.

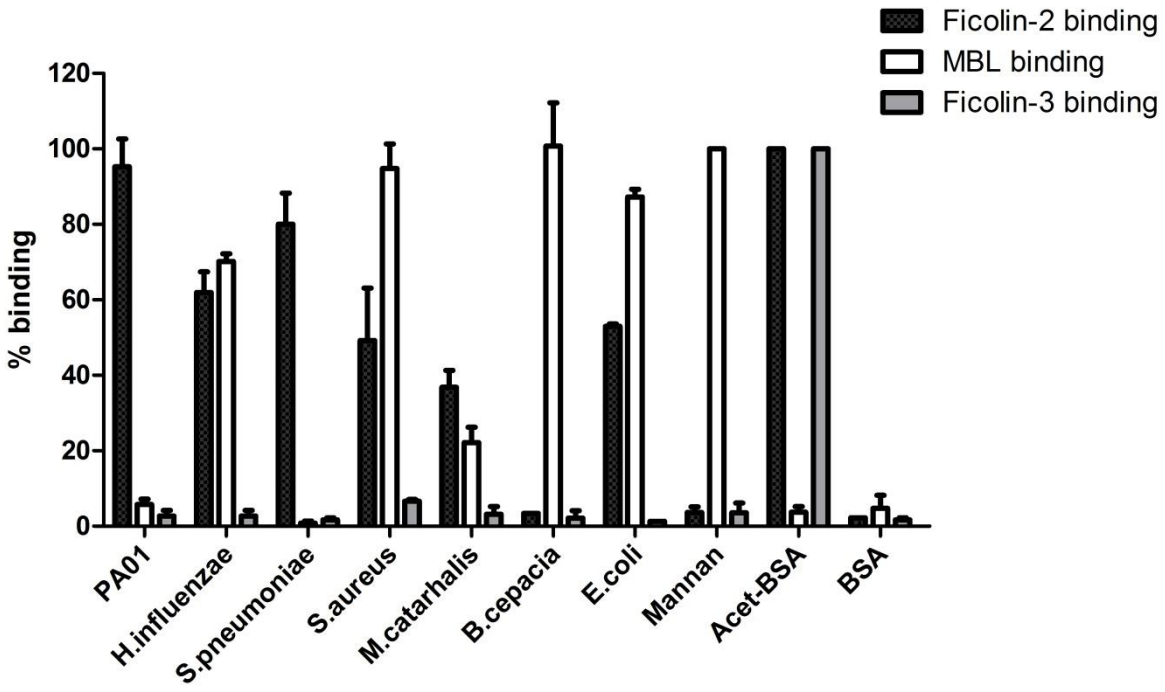

Figure 5.4. Binding of lectin pathway components to respiratory pathogens. Binding is expressed as a percentage of the positive control control (acetylated BSA for ficolin-2 and mannan for MBL). Binding assays represent the mean with standard error (SE) of 3 independent experiments performed in duplicate.

Ficolin-2 binding to *P. aeruginosa* was dose dependent and partially inhibited by N-acetylglucosamine and EDTA consistent with binding via for fibrinogen-like domain (figure 5.5, $p<0.0001$ by ANOVA). Mannose binding lectin by contrast did not bind to PA01 but did bind to *B. cepacia* and *S. aureus* (figure 3.12). No binding of ficolin-3 to any of the pathogens investigated was found. *P. aeruginosa* binding assays were

performed by by microplate and flow cytometry based methods to confirm that ficolin-2 bound specifically to PA01. Dose dependent binding of ficolin-2 to *P. aeruginosa* was observed by flow cytometry (figure 5.5). In each case, where recombinant ficolin-2 was added, mean fluorescence was significantly increased relative to control fluorescence ($p<0.05$ by students t test).

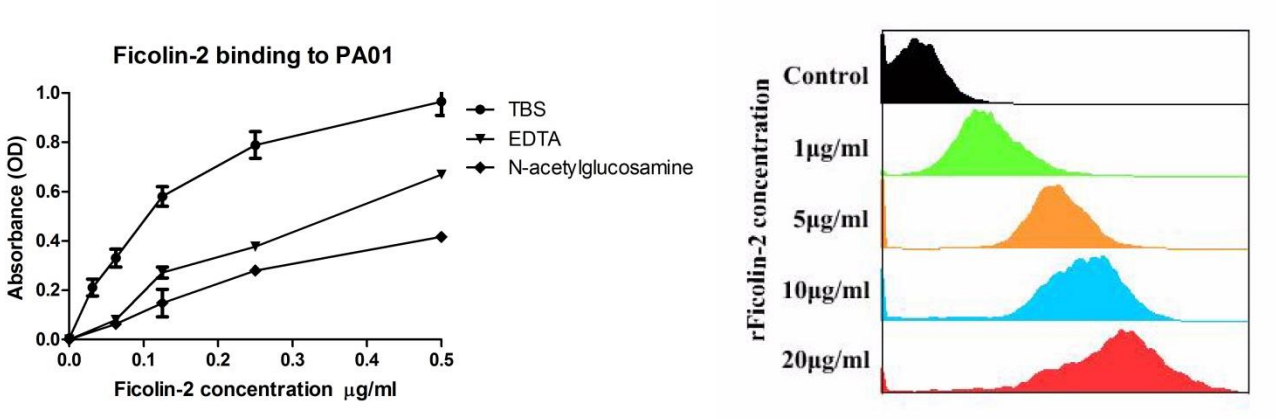

Figure 5.5. Binding of ficolin-2 to *P. aeruginosa* using both microplate and flow cytometry based binding assays. For the left graph, results are mean with standard error of the mean of 3 independent experiments.

Binding was subsequently investigated in a range of environmental and clinical strains of *P. aeruginosa* (figure 5.6). No significant difference in % binding of ficolin-2 was observed between environmental and clinical strains ($p=0.5$) and ficolin-2 bound to all of the strains investigated, while minimal binding of MBL was observed, figure 5.6.

These results suggest that ficolin-2 binding to *P. aeruginosa* is conserved across multiple strains and that adaptations to chronic infection by clinical *P. aeruginosa* isolates from patients with cystic fibrosis do not include evading binding by ficolin-2.

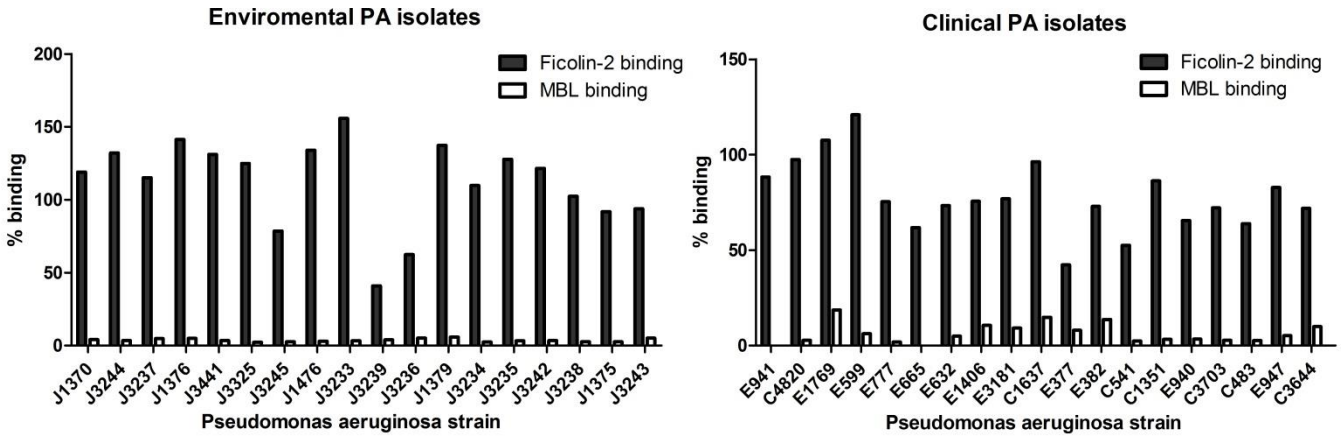

Figure 5.6. Binding of *P. aeruginosa* environmental and clinical isolates. Binding is expressed as a percentarge of a positive control (Acetylated BSA for ficolin-2 and Mannan for mannose binding lectin).

Binding of Ficolin-2 was associated with a dose dependent increase in complement C4 activation on PA01 using the Petersen assay with a strong correlation between ficolin-2 serum concentration and C4 deposition in this lectin pathway complement assay ($r=0.73$, $p<0.0001$) while MASP-2 serum concentration did not correlate with C4 deposition ($r=0.13, p=0.5$)- figure 5.7.

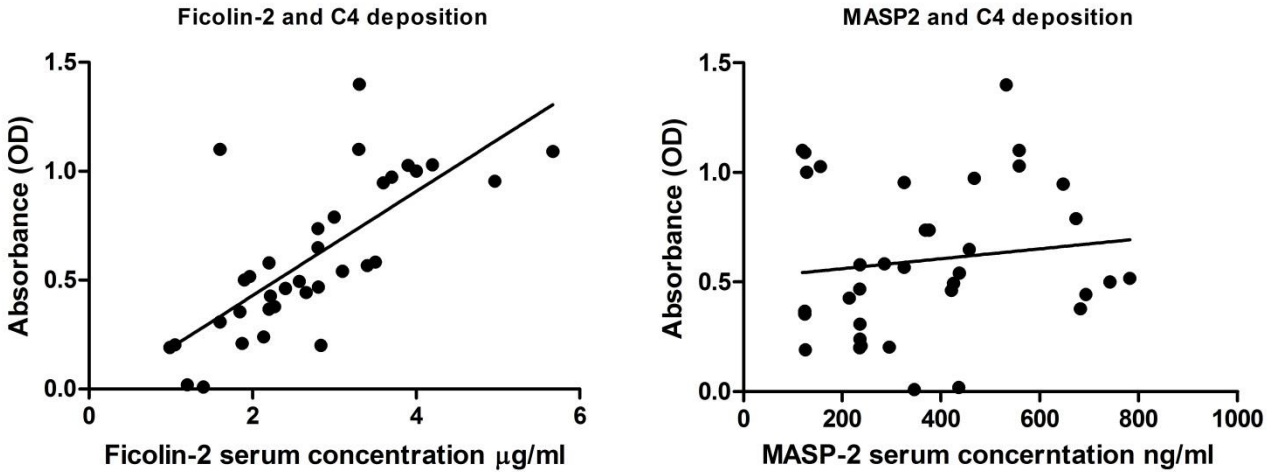

Figure 5.7. Complement activation using a lectin pathway C4 deposition assay (Pedersen et al, 2004). Increasing concentrations of ficolin-2 resulted in a significant increase in C4 deposition on PA01.

Confirmation of the binding results was obtained by depletion assay showing the same spectrum of microbial specificity, figure 5.8. This demonstrated binding of MBL to *S. aureus*, *B. cepacia* and *E. coli* with no significant binding to the other pathogens identified. No binding of ficolin-3 to any of the pathogens was observed, but binding to N-acetylglucosamine-sepharose was demonstrated. The spectrum of ficolin-2 binding observed was similar to the direct microplate assay and included specific binding to PA01, *H. influenzae*, *S. aureus* and *S. pneumoniae*.

Note that in these assays, positive binding is indicated by a low optical density, as the lectin pathway components are depleted from samples by binding, following which the remaining MBL, ficolin-2 or ficolin-3 were quantified by ELISA.

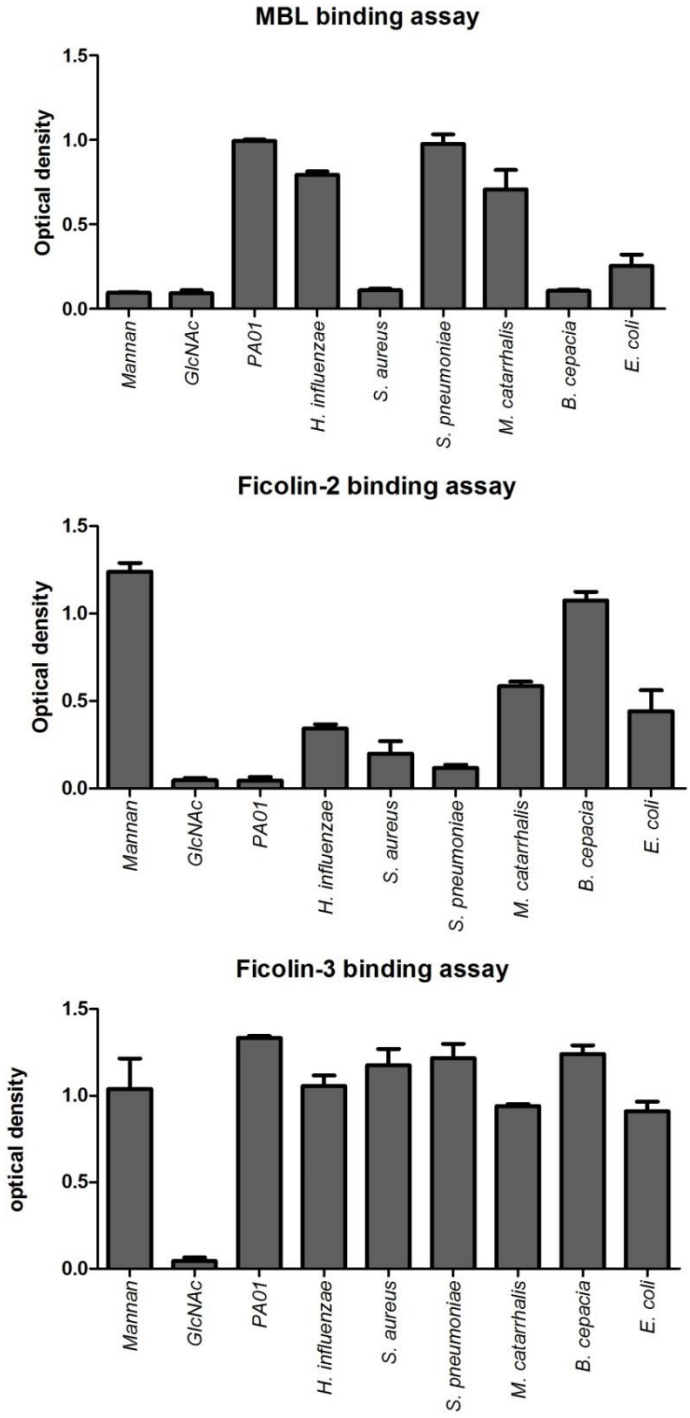

Figure 5.8. Depletion binding assay. Values are expressed as relative binding compared to the negative control (1.0). Values <0.25 were regarded as positive binding. Binding assays represent the mean (SE) of 3 independent experiments performed in duplicate.

Complement activation using C3 activation on microtitre plates confirmed that the alternative pathway was the dominant pathway for complement activation at high serum dilutions (20%) with only a small contribution from the lectin pathway (figure 3.17). For these experiments, microtitre plates were coated overnight with 1 x 10^8 cfu/ml of PA01 in coating buffer. Plates were washed 3 times in 10mM Tris-HCl, 140mM NaCl, pH 7.4. The indicated concentration of pooled human serum was diluted in 10mM Tris-HCl, 140mM NaCl, pH 7.4 containing 10mM EGTA and 10mM Mg^{2+} (for selective study of the alternative pathway), 100mM GlcNac (depletion of the lectin pathway of complement by inhibiting the binding of Ficolin-1, Ficolin-2, ficolin-3 and mannose binding lectin). 10mM EDTA was used as a negative control as this is known to inhibit all 3 pathways of complement activation. 100mM mannose was used to selectively inhibit binding of mannose binding lectin without affecting binding of the other lectin pathway components. Experiments were performed using 20% serum, 0.5% serum and 20% serum diluted in sodium polyanethole sulfonate at a concentration of 0.6mg/ml which selectively inhibits the classical and alternative pathways (see chapter 2).

Serum samples diluted in the appropriate conditions were incubated for 1 hour at 37°C followed by washing 3 times in wash buffer. Complement activation was detected by incubation with mouse anti-complement C3 (HAV 003-05, Bioporto) followed by anti-mouse HRP detection antibody. Complete inhibition of alternative pathway activity was defined as an optical density of C3 activation equivalent to the negative control (C3 depleted serum).

20% serum dilution was used given the results of neutrophil phagocytosis assays displayed in chapter 2 which showed effective opsonisation of PA01 for phagocytosis at this serum concentration. Depletion of the lectin pathway with N-acetylglucosamine resulted in only an 11.5% reduction in C3 deposition. Selective depletion of ficolin-2 with anti-ficolin-2 antibody produced a similar reduction (mean 11%) consistent with ficolin-2 being the major lectin pathway component responsible for complement activation on PA01 (figure 5.9). C1q depletion has little effect under any of the conditions testing indicating that in this serum pool and under these experimental conditions, the classical pathway was not a major contributor to C3 activation on PA01.

Under conditions of low serum dilution the alternative pathway was less active, with lower values obtained in the presence of EGTA-Mg which selectively inhibits the classical and lectin pathways allowing the contribution of the alternative pathway to be determined. Under these conditions lectin pathway and ficolin-2 depletion had a more marked effect. Figure 5.9 illustrates these results at 0.5% serum dilution, the lowest serum dilution at which C3 deposition could be detected in this assay). Similarly, in the presence of 0.6mg/ml SPS, the alternative pathway was inhibited and the lectin pathway was responsible for majority of complement activation. Values are expressed as a percentage of the positive control (a pool of serum from healthy donors). It should be noted that the overall C3 deposition was significantly lower at low serum dilutions and in the presence of SPS compared to 20% serum.

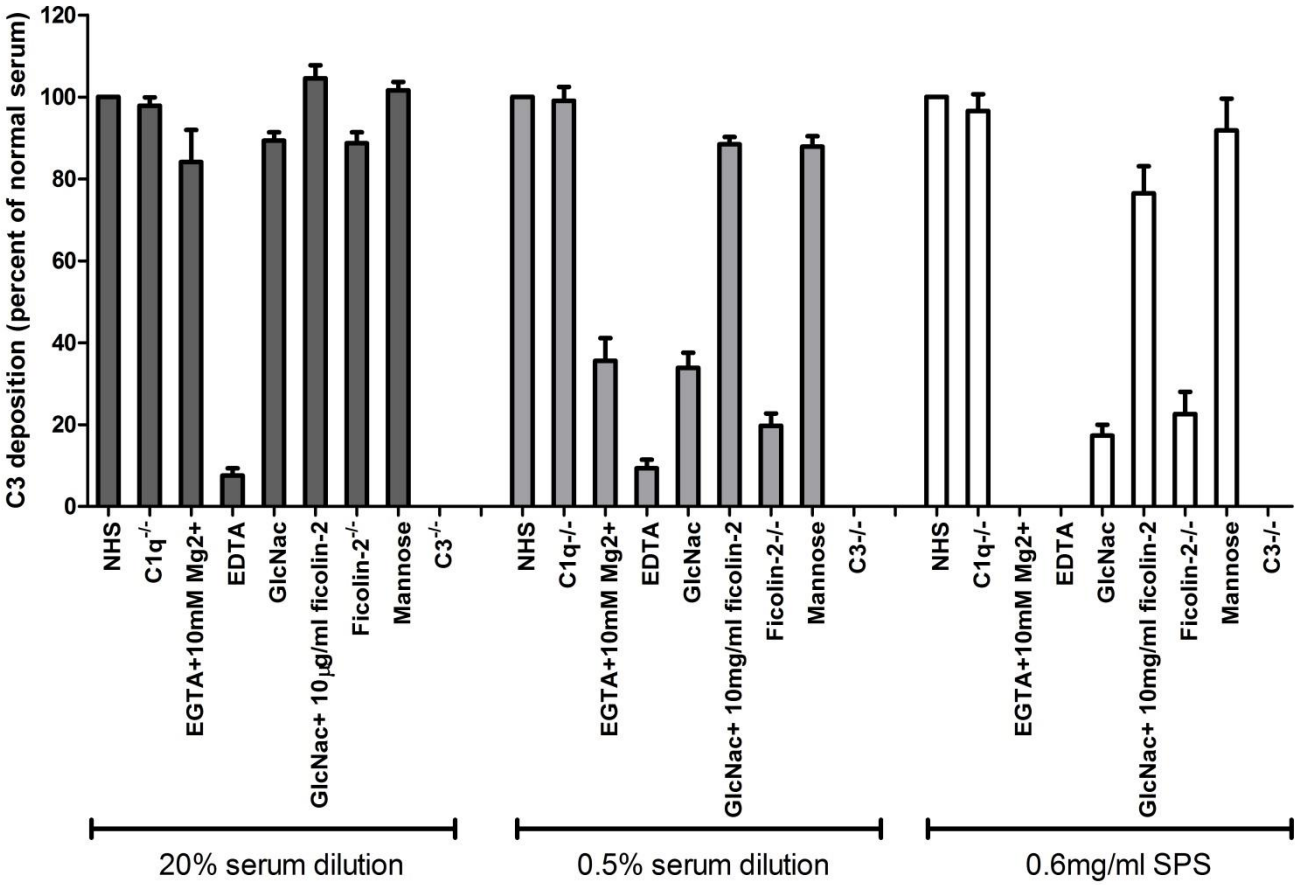

Figure 5.9. Determining the impact of the individual complement pathways contributing to C3 deposition on PA01. Data represent the mean of 3 independent experiments performed in duplicate.

5.4 Ficolin-2 enhances phagocytosis of *Pseudomonas aeruginosa* strain PA01

PA01 was opsonised in the presence of low serum concentrations of C1q^{-ve}, MASP 2^{-ve} or no serum at either low serum dilution (0.5% serum for 1 hour at 37°C) or in the presence of 0.6mg/ml SPS which was found to selectively inhibit the alternative pathway without affecting the lectin pathway for 1 hour at 37°C. Sera were depleted of all lectin pathway components by affinity chromatography as described in the materials and methods and then supplemented with increasing concentrations of Ficolin-2 or mannose binding lectin. As show in figure 5.10, increasing concentrations of ficolin-2 caused an increase in neutrophil phagocytosis of PA01, as measured by both mean fluorescence and % of positive cells. Data are displayed for experiments run using opsonisation with 1 hour at 37ºC with 0.5% serum with phagocytosis permitted for 30 minutes.

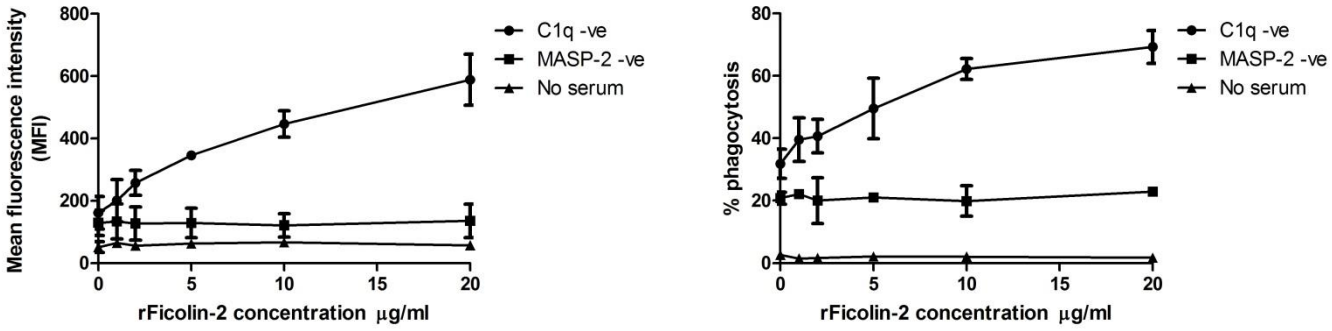

Figure 5.10. Enhancement of phagocytosis of *P. aeruginosa* strain PA01 with recombinant ficolin-2 supplementation to serum. Results are the mean of 5 independent experiments and are presented as mean with standard error of the mean.

($p<0.0001$ compared C1q-ve serum with both MASP-2 deficient and no serum by two way ANOVA).

Statistically significant differences were observed for phagocytosis (compared to baseline with no supplemental rficolin-2) at 5µg/ml and higher ($p<0.05$). MASP-2 depleted serum was associated with higher rates of phagocytosis than no serum, consistent with the presence of opsonins other than complement present in serum ($p<0.0001$). No enhancement effect was seen adding ficolin-2 to MASP-2 deficient serum suggesting ficolin enhancement of phagocytosis is dependent on complement activation. Supraphysiological doses of ficolin-2 (10 and 20µg/ml) were used in these experiments to demonstrate if there was a dose dependent increase in complement activation with higher concentrations of ficolin-2, and also because it was not known if recombinant ficolin-2 was as efficient at activating complement as native ficolin-2.

No effect was seen by supplementing sera with recombinant mannose binding lectin at any dose. (figure 5.11).

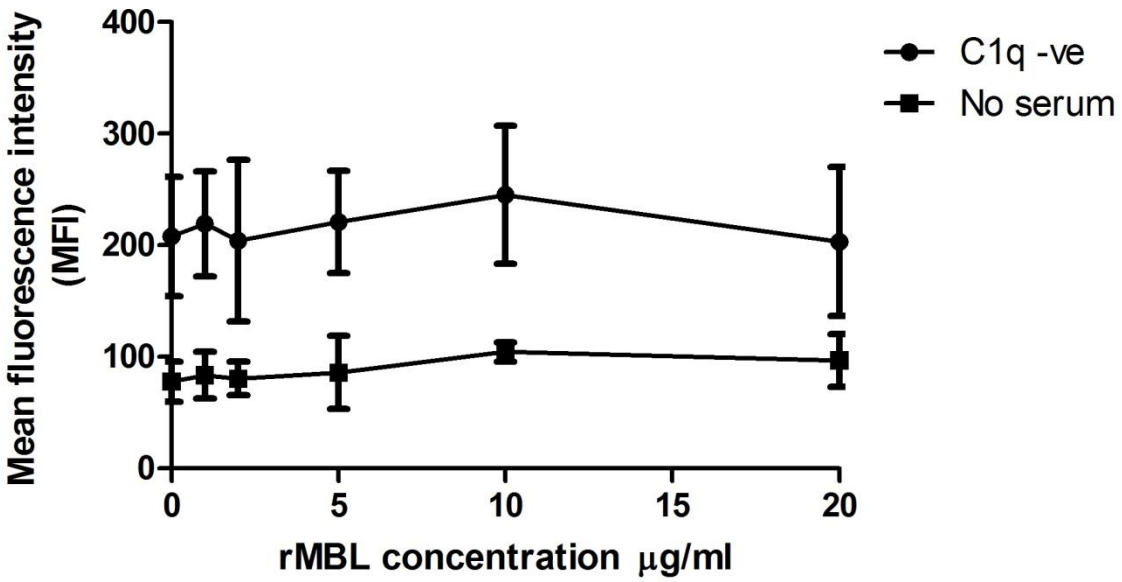

Figure 5.11. Supplementation of serum with recombinant mannose binding lectin at increasing concentrations does not enhance phagocytosis of *P. aeruginosa* stain PA01. Experiments with MASP-2 deficient sera were not performed to save reagents as no effect was observed with supplementing C1q-ve sera, no effect would be expected in the absence of MASP-2. Data are the mean of 3 independent experiments with standard error of the mean.

Microscopy confirmed that the observed results were due to phagocytosis with large numbers of FITC-labelled PA01 visible within neutrophils, figure 5.12. Images were obtained by simultaneous phase contrast and fluorescence microscopy after adhering neutrophils to glass slides.

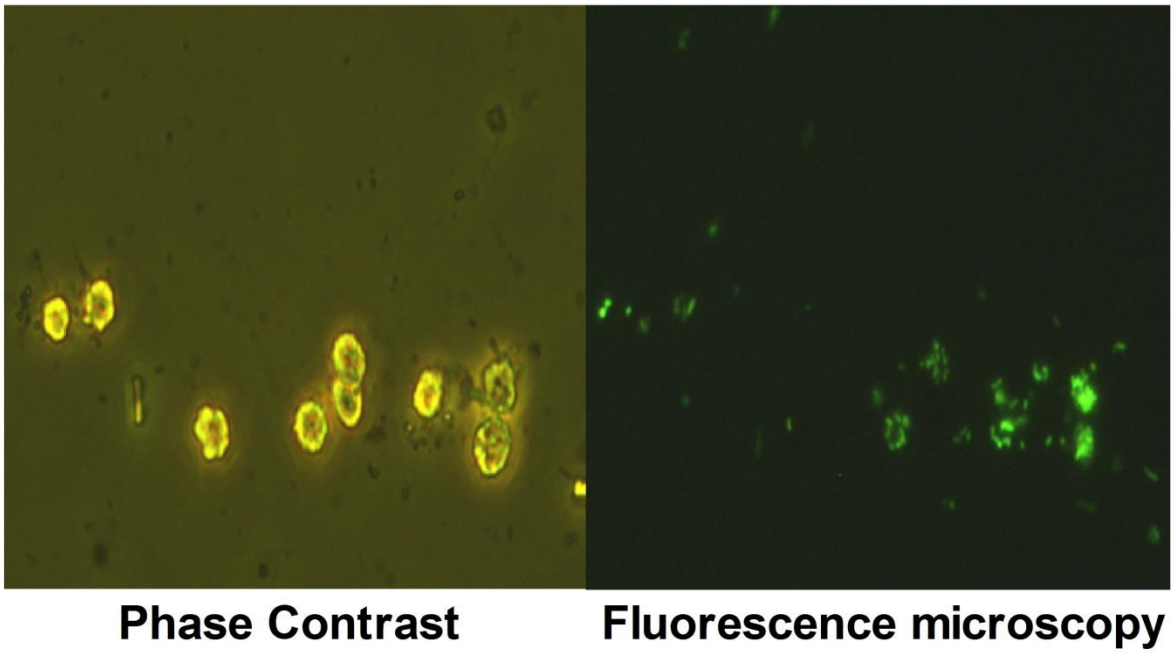

Figure 5.12. Phase contrast and fluorescence microscopy demonstrating phagocytosis of FITC labelled PA01 by neutrophils. In this experiment, PA01 was opsonised with 10µg/ml ficolin-2 in 0.5% serum prior to incubation with healthy donor neutrophils for 30 minutes.

Representative flow cytometry plots, showing the increasing % phagocytosis with increasing ficolin-2 concentrations are shown (figure 5.13).

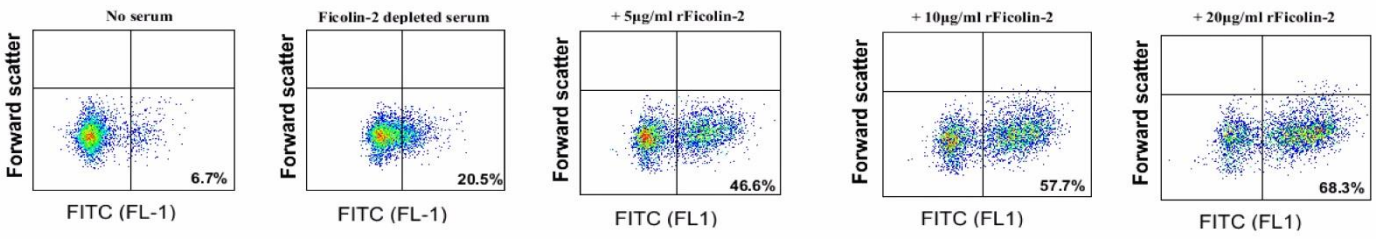

Figure 5.13. Representative flow cytometry data from phagocytosis experiments using FITC-labelled PA01. The threshold for FITC positive cells was set by running neutrophils not exposed to PA01 and setting the threshold at 0% FL-1 positive cells.

The results suggest that under appropriate conditions of low serum concentration with inhibition of the alternative complement pathway, ficolin-2 is able to cause complement activation and enhance opsonophagocytosis of *P. aeruginosa* strain PA01.

5.5 Neutrophil killing of *Pseudomonas aeruginosa*

Neutrophil killing required opsonisation with serum and the presence of neutrophils. In this experiment, PAO1 was opsonised in C5 depleted sera obtained from Sigma, to prevent formation of the membrane attack complex ensuring that all killing was due to neutrophils rather than complement mediated killing. After exposure of opsonised *P. aeruginosa* to neutrophils for 90 minutes at 37ºC, the supernatants were aspirated and the neutrophils lysed with 0.1% Triton x-100. Serial dilutions of the supernatants and cells were made and 100µl samples plated on LB agar overnight at 37°C in

triplicate. Bacterial load in the supernatants (non-phagocytosed bacteria) and in the cell lysates (phagocytosed bacteria that were not killed) was determined by colony counts.

As expected, incubation with 10µg/ml gentamicin for 60 minutes resulted in 100% killing (not shown). Following opsonisation with 50% C5a depleted serum, incubation with neutrophils caused a progressive reduction in viable *P. aeruginosa* with a reduction equivalent to 97% killing following opsonisation with C5 depleted serum, and a reduction equivalent to 88% killing following opsonisation with C5 depleted and GlcNAc depleted serum (to remove lectin pathway components)- figure 5.14. This difference was not statistically significant ($p>0.05$). When this assay was repeated after opsonisation in the presence of 0.6mg/ml SPS to inhibit the classical and alternative pathways, killing was still observed in 50% serum (a log unit reduction equivalent to 72% killing at 90 minutes). This was reduced by depletion of lectin pathway components with GlcNAc (log unit reduction equivalent to 19% at 90 minutes). This was restored to 76% with the addition of 20µg/ml ficolin-2. The differences between 50% $C5^{-/-}$ serum and 50% $C5^{-/-}$/GlcNAc depleted serum and 50% $C5^{-/-}$/GlcNAc depleted serum +20µg/ml ficolin-2 were statistically significant ($p<0.05$). All other comparisons, $p>0.05$.

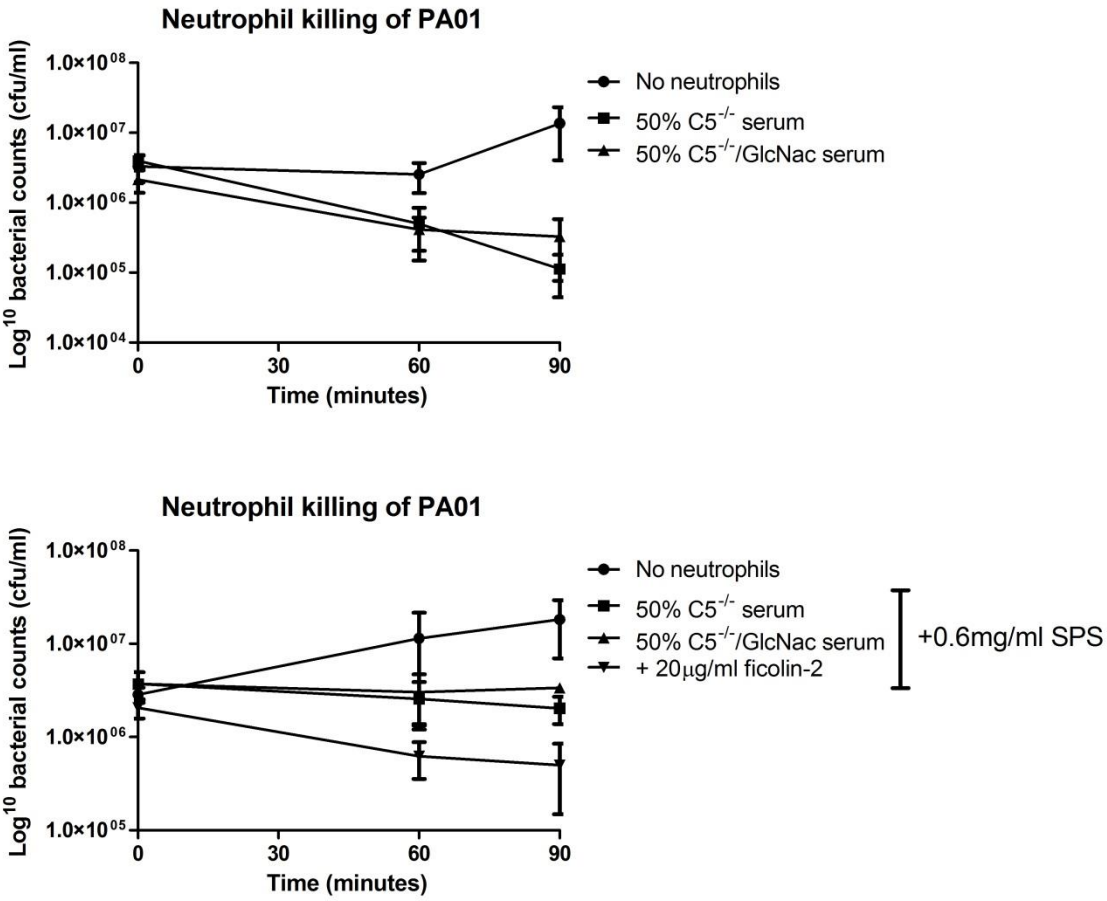

Figure 5.14. Killing of *P. aeruginosa* following incubation with neutrophils from healthy donors. No neutrophils indicates PA01 incubated with 50% C5 depleted serum without neutrophils for 90 minutes. +20μg/ml ficolin-2 refers to reconstitution of 50% $C5^{-/-}$/GlcNac depleted serum with 20+20μg/ml recombinant ficolin-2. Data are shown as the mean (SE) from 5 independent experiments.

5.6 Macrophage phagocytosis

Similar results were obtained with macrophage phagocytosis as with neutrophil phagocytosis. Ficolin-2 supplementation during opsonisation enhances macrophage phagocytosis in a dose dependent manner, with the requirement for serum and MASP-2. This is shown below in figure 5.15. The difference between C1q and the other curves was statistically significant (p=0.01). Representative flow cytometry plots show monocyte derived macrophages exposed to FITC-labelled *P. aeruginosa* strain PA01 opsonised with the indicated sera or control (buffer) depleted of lectin pathway components and then supplemented with recombinant ficolin-2 at the indicated concentration.

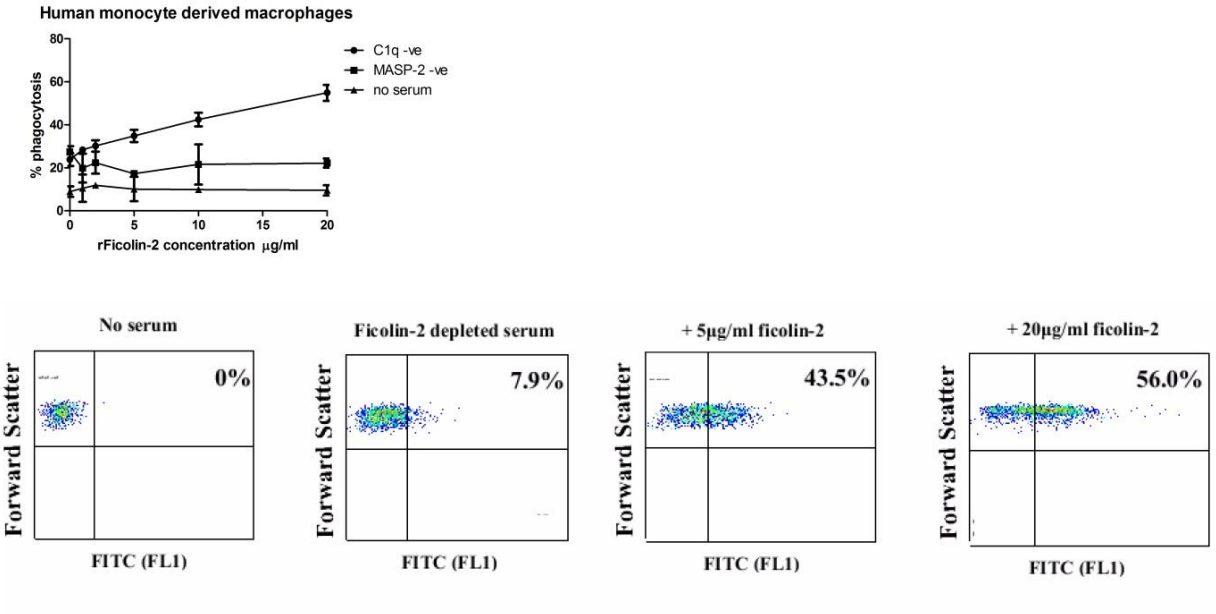

Figure 5.15. Phagocytosis of PA01 by human monocyte derived macrophages is enhanced in the presence of rFicolin-2. Data are shown as the mean (SE) from 5 independent experiments.

5.7 Peripheral and sputum neutrophils from bronchiectasis patients and controls demonstrate significant neutrophil dysfunction in the bronchiectasis airway

28 patients with non-cystic fibrosis bronchiectasis were studied for peripheral blood and sputum neutrophil studies. 5 patients underwent sputum induction and blood sampling but were ultimately excluded as sputum samples were of poor quality/contained too few neutrophils (N=4) or because the patient was subsequently found to be ineligible (N=1). 23 patients therefore completed the study.

Characteristics of the included patients are shown below, table 5.1. All patients classified as severe bronchiectasis were chronically colonised with *P. aeruginosa*.

	Severe	Moderate	Mild	p-value
N	8	8	7	
Age (years)	63.3 (11.5)	65.4 (8.2)	69 (7.6)	0.3
Gender (male)	5 (62.5%)	5 (62.5%)	5 (71.4%)	0.8
FEV_1 %predicted	56.4 (20.9)	72.4 (26.4)	77.7 (12.9)	0.2
FVC % predicted	71.8 (28.0)	81.5 (26.5)	87.9 (39.6)	0.6
FEV_1/FVC	62.7 (7.4)	71.1 (12.0)	72.7 (11.9)	0.9
BMI kg/m^2	23.9 (4.1)	28.2 (5.9)	25.8 (5.0)	0.3
%Hospitalised in previous year	100%	25%	0%	<0.001
Outpatients exacerbations/yr	4.7 (2.0)	3.5 (1.5)	1.8 (0.9)	0.2
Chronically colonised	100%	100%	0%	<0.0001
% *Pseudomonas aeruginosa*	100%	0%	0%	<0.0001
Quality of life (SGRQ)	62.9 (11.8)	44.1 (23.0)	35.6 (18.8)	0.03

Table 5.1. Characteristics of patients undergoing sputum and peripheral blood neutrophil studies. Abbreviations: SGRQ= St. Georges Respiratory Questionnaire, FEV_1= forced expiratory volume in 1 second, FVC= forced vital capacity, BMI= body mass index. Yr= year.

Compared with healthy control subjects, peripheral blood neutrophils from patients with bronchiectasis did not show reduced phagocytosis of FITC-labelled *E.coli* or *P.aeruginosa*, figure 5.16. In this experiment, organisms were opsonised for 1 hour with 20% pooled donor serum followed by incubation with patient or healthy donor neutrophils for 30 minutes. Phagocytosis was quantified by flow cytometry.

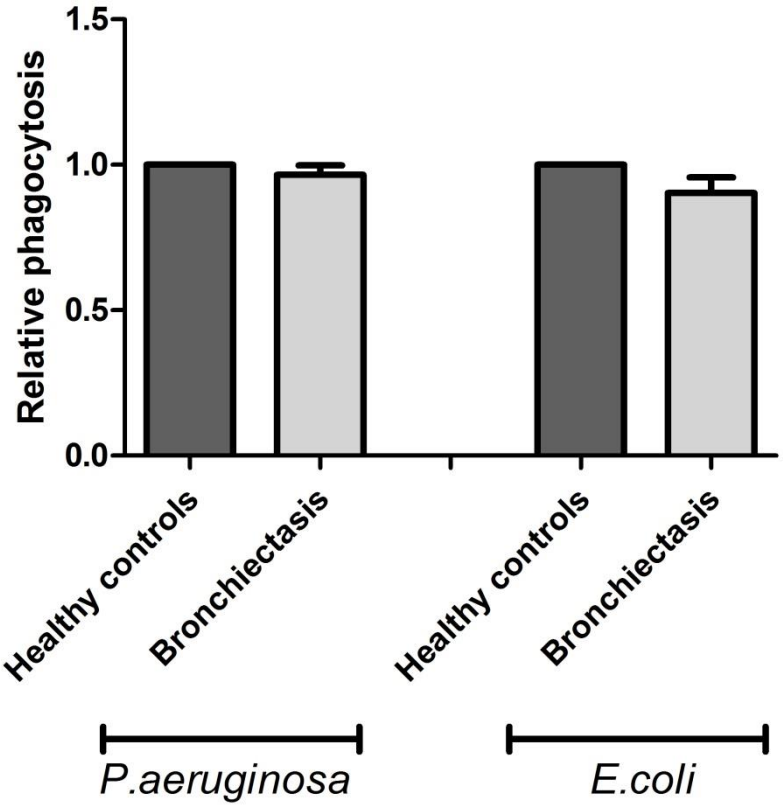

Figure 5.16. Phagocytosis by peripheral blood neutrophils from bronchiectasis patients and healthy control subjects. Measurements were performed by flow cytometry as described in the text. Phagocytosis (mean fluorescence intensity) is expressed as % of the mean value for 9 healthy controls. N=23 for bronchiectasis patients.

Receptor expression was similar between peripheral blood neutrophils from healthy donors and from patients with bronchiectasis, with no significant differences within the bronchiectasis sub-groups, figure 5.17. Results are expressed as a ratio of expression (mean fluorescence by flow cytometry) on neutrophils from 9 healthy controls subjects, indicated by the white bars in figure 5.17.

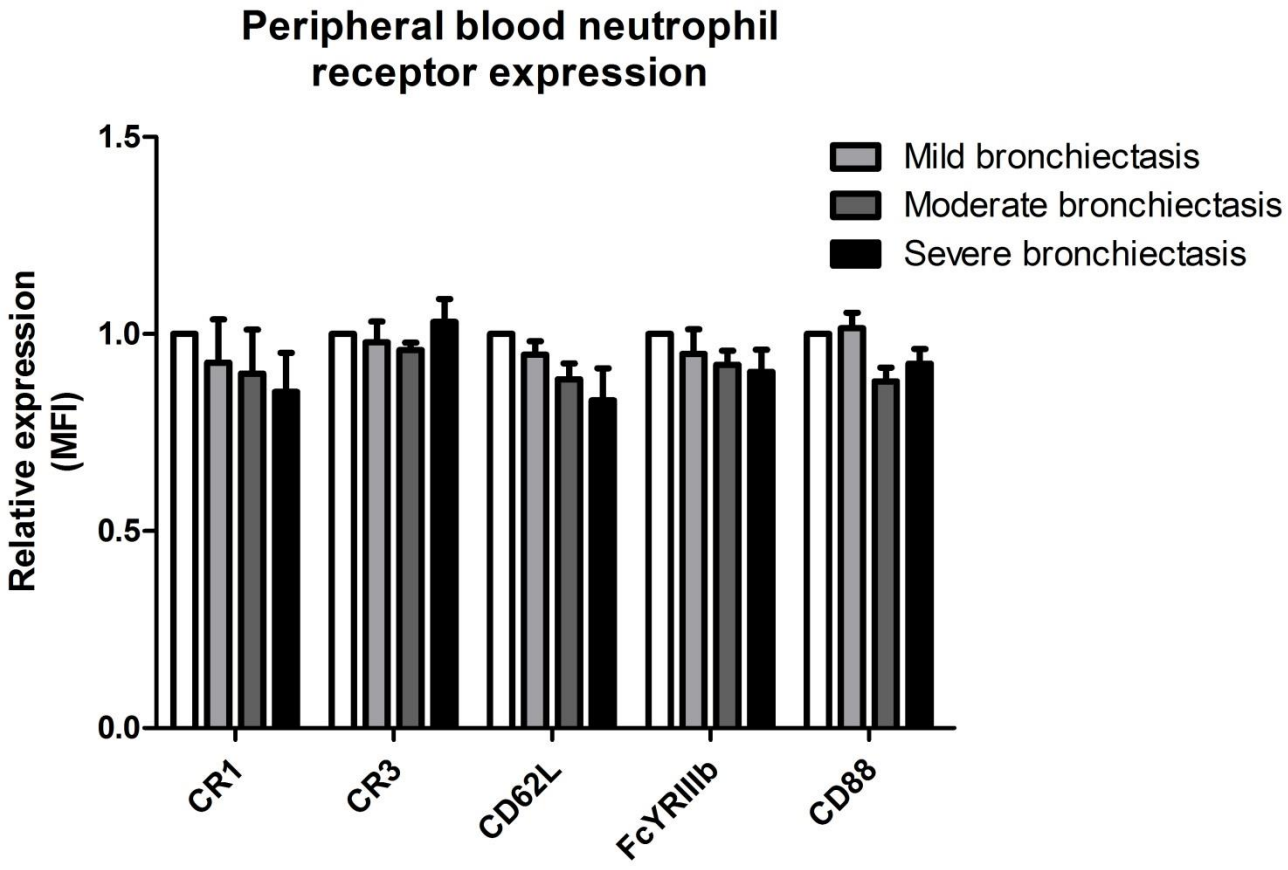

Figure 5.17. Neutrophil receptor expression in peripheral blood neutrophils between healthy control subjects (N=9) and bronchiectasis patients (N=23). Values are expressed as relativel expression compared to healthy control subjects (1.0). Abbreviations: CR1= complement receptor 1, CR3= complement receptor 3

Compared to healthy peripheral blood neutrophils, all patients sputum neutrophils demonstrated reduced expression of CR1, consistent with previous reports from cystic fibrosis (Berger et al 1989). There was also reduced expression of FCγRIIIb in patients with bronchiectasis, and reduced expression of FCγRIIIb correlated with the severity of bronchiectasis, p=0.01 by Kruskal-wallis test.(figure 5.18)

Sputum neutrophil studies showed substantial variation in receptor expression and neutrophil phagocytosis between individual bronchiectasis patients. Again, in figure 5.18, results are expressed as a ratio of peripheral blood neutrophil receptor expression from 9 healthy control subjects, except that for sputum neutrophil studies the control neutrophils were maximally activated with platelet activating factor followed by fMLP as described in chapter 2.

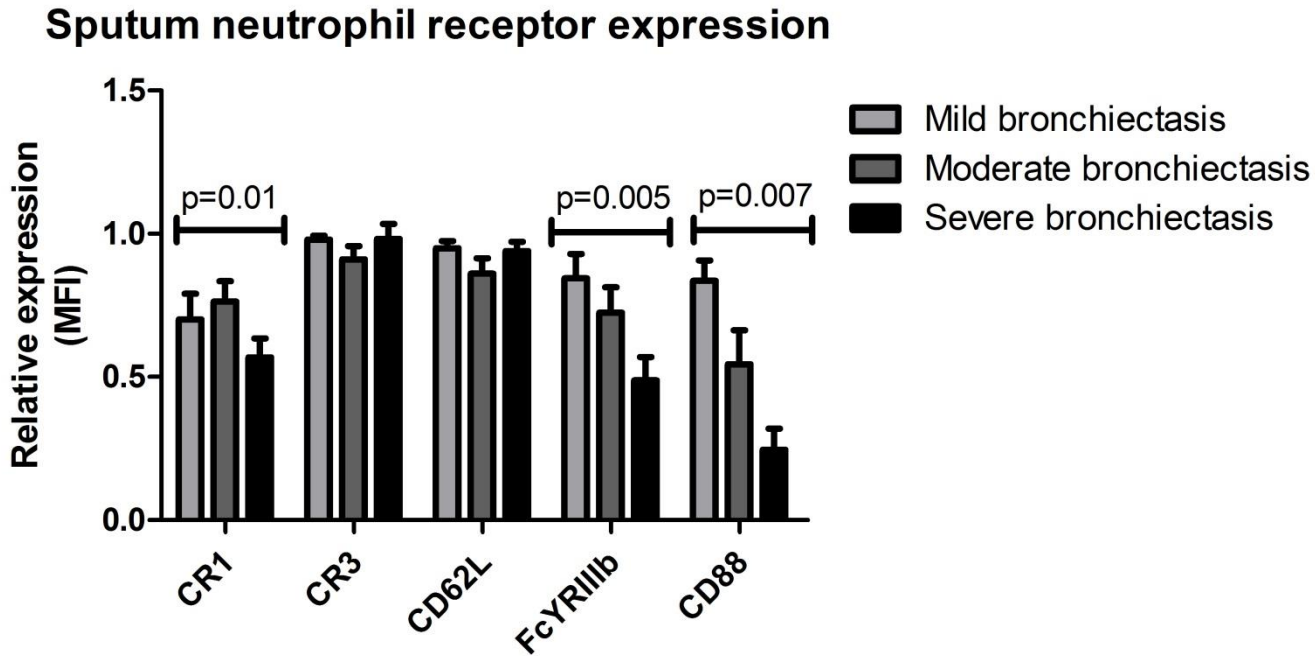

Figure 5.18. Sputum neutrophil receptor expression in patients with bronchiectasis compared to maximally activated peripheral blood neutrophils from 9 healthy controls. Abbreviations: PA= *P. aeruginosa*. CR1= complement receptor 1, CR3= complement receptor 3. Data are shown at the mean (SE) from 23 patients with bronchiectasis and 9 healthy controls. Statistically significant differences are highlighted.

Patients demonstrated similar expression of CR3 and CD62L to activated peripheral blood neutrophils, with no significant differences observed according to severity of bronchiectasis ($p>0.05$). Treatment of sputum neutrophils with platelet activating factor and fMLP did not increase CD11b expression or reduce CD62L expression, suggesting that airway neutrophils are maximally activated, figure 5.19.

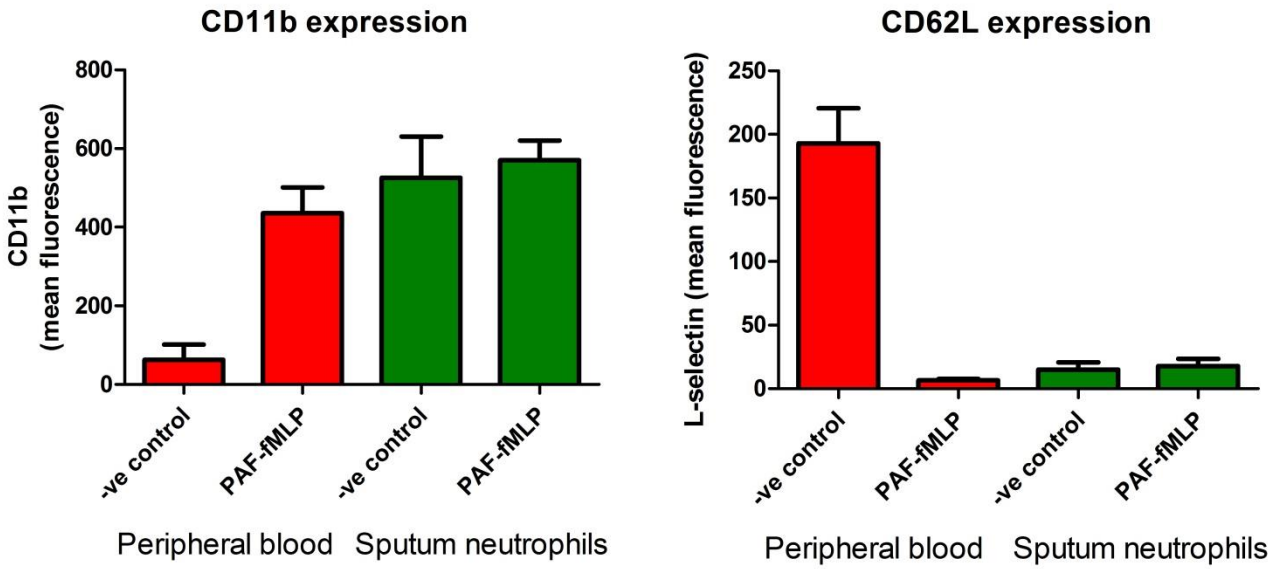

Figure 5.19. CD11b expression and C62L expression as markers of neutrophil activation. Mean fluorescence for peripheral blood neutrophils is shown in red.

After treatment with the negative control (phosphate buffered saline) neutrophils show low expression of CD11b and high expression of CD62L. Following activation with platelet activating factor (PAF) followed by fMLP, there was a significant upregulation of CD11b ($p<0.0001$) and shedding of CD62L ($p<0.0001$). In contrast, in sputum neutrophils (green bars), prior to treatment sputum neutrophils had high

expression of CD11b and low expression of CD62L. Further treatment with PAF-fMLP did not significantly affect expression of these markers ($p>0.05$ for both comparisons).

Sputum neutrophil phagocytosis varied significantly. There was no difference between patients with mild and moderate bronchiectasis in terms of sputum neutrophil phagocytosis but there was a significant reduction in phagocytosis in patients with severe bronchiectasis and *P. aeruginosa* colonisation compared to the other two groups ($p=0.004$ for phagocytosis of *P. aeruginosa* and $p=0.008$ for phagocytosis of *E.coli*)- figure 3.20.

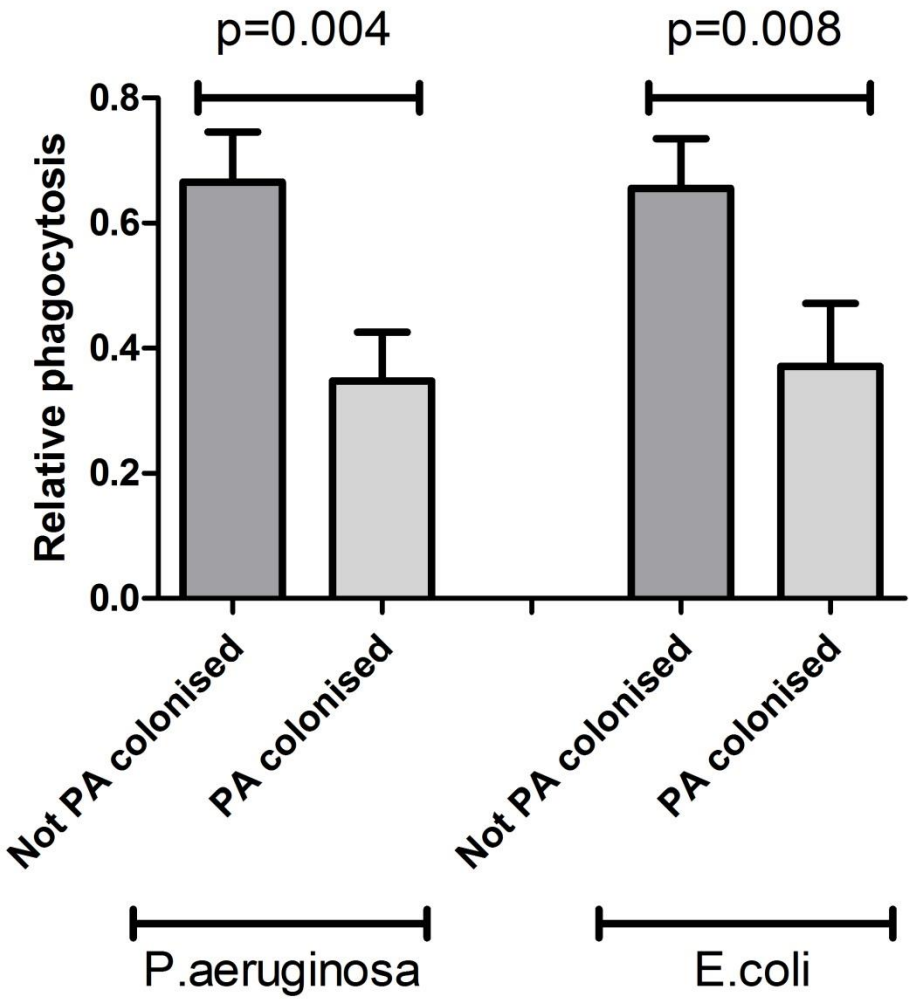

Figure 5.20. Sputum neutrophil phagocytosis. The mean fluorescence intensity and standard error of the mean are displayed relative to the values obtained for maximally activated peripheral blood neutrophils from 9 healthy donors (N=23 for bronchiectasis patients). Abbreviations- PA= *P. aeruginosa*.

CD88 expression was lower in patients with severe bronchiectasis compared to mild/moderate bronchiectasis. (Figure 5.18). Expression was reduced to 83% (standard deviation 20.3) of control in mild bronchiectasis, compared to 54%

(standard devation 33.7) of control in moderate bronchiectasis and 24.6% (standard deviation 20.8) of control in severe patients (p=0.007).

CD88 is the receptor for complement component C5a. As one of the major functions of ficolin-2 is the activation of complement and therefore the generation of C5a, we sought to investigate the importance of CD88 in bronchiectasis neutrophils in more detail.

5.8 Neutrophil elastase is responsible for loss of CD88 on sputum neutrophils

There was a significant correlation between CD88 expression and phagocytosis of *P. aeruginosa* (r=0.59,p=0.002) and *E. coli* (r=0.63,p=0.0009), (figure 5.21) and a significant correlation between CD88 expression and oxidative burst in sputum neutrophils (r=0.54,p=0.006). Oxidative burst was measured by the dihydrorhodamine method (see chapter 2).

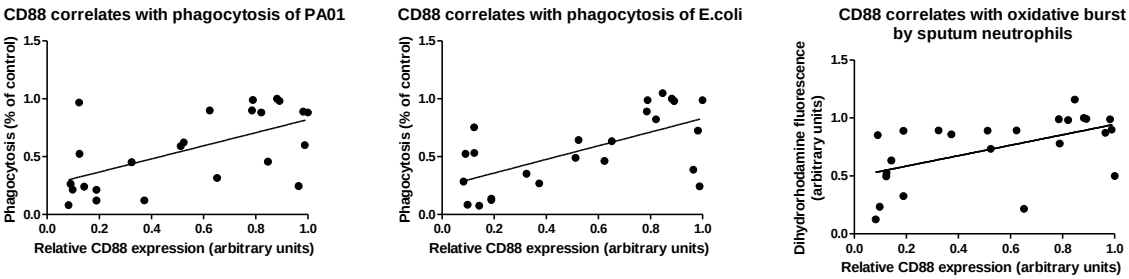

Figure 5.21. Correlations between sputum neutrophil CD88 expression and phagocytosis of *P. aeruginosa* and *E.coli* labelled with FITC. Phagocytosis is expressed as a ratio of the phagocytosis observed in peripheral blood neutrophils as before. n=23 patients with bronchiectasis.

A pool of sputum from 5 patients with non-CF bronchiectasis caused loss of CD88 from healthy neutrophils after 30 minutes incubation, while a pool of induced sputum from the 9 healthy donors without lung disease did not cause any significant reduction in neutrophil expression of CD88. At the dilutions used (up to 10% v/v sputum sol), sputum sol treatment for 30 minutes did not induce neutrophil apoptosis or necrosis as demonstrated by annexin-V and PI staining. (figure 5.22).

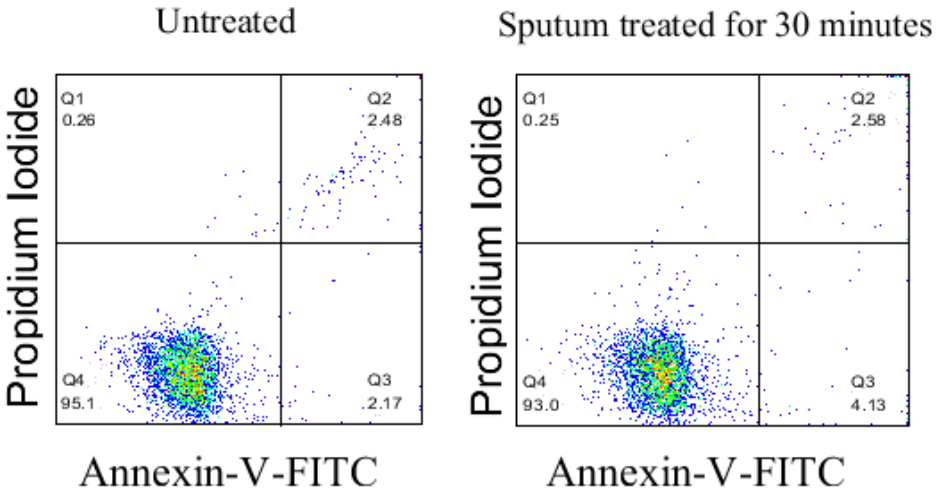

Figure 5.22. Flow cytometry assessment of apoptosis in peripheral blood neutrophils from healthy controls treated for 30 minutes with a pool of sputum from patients with bronchiectasis. The X-axis shows Annexin-V-FITC staining and the Y-axis shows propidium iodide staining. The quadrants shows Q1- $AnnV^{-ve}/PI^{+ve}$= necrotic cells, Q2- $AnnV^{+ve}/PI^{+ve}$= secondary necrotic cells. Q3- $AnnV^{+ve}/PI^{-ve}$= apoptotic cells, Q4- $AnnV^{-ve}/PI^{-ve}$= viable cells.

Initially I hypothesised that C5a present in sputum would be responsible for CD88 downregulation by bronchiectasis sputum sol, since it is known that C5a binding to its receptor causes rapid internalisation of CD88 and reduced surface expression (Ward, 2004). As expected, C5a treatment of healthy neutrophils led to downregulation of CD88. (figure 5.23)

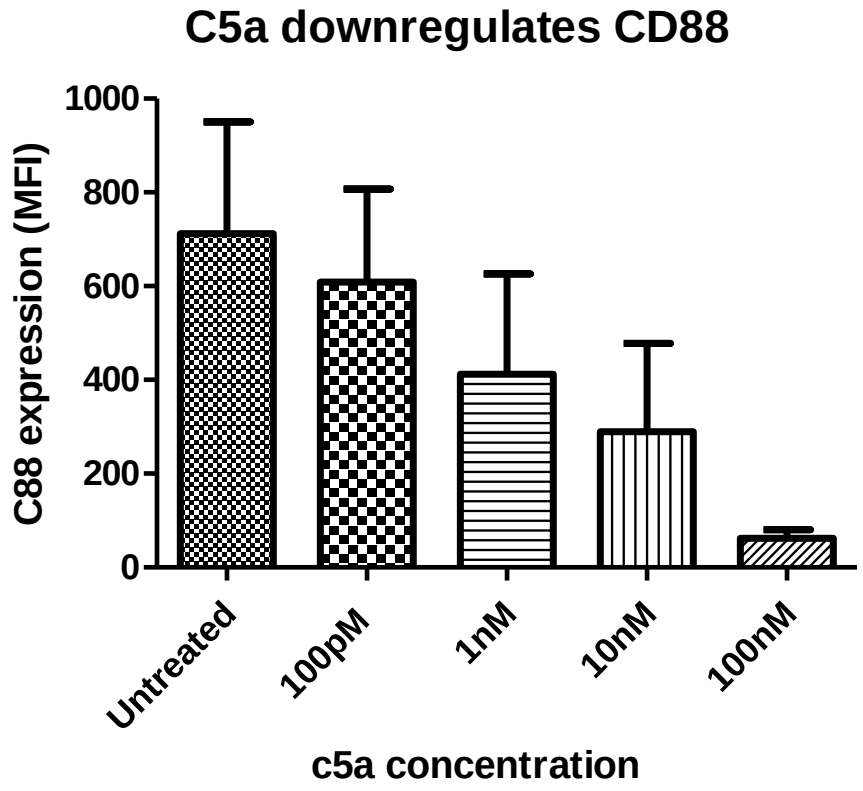

Figure 5.23. Downregulation of CD88 in response to increasing concentrations of recombinant complement C5a. Results show the mean (SE) of 3 separate experiments. The mean fluorescence intensity was significantly different to untreated neutrophils at 100pM ($p=0.02$) and at all other concentrations ($p<0.0001$).

Loss of CD88 was, however, observed after exposure of neutrophils to highly diluted (0.6%) sputum sol that contained no detectable C5a by ELISA.

Neutrophils from healthy donors were treated with increasing doses of purified neutrophil elastase. The results demonstrated a dose dependent reduction in CD88 expression in elastase treated neutrophils. Importantly, marked CD88 cleavage was demonstrated at levels of neutrophil elastase activity (<0.5µg/ml) lower than those present in patients with severe bronchiectasis and other disorders such as COPD or cystic fibrosis (Stockley 1984, Nunley 1999), figure 5.24.

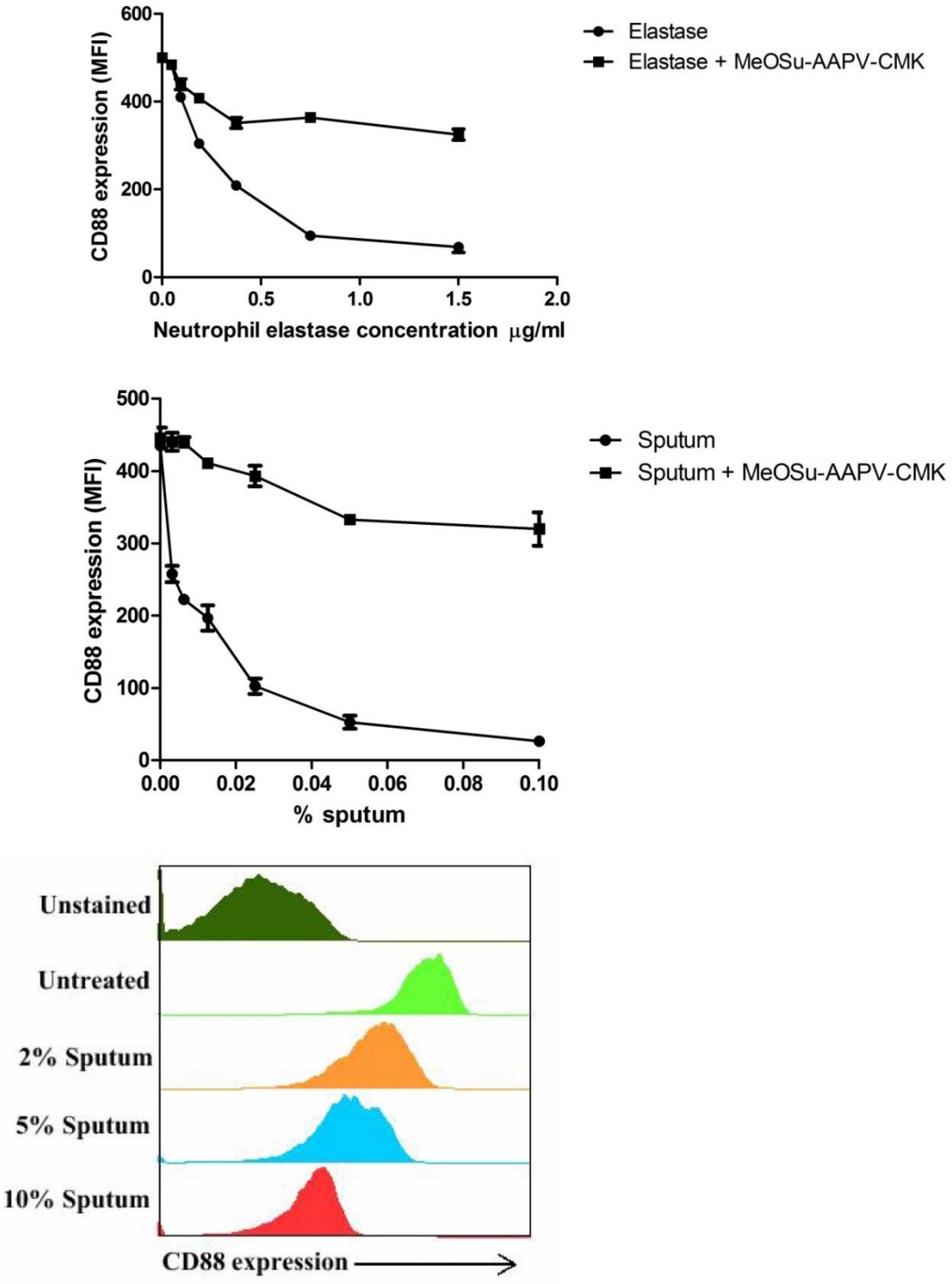

Figure 5.24. Neutrophil elastase and CD88 expression on neutrophils. Elastase or sputum sol treatment of neutrophils causes dose dependent loss of CD88 from neutrophils. This loss is inhibited by the specific neutrophil elastase inhibitor - methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MeOSu-AAPV-CMK). Data are shown as mean (SE) from 3 individual experiments for elastase and mean (SE) from 3 individual pools of sputum sol for sputum studies.

Treatment with another neutrophil protease, Cathepsin G, did not affect CD88 expression, nor did activation with fMLP (figure 5.25).

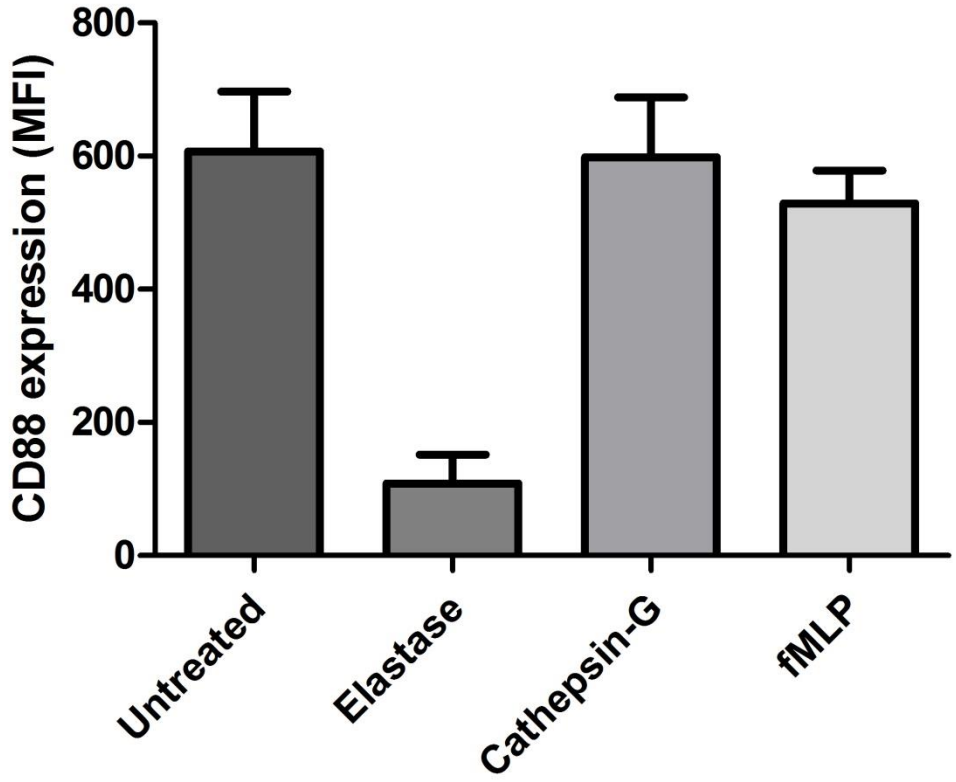

Figure 5.25. Treatment with 1µg/ml neutrophil elastase caused a statistically significant loss of CD88 from neutrophils ($p<0.0001$) while the protease cathepsin-G and fMLP had no effect on CD88. Results are the mean of 3 independent experiments and results are expressed as mean fluorescence with standard error of the mean

The reduction in CD88 expression on donor neutrophils by elastase was completed abrogated by addition of a specific inhibitor of neutrophil elastase N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone at a concentration of 1mM.

(figure 5.24). This concentration of N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was sufficient to fully inhibit elastase activity in sputum sol samples at the dilutions used in a microplate assay using the synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MeoSAAPvN) (figure 3.26).

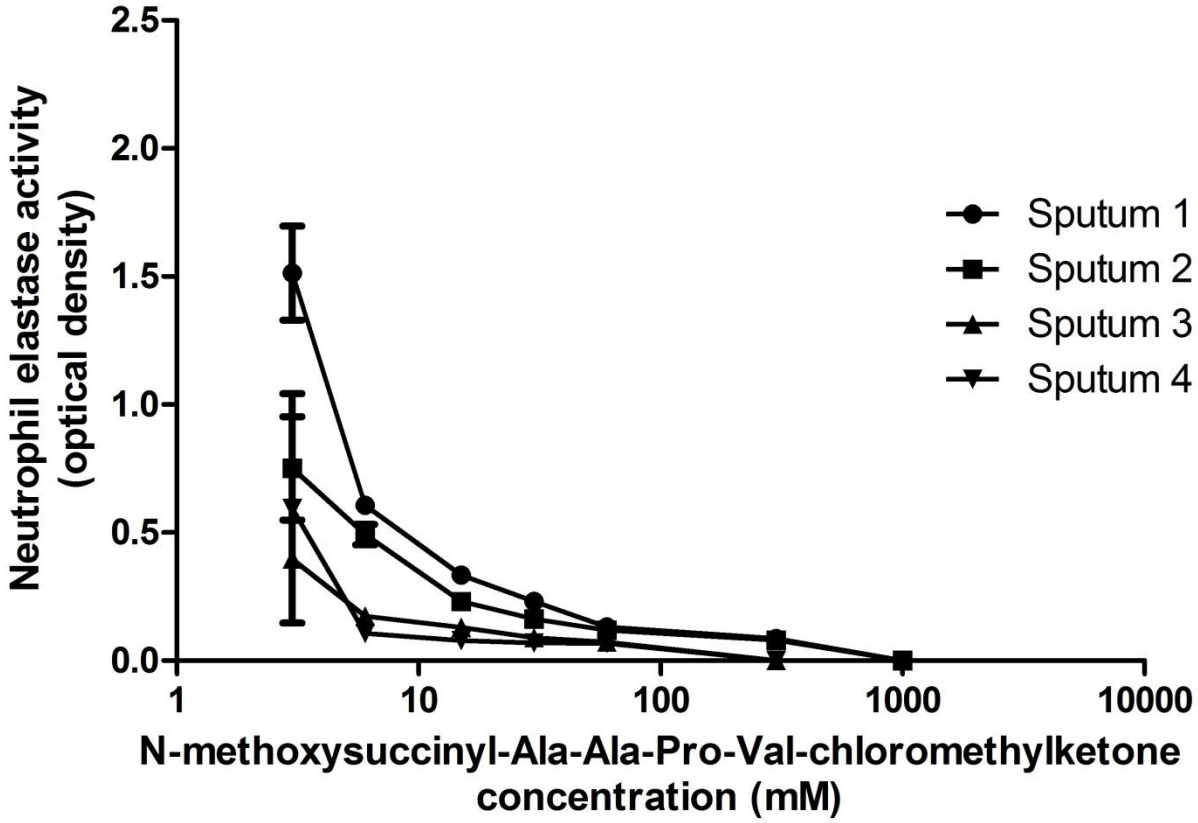

Figure 5.26. Inhibition of sputum neutrophil elastase activity with the neutrophil elastase inhibitor N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone. Results are mean of 3 independent experiments using four representative patient samples.

Pre-treatment of sputum samples with N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone prevented cleavage of CD88 when sputum samples were

incubated with neutrophils confirming that elastase was primarily responsible for loss of CD88 induced by sputum sol, figure 5.24.

Treatment with leukocyte elastase impaired C5a mediated enhancement of phagocytosis and oxidative burst, implicating CD88 cleavage by elastase in airway neutrophil dysfunction in bronchiectasis.

C5a is known to prime neutrophils for activation, superoxide production and phagocytosis (Mollnes 2002). To determine if elastase cleavage of CD88 has functional consequences for bronchiectasis neutrophils, we studied superoxide production by dihydrorhodamine fluorescence following treatment of neutrophils with C5a (1nM) followed by fMLP (0.1 µmol/L). Since platelet activating factor (PAF) also primes neutrophils but by a mechanism that does not involves CD88, treatment with PAF (1µmol/L) followed by fMLP (0.1 µmol/L) was used for comparison. Neutrophils were incubated at 37°C for 30 minutes in either PBS (control) or 1.5µg/ml purified neutrophil elastase prior to priming and activation.

The data demonstrated a typical priming response to c5a in the untreated neutrophils, with a significant increase in fluorescence between the C5a-fMLP treated neutrophils compared to PBS-fMLP treated neutrophils. In contrast, the difference between C5a-fMLP and PBS-fMLP treated neutrophils was not statistically significant following elastase treatment of neutrophils, suggesting a failure of CD88 dependent priming, figure 5.27.

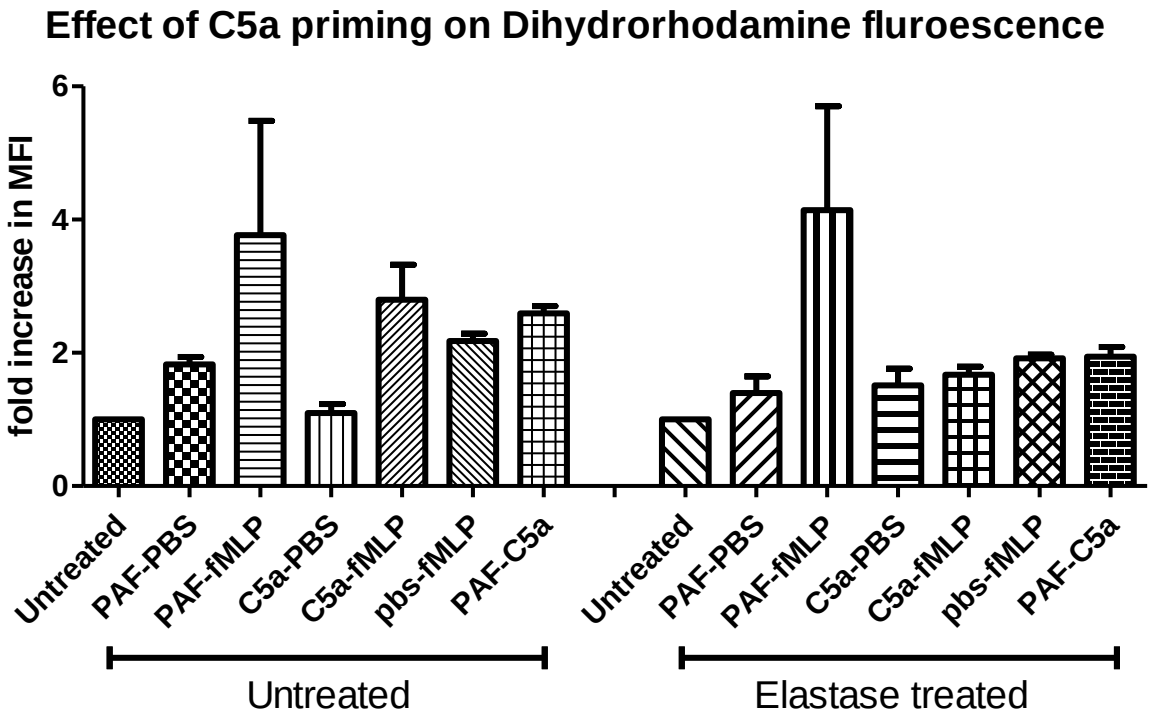

Figure 5.27. The effect of neutrophil elastase pre-treatment on oxidative burst. Data are presented as fold increase from control (1). Differences were statistically significant for PAF-fMLP treated neutrophils in both conditions ($p<0.05$) and for C5a-fMLP, pbs-fMLP and PAF-C5a treated neutrophils in untreated neutrophils ($p<0.05$) but not in elastase treated neutrophils. All other comparisons $p>0.05$. Data are shown as mean (SE) from 3 independent experiments. PAF= platelet activating factor, fMLP=N-formyl-methionyl-leucyl-phenylalanine, PBS= phosphate buffered saline.

30 minutes pretreatment of neutrophils with 100pM to 10nM recombinant C5a induced a dose dependent increase in phagocytosis after exposure to *E.coli* or *P.aeruginosa* ($p<0.0001$ for both by Kruskal-wallis test). Elastase treatment

(1.5µg/ml purified elastase or PBS control) for 30 minutes resulted in a reduction in phagocytosis, but also prevented the enhancement of phagocytosis induced by C5a (p=0.5 for *E.coli* and p=0.3 for *P.aeruginosa* by Kruskal-wallis test). Figure 5.28

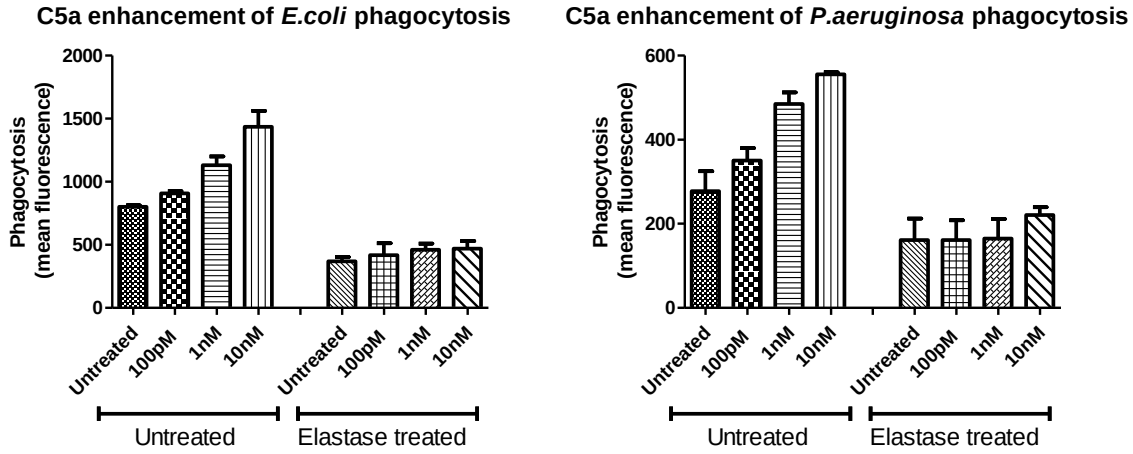

Figure 5.28. The effect of elastase cleavage of CD88 on C5a enhancement of neutrophil phagocytosis.

5.9 Opsonisation with Ficolin-2 enhances phagocytosis of *P. aeruginosa* by sputum neutrophils

Despite the neutrophil receptor and functional changes described above, sputum neutrophils responded similarly to peripheral blood neutrophils to serum opsonins. Increasing serum concentration increased the rate of phagocytosis over 30 minutes and phagocytosis was prevented by treatment of cells with cytochalasin-D as described for peripheral blood neutrophils.

Opsonisation of PA01 with increasing Ficolin-2 concentrations resulting in a dose dependent increase in phagocytosis as measured by both mean fluorescence (p=0.001

by two way ANOVA) and % phagocytosis (p=0.006 by two way ANOVA) (figure 5.29). This opsonisation was MASP-2 and serum dependent. Representative flow cytometry plots are shown.

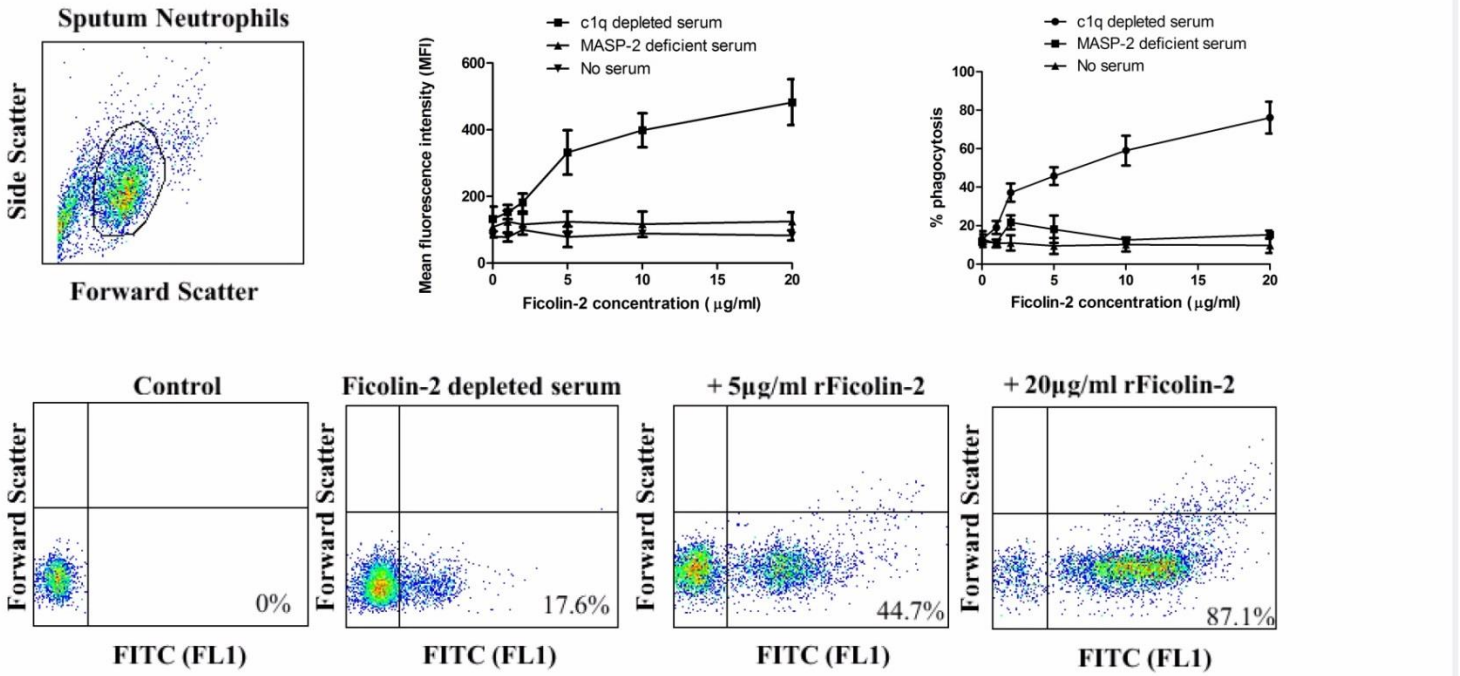

Figure 5.29. Sputum neutrophil phagocytosis is enhanced by opsonisation with recombinant ficolin-2 in the presence of serum and MASP-2. The image in the top left shows forward and side scatter properties of isolated sputum neutrophils. There is a clearly identified neutrophil population with associated debris which was a typical finding after sputum neutrophil preparation. In the presence of complement and recombinant ficolin-2 there was a dose dependent increase in phagocytosis quantified by mean fluorescence and percentage of FITC positive cells. This is reflected in the lower panels which show representative flow cytometry data. The first panel shows neutrophils prior to exposure to bacteria, with a threshold set at 0% phagocytosis.

Since neutrophil elastase was found to be major contributor to neutrophil dysfunction, we tested whether neutrophil elastase affected ficolin-2 binding to PA01. Non-reduced western blotting after incubation with 0.5µg/ml elastase for 1 hour suggested a minor effect of elastase on oligomerisation of ficolin-2, with qualitatively fewer high order multimers and more monomers compared to untreated ficolin-2 (figure 5.30). This did not have a significant impact on the amount of bound ficolin-2 in a solid-phase binding assay of PA01 ($p>0.05$ for comparisons at all concentrations).

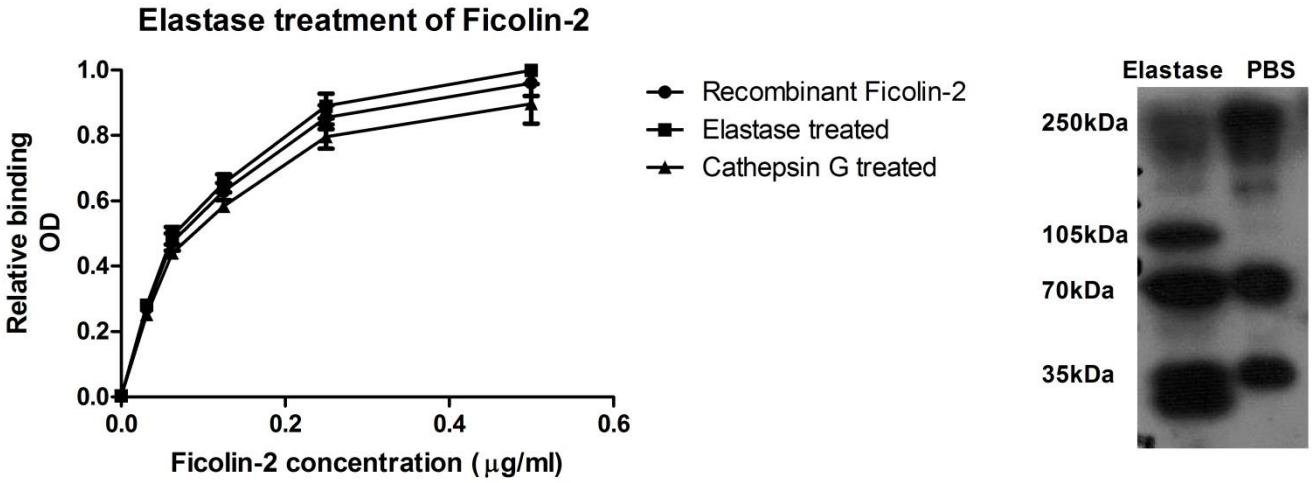

Figure 5.30. No effect of neutrophil elastase or Cathepsin G on binding of recombinant ficolin-2 to PA01.

5.10 Interaction between ficolin-2 and pentraxin-3 enhances phagocytosis of *Pseudomonas aeruginosa*

It has been reported that Ficolin-2 dependent complement activation can be enhanced by interaction with pentraxins. To investigate this further, I investigated binding of Ficolin-2 to Pentraxin-3, serum amyloid P and C-reactive protein. Significant binding to all three pentraxins was observed, Figure 5.31A. Opsonisation of PA01 in the presence of ficolin-2 and pentraxin-3 (10µg/ml) resulted in significantly greater deposition of C3 by flow cytometry (figure 5.31B) and phagocytosis of FITC-PA01 (figure 5.31B). In view of a previous report (Zhang et al 2009) that C-reactive protein could enhance phagocytosis of *P. aeruginosa* in collaboration with ficolin-2, assays were repeated adding CRP at 10µg/ml along with ficolin-2. No effective on complement activation or phagocytosis was observed.

Pentraxin-3 was found to be present in induced sputum in significant quantities. Pentraxin-3 was measured in induced sputum from 30 stable patients with bronchiectasis and 30 healthy control subjects. Higher levels of pentraxin-3 were demonstrated in bronchiectasis sputum compared to healthy control subjects. ($p=0.03$), figure 5.31C.

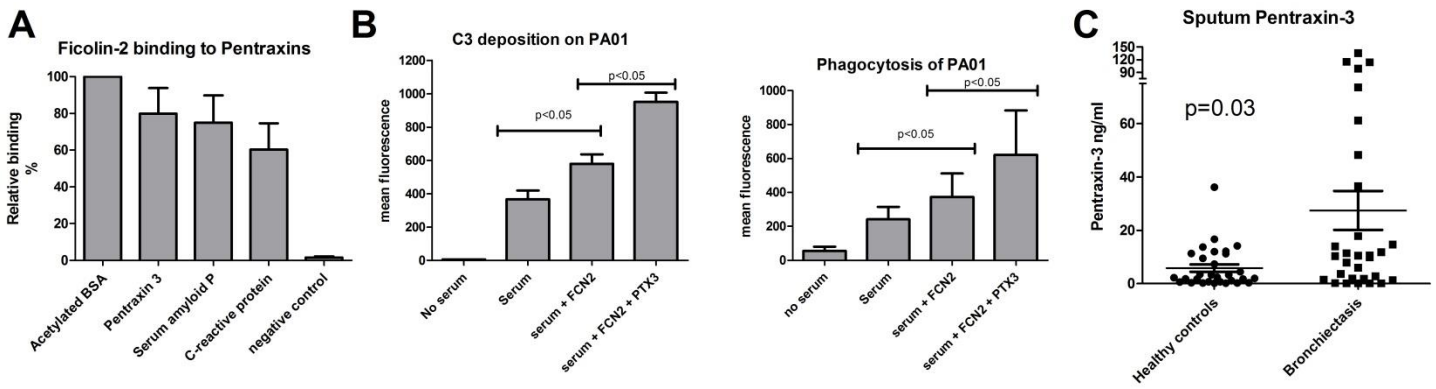

Figure 5.31. Interaction of ficolin-2 with pentraxins boost complement deposition and phagocytosis of PA01. Figure A- % binding is shown compared to the positive control (binding to Acetylated BSA-100% and the negative control, BSA). Significant binding of each Pentraxin was statistically significant compared to the negative control ($p<0.0001$). Data in figure 5.31A and 5.31B shown the mean (SE) from 3 independent experiments. Figure 5.31C, n=30 in each group.

5.11 Discussion

In this chapter, we demonstrate a role for ficolin-2 in the opsonophagocytic clearance of bacteria by neutrophils and macrophages and specifically demonstrate a role in the clearance of *P. aeruginosa.* Enhancement of phagocytosis was demonstrated in both peripheral blood neutrophils and, importantly, in airway neutrophils from patients with bronchiectasis when opsonisation was enhanced with recombinant ficolin-2. Enhancement of phagocytosis was dependent on the presence of MASP-2 and C3, indicating that complement activation through the lectin pathway was the underlying mechanism.

Ficolin-2 bound to a wide range of clinically relevant bacteria, and binding was accompanied by complement activation. In keeping with our clinical findings, ficolin-2 was the critical regulator of complement activation, with the amount of C3 deposited on cells dependent on the ficolin-2 serum concentration and not on the concentration of MASP-2. This would suggest that the enzyme MASP-2 is present in excess in serum and that it is engagement of the fibrinogen-like domain of ficolin-2 to pathogens that is required for complement activation. Having demonstrated enhanced phagocytosis and killing of PA01 in healthy neutrophils we went on to investigate airway neutrophils in bronchiectasis.

Neutrophils are the major inflammatory cell present in the bronchiectasis airways (Eller et al 1994). The number of neutrophils migrating to the airway is increased in stable patients and the numbers increase further with bacterial infection and exacerbation (Chalmers et al 2011). Despite the large numbers of neutrophil phagocytes in the airway, these cells fail to effectively phagocytose and kill bacteria

leading to chronic bacterial colonisation (Angrill 2002). The reasons for this in bronchiectasis are unknown. The present study provides a large amount of data on neutrophil function in bronchiectasis. We found no differences in complement receptor 1, complement receptor 3, CD62L, CD88 and FcγRIIIb expression on peripheral blood neutrophils between healthy controls and patients with bronchiectasis. Healthy and bronchiectasis peripheral blood neutrophils had similar phagocytic ability. Together these suggest that neutrophils are relatively normal prior to migrating to the bronchiectasis airway. This is consistent with the previous work by Pasteur et al, who also did not find evidence of abnormal neutrophil function in a significant number of patients with bronchiectasis (Pasteur et al 2000).

There were, however, marked differences between peripheral blood and airway neutrophils. Airway neutrophils, isolated from sputum, had reduced expression of complement receptor 1, FcγRIIIb and CD88 compared to peripheral blood neutrophils from the same patients. Down-regulation of these receptors correlated with the severity of bronchiectasis, with the lowest levels observed in patients with *P. aeruginosa* infection. Similarly, patient's airway neutrophils had reduced phagocytosis of *E. coli* and *P. aeruginosa* compared to peripheral blood neutrophils. The present study is the first to describe neutrophil elastase mediated reduction in CD88 expression on the surface of neutrophils, the receptor for complement component C5a. Complement component c5a is a potent regulator of the inflammatory response (Guo and Ward 2005) produced by the cleavage of component c5 during complement activation via the classical, lectin or alternative pathways. C5a exerts multiple pro-inflammatory effects via its receptor CD88. CD88 is expressed on monocytes and bronchial epithelial cells, but is most abundant on

neutrophils where the effects of C5a are consequently most pronounced (Chenoweth and Hugli 1978). C5a primes neutrophils to enhance functional responses such as phagocytosis and the generation and release of granule enzymes and superoxide anions which are critical to bacterial killing (Mollnes 2002).

Generation of c5a causes local vasodilation, oedema and is a powerful chemoattractant for neutrophils and other leucocytes (Marder et al 1985). C5a also protects neutrophils from apoptosis (Perianayagam et al 2002). When regulated, these processes are protective against bacterial infection and beneficial to the host. It has been reported that the very high levels of c5a generated during sepsis can be harmful to neutrophils by suppressing chemotaxis and phagocytosis (Huber-Lang et al 2002, Ward 2004).

The c5a receptor, CD88 is nevertheless critical to host defence, as demonstrated by markedly increased neutrophil influx and failed bacterial clearance in CD88 deficient mice infected with *P. aeruginosa* (Hopken et al 1996).

This study found that neutrophil elastase caused loss of CD88 by neutrophils and impaired CD88 mediated functions of C5a. Elastase is already known to damage epithelial cells, slowing ciliary beat frequency and promoting mucous hypersecretion in bronchiectasis (Voynow, 1999, Amitani, 1991). Elastase is directly pro-inflammatory. Destruction of elastin, basement membrane collagen and proteoglycans by elastase and other neutrophil proteases contributes directly to disease progression (Shun, 2000, Stockley, 1984).

Importantly however, there is strong evidence that elastase also impairs neutrophil phagocytosis by multiple mechanisms. Elastase cleaves FcγRIIIb, and has also been

shown to cleave complement receptor 1 in patients with cystic fibrosis (Voglis, 2009, Berger, 1989). Elastase also cleaves iC3b from the surface of pathogens leading to an important opsonin/receptor mismatch.(Tosi et al, 1990). The present study identified a novel mechanism of elastase induced neutrophil dysfunction in bronchiectasis. Elastase treatment of neutrophils led to a reduction in CD88 expression. Incubation of neutrophils with bronchiectasis sputum also reduced CD88 expression and this could be inhibited by neutrophil elastase inhibitors. Elastase or sputum treatment impaired complement C5a induced enhancement of phagocytosis and oxidative burst.

Despite the marked neutrophil dysfunction identified in bronchiectasis, ficolin-2 was still able to enhance phagocytosis of *P. aeruginosa*, adding validity to our hypothesis that ficolin-2 may be important in defence against *P. aeruginosa* in the airway.

We went on to investigate the role of the pentraxins and their interaction with ficolins in phagocytosis of *P. aeruginosa.* Zhang et al demonstrated enhanced complement mediated killing of *P. aeruginosa* in the presence of C-reactive protein under inflammatory conditions, demonstrating the ability of Ficolin-2 to enhance C4 deposition and phagocytosis of *P. aeruginosa* (Zhang et al 2009). In the present study, we investigated the effect of the pentraxins on complement activation and phagocytosis of *P. aeruginosa* and found enhancement of phagocytosis in the presence of pentraxin-3, but not c-reactive protein. This is consistent with the work of Ma et al who have demonstrated the interaction between pentraxin-3 and ficolin-2 could enhance complement activation on *Aspergillus fumigatus*. In this study, we found that pentraxin-3 could enhance complement activation on *P. aeruginosa* and phagocytosis (Ma et al 2011). Importantly, pentraxin-3 was present in induced

sputum from patients with bronchiectasis raising the possibility that this interaction is relevant in-vivo. Unlike CRP, where the collaboration is reported to only occur at low pH and in inflammatory conditions (Zhang et al 2009), ficolin-2 collaborated with Pentraxin-3 at physiological pH and osmotic conditions in the present study. Pentraxin-3 is critical to host defence against *P. aeruginosa* (Garlanda 2002). Mice lacking pentraxin-3 are more susceptible to *P. aeruginosa* infection and pentraxin-3 has been evaluated as a possible novel therapy for chronic *P.aeruginosa* infections (Moalli 2011). The apparent interaction between ficolin-2 and pentraxin-3 is an important area for future study.

Complement activation in human serum against *P. aeruginosa* occurs largely by the alternative pathway with a small contribution from the lectin pathway (Younger et al 2003). In mice, MASP-2 deficiency which completely prevents lectin pathway activation did not lead to increased susceptibility to *P. aeruginosa* pneumonia (Kenawy et al 2012). This is in contrast to work in mice by Moller-Kristensen which found that MBL knockout mice had increased susceptibility to *P. aeruginosa* infection following burn injury (Moller-Kristensen et al 2006), and to the increasing body of evidence of a role for the lectin pathway against *P. aeruginosa* infection in humans (Garred et al 1999, Zhang et al 2009, Chalmers et al 2011, Haerynck et al 2012). Several important differences between mice and humans may explain these contradictory findings. In mice, there are only 2 ficolins:.Ficolin A is present in liver and blood plasma and is analogous to ficolin-2 in humans, while ficolin B (60% identical) is expressed in bone marrow and spleen and is associated with macrophages, analogous to ficolin-1 (Fujimori et al 1998). Ficolin-3 is absent in

mice (Endo et al 2004). Although ficolin-A in mice is analogous ficolin-2 in humans, there are significant differences in binding specificity (Hummelshoj et al 2012). Mannose binding lectin is represented by two proteins (MBL-A and MBL-C) in mice compared to a single molecule in humans (Liu et al 2001). In addition, given the emerging evidence of a role for pentraxins in amplifying the lectin pathway, it important to note differences between human and murine pentraxins. In humans, C-reactive protein is the major acute phase reactant in human plasma (Smith 1995), in contrast to mice where serum amyloid P is the major acute phase reactant and CRP is virtually absent (Yuste 2007). Pentraxin-3 is present in mice and has been shown to be critical to response against *P. aeruginosa* as well as the fungal pathogen *Aspergillus fumigatus* (Garlanda 2002). Thus, while there are many similarities between murine and human lectin pathway components, there are critical differences that mean studies in mice cannot be easily extrapolated to humans.

It is interesting to speculate on how defects in the lectin pathway of complement lead to increased susceptibility to infection in patients with chronic lung disease such as non-CF bronchiectasis, cystic fibrosis and COPD. Low ficolin-2 and MBL levels are common in the general population and do not appear to significantly increase the risk of infections with *P. aeruginosa* or other micro-organisms in otherwise healthy individuals. The "double-hit" hypothesis holds that the lectin pathway of complement is redundant and that a second innate immune defect is required before lectin pathway deficiency becomes important (Fevang et al 2005). This explains the apparent importance of MBL deficiency in diseases as diverse as common variable immunodeficiency (Fevang et al 2005), HIV infection (Catano et al 2008) and CF (Chalmers et al 2011). An alternative hypothesis, that is supported by our finding that

pentraxin-3 enhances complement activation, is that the lectin pathway is important in the context of chronic inflammation. We speculate that local and systemic release of pentraxins may enhance complement activation via the lectin pathway at inflammatory sites such as the lung, under conditions that inhibit the other components of the complement pathway (Zhang et al 2009, Gout et al 2011, Ma et al 2011). Unique features of the lectin pathway are its resistance to high salt concentrations, neutrophil proteases and inhibitors such as SPS (Petersen et al 2001, Palarasah et al 2011). The high-salt hypothesis in cystic fibrosis proposes that high ionic strength disables multiple anti-bacterial mechanisms in the bronchiectasis airway (Bals et al 1998). Whether this hypothesis is relevant to the development of CF-associated bronchiectasis is a matter of debate (Matsui et al 1998), and the osmotic conditions of the non-CF bronchiectasis airway have not been described. It is true, however, that Ficolin-2 is resistant to high salt concentrations and ionic strengths that inhibit the alternative and classical pathways.

In summary therefore, the present study links 3 important mechanisms with *P. aeruginosa* colonisation in bronchiectasis: low expression of ficolin-2, the collaboration between ficolin-2 and pentraxin-3 and the loss of CD88 from airway neutrophils. CD88 and pentraxin-3 are already known to be critical to defence against *P. aeruginosa* but this is the first demonstration of the relevance of this in bronchiectasis (Hopken et al 1996, Garlanda et al 2002).

The in-vitro studies used in the present report were established methods identified from the literature for studying the lectin pathway and took advantage of the growing knowledge in this area (Petersen et al 2001, Palarasah et al 2011, Ma et al 2011) .

This improves external validity and reproducibility of the findings and strengthens the conclusions.

Conclusion

Ficolin-2 enhances phagocytosis of *P. aeruginosa* through complement activation under conditions of low serum concentration. In-vitro, ficolin-2 collaborates with pentraxin-3 and enhances complement mediated phagocytic clearance of *P. aeruginosa*. Significant neutrophil dysfunction was found in bronchiectasis airway neutrophils with neutrophil elastase implicated both in-vivo and in-vitro. CD88 cleavage by neutrophil elastase further impairs complement mediated defence against *P. aeruginosa* but opsonophagocytosis is still enhanced by ficolin-2 in dysfunctional bronchiectasis airway neutrophils. Ficolin-2 should be evaluated as a new therapeutic target in bronchiectasis.

Chapter 6

Mannose binding lectin and disease severity in bronchiectasis

6.1 Introduction

As previously discussed, bronchiectasis is a chronic inflammatory lung disease characterised by permanent dilatation of the bronchi (Pasteur, 2010). Patients suffer daily cough, sputum production and recurrent respiratory infections (Pasteur, 2010). Central to the pathogenesis of bronchiectasis is a vicious cycle of failed bacterial clearance, airway inflammation and airway structural damage (Cole, 1986). Patients become chronically colonised with pathogens due to a failure of host immune defences (Fuschillo, 2008). Recognised immunological defects associated with bronchiectasis include antibody deficiencies (common variable immunodeficiency, x-linked agammaglobulinaemia, IgG subclass deficiency), neutrophil and macrophage defects and secondary immunodeficiency (such as following chemotherapy or transplantation), but from published data, only around 7% of adult patients with bronchiectasis have an identifiable primary immunodeficiency (Nicotra et al, 1995, Pasteur et al, 2000, Kelly et al, 2003).The cause of adult bronchiectasis in the majority of adult patients is unknown.

Mannose binding lectin (MBL) is a soluble pattern recognition molecule of the innate immune system (Kilpatrick, 2002). MBL binds to glycoconjugates containing mannose, fucose or N-acetylglucosamine on the surface of a wide range of clinically important bacteria, viruses and fungi, activating the lectin pathway of complement (Neth et al, 2000, Moller-Kristensen et al, 2006). Through activation of the associated enzyme MBL-associated serine protease-2, MBL promotes opsonisation

of pathogens for phagocytosis (through complement component C3 or direct interaction with phagocytes), direct cell lysis (through generation of the membrane attack complex) and can also promote inflammatory cell chemotaxis and activation through generation of complement component C5a (Thiel et al, 1997, Wallis, 2007). MBL may also have a role in the resolution of inflammation in the lung through the clearance of apoptotic cells and in suppressing pro-inflammatory cytokine secretion (Hodge et al, 2010, Wang et al, 2011).

Deficiency in mannose binding lectin is one of the most common immunological defects in humans, affecting 10%-30% of the population depending on the definition of deficiency used (Eisen, 2010). MBL deficiency has been associated with recurrent respiratory infections, and is associated with the presence of bronchiectasis in patients with common-variable immunodeficiency (Fevang et al, 2005, Litzman et al, 2008)

In addition, although mannose binding lectin is not the cause of cystic fibrosis, several studies have found evidence that MBL deficiency modifies the course of disease in cystic fibrosis, leading to a more rapid decline in FEV_1, earlier acquisition of *Pseudomonas aeruginosa*, infection with *B. cepacia*, and death (Garred et al, 1999, Dorfman et al, 2008, McDougall et al, 2011, Chalmers et al, 2011).

We therefore sought to investigate whether mannose binding lectin deficiency was associated with disease severity in adult non-cystic fibrosis bronchiectasis.

6.2 Patient characteristics

The study included 470 patients with non-cystic fibrosis bronchiectasis and 414 healthy control subjects. The characteristics of the study population are shown in Table 6.1 (repeated from the previous chapter). There were no significant differences in demographics or co-morbid illnesses between bronchiectasis patients and controls. The majority (80.4%) of the cohort had idiopathic/post-infective bronchiectasis. None of the control subjects had a history of chronic respiratory disease and none were prescribed inhaled corticosteroids (table 6.1).

	Bronchiectasis cohort	Control cohort	p-value
N	470	414	
Age	67 (58-75)	66 (56-77)	0.2
Gender (% female)	259 (55.1%)	211 (51.0%)	0.2
BMI kg/m^2	25.4 (21.9-29.3)	Not recorded	n/a
Co-morbidities			
Chronic cardiac disease	108 (23.0%)	99 (23.9%)	0.7
Cerebrovascular disease	46 (9.8%)	42 (10.1%)	0.9
Chronic renal impairment	32 (6.8%)	33 (8.0%)	0.5
Diabetes mellitus	58 (12.3%)	41 (9.9%)	0.3
Causes of bronchiectasis			
Idiopathic/post infective	378 (80.4%)	Not applicable	
Previous ABPA	41 (8.7%)		
Connective tissue disease	28 (6.0%)		
Inflammatory bowel disease	14 (3.0%)		
Others	9 (1.9%)		
Medications			
Inhaled corticosteroid use	196 (41.7%)	0 (0%)	<0.0001

Table 6.1. Clinical characteristics of the study population. Note this is a duplication of table 3.1 and is included here for ease of reference.

6.3 Frequency of MBL deficiency in non-CF bronchiectasis patients and controls

The expected relationships between MBL haplotypes and serum levels were observed and are shown in figure 6.1. Patients with high expressing haplotypes had a median serum MBL of 2100ng/ml (1400-3200ng/ml), compared to 600ng/ml (400-1300ng/ml) in intermediate expressing haplotypes and 0ng/ml (0-200ng/ml) in patients with the low expressing MBL groups.

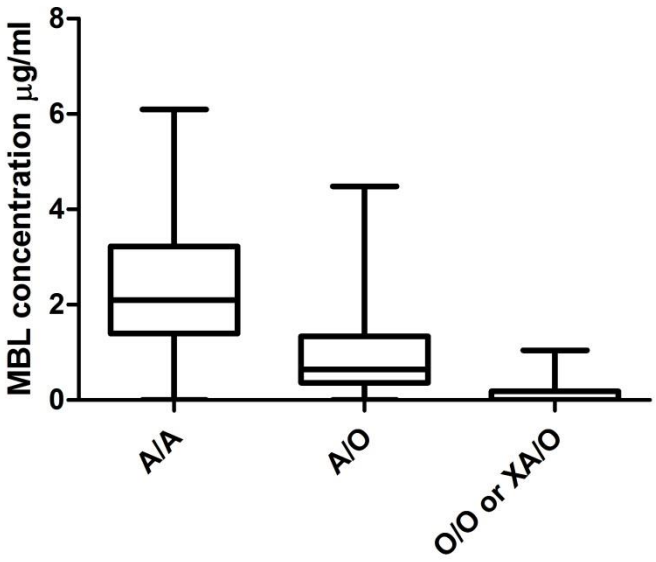

Figure 6.1. The relationship between MBL haplotypes and MBL serum levels. (all comparisons $p<0.0001$).

In examining genotype frequencies, there were no significant differences between bronchiectasis patients and controls. Table 6.2 shows the frequency of MBL haplotypes and the serum level comparisons between bronchiectasis patients and controls.

MBL group N	**Bronchiectasis** 470	**Healthy controls** 414	**p-value**
Low expressing groups YO/YO XA/YO	55 (11.7%) 18 (3.8%) 37 (7.9%)	42 (10.1%) 14 (3.4%) 28 (6.8%)	0.5 0.8 0.5
Intermediate expressing groups YA/YO XA/XA	135 (28.7%) 111 (23.6%) 24 (5.1%)	145 (35.0%) 117 (28.3%) 28 (6.7%)	0.06 0.1 0.3
High expressing groups YA/XA YA/YA	280 (59.6%) 119 (25.3%) 161 (34.3%)	227 (54.8%) 95 (22.9%) 132 (31.9%)	0.2 0.4 0.5
Serum levels <500ng/ml <200ng/ml	 128 (27.2%) 88 (18.7%)	 122 (29.5%) 79 (19.1%)	 0.4 0.8

Table 6.2. Genotype frequencies and serum levels in patients with non-CF bronchiectasis and controls.

6.4 MBL deficiency and chronic bacterial colonisation

Chronic bacterial colonisation was present in 76.8% of the population. *Haemophilus influenzae* was the most frequently isolated pathogen (141 patients, 30%), followed by *Pseudomonas aeruginosa* (68 patients- 14.5%), *M. catarrhalis* (54 patients- 11.5%), enteric gram negative organisms (46 patients- 9.8%), *S. aureus (*43 patients- 9.1%), *S. pneumoniae* (30 patients- 6.4%) (note that percentages add up to greater than 100% as some patients were colonised with more than one pathogen).

There was a higher frequency of bacterial colonisation (85.4%) in the MBL deficient group as determined by genotype (table 6.3) and serum levels (table 6.4). Differences between the intermediate expressing genotype group and the high expressing group were not statistically significant, p=0.4. Using both serum level cut-offs, there was a higher frequency of bacterial colonisation, *H.*influenzae and *P.aeruginosa* colonisation in the MBL deficient group, but this was not evident when the patients with the low expressing genotype were excluded, suggesting this effect was entirely due to these patients (p=0.9 for bacterial colonisation, p=0.9 for *H.influenzae* and

p=0.7 for *P.aeruginosa*). After adjustment for age, radiographic severity and FEV_1 % predicted, the logistic regression analysis did not show a statistically significant effect of MBL deficient genotypes on chronic colonisation – adjusted odds ratio (AOR) 1.85 95% 0.96-3.59,p=0.06. The relationship between MBL deficient genotypes and *P.aeruginosa* was not statistically significant after adjustment for age, radiographic severity and FEV_1 % predicted, AOR 1.25 95% CI 0.55-2.83,p=0.6.

There were no statistically significant relationships with other bacterial species and MBL deficiency, although the numbers in each group were small. Only one patient was chronically colonised with *B. cepacia* and this patient had O/O genotype.

Severity marker	MBL deficient	Intermediate group	High expressing group	p-value
N	55	135	280	
Age	63 (53-70)	65 (55-71)	63 (52-72)	0.9
Bacteriology				
Chronic colonisation	47 (85.4%)	82 (60.7%)	183 (65.4%)	0.004
H.influenzae	26 (47.3%)	36 (26.7%)	79 (28.2%)	0.01
P.aeruginosa	19 (34.5%)	13 (9.6%)	36 (12.9%)	<0.001
Pulmonary function				
FEV_1 % predicted	71.0% (43.8-85.6%)	71.4% (50-86.3%)	76.2% (56.7%-91.9%)	0.1
FVC % predicted	78.9% (60.9-96.2%)	84.6% (71.1-102%)	84.6% (70.9-99.8%)	0.2
Exacerbations and QOL				
Annual exacerbation frequency*	3 (2-6)	2 (1-4)	2 (1-4)	0.0002
Hospitalisations*	19 (34.5%)	29 (21.5%)	63 (22.5%)	0.1
SGRQ	55.4 (36.2-66.4)	46.6 (24.3-61.4)	41.6 (24.3-61.4)	0.001
LCQ	13.1 (9.4-17)	14.8 (11.5-17.7)	14.5 (10.5-17)	0.02
HRCT score	4 (3-15)	3 (2-6)	3 (2-6)	0.001
BMI kg/m^2	26.7 (23.6-30.9)	25.7 (21.8-29.6)	24.9 (21.7-28.7)	0.2

Table 6.3. Markers of severity in patients with MBL deficiency according to genotype. *in the year prior to the study. Data during prospective follow-up are presented under long-term prognosis. FEV_1= forced expiratory volume in 1 second, FVC= forced vital capacity. SGRQ= St. George's Respiratory Questionnaire, LCQ= Leicester cough questionnaire, HRCT= high resolution computed tomography, BMI= body mass index.

6.5 Pulmonary function and radiological severity of bronchiectasis

There was no significant relationship between MBL deficiency, defined using genotype or serum levels, and either FEV_1 % predicted or FVC % predicted (see tables 6.3 and 6.4). MBL deficient patients had more severe radiological bronchiectasis using the modified Reiff score (table 6.3), as did patients with an MBL <200ng/ml. This difference was not evident using serum levels <500ng/ml. There were no significant differences between intermediate and high expressing genotypes.

6.6 Exacerbation frequency, hospitalisations and quality of life

Baseline exacerbation frequency was higher in the MBL deficient group using genotype, mean per patient/year of 4·2 (standard deviation 3·4) in the deficient group vs 2·6 (2·5) and 2·7 (2·8) in the intermediate and high expressing groups,p=0·001 (table 6.3). There was no difference between the intermediate and high expressing genotype groups. Patients with serum levels <200ng/ml had more frequent exacerbations compared to those with higher MBL levels. (table 6.4). For quality of life, this secondary care population of bronchiectasis patients had marked impaired of quality of life. SGRQ scores were higher for MBL deficient patients by genotype (table 6.3). The difference was greater than the 4 point minimal clinically important difference,p=0·0069. There was no significant difference between intermediate and high expressing MBL genotypes (p=0·35). Using the Leicester cough questionnaire, patients with MBL deficient genotypes had more severe cough symptoms (table 3),p=0·029, with differences greater than the 1·3 minimal clinically important difference. There was no significant difference between the intermediate and high expressing groups, p=0·49. Serum levels did not show significant relationships with SGRQ but were associated with LCQ scores.

Severity marker	MBL "deficient" Serum <500ng/ml	MBL sufficient Serum ≥ 500ng/ml	p-value	MBL deficient Serum <200ng/ml	MBL sufficient Serum ≥ 200ng/ml	p-value
N	128	342		88	382	
Age	63 (53-72)	64 (52-72)	0.7	63 (56-71)	64 (52-72)	1.0
Bacteriology						
Chronic colonisation	94 (73.4%)	218 (63.7%)	0.04	67 (76.1%)	245 (64.1%)	0.03
H.influenzae	47 (36.7%)	94 (27.5%)	0.05	35 (39.8%)	106 (27.7%)	0.02
P.aeruginosa	29 (22.7%)	39 (11.4%)	0.002	22 (25.0%)	46 (12.0%)	0.002
Pulmonary function						
FEV_1 % predicted	71.7% (45.7-90%)	73.8% (55.7-90.9%)	0.2	71.4% (46.9-90)	73.7% (55.2-90.9)	0.3
FVC % predicted	84.9% (66.7-101%)	83.3% (70.7-99%)	0.9	80.7% (66.3-100)	84.5% (70.8-100)	0.3
Exacerbations and QOL						
Annual exacerbation frequency	3 (1-4)	2 (1-4)	0.1	3 (1-5)	2 (1-5)	0.03
Hospitalisations	39 (30.5%)	72 (21.0%)	0.03	32 (36.4%)	79 (20.7%)	0.002
SGRQ	48.8 (25.2-63.4)	44.1 (28.7-61.5)	0.6	49.6 (29.4-63.8)	44.1 (27.2-62.4)	0.1
LCQ	13.6 (10.6-17)	14.7 (10.0-17.9)	0.06	13.7 (9.5-17.8)	14.5 (10.6-17.6)	0.07
HRCT score	3 (2-9)	3 (2-6)	0.4	4 (2-12)	3 (2-6)	0.03
BMI kg/m^2	26.6 (22.6-30.2)	25.1 (21-7-28.6)	0.07	26.0 (22.2-29.7)	25.3 (21.8-29.1)	0.3

Table 6.4. Markers of severity in patients with MBL deficiency according to serum levels. Abbreviations: FEV_1= forced expiratory volume in 1 second, FVC= forced vital capacity. SGRQ= St. George's Respiratory Questionnaire, LCQ= Leicester cough questionnaire, HRCT= high resolution computed tomography, BMI= body mass index.

6.7 Measures of airways inflammation

Patients with MBL deficient genotypes had higher levels of airway inflammation ($p<0.05$ for all markers tested) overall. These differences were primarily due to higher bacterial loads in sputum for patients with MBL deficiency compared to those with intermediate and high expressing MBL genotypes (median cfu/ml $3.3x10^7$ (3.4×10^5-1.3×10^8) vs. 5.7×10^6 (1.5×10^5-6.2×10^7 vs. 4.8×10^6 (1.5×10^5- 6.0×10^7)),p=0.008.

After accounting for this by analysing groups according to bacterial load (figure 6.2), significantly higher levels of each marker were identified in the MBL deficient group at 10(8) cfu/ml or greater ($p<0.05$).

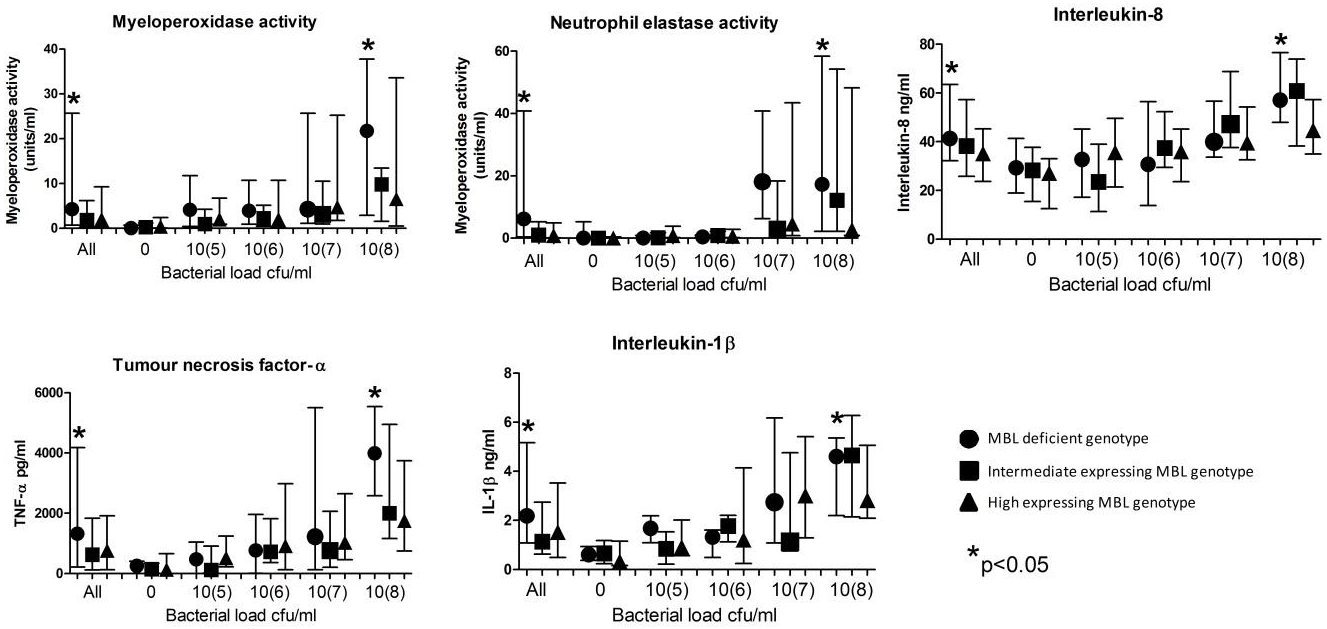

Figure 6.2. Measures of airway inflammation in MBL groups stratified by airway bacterial load. Data are presented as median (symbols) with interquartile range (bars). *comparisons by Kruskal-wallis test, all other comparisons $p>0.05$. X-axis shows log(10) bacterial load in cfu/ml.

6.8 Long term prognosis

*Longitudinal measurement of MBL serum levels*As previously reported, MBL serum levels are largely genetically determined and there was no statistically significant variation in MBL serum levels comparing the start, middle and end of the study ($p>0{\cdot}05$ for all comparisons, figure 4.3). In sub-analyses, changes in serum MBL over time did not correlate with deteriorating lung function or changes in bacterial colonisation but there was a significant increase in MBL serum levels at the onset of exacerbations (figure 6.3). The authors studied 24 patients with high expressing haplotypes, 26 patients with intermediate expressing haplotypes and 18 patients with MBL deficient haplotypes who attended for blood and sputum sampling at the onset of exacerbations and repeat measurement at completion of 14 days antibiotics. In the high expressing MBL group, MBL increased by a mean of 27·3% at start of exacerbation, $p=0.007$). The same pattern was observed in the intermediate expressing group, mean increase 20·2% but the difference was not statistically significant ($p=0{\cdot}37$). MBL deficient patients failed to upregulate MBL serum levels significantly during exacerbation (figure 6.3).

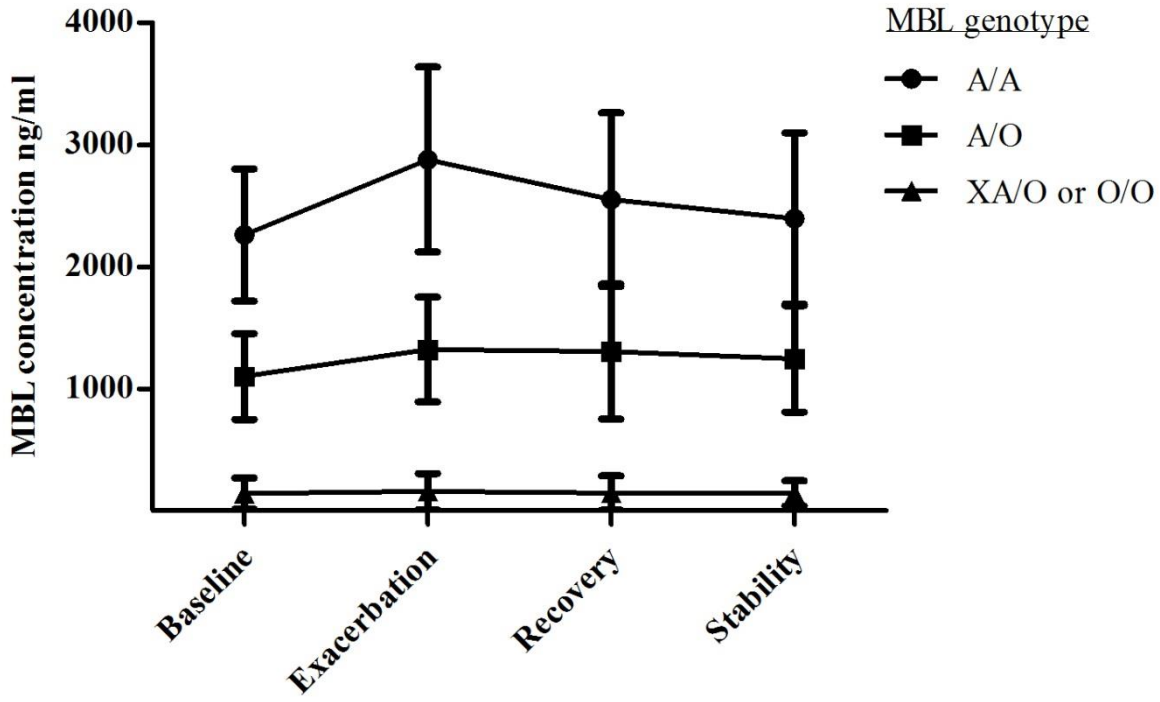

Figure 6.3. Repeat measurement of MBL serum levels during the study. A/A indicates the high expressing MBL haplotypes, A/O indicates the intermediate expressing MBL haplotypes. XA/O and O/O indicate the deficient MBL haplotypes. Blood samples were taken at recruitment into the study (baseline) on day 1 (onset of exacerbation) and day 14 (end of exacerbation) and repeated when clinically stable at least 3 months after exacerbation (stability). Data are shown as mean with (95% CI). Increase in MBL during exacerbation was only significantly different for the high expressing (A/A) haplotypes (see text).

Exacerbations and hospitalisation

Patients with MBL deficiency had a higher frequency of exacerbations and hospitalisations during follow-up. MBL deficient patients had a mean exacerbation

rate (per patient per year) of 2·7 (standard deviation 1·8) compared to patients with intermediate (mean 1·9 per patient per year, SD 1·2) or high expressing MBL genotypes (mean 1·9 per patient per year SD 1·3),p<0·0001. The mean difference between the low expressing and the other two groups was 0.72 95% CI 0.34-1.1 exacerbations per patient per year. A higher proportion of MBL deficient patients were hospitalised on at least 1 occasion during follow-up (49·1% in the MBL deficient group compared to 31·1% in the intermediate MBL group and 31·4% in the high expressing group, p=0·032).

Mortality

There were 42 deaths during follow-up, with 59.5% of these deaths deemed to be directly related to bronchiectasis or respiratory infections. In this exploratory analysis, there were 8 deaths in the group with MBL deficient genotypes, (14.5%), 10 deaths in the intermediate expressing group (7.4%) and 24 deaths in the high expressing MBL group (8.6%). Figure 6.4 shows the kaplain-meier analysis. The difference between the groups was not statistically significant by log-rank test (p=0.4). A cox-proportional hazard regression analysis adjusting for age, FEV_1, bacterial colonisation and radiological severity of bronchiectasis did not establish significantly higher mortality in the MBL deficient group (hazard ratio 1.58 95% CI 0.73-3.4,p=0.4). Similarly, although there was a trend towards more rapid decline in FEV_1 over four years in the MBL deficient group, this difference was not statistically significant (p=0.3).

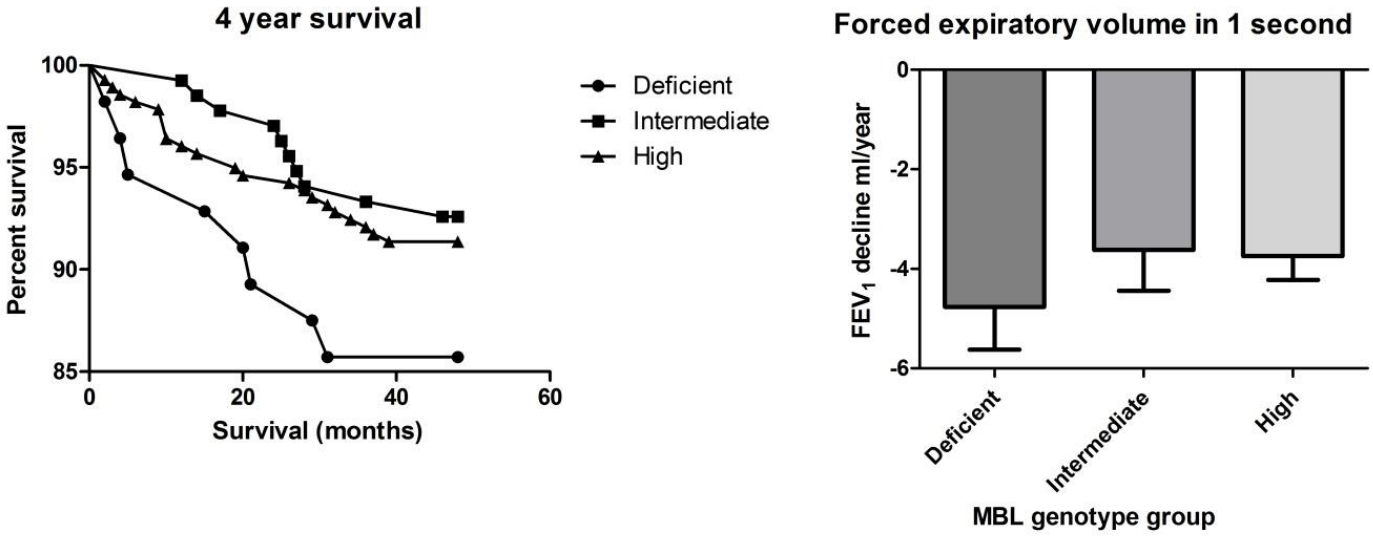

Figure 6.4. Long term prognosis in patients with bronchiectasis according to MBL2 genotype. A- survival over 4 years, B- Lung function decline over 4 years.

6.9 MASP-2 and Ficolin-3

There was no significant relationship between MASP-2 and Ficolin-3 serum levels with any of the clinical markers of severity investigated in this study. A summary of this data is shown, figure 6.5. All of the comparisons were not statistically significant ($p>0.05$).

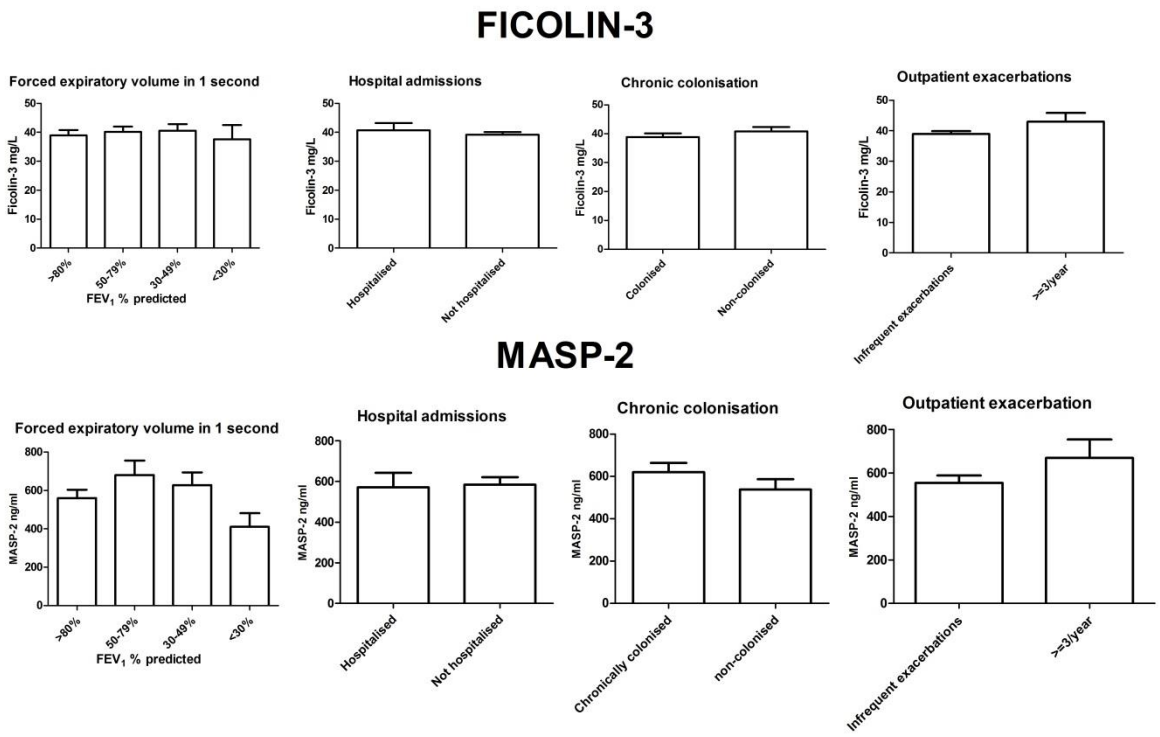

Figure 6.5. Relationship of ficolin-3 and MASP-2 serum levels with clinical severity in bronchiectasis. All comparisons $p>0.05$.

22 patients with bronchiectasis were heterozygous for the MASP-2 D120G polymorphism, with 1 patient homozygous and showing complete MASP-2 deficiency. There was a strong statistically significant relationship between the MASP-2 SNP and MASP-2 serum levels ($p<0.0001$), figure 6.6. 9 patients with bronchiectasis were heterozygous for the FCN3 polymorphism (L117fs). Ficolin-3 serum levels were significantly lower in this group ($p<0.0001$).

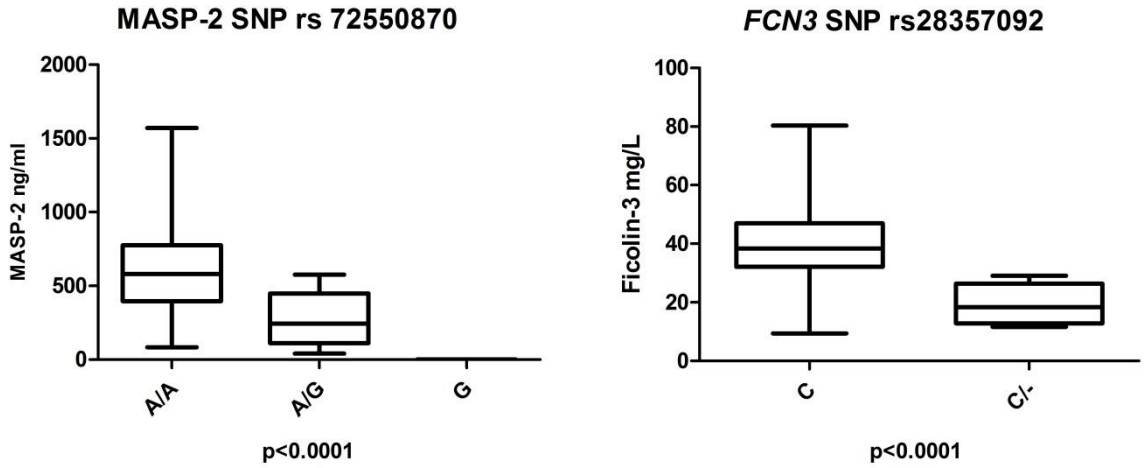

Figure 6.6. The relationship between MASP-2 and FCN3 SNP's with serum levels. $p<0.0001$ by Mann-Whitney U test comparing MASP-2 A/A with A/G and comparing FCN3 C with C/-.

There were insufficient patients with bronchiectasis and these MASP-2 and FCN3 SNP's to make meaningful statistical comparisons or conclusions about an effect on phenotype.

6.10 Co-inheritance of lectin pathway single nucleotide polymorphisms

As this study has identified a severity phenotype associated with SNP's in both the ficolin-2 and MBL genes, one hypothesis would be that co-inheritance of both low expressing ficolin-2 haplotypes and MBL deficiency combined would lead to a very severe phenotype. The study sample size was not sufficiently large to investigate conclusively whether this was the case.

No patients had combined MBL deficiency and two low expressing ficolin-2 haplotypes. 3 patients had MBL deficiency and a low/intermediate expressing ficolin-2 haplotype and 9 patients had MBL deficiency and intermediate/intermediate expressing ficolin-2 status. 7 patients had MBL deficiency based on genotype and a low serum ficolin-2 concentration. 6 patients had both MBL deficiency and heterozygosity for the MASP-2 D120G polymorphisms. The modifying effect of MASP-2 heterozygosity was investigated based on the previous findings of modification of MBL effects by two gene interactions (Olesen et al 2004, Dorfman et al 2008). 8 patients had one of the low or intermediate expression ficolin-2 haplotypes and heterozygosity for MASP-2 D120G.

The characteristics of these small numbers of patients are shown in table 6.5.

Severity marker	MBL deficient Ficolin-2 SNP's	MBL deficient Ficolin-2 serum <1600ng/ml	MBL deficient + MASP-2 heterozygous	Ficolin-2 low or intermediate expression + MASP-2 heterozygous
N	12	7	6	8
Age (years)	49 (9.2)	53.9 (24.1)	69.2 (16.5)	61.5 (17.8)
Aetiology				
Idiopathic/post infective	6	4	4	6
Previous ABPA	2	2	1	0
Connective tissue disease	1	1	0	1
Inflammatory bowel disease	2	0	1	1
Others	1	0	0	0
Bacteriology				
Chronic colonisation	11 (91.7%)	7 (100%)	4 (66.7%)	5 (62.5%)
H.influenzae	6 (50.0%)	3 (42.9%)	2 (33.3%)	4 (50.0%)
P.aeruginosa	5 (41.7%)	5 (71.4%)	1 (16.7%)	2 (25.0%)
Pulmonary function				
FEV_1 % predicted	72.5% (18.0)	50.5% (19.8)	71.6% (17.9)	77.5% (22.3)
FVC % predicted	85.7% (22.1)	79.8% (25.0)	84.0% (23.4)	86.9% (20.0)
Exacerbations and QOL				
Annual exacerbation frequency (median-IQR)	3 (2-8)	5 (3-8)	3 (2-4)	3 (1-5)
Hospitalisations	5 (41.7%)	6 (85.7%)	1 (16.7%)	3 (37.5%)
Reiff score (median-IQR)	5 (3-12)	12 (6-15)	3 (2-4)	4 (2-5)

Table 6.5. Characteristics of patients groups carrying more than one lectin pathway deficient single nucleotide polymorphism, or patients with MBL deficiency and low serum levels of ficolin-2. Abbreviations: FEV_1= forced expiratory volume in 1 second, FVC= forced vital capacity. SGRQ= St. George's Respiratory Questionnaire, LCQ= Leicester cough questionnaire, HRCT= high resolution computed tomography, BMI= body mass index.

6.11 Discussion

This study demonstrated a significant relationship between MBL deficiency and disease severity in a large cohort of patients with non-cystic fibrosis bronchiectasis.

Deficiency of mannose binding lectin arises from variants in exon-1 and in the promoter region of the *MBL2* gene. The exon-1 alleles designated B, C and D have a

profound dominant effect of MBL serum levels. Patients homozygous for exon-1 mutations or compound heterozygous for an exon 1 mutation and the X/Y promoter polymorphism, have very low serum MBL levels, typically less than 200ng/ml (Kilpatrick, 2002, Eisen, 2010, Chalmers et al, 2011).

This study identified that these patients, with the lowest serum MBL levels, have a higher frequency of chronic bacterial colonisation with a higher incidence of both *Haemophilus influenzae* and *P. aeruginosa*. Patients with MBL deficiency had more severe bronchiectasis as assessed by radiological scoring, and a higher frequency of outpatient exacerbations and hospitalisations for severe exacerbations over the period of follow-up. These patients also had greater impairment of quality of life and worse cough severity as assessed by the Leicester cough questionnaire. The differences in spirometry values were not statistically significant. Patients with MBL deficiency had significantly higher measures of neutrophil mediated airway inflammation, primarily related to higher bacterial loads present in the airways of patients with MBL deficiency. An exploratory analysis suggested a trend towards higher mortality rates and a faster decline in lung function in the MBL deficient group. Our study was not powered for this end-point and larger multicentre studies are needed to investigate this.

Importantly, this study did not find any evidence of an effect of intermediate expressing MBL genotypes on disease severity, with no significant differences observed in these markers of disease severity between intermediate expressing and high expressing MBL genotypes.

When we used definitions of serum MBL deficiency determined a priori (serum level <500ng/ml or <200ng/ml) we observed significant relationships between serum MBL and bacterial colonisation, hospitalisations, exacerbations and radiological severity. These differences were, however, entirely due to the presence of the most severe MBL deficient patients in this group, as exclusion of patients with low expressing MBL genotypes abolished these relationships. We therefore conclude that patients with intermediate expressing MBL genotypes are not at higher risk of severe disease.

We previously reported no differences between bronchiectasis patients and controls in serum MBL concentrations and have now confirmed these findings in a larger cohort (Kilpatrick, 2009). One previous study has investigated the role of impact of MBL deficiency on disease severity in 133 patients with non-CF bronchiectasis (MacFarlane, 2012). This study measured serum MBL levels but did not perform genotyping. This study found no difference in disease severity between patients with serum levels <600ng/ml compared to those with >600ng/ml. Patients with serum levels <100ng/ml had a trend towards more severe disease with more *P. aeruginosa* and *H.influenzae* and a higher frequency of exacerbations, but this group included only 13 patients and was underpowered to show significant differences. The results appear to therefore support the findings of our present study, indicating that only patients with severe MBL deficiency have a worse phenotype. There were other differences between the MacFarlane study and the current study. MacFarlane et al performed a retrospective study of patients having MBL measurements for clinical reasons and therefore included a much higher frequency of patients colonised with *P.aeruginosa* than has been typically reported in the literature (MacFarlane, 2012).

Recently an association between MBL levels and the progression of allergic bronchopulmonary aspergillosis and other aspergillus related lung diseases have been reported (Lambourne et al, 2009, Harrison et al, 2012). The present study excluded patients with active ABPA, and the group of patients reporting a past history of ABPA in this study was too small to make realistic conclusions.

Several large studies have now confirmed a relationship between mannose binding lectin deficiency and disease severity in cystic fibrosis. A meta-analysis of studies in adults with cystic fibrosis demonstrates that MBL deficiency is associated with early acquisition of *P. aeruginosa*, increased infections with *B. cepacia* and increased mortality (Chalmers et al, 2011). Importantly, the largest studies suggest that the poor prognosis associated with variant MBL alleles is only evident in those with low expressing MBL genotypes (O/O and XA/O) (Dorfman et al, 2008, McDougall et al, 2010). Our meta-analysis found no significant effect on lung function or markers of CF severity with the intermediate expression genotypes (Chalmers et al, 2011). This supports the findings of the present study that poor prognosis is associated with the low expressing genotypes but that intermediate MBL expression is not associated with greater disease severity.

The mechanism by which MBL predisposes to more severe disease is not entirely clear although it has been reported that MBL binds *P. aeruginosa*, *S. aureus* and *B. cepecia* along with other clinically relevant bacteria leading to complement activation and enhanced clearance (Neth et al 2000, Moller-Kristensen 2006). MBL deficient mice are susceptible to more severe infections with *P. aeruginosa* (after burn injury) and *S. aureus* than wild-type mice (Shi et al, 2004, Moller-Kristensen et al, 2006). Furthermore, although MBL is a serum protein, it accumulates in the lung

during inflammatory diseases in quantities sufficient to promote phagocytosis and cause complement activation (Reading et al, 1997, Fiddler et al, 2009). It is therefore possible that MBL deficiency leads to a failure to clear microorganisms through reduced opsonophagocytosis.

There are, however, other potential mechanisms not related to MBL's role in bacterial clearance. MBL appears to be important in clearance of apoptotic cells, a key mechanism for the resolution of inflammation (Haslett, 1999). Hodge et al observed low levels of MBL in the airway of subjects with COPD (Hodge et al, 2008) and, importantly, this correlated with reduced apoptotic cell clearance. Azithromycin was found to enhance mannose receptor expression on macrophages and restore defective apoptotic cell clearance (Hodge et al, 2008). The importance of MBL was confirmed in smoke-exposed mice, where treatment with MBL improved the resolution of cigarette smoke induced inflammation (Hodge et al 2010).

The relationship of MBL with *P. aeruginosa* and other organisms is controversial. MBL has been shown to bind to *P.aeruginosa* in some studies and not in others (Neth et al, 2000, Moller-Kristensen et al, 2006). We investigated MBL binding extensively as described in chapter 3.1 and found no binding of MBL to *P. aeruginosa* by any of the methods tested. Studies in MASP-2 deficient mice, which are unable to activate complement via the lectin pathway, demonstrate no significant role for the lectin pathway in defence against *P.aeruginosa* in mice (Kenawy, 2011). The alternative pathway is the major mechanism for defence against *P.aeruginosa* in health (Younger et al, 2003). Despite this, both this study and the literature from cystic fibrosis suggest a relationship between MBL deficiency and *P. aeruginosa*. During acute inflammation there is increased production of acute phase reactants,

including the pentraxins: C-reactive protein, serum amyloid P and pentraxin-3. Recent data suggest that these interact with MBL to enhance the lectin pathway (Ma et al, 2011). This may explain why MBL deficiency becomes important during inflammatory diseases such as bronchiectasis, or following burn injury as reported by Moller-Kristensen et al (Moller-Kristensen et al, 2006).

What are the implications of this study? The identification of a group of patients at higher risk of exacerbations and poor outcome should stimulate further research to improve outcomes in bronchiectasis. Currently, there are few evidence-based treatments for this disease (Pasteur, 2010). MBL replacement therapy has been developed and has been suggested as a new therapeutic avenue for diseases associated with MBL-deficiency (Garred et al, 2002). Hodge et al demonstrated that azithromycin could restore the failure of apoptotic cells associated with MBL deficiency, and raises the question of whether future trials should target long-term macrolide treatment for patients with MBL deficiency to improve outcome.

Limitations of our analysis must be acknowledged. The number of patients with severe MBL deficiency was small (n=55) and larger studies arising from multicentre collaborations are now needed. Others have found that the impact of MBL can be modified by other genes including TGF-beta and can be influenced by linkage disequilibrium with other genes including other collectins (Dorfman et al, 2008, Garcia-Laorden et al, 2012). Our study was designed in 2008 before these effects were described: future studies of MBL in bronchiectasis should take these influences into account.

Conclusion

Mannose binding lectin deficiency is associated with disease severity in non-cystic fibrosis bronchiectasis.

CHAPTER 7

General discussion and future work

7.1 Ficolin-2 in bronchiectasis

The lectin pathway of complement has now been linked with diverse processes including innate immune response to bacteria (Kilpatrick and Chalmers, 2012), viruses (Pan et al, 2012) and fungi (Ma et al, 2009), the clearance of apoptotic cells (Kuraya et al, 2005), coagulation (La Bonte et al, 2012) and ischaemic reperfusion injury (Schwaeble et al, 2011). Investigations in these areas are at an early stage but suggest that this pathway may be critical to many pathologies and therefore of relevance to a number of human diseases.

Compared to the intensively studied mannose binding lectin, little is known to date about the physiological function, the regulation or disease associations of the ficolins in humans (Kilpatrick and Chalmers, 2012).

This thesis has described an association between single nucleotide polymorphisms causing low serum levels of ficolin-2 and disease severity in bronchiectasis. Defining low levels of ficolin-2 was challenging because no established or accepted definition of "deficiency" exists and because large variations in ficolin-2 serum levels are observed, even among patients with the same genotype (Hummelshoj et al, 2005). We attempted to correlate serum levels with combinations of *FCN2* haplotypes and found that the serum level could be predicted with a greater degree of accuracy and that low serum levels could therefore be identified most accurately using a combination of low, intermediate and high expressing haplotypes, with the lowest levels observed in patients with 2 low expressing haplotypes or with a combination

of low and intermediate expressing alleles. This analysis should be replicated in a non-diseased cohort in larger numbers of patients but has the potential to become the standard method for defining the functional relevance of *FCN2* genotypes.

We found this method to be significantly more predictive of ficolin-2 serum levels, using the area under the receiver operator characteristic curve, than using individual SNP's alone. This approach is used successful in research with the closely related molecule mannose binding lectin where haplotypes can be used to predict serum levels and deficiency with a high degree of accuracy (Kilpatrick, 2002).

Having defined low levels of ficolin-2 in this way, we demonstrated a correlation between low expressing ficolin-2 genotypes and serum levels with accepted markers of disease severity, including exacerbation frequency, hospitalisation for severe exacerbations and health related quality of life. There was also a striking relationship between low ficolin-2 levels and *P. aeruginosa* colonisation.

There was also an apparent different in the frequency of ficolin-2 haplotypes between patients with bronchiectasis and controls. We used spouses and partners of patients attending the clinics at the Royal Infirmary of Edinburgh to provide a population that was largely matched by age, gender and to some extent environment and socioeconomic status. We observed lower levels of serum ficolin-2 in patients than in controls, confirming previous findings in a pilot cohort (Kilpatrick et al, 2009), and we observed a higher frequency of the high expressing AGGTG haplotype in controls. Possible explanations for this are that high ficolin-2 levels protect against the development of bronchiectasis. This is speculative, but as the true aetiology of bronchiectasis is unknown, this warrants further study. Perhaps a more

likely explanation given our other findings is that patients attending a specialist clinic at the Royal Infirmary of Edinburgh are more likely to be selected on the basis of more severe disease. Therefore the low frequency of patients with the AGGTG haplotype may reflect a reduced severity in these patients rather than a true increased susceptibility to bronchiectasis. This hypothesis requires further study.

Future work in this area will focus on replicating the findings of the present study in a larger cohort of patients with bronchiectasis from different centres. The present study is the largest genetic study of non-CF bronchiectasis that the authors are aware of and one of the only studies to describe genetic associations with disease severity. Previous studies have investigated the role of CFTR mutations (the cause of cystic fibrosis) in patients with diffuse bronchiectasis not due to cystic fibrosis and suggested that this may modify the disease presentation to some extent (Bienvenu et al, 2010). Multicentre collaborations are now underway in the United States (Aksamit et al, 2012) and within the UK with genetic analysis of large patients groups one of the stated aims of such collaborations (De Soyza et al, 2012). This may provide the opportunity to replicate the present findings on a large scale. There are several end-points associated with survival, such as decline in lung function and particularly survival that will only be adequately addressed by large, well powered and longitudinal studies involving multiple centres.

Prior to these confirmatory studies, it would be inappropriate to make any recommendations about the implications of our findings to clinical practice. There is no evidence at this stage that patients with low expressing ficolin-2 haplotypes

should be managed differently. If our results are confirmed in a larger cohort, it may be that such patients would benefit from more intensive follow-up, surveillance for bacterial colonisation, *P. aeruginosa* eradication and perhaps long term prophylactic antibiotic therapy. There is an ongoing debate in the literature about how best long term antibiotic therapy should be targeted in bronchiectasis and better ways of predicting long term outcome in this disease are needed (Wilson and Wells, 2012).

To aid further studies of ficolin-2 and susceptibility to disease it would be beneficial to study a large cohort of healthy individuals for ficolin-2 serum levels and to identify the optimal method of defining low ficolin-2 expression. We have made an attempt to do so in the present study, but it is likely that other *FCN2* single nucleotide polymorphisms affecting serum levels will be identified. Within the currently accepted haplotype groups there is wide variation in ficolin-2 serum levels and a dedicated genetic study examining large numbers of *FCN2* SNP's and serum levels in healthy individuals would help to better define this. We attempted to identify some non-genetic factors that may influence circulating ficolin-2 levels, but did not identify a significant impact of hormonal or inflammatory stimuli, in-vitro or in-vivo. Further studies to identify the regulators of ficolin-2 expression in humans are needed.

This study utilised traditional culture based microbiological techniques to identify and quantify bacterial species in sputum samples and to define colonisation. Newer molecular techniques utilising the bacterial 16s rRNA gene have been developed and are transforming our view of respiratory tract microbiology (Blaser, 2010). It was

traditionally held that the healthy airway is sterile but this appears not to be the case with a very diverse microbiome evident in healthy control subjects (Charlston et al, 2011). All such studies should be treated with a degree of caution at this stage as the upper airway is not sterile and some of these studies may be confounded by contamination from the upper respiratory tract. Nevertheless, studies in cystic fibrosis and chronic obstructive pulmonary disease reveal extraordinary diversity of bacterial species within respiratory samples, with often >1,000 individual 16S rRNA sequences identified in each sample (Sze et al, 2012, Fodor et al, 2012). Less than 1% of bacteria can be cultured using traditional laboratory methods as employed in the present thesis. It appears that in patients in whom a single organism, such as *Haemophilus influenzae* is cultured from sputum we may be missing at least 99.99% of the bacterial composition of the lower respiratory tract (Zemanick et al, 2011). The clinical relevance of these other organisms is yet to be defined. Future work will certainly involve trying to correlate innate immune defects in bronchiectasis with the findings of these novel microbiological assays. Airway infection is a major clinical problem in cystic fibrosis related bronchiectasis and chronic obstructive pulmonary disease. A logical extension of the present work would be to test for single nucleotide polymorphisms of the ficolin-2 gene in these populations, where *P. aeruginosa* colonisation is common. A small study has already been published suggesting a relationship between SNP's in *FCN2* and earlier *P. aeruginosa* colonisation and larger studies are now needed (Haerynck et al, 2012).

The surprisingly high prevalence of *P. aeruginosa* in patients with low expressing ficolin-2 haplotypes led us to investigate the role of ficolin-2 in opsonophagocytosis of *P. aeruginosa* and other bacteria in in-vitro. These studies were complicated by

the lack of an established assay for selectively studying the lectin pathway of complement, while inhibiting the classical and alternative pathways. We used C1q depleted sera to prevent activation of the classical pathway and either high ionic strength or low serum dilutions to inhibit the alternative pathway. These assays resulted in conditions which permitted study of the lectin pathway in relative isolation. Without these conditions the alternative pathway was found to be the dominant pathway for complement activation on *P. aeruginosa* with only a small contribution from the lectin pathway. These results would suggest that in the absence of another immunodeficiency, ficolin-2 deficiency would not greatly increase susceptibility to systemic *P. aeruginosa* infection. This is consistent with recent findings from a mouse model of pneumonia in which the lectin pathway of complement was found to be non-essential for house defence against intranasal *P. aeruginosa* infection in MASP-2 knockout and wild type mice (Kenawy et al, 2012). In bronchiectasis, however, the infection is not systemic but localised and affects the bronchial mucosa. The mechanisms of host defence in this context are less well understood but it is known that serum components are present at much lower concentrations and that the alternative pathway is relatively inactive (Younger et al, 2003). We therefore hypothesise that ficolin-2 may diffuse into the lung during acute and chronic inflammation and enhance opsonophagocytosis of *P. aeruginosa* and other organisms. The animal models of bronchiectasis are limited and so further work to determine the contribution of ficolin-2 to airway mucosa defence will require in-vitro studies. Specifically, it would be important to establish the microbial spectrum against which ficolin-2 is active and whether susceptibility to infections extend beyond *P. aeruginosa*.

Ficolin-2 binding to carbohydrate ligands is necessary for complement activation via the lectin pathway. We did not find any activity of ficolin-2 in the absence of complement and further study of how ficolin-2 binding triggers complement activation would be important. Similarly, we have not yet identified the ligand(s) on *P. aeruginosa* and other bacteria to which ficolin-2 binds. This would be important if the therapeutic potential of ficolin-2 is to be realised. Ultimately, I hope a greater understanding of how ficolin-2 is able to opsonise and promote clearance of bacterial pathogens may lead to the development of a new therapeutic approach to this disease that presenting has few effective treatments.

7.2 Mannose binding lectin

This study also observed a significant association between mannose binding lectin deficiency and disease severity in bronchiectasis. The finding of a strong association with *P.* aeruginosa infection, chronic colonisation and the frequency of exacerbations would be consistent with data from cystic fibrosis (Chalmers et al, 2011). Several large studies in cystic fibrosis, which were consolidation into a meta-analysis in chapter 1 of this thesis, support the view that MBL deficiency is associated with a more rapid progression of the disease, worse lung function and premature death.

The present study is the first demonstration of this disease modifying effect in bronchiectasis. As with the data on ficolin-2, confirmation in an independent and larger cohort is required before any recommendations about clinical practice can be suggested.

The results of our in-vitro studies raise important questions about how MBL may be modifying disease severity in bronchiectasis. MBL deficiency was found to be associated with a higher frequency of *P. aeruginosa* colonisation in patients, and yet in-vitro MBL did not bind to *P. aeruginosa* and opsonisation with rMBL did not enhance neutrophil or macrophage phagocytosis of *P. aeruginosa*. Ficolin-2 was found to be the major lectin pathway component responsible for opsonisation of *P. aeruginosa* with no significant contribution from MBL. How then, can this association be explained?

Although not involved in host defence against *P. aeruginosa*, MBL does bind to and opsonise a number of important pathogens including *Aspergillus fumigatus* (Crosdale et al, 2001), multiple viruses include influenza virus (Kase et al, 1999), and bacteria including *S. aureus* (Shi et al, 2004). It is possible to susceptibility to other pathogens such as viruses causes recurrent exacerbations and that this leads to progressive lung damage, with *P. aeruginosa* colonisation an indirect consequence of the lung damage rather than being directly due to a failure of phagocytic clearance.

Similarly, MBL has been shown to bind to, and enhance the clearance of apoptotic cells (Kuraya et al, 2005). MBL deficient mice have a defect in apoptotic cell clearance in-vivo although unlike C1q deficiency which is associated with autoimmunity, MBL deficiency does not appear to contribute to autoimmunity (Stuart et al, 2005). Apoptotic cell clearance by macrophages is a critical mechanism for the resolution of inflammation as failure to clear these cells results in secondary necrosis and release of pro-inflammatory intracellular contents into surrounding tissues (Savill et al, 2002). This is thought to be an important contributor to inflammatory diseases and autoimmunity. The neutrophil is the dominant cell type in

the airway of patients with bronchiectasis and a potentially important future area of study would be to establish if MBL is involved in clearance of apoptotic neutrophils by macrophages (Rossi et al, 2007). Failure of this mechanism in the bronchiectasis airway would lead to enhanced lung damage by neutrophil granule contents. I speculate that our findings of more severe lung disease and chronic colonisation with bacteria may be a consequence of this process, rather than a failure of opsonophagocytosis of bacteria, since we were unable to demonstrate any such defect in-vitro with MBL.

MBL deficiency is potentially clinically important because of the availability of therapies that can reverse the basic defect associated with MBL deficiency. Recombinant MBL has been successfully administered to mice under experimental conditions to rescue phenotypes associated with MBL deficiency. MBL-null mice infected with *S. aureus* had reduced survival compared to wild-type but this was reversed by the administration of rMBL (Shi et al, 2005).

In early trials in humans, plasma derived MBL and rMBL administered to restore MBL to normal levels (>1000ng/ml) in those with MBL deficiency was shown to be safe and was not immunogenic (Valdimarsson et al, 2004). Recent data suggest that although administration of MBL replacement restored serum levels to normal, higher than normal doses were required to restore normal complement activation capacity (Brouwer et al, 2009). MBL has to be given as regular infusions, making chronic treatment potentially impractical. There have been no phase II or phase III trials of MBL replacement therapy and the only limited evidence of efficacy comes from uncontrolled case reports of patients given MBL replacement on compassionate grounds (Garred et al, 2002).

If MBL deficiency identifies a sub-group of patients at risk of bacterial colonisation, airway inflammation and disease progression, this would argue strongly for a targetted approach to treatment. There is evidence that azithromycin can reverse the phagocytic defects caused by MBL deficiency in-vitro and in-vivo (Hodge et al, 2008). Azithromycin causes up-regulation of mannose receptor on macrophages which enhances the uptake of apoptotic cells, reversing a failure of apoptotic cell clearance demonstrated with MBL deficiency (Hodge et al, 2008). It is now known that azithromycin is an effective treatment for bronchiectasis and that long term azithromycin reduces exacerbations in COPD (Wong et al, 2012, Albert et al 2011). The optimal group of patients to benefit from this treatment is unknown. Macrolides have a number of side effects including not just gastrointestinal effects but also a significant risk of hearing impairment (Albert et al, 2011) and in some studies, cardiovascular events (Jespersen et al, 2006). It is therefore unlikely that azithromycin will be a treatment suitable for all patients with bronchiectasis, but may be targeted to patients most at risk of complications. A pharmacogenetic approach to treatment, in which MBL deficient patients are given azithromycin to reverse their increased risk of disease progression, is the ultimate objective of this work.

7.3 Neutrophil function in bronchiectasis

Bronchiectasis is a neutrophil driven disorder. Neutrophils are the most frequent cell type identified in the bronchiectasis airway (Eller et al, 1994, Angrill et al, 2002), and neutrophil derived granule products and in particular, neutrophil elastase have

been directly linked to disease progression and severity (Tsang et al, 2000, Lloberes et al, 1992). Despite this, relatively little is known in the literature about neutrophil function in non-CF bronchiectasis. Pasteur et al found no abnormal values of peripheral neutrophil surface markers in bronchiectasis (Pasteur et al, 2000) and Voglis et al demonstrated phagocytic dysfunction in sputum neutrophils in bronchiectasis induced by exposure to neutrophil neutrophil peptides (α-defensins) (Voglis et al, 2009). Our study is therefore one of the first to extensively study neutrophil function in non-CF bronchiectasis. We found no difference in peripheral blood neutrophil receptor expression or phagocytosis in bronchiectasis compared to healthy controls. This finding is not unexpected, as the majority of patients with idiopathic bronchiectasis do not have an increase in susceptibility to infection out-with the respiratory tract and therefore a generalised neutrophil disorder or neutrophil dysfunction would not be expected in these patients. Sputum neutrophils, however, showed significant changes compared to peripheral blood neutrophils. Reduced levels of the phagocytic receptor FcγRIIIb and complement receptor 1 were demonstrated, with an apparent correlation between the level of expression and the severity of lung disease. Furthermore, we demonstrated a reduction in CD88, the C5a receptor that correlated with the severity of lung disease and was found to be due to cleavage through neutrophil elastase.

There are certainly limitations to the methods used in these neutrophil studies. Significant numbers of neutrophils could not be obtained from healthy control sputum and so comparisons were made with maximally activated peripheral blood neutrophils. Although this is an approach that has been used previously (Berger et al, 1989) it is not a direct comparison of like with like and a superior study design

would be to use cells obtained by bronchoalveolar lavage, where cells could also be obtained from healthy controls.

Our study was concerned primarily with opsonophagocytosis and complement activation through the lectin pathway of complement and so our studies of neutrophil surface markers focussed on those involved in neutrophil adhesion, activation and phagocytosis. It is therefore likely we present a very incomplete view of the neutrophil dysfunction in the airway in bronchiectasis. Further studies will be needed to investigate neutrophil properties such as chemotaxis (Yoshikawa et al, 2007) and neutrophil extracellular trap formation (Young et al, 2011) which may be equally important as phagocytosis in the pathophysiology of chronic lung disease.

Neutrophil elastase appears to be a key mediator of disease severity in bronchiectasis and was found to be strongly associated with bacterial infection in this study. Elastase was reduced by short or long term antibiotic treatment, suggesting the hypothesis that antibiotic treatment may, to some extent, reverse the defects seen in bronchiectasis neutrophils. An important area for future study would be to investigate whether the abnormalities we observed in sputum neutrophils could be reversed by antibiotic or anti-inflammatory treatment. Neutrophil elastase has long been recognised as a target for therapy in chronic lung disease and oral neutrophil elastase inhibitors are currently undergoing phase I and phase II clinical trials (Stevens et al, 2011). We await the results of such trials with interest.

Failure of bacterial clearance in bronchiectasis is likely to be multi-factorial and a greater understanding of the mechanisms underlying chronic bacterial colonisation, including the role of ficolin-2, MBL and other innate immune molecules are needed

In conclusion, I have shown that single nucleotide polymorphisms in the lectin pathway of complement activation molecules ficolin-2 and mannose binding lectin are associated with disease severity and bacterial colonisation in bronchiectasis. The clinical and therapeutic implications of these findings require further study but may open a new avenue for treatment in this orphan disease.

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APPENDIX 1

Published manuscripts arising from this thesis