IMMUNE ACTIVATION IN ALPHA-1 ANTITRYPSIN DEFICIENCY RELATED AND USUAL CHRONIC OBSTRUCTIVE PULMONARY DISEASE

By

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A Thesis submitted to the University of Birmingham for the degree of DOCTOR OF MEDICINE

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October 2017

Abstract

A polyclonal increase in free light chains (FLCs) has been observed in a number of chronic inflammatory and autoimmune conditions and may be considered a biomarker of adaptive immune activation. The aim of this study was to examine whether FLCs could be a useful clinical biomarker in alpha-1 antitrypsin deficiency (A1ATD) and chronic obstructive pulmonary disease (COPD).

Combined (κ & λ) FLCs (cFLCs) were measured in patients with A1ATD and usual COPD in the stable state and assessed for association with clinical phenotype, disease severity, bacterial colonisation and mortality. The relationship of FLCs to total immunoglobulin levels was also examined in the COPD cohort. In addition, FLCs were measured in a small cohort of patients with bronchiectasis to further examine the relationship to bacterial colonisation.

Circulating cFLCs were static in the stable state in both A1ATD and usual COPD. Levels were significantly higher in patients with chronic bronchitis and airway bacterial colonisation in A1ATD. After adjusting for renal function and age the relationship between cFLCs and lung function was weak, however cFLC levels greater than normal significantly associated with mortality in both COPD cohorts. In conclusion, cFLCs may be a promising biomarker for risk stratification in A1ATD and COPD.

Dedication

This thesis is dedicated to my grandfather Dr Hugh Brebner.

Acknowledgments

I would like to thank all the members of the ADAPT team whom without this would not have been possible. Anita Pye and Rebecca Bray for all the administrative help and support in searching historical patient records. Ross Edgar and Diane Griffiths for navigating me through the clinical databases. Michael Ungurs and Pratibha Bharadwa for their assistance in sample collection and performing the microbiological tests on sputum samples. Dr Peter Nightingale for his guidance on statistical analyses.

I would also like to thank Anne Burmeister, Lakhvir Assi and The Binding Site Group Ltd for performing the free light chain assays on all the patient samples.

Finally, I would like to thank my supervisors Professor Robert Stockley and Dr Alice Turner for their great support throughout my research.

My research was funded through a non-commercial educational grant from Grifols.

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CHAPTER 1: Introduction

1.1 Chronic Obstructive Pulmonary Disease[1]

Chronic obstructive pulmonary disease (COPD) is a major global public health problem with a significant associated economic burden on both developing and higher income countries. In 2004, COPD was the fourth leading cause of death worldwide (2) and due to projected increases in tobacco use, it has been predicted by the World Health Organisation (WHO) that it will become the third leading cause by 2030 (3). The Global Initiative for COPD (GOLD) has defined it as *"a common preventable and treatable disease which is characterised by persistent airflow limitation that is usually progressive and associated with enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases"* (4).

The pathophysiology of COPD is complex resulting from a variety of gene-environment interactions and there is there is considerable phenotypic heterogeneity expressed amongst disease sufferers (5). Within the last decade, studies have shown the prevalence of COPD to be much higher than previously realised (6, 7) and the proportion of patients with COPD that remain undiagnosed has been reported to be as high as 66- 73% (8, 9). The prevalence of COPD increases with age. A large international population based study found the prevalence of at least moderately severe COPD was usually less than 5% in people aged 40-49 but in those over 70 it ranged from 19 - 47% in men and 6 - 33% in women (6).

[1] Excerpts of this chapter have been previously published (1. Brebner JA, Turner AM. Early chronic obstructive pulmonary diease: Beyond spirometry. World Journal of Respirology. 2013;3(3):57 - 66.)

For the purpose of this thesis I will refer to non-alpha-1-antitrypsin deficiency (A1ATD) related COPD as 'usual' COPD.

1.1.1 Clinical features of COPD

In the early stages of COPD, patients can be relatively asymptomatic. As the disease progresses key symptoms emerge including chronic cough, dyspnoea and sputum production (4). Some patients produce sputum daily and the generally accepted Medical Research Council (MRC) definition of chronic bronchitis is *"a cough with sputum production for at least 3 months of 2 consecutive years"* (10). Breathlessness is usually progressive and exacerbated by exertion. Patients may also complain of wheezing, chest tightness, weight loss and fatigue (4). Weight loss in particular is usually a symptom of more advanced disease and a low body mass index (BMI) has been shown to be a predictor of mortality (11). There can often be discordance between symptomatic burden and severity of airflow limitation (12). A study specifically designed to explore whether symptoms predict the presence of COPD found that 92% of the smokers with airflow obstruction reported symptoms such as cough, dyspnoea, sputum production and wheeze. However, the same symptoms were also reported in 76% of smokers with normal spirometry (13).

Patients with COPD may suffer from intermittent 'exacerbations' of their lung disease. An exacerbation has been defined as: *"A sustained worsening of the patient's condition from the stable state and beyond normal day-to-day variations that is acute in onset and may warrant additional treatment in a patient with underlying COPD"* (14).

Frequent exacerbations have been shown to impact significantly on quality of life and are associated with a more rapid clinical decline in lung function and increased risk of mortality (15-17). More recently the contribution of exacerbations and comorbidities to the disease burden in COPD has been highlighted. Formal tools for assessing symptoms and health status such as the COPD assessment test (CAT) have now moved from being primarily used as an end-point in clinical trials to part of the routine assessment of patients with COPD (4).

1.1.2 Diagnosis and Treatment

1.1.2.1 Diagnosis

Spirometry is the primary tool for the diagnosis of COPD. The GOLD and joint American Thoracic Society (ATS) and European Respiratory Society (ERS) guidelines advise physicians to consider spirometry in patients presenting with symptoms suggestive of COPD with a history of exposure to a risk factor such as smoking or occupational dust. In the context of such symptoms, the presence of a post-bronchodilator FEV_1/FVC ratio <0.7 demonstrates incompletely reversible airflow limitation and hence a diagnosis of COPD (FEV_1: Forced expiratory volume in 1 second, FVC: Forced vital capacity) (4, 18). The FEV_1 as a percentage of its predicted value for the patient's sex, age and height is used to sub-classify patients with respect to the severity of their airflow obstruction (mild $FEV_1 \geq 80\%$, moderate $\geq 50\%$ $FEV_1 < 80\%$, severe $\geq 30\%$ $FEV_1 < 50\%$, very severe $\leq 30\%$)(19). Advances in technology, including the advent of simple to use hand held spirometers makes it a convenient test which can be easily performed in the outpatient clinical setting.

Although this fixed cut-off is easy to remember it does not take in to consideration the fact that the FEV_1/FVC ratio reduces with age (20) resulting in the potential over-diagnosis of COPD in the elderly population (21) and under-diagnosis in the young (22). Other approaches to interpreting spirometric values have therefore been advocated, in particular the use of statistically derived 'lower limit of normal (LLN)' reference values (23). In comparison to the fixed ratio method this has been shown to reduce the number of people potentially misclassified as having significant airflow obstruction (24-26).

In addition to spirometry other lung function tests can provide further information in the diagnosis and phenotyping of patients with COPD. Reductions in the transfer factor of the lung for carbon monoxide (TLCO) are deemed more reflective of alveolar destruction which is an important feature of COPD. Indeed, it has been recognised that smokers with an isolated reduction in TLCO are more likely to go on to develop airflow obstruction compared to smokers with a normal TLCO (27).

High resolution computed tomography (HRCT) scans are also pivotal in the more detailed assessment of patients with COPD (28). In particular they can provide important phenotyping information by examining the extent, type and distribution of emphysema. Emphysema can be centrilobular, panlobular or paraseptal (Figure 1.1) and is seen as areas of low attenuation within the lung fields of CT images. More advanced scanning techniques whereby the degree of emphysema present is quantified (by declaring lung tissue below a certain threshold density as emphysematous) have evolved (28). There is significant heterogeneity exhibited amongst patients with respect to the extent and distribution of their emphysema. How this relates to the phenotypic presentation and prognosis is an area of increasing interest (29).

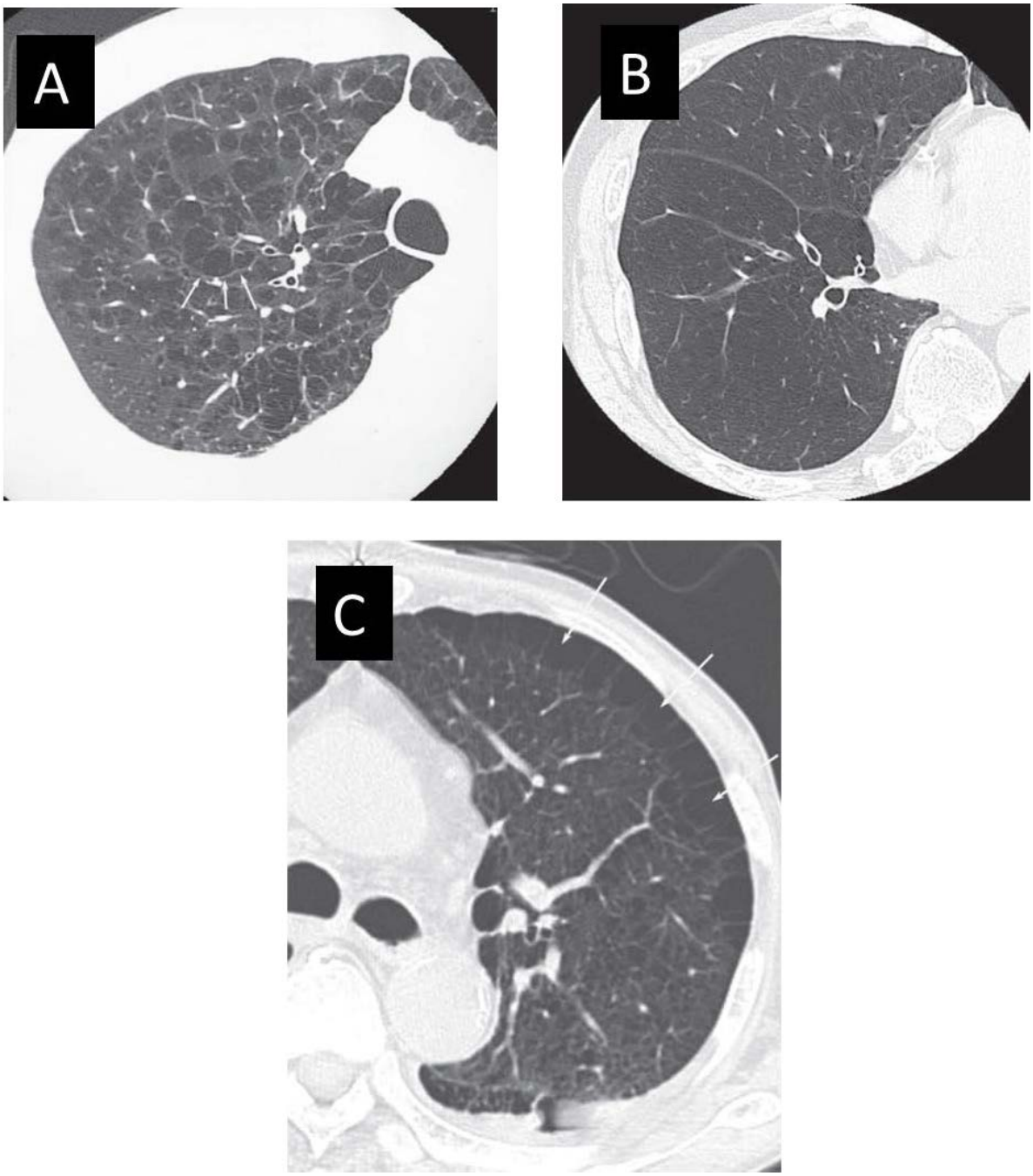

Figure 1.1: CT scan findings demonstrating different subtypes of emphysema (images reprinted with permission[2])

The subtypes of emphysema relate to the lobular anatomy of the lung. Image A shows centrilobular emphysema with central areas of low attenuation. In panlobular emphysema (image B) there is more generalised low attenuation. Image C shows paraseptal emphysema with arrows highlighting the subpleural well defined cystic areas of low attenuation.

[2] Reprinted from 30. Takahashi M, Fukuoka J, Nitta N, Takazakura R, Nagatani Y, Murakami Y, et al. Imaging of pulmonary emphysema: a pictorial review. Int J Chron Obstruct Pulmon Dis. 2008;3(2):193-204. With permission from Dove Medical Press Ltd.

Indeed, one potential pitfall of diagnosing COPD using spirometry alone is the recognition that emphysema and airflow obstruction do not necessarily go hand in hand. In a study of 80 current smokers who underwent HRCT scanning and lung function tests, 20 were found to have radiological emphysema but only 5 had evidence of airflow obstruction (defined by the authors as a low FEV_1 and/or a low MEF50 (maximum expiratory flow after 50% expired forced vital capacity) using LLN cut-off) (31). A more recent study followed up current and heavy smokers who had participated in a lung cancer screening trial. 1391 individuals had no evidence of airflow obstruction at baseline (FEV_1/FVC >0.7) but 21.9% progressed to developing obstruction over a mean period of 3 years. More severe baseline radiological emphysema (quantified by a lower Perc15 value) was found to be a risk factor for developing airflow obstruction at follow up (32). The radiation and cost involved in performing HRCT scanning are likely to limit its utility in the early investigation of patients in clinical practice and many of the patients in these studies were asymptomatic. However, it does serve to highlight the point that radiological evidence of smoking related lung damage and lung function parameters can be discordant.

1.1.2.2 Treatment

Smoking cessation is essential in the management of COPD. Successful smoking cessation has been shown to halve the rate of FEV_1 decline in patients with mild to moderate COPD, returning it to a level comparable with never smokers (33). The main available drug treatments are short and long acting inhaled bronchodilators and inhaled steroids. Inhaled treatments have been shown to improve symptoms and reduce exacerbations however there is lack of disease modifying pharmacological options shown to prevent lung function

decline (4). All patients in the UK should be offered pneumococcal and annual influenza vaccinations (34). However the international GOLD guidelines states *'Decisions about vaccination in COPD patients depend on local policies, availability and affordability'* (4).

Exacerbations considered to be bacterial (guided by clinical symptoms such as sputum purulence) should be treated with antibiotics (4, 34) which have been shown to improve symptom resolution and prolong the time to next exacerbation (35). Short-courses of oral steroids are also advised and have been shown to improve lung function as measured by FEV_1 and reduce hospital length of stay (36). As disease advances, the potential benefits of exercise encouraged by formal pulmonary rehabilitation courses are well recognised (37).

Long term oxygen therapy (>15 hours per day) improves survival in patients with severe hypoxia at rest (38, 39). Patients with bullous disease or upper lobe predominant emphysema may be appropriate for surgical bullectomy or lung volume reduction surgery (40). However patient selection is important as there is a higher risk of death in patients with very poor lung function (FEV_1 <20% predicted or gas transfer of <20% predicted) (41). Appropriate patients who remain symptomatic with a poor quality of life despite maximal medical treatment should be considered for lung transplantation (34). National transplant centres have specific referral criteria and many patients may not be suitable candidates due to age, comorbidity, BMI, smoking status, osteoporosis or co-existent infection with HIV or hepatitis. The importance of good palliative care for patients with end-stage disease has also been highlighted in international guidelines (4). Figure 1.2 demonstrates the relative value of some of the treatments and interventions outlined above in the management of COPD.

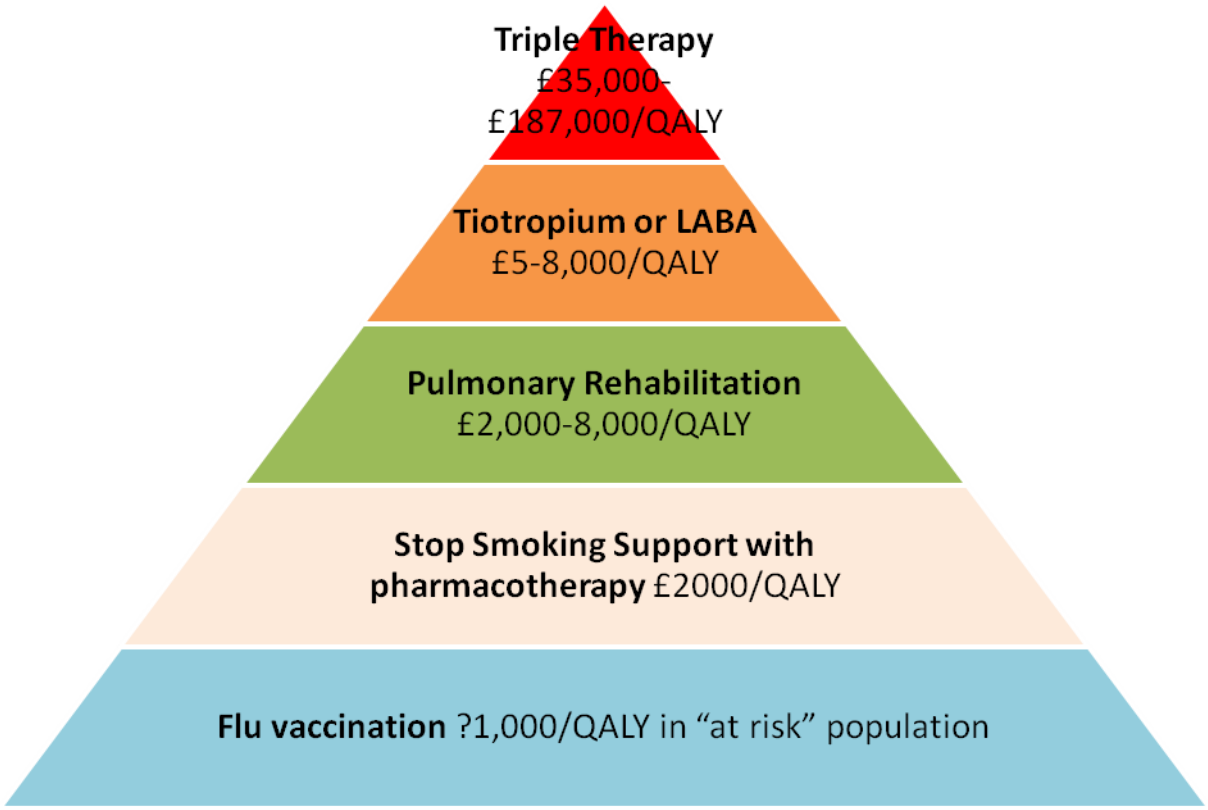

Figure 1.2: COPD 'Value' Pyramid

This 'Value' Pyramid was designed by the London Respiratory Team and demonstrates the relative value of different treatments and interventions in the management of COPD (42). Triple therapy refers to the use of a steroid/LABA inhaler and a long acting muscarinic antagonist inhaler (LAMA such as Tiotropium). QALY: Quality Adjusted Life Year, LABA: Long acting beta agonist.

1.1.3 Pathogenesis of COPD

The pathophysiology of COPD has not been entirely elucidated but is known to involve complex genetic and environmental interactions. The immune response within the lung to inhaled smoke and noxious particles is known to involve cells of both the innate and adaptive immune systems (43). However, many questions remain regarding the precise nature of this immune response, in particular, what drives the on-going airway inflammation following smoking cessation. The apparent self-perpetuating inflammation in COPD may represent a breakdown in 'self-tolerance' raising the possibility that there is an autoimmune component to the pathogenesis of this disease (44, 45).

The role of the innate immune response in COPD, primarily the infiltration of neutrophils and macrophages is well defined (46). However, it is the less clearly defined adaptive immune response outlined in Figure 1.3 that is of particular interest for this thesis. The induction of adaptive immune activation starts with the ingestion of antigen by immature dendritic cells. Dendritic cells perpetuate the innate immune response through the secretion of cytokines and play a key role in presenting antigen to T lymphocytes thereby promoting their proliferation and differentiation into antigen specific effector cells. Activated helper T cells can then promote B cell activation and the production of antibody-secreting mature B cells (47).

The adaptive immune response in COPD is evidenced by the presence of T cells, B cells and 'lymphoid follicles' within the lungs of patients with COPD (48-52). These lymphoid follicles are comprised of B cells and follicular dendritic cells, surrounded by CD4+ T cells (52). They

contain germinal centres which is where B cell proliferation and differentiation into mature antibody-secreting plasma cells and memory B cells occurs (53). The number of CD8+ T cells and B cells present in both the central and peripheral airways of patients with COPD have been shown to correlate with airflow obstruction as measured by FEV_1 % predicted (48-51). Although these correlations do not prove causality it certainly suggests a role of T and B cell responses in the perpetuation of inflammation in patients with COPD. It has been hypothesised that mature B cells within the lymphoid follicles may produce auto-antibodies that promote pulmonary inflammation and the subsequent destruction of lung tissue causing emphysema. Lee *et al* reported an increase in circulating anti-elastin antibodies in patients with emphysema compared to controls. Furthermore, they isolated anti-elastin antibody secreting B cells from the peripheral lung of patients with emphysema (54). Polverino *et al* found B-cell-activating factor (BAFF – a member of the tumour necrosis factor family) expression was increased in the peripheral lungs of patients with COPD. BAFF improves B-cell survival and promotes B cell maturation, and its over-expression has been reported in a number of autoimmune diseases (55-57).

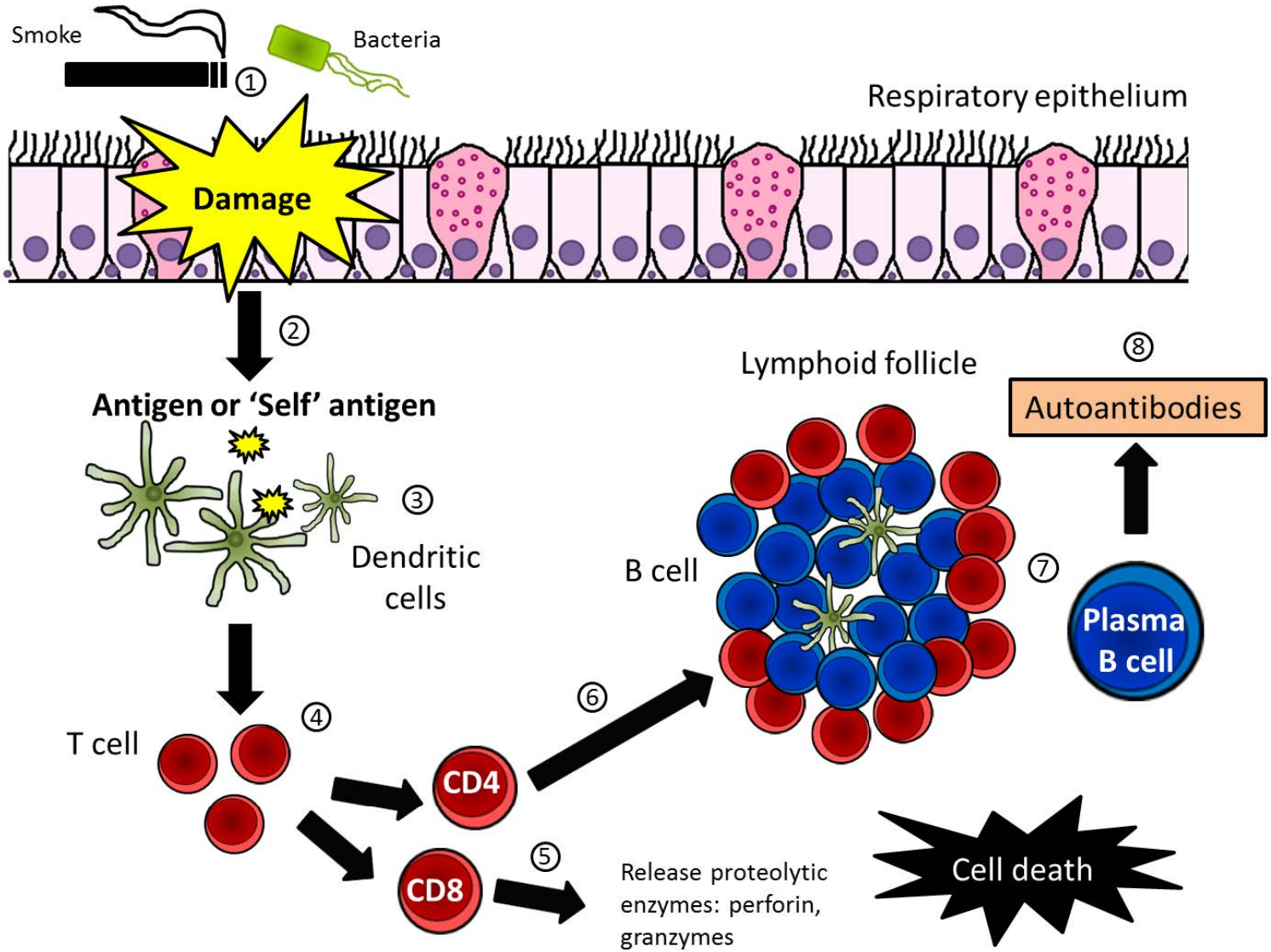
Smoke
Bacteria
①
Respiratory epithelium
Damage
②
Antigen or 'Self' antigen
③
Dendritic cells
T cell
④
CD4
CD8
⑤
Release proteolytic enzymes: perforin, granzymes
⑥
Lymphoid follicle
B cell
⑦
Plasma B cell
⑧
Autoantibodies
Cell death

Figure 1.3: The adaptive immune response in COPD

This figure shows a simplified overview of the adaptive immune response within the lung of a patient with COPD (43, 45). The antigenic trigger is not known but it is thought that smoke constituents and infective agents may play a role ①. Another theory is that the damage caused to the lung through inflammation and oxidative stress may produce so called 'self' antigens causing a breakdown in self-tolerance ②. Dendritic cells ingest antigen ③ before presenting it to T cells ④ which proliferate and differentiate into antigen specific effector T cells. CD8+ cytotoxic T cells cause apoptosis and cell death of targeted cells by releasing proteolytic enzymes such as perforin and granzymes ⑤. CD4+ helper T cells play a role in promoting B cell proliferation and antibody isotype switching ⑥. Within lymphoid follicles B cells mature into antibody-secreting plasma cells and memory B cells ⑦. There is some evidence to suggest that mature plasma cells secrete auto-antibodies ⑧ thereby promoting further pulmonary inflammation and tissue damage (54, 58).

At present, the antigenic stimulus to the adaptive immune response in COPD is unknown. Viral and bacterial infections have been implicated and the bacterial colonisation of the lower airways of patients with COPD is one potential explanation for the chronic inflammatory response (59). Another hypothesis is that tissue destruction and oxidative stress brought about by the initial environmental insult results in the modification of self-proteins, producing antigens that are recognised as foreign thereby triggering the adaptive immune response (46, 60).

1.2 Alpha-1 antitrypsin deficiency (A1ATD)[3]

Alpha-1 antitrypsin deficiency (A1ATD) is an under-recognised hereditary disorder first described by Laurell and Eriksson in 1963, after they linked the absence of an α1 globulin band on serum protein electrophoresis to the presence of premature emphysema (62). Alpha-1 antitrypsin is a 52 kDa, single chain glycoprotein, with a 394 amino acid sequence (63). It is synthesised predominantly in the liver and functions as a serine proteinase inhibitor or 'serpin', providing essential protection to lung tissue against the actions of proteolytic enzymes such as neutrophil elastase (NE) and proteinase 3 (PR3).

1.2.1 Clinical features of A1ATD

Deficiency of circulating A1AT is associated with multiple conditions including emphysema, hepatic cirrhosis (64), panniculitis (65), bronchiectasis (66) and vasculitis (67).

[3] Excerpts of this chapter have been previously published (61. Brebner JA, Stockley RA. Recent advances in alpha-1-antitrypsin deficiency-related lung disease. Expert review of respiratory medicine. 2013;7(3):213-29; quiz 30.)

1.2.1.1 Clinical manifestation of A1ATD in the lung

1.2.1.1.1 *Emphysema*

The premature onset of emphysema was the first identified clinical manifestation of A1ATD described in 1963. The classical distribution has a lower zone predominance (68) however all zones can be affected. In one study of 102 A1AT deficient patients, 65 (64%) had predominantly basal changes and 37 (36%) had a greater degree of apical emphysema (69). It was also noted in this study that basal emphysema had a closer association with FEV_1 impairment than apical emphysema, confirming previous observations in usual COPD (70).

1.2.1.1.2 Bronchiectasis

Initial reports of the incidence of bronchiectasis in A1AT deficient individuals were as high as 43% although based on small study populations (66, 71). In a larger study of 74 severely deficient subjects, Parr *et al* reported the incidence of "clinically relevant" bronchiectasis to be 27% which is similar to the reported incidence in usual COPD (72). Using a different approach, Cuvelier *et al* looked at the frequency of deficiency alleles in 202 patients with known bronchiectasis compared to a control group and concluded that there was no association. They did however, note a higher Z allele frequency in bronchiectasis associated with emphysema (73). Whether or not A1ATD is an independent risk factor for bronchiectasis or that bronchiectasis simply occurs in association with emphysema remains unresolved and requires a sufficiently powered case control study with usual COPD (74).

1.2.1.1.3 Small airways disease and bronchodilator reversibility

A1ATD can be associated with varying degrees of airflow obstruction even varying within individual patients. A study of 1052 subjects with A1ATD from the National Heart, Lung and

Blood Institute (NHLBI) Registry found that 82% reported symptoms of wheeze without a cold and 70% described 'attacks of wheezing' associated with breathlessness. The onset of these attacks was around 31 years of age, therefore the initial diagnosis of asthma given in young A1AT deficient individuals is unsurprising. The study found that 49% of patients demonstrated significant reversibility at some stage of their follow up, and the average increase in the FEV_1 was 382ml (+/-180). Reversibility (defined as an FEV_1 increase of ≥ 12% and at least 200ml post bronchodilator) was even seen in 12.5 % of patients with a normal FEV_1 suggesting bronchial hyper-responsiveness may be an early feature of the disease process (75). Analysis of data from the UK and US A1AT registries has shown that bronchodilator reversibility is associated with a more rapid decline in FEV_1 (76, 77). The difficulty in distinguishing asthma and COPD in A1AT deficient patients is particularly challenging, and recognising overlap between the two conditions is important to ensure appropriate therapeutic strategies are used to prevent an accelerated decline in lung function (78).

1.2.1.1.4 Clinical phenotypes

Emphysema and airflow obstruction often coexist, however as seen in usual COPD, subsets of patients with either marked emphysema and preserved spirometry or severe airflow obstruction with relatively little parenchymal destruction are identifiable. The lung disease associated with A1ATD exhibits considerable heterogeneity and the recognition of distinct clinical phenotypes is important as this may lead to more individualised therapy. This pathological disparity was first postulated by Parr *et al* partly reflecting the distribution of the emphysema with apical disease having little effect on FEV_1 (69). Holme *et al* confirms

this in a prospective study of individuals identified with physiological discordance (normal FEV_1 and low diffusing capacity and vice versa) (79).

An observational study of 745 patients with severe A1ATD compared subjects with emphysema, chronic bronchitis and 'asthma overlap'(80). This study found chronic bronchitis patients were younger, with a lower number of pack years and had better preserved lung function. A greater proportion of 'asthma overlap' patients were women (55.2%) compared to the emphysema group (34.8%). This highlights the importance of patient characterisation in the study of A1ATD.

1.2.2 Diagnosis and treatment of A1ATD

1.2.2.1 Diagnosis

The first step in the diagnosis of A1ATD is the assessment of serum A1AT level which can be performed by most clinical laboratories. If found to be low, more detailed testing of the A1AT phenotype and genotype are then performed to confirm which deficient alleles have been inherited (81). The A1AT protein is encoded for by the *SERPINA1* gene which is composed of seven exons on the long arm of chromosome 14 (14q31-32.3) (63). Inheritance occurs in an autosomal co-dominant manner. The traditional Pi (proteinase inhibitor) nomenclature uses alphabetical abbreviations to denote the speed of migration of the different allelic variants on gel electrophoresis (82). The M allele is the 'normal' variant and the commonest deficiency variants are S and Z which migrate more slowly. The Pi phenotype refers to the type of circulating A1AT identified by serum electrophoresis whereas genotyping relies on the use of specific probes to identify abnormal allelic sequences. The

majority of individuals are homozygotes for the normal M allele. The majority of individuals with 'severe pathophysiological deficiency', classified by a serum A1AT level below the putative protective threshold (11µM), have the PiZZ genotype (83). Rarely individuals can also inherit 'null' alleles which do not produce any detectable A1AT protein by routine quantitative methods and hence cannot be phenotyped. The commonest deficient phenotype is PiZ, which includes both PiZZ and PiZnull genotypes (61). There is however a large number of other rare genetic variants with over 100 alleles having been identified. Some are similarly deficiency alleles such as M_{malton} and $M_{procida}$ and some are dysfunctional variants such as the F variant which results in A1AT with a reduced capacity to bind to NE (84).

In 2003, a joint statement from the American Thoracic and European Respiratory Societies recommended genetic screening for all symptomatic adults with COPD or adult onset asthma with incompletely reversible airflow obstruction (83). However, the majority of severely A1AT deficient patients predicted epidemiologically remain undiagnosed. The concept of population based screening has been entertained, but at present only targeted testing of high risk groups is recommended (85, 86).

1.2.2.2 Treatment

The current mainstay of treatment of A1AT related COPD is similar to that of usual COPD (85). This involves the use of inhaled bronchodilators and steroids, pulmonary rehabilitation, long term oxygen therapy, antibiotics and steroids during exacerbations in addition to smoking cessation advice and preventative influenza and pneumonia vaccinations. Patients with end-stage disease are referred if appropriate for transplantation.

The evidence for unilateral and bilateral lung volume reduction surgery (LVRS) is limited to a number of case series. Compared to the outcomes in non-A1ATD related emphysema, the magnitude of improvement seen post LVRS, appears to be less and is not sustained for the same length of time (87-89). A case series assessing endobronchial valve (EBV) placement as an alternative to surgical LVRS demonstrated a median FEV_1 improvement of 0.575L to 0.905L (p= 0.028) with an associated improvement in BODE index (90). Studies with larger numbers and longitudinal data will be needed before any firm conclusions can be drawn of long term benefit, but this approach may play a role in bridging younger patients to transplant.

1.2.2.2.1 Augmentation

Intravenous administration of A1AT derived from pooled human plasma is only available in some countries as doubts remain over its efficacy and cost effectiveness. Augmentation therapy can certainly increase and sustain serum levels above the accepted protective threshold (11μM, 80mg/dl or~57mg/dl by nephelometry) at a dose of 60mg/kg plasma derived A1AT once weekly and increase the anti-NE capacity in the epithelial lining fluid of the lungs (91). Evidence regarding the clinical efficacy of augmentation therapy is less clearly defined. The majority of evidence is based upon observational cohort studies and three randomised controlled trials (results summarised in Table 1.1) (92-94).

The reasons that only a small number of randomised controlled trials have been carried out are likely to reflect a number of challenges including the relative rarity of the disease, ethical justification for IV placebo arms, high cost, and need for a long duration of follow up (95, 96).

Study	Study design	Methods	Follow up	Primary outcomes
1. Dirksen *et al* 1999 (92)	RCT	56 PiZZ patients 4 weekly infusions of A1AT or placebo. Monitored with spirometry and annual CT densitometry (PD15).	3 yrs	Annual loss of lung density (mean ± SEM) in placebo group was 2.6 ± 0.41g/L/yr compared to 1.5 ± 0.41g/L/yr in A1AT Rx group (p = 0.07)
2. Dirksen *at al* 2009 (93)	RCT	77 PiZZ/PiZnull patients Weekly infusions of A1AT or placebo	2 – 2.5 yrs	A trend towards a beneficial effect of augmentation on decline in lung density was seen (p = 0.049-0.084). No difference in exacerbation frequency but a reduction in exacerbation severity was observed
3. Stockley *et al* 2010 (95)	Post hoc analysis of 1 and 2.	119 patients included in combined analysis of PD15 densitometry data. Results adjusted for lung volume.	Mean 2.5 yrs	Least squares mean change in lung density (PD15) from baseline to last CT scan in placebo group was -6.379g/L compared to -4.082g/L in A1AT Rx group (p=0.006)
4. Chapman *et al* 2015 (94)	RCT	180 patients (serum A1AT <11µM). Weekly infusions of A1AT or placebo	2 yrs	Annual loss of lung density at TLC (mean ± SEM) in placebo group was -2.19 ± 0.23g/L/yr compared to -1.45 ± 0.25g/L/yr in A1AT Rx group (p=0.03). No difference seen at FRC.

Table 1.1: Summary of randomised controlled trials comparing A1AT augmentation to placebo in patients with severe A1ATD.

Study 1 and 2 demonstrated a trend towards a beneficial effect of augmentation therapy on the annual decline in 15th percentile lung density (PD15). A post-hoc integrated analysis which combined the result of 1 and 2 and adjusted for lung volume found a significant difference in lung density change in the augmentation group compared to placebo. This finding was replicated in a larger randomised controlled trial (RCT) (Study 4) looking at annual lung density change at total lung capacity (TLC). FRC: Functional Residual Capacity, Yr: Year, SEM: standard error of the mean, Rx: treatment).

The variability of lung function and damage even in smokers with A1ATD indicates that management should be individualised. Smoking cessation slows or stops progression and other lung function can decline even when the FEV_1 remains stable. Thus, assuming augmentation therapy is effective it will remain critical to assess progression accurately through a variety of methods and only instigate therapy when clear evidence of decline above age related changes, is confirmed. Biomarkers that could identify potential so-called 'rapid-decliners' are therefore an attractive prospect and could be particularly beneficial in the field of A1ATD in establishing which patients may benefit from augmentation therapy.

1.2.3 Pathogenesis of A1ATD

'Serpins' such as A1AT are structurally composed of three β sheets (A-C) and eight or nine alpha helices (A-I) with an exposed mobile reactive loop containing residues that act as pseudosubstrates for the targeted proteinase (97, 98). In the case of A1AT, a methionine residue at position 358 in the polypeptide chain is critical for the interaction with neutrophil elastase (NE). The process of proteinase binding brings about a conformational change within the A1AT protein, whereby the enzyme cleaves the reactive centre loop, which moves to the opposite pole of the protein taking the tethered proteinase with it, before being inserted into β sheet A. The structural deformation of the proteinase that occurs as a result is key to the inhibitory function of the serpin (99). Serpins have a metastable native state that becomes more stable during proteinase inhibition. This makes them prone to aberrant structural formation and protein misfolding as a result of genetic mutations (100).

1.2.3.1 Polymerisation

The Z mutation occurs due to the substitution of lysine for glutamic acid at position 342 in the polypeptide chain. The resulting structural change promotes the interaction of the reactive centre loop of one molecule and the gap in the β sheet A of another causing molecular linkage or so-called 'loop sheet polymerisation' (101).

Intracellular accumulation of Z-variant A1AT polymers within the endoplasmic reticulum (ER) of hepatocytes can lead to neonatal hepatitis, hepatic cirrhosis and hepatocellular carcinoma (102). The resulting lack of circulating A1AT predisposes individuals to proteolytic attack of their lung tissue resulting predominantly in emphysema. However, this explanation of the pathogenesis of lung disease in A1ATD is over-simplified as we now recognise that the manifestation relies on a complex combination of these mechanisms and other environmental and genetic factors.

1.2.3.2 Mechanisms of lung disease in A1ATD

Figure 1.4 summarises the different mechanisms by which lung tissue is damaged in A1ATD.

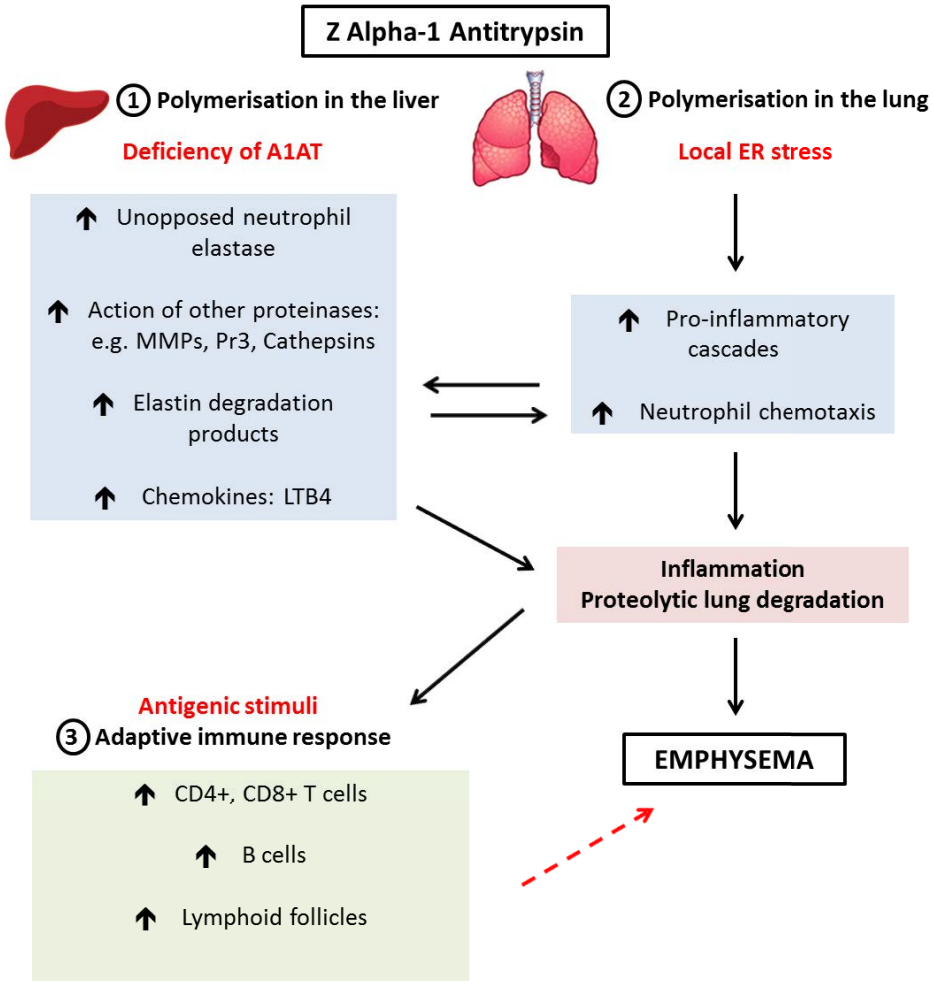

Figure 1.4: Pathogenic mechanisms responsible for the development of emphysema in A1ATD.

1. Polymerisation in the liver results in serum and lung deficiency, causing unopposed neutrophil elastase activity as a result of the recruited neutrophils, which in turn can activate other classes of enzymes in addition to other uninhibited serine proteinases. The net result is proteolytic degradation of the lung tissue leading to emphysema. In addition, neutrophil elastase-derived peptide and chemokines can amplify the neutrophilic load and accelerate parenchymal damage through further release of proteinases.
2. Polymerisation within the lung leads to local ER stress and establishment of a post-inflammatory cascade including increased neutrophil recruitment. Lung polymers are also chemoattractants, recruiting and localising neutrophils further.
3. The loss of the anti-inflammatory properties of A1AT promotes an adaptive immune response within the lung through the production of antigens as a result of inflammation and oxidative stress. An increase in T cell and B cells and lymphoid follicles are seen, however, exactly how they contribute to further parenchymal destruction is not fully understood.

A1AT: Alpha-1-antitrypsin, MMP: Matrix metalloproteinase, Pr3: Proteinase 3, LTB4: Leukotriene B4, ER: Endoplasmic reticulum.

1.2.3.2.1 Proteinase: antiproteinase theory

Our knowledge of the pathophysiology of emphysema in A1ATD started with the 'proteinase: antiproteinase' theory. Following the observations made by Laurell and Eriksson it was subsequently demonstrated that neutrophil elastase could reproduce changes in animal models suggestive of emphysema (103). The development of this animal model and the recognition of A1AT as an NE inhibitor formed the basis of the proteinase:antiproteinase hypothesis whereby imbalance in favour of NE (104) leads to excessive tissue destruction and hence emphysema.

There is extensive literature on the role of NE in the pathogenesis of emphysema in A1ATD, but other proteinases may also be relevant. Proteinase 3 (PR3) is a serine proteinase found in the azurophil granules of neutrophils which also causes emphysema in animal models (105). PR3 activity has been shown to be greater than NE in sputum of A1AT deficient individuals especially during exacerbations suggesting a potential role (106). Matrix metalloproteinases (particularly MMP-12) and cysteine proteinases such as Cathepsin B have also been implicated as having a direct role in proteolytic alveolar destruction (107, 108). Interestingly, NE has been shown to upregulate MMP-2 and Cathepsin B expression *in vitro* (109) as well as inactivating their relevant inhibitors resulting in further enzyme/inhibitor imbalance and a *'proteinase cascade'* (110).

1.2.3.2.2 Chemotactic mediators

High neutrophil counts have been observed in broncheoalveolar lavage (BAL) specimens from A1AT deficient patients (111). Neutrophils are key effector cells in airway inflammation and have the potential to accelerate parenchymal damage through release of their

proteinases. Multiple factors affecting neutrophil recruitment and activation have been suggested. Leukotriene B4 (LTB4) is a potent neutrophil chemotactic mediator found in increased concentration in the sputum of A1AT deficient patients (112). Furthermore studies of sputum chemotactic activity identified both LTB4 and interleukin 8 (IL8) as significant contributors to chemotaxis (113) although only the former correlated with the total migratory potential. *In vitro* experiments demonstrated that LTB4 is released following the binding of alveolar macrophages to NE, supporting the concept that a proteinase imbalance could promote neutrophilic inflammation through the excess production of this chemokine (114). A reduction in sputum LTB4 concentrations in A1AT deficient individuals occurs in response to augmentation therapy (115) confirming this as a likely mechanism.

In a murine emphysema model, elastin degradation products found in BAL fluid have been shown to be chemotactic for monocytes. Using a monoclonal antibody (mAb) to inhibit these elastin fragments, eliminated the chemotaxis *in vitro*. Administration of the same mAb at the time of cigarette exposure was also shown to reduce the accumulation of macrophages in the lung and abrogated the development of emphysema *in vivo* (116). Since elastin degradation is likely to be a key process in the development of emphysema in A1ATD, it is possible that elastin fragments also play a role in amplifying neutrophilic inflammation but direct evidence is lacking.

1.2.3.2.3 A1AT polymerisation and the lung

Z A1AT polymers have been identified in the BAL fluid from A1AT deficient patients (117). This extra-hepatic polymerisation may serve to exacerbate the deficiency further in these individuals, given the lack of functional anti-proteinase activity of polymerised A1AT. The Z

polymers have also been shown to activate neutrophils, manipulate neutrophil shape, promote adhesion and stimulate myeloperoxidase (MPO) release *in vitro* (118). Mahadeva *et al* subsequently demonstrated that polymers of A1AT co-localise with neutrophils in the interstitium of PiZ individuals which may also add to the connective tissue degradation. Furthermore, Mahadeva and colleagues also showed that instilling polymers into the lungs of mice resulted in a significant neutrophil influx (119) which may reflect a direct or indirect chemoattractant process.

Although the majority of A1AT is produced in the liver, it is also synthesised by other cells including bronchial and alveolar epithelial cells (120) supported by the presence of Z A1AT polymers in the BAL fluid of a patient 9 years post liver transplant (121). The potential pro-inflammatory properties of polymerised, locally produced, Z A1AT in the lung may partly explain the progression of emphysema despite augmentation therapy in some individuals or the delay in efficacy seen in clinical trials (92, 93).

The importance of recognising these so called 'gain of function' effects within the lung including the concept of 'ER stress' (brought about by the accumulation of misfolded Z A1AT protein in the ER) and the activation of associated inflammatory signalling pathways has been emphasised in recent years (122). ER stress can have multiple effects including NFκB activation, promotion of ER associated degradation and apoptosis (123).

1.2.3.2.4 Role of the adaptive immune system

In addition to the unopposed action of proteases and gain of function effect of polymerisation, the adaptive immune system is now thought to play an important role in the pathogenesis of lung damage in A1ATD. There is growing evidence of the anti-inflammatory

role of A1AT in addition to its protease inhibitory function (124). It has been hypothesised that the absence of A1AT can therefore promote an adaptive immune response within the lung through the production of antigenic material as a result of inflammation and oxidative stress (125). Baraldo *et al* examined explanted lungs from patients with severe A1ATD and found an increase in CD4+ and CD8+ T cells, B cells and lymphoid follicles compared to controls. Interestingly the B lymphocytes identified within the lymphoid follicles were monoclonal suggestive of an immune response to a specific antigen. Moreover, the number of CD4+ T cells and B cells correlated with the degree of airflow obstruction within these patients (125). Similar findings have been found in usual COPD as previously discussed suggesting this is a common pathogenic mechanism to both usual and A1ATD related COPD.

1.3 Biomarkers: why are they needed in COPD and A1ATD?[4]

A 'biomarker' is defined as *"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"*(127). One of the main drives behind establishing validated biomarkers is their utility as surrogate end points in clinical trials. Decline in FEV_1 has historically been used as the primary outcome measure for pharmacological studies in COPD, however its' limitations in this role are well recognised. FEV_1 is a non-specific marker of COPD, can have significant day to day variation, doesn't identify phenotypic subgroups and does not usually respond to short-term therapeutic strategies (128). As a significant amount

[4] Excerpts of this section have been previously published (61. Ibid., 126. Hampson JA, Turner AM, Stockley RA. Polyclonal free light chains: promising new biomarkers in inflammatory disease. Current Biomarker Findings. 2014;4:139-49.)

of damage must be done to airways before FEV_1 is altered, biomarkers that correlate with FEV_1 have been described as being "*reflective rather than predictive"* (128). The use of quantitative CT scanning has been supported as an alternative end-point to FEV_1 particularly in the field of A1ATD (95). However, the potential drawbacks include radiation exposure to the patient from sequential scanning and the limited number of centres with adequate expertise to ensure consistency of acquisition of densitometry data. Biomarkers that can be measured using a simple blood test are therefore an attractive prospect.

The ECLIPSE study was a large prospective observational study, which aimed to identify novel surrogate markers superior to FEV_1 that would predict disease progression in COPD and help phenotype patients (129). Severe A1ATD was one of the exclusion criteria for enrolment in this study. Table 1.2 summarises the blood biomarkers that were investigated in the ECLIPSE trial and the associations found (130). Despite a large number of subsequent publications only fibrinogen has since been accepted by the U.S Food and Drug Administration as a prognostic biomarker for COPD clinical trials (131). One of the main issues highlighted as a potential reason for this is lack of adequate validation (130). Ideally a biomarker should be central to the pathophysiology of the disease process, relate to disease activity and severity, be stable and fluctuate only with disease flares, predict disease progression and be sensitive to treatments known to be effective (128).

Studies	Biomarker	Associations
(132-134)	WCC	COPD presence, exacerbation risk, mortality and is stable at follow up
(132, 133, 135)	CRP	COPD presence, disease severity (FEV_1), mortality
(132, 133)	IL6	COPD presence, mortality
(133, 135)	IL8	Disease severity (FEV_1), mortality
(133, 136, 137)	Fibrinogen*	COPD presence, disease severity (FEV_1), exacerbation frequency, symptoms (MRC dyspnoea scale), exercise capacity (6MWT), BODE index and mortality
(133, 138)	Surfactant protein D	COPD presence, exacerbation risk, mortality and is responsive to steroid treatment
(135, 139, 140)	CC-16	COPD presence, FEV_1 decline, depression
(11, 141)	CCL-18	COPD presence and mortality
(142)	Vitamin D	Disease severity (FEV_1 and emphysema severity), exercise capacity (6MWT) and bronchodilator response
	Biomarker panels	
(132)	Inflammome: WBC, CRP, IL6, IL8, fibrinogen, TNFα	Exacerbation risk, mortality

Table 1.2: Summarising the blood biomarkers investigated in the ECLIPSE trial

WCC: white cell count, CRP: C reactive protein, IL6: interleukin 6, IL8: interleukin 8, CC-16: clara cell secretory protein 16, CCL-18: CC-chemokine-ligand-18, FEV1: forced expiratory volume in 1 second, 6MWT: 6 minute walk test, BODE index: Body mass index, airflow Obstruction, Dyspnoea and Exercise, MRC: Medical Research Council. (Adapted from (130)).

The term 'heterogeneity' is used time and again with respect to the clinical presentation, CT findings, response to treatments and prognosis in patients with COPD. It follows that the underlying pathogenesis of the lung disease in individuals may differ as a result of different gene-environment interactions. Identifying specific subgroups of patients that may have either a preferential or disadvantageous prognosis or response to specific immunomodulatory treatments is therefore key in the future approach of managing COPD. The use of a biomarker or indeed a panel of biomarkers to help delineate key subgroups is therefore an attractive prospect. To date the potential of free light chains as a novel biomarker in patients with COPD has not been explored.

1.4 Free Light Chains[5]

1.4.1 Background

An essential component of adaptive immunity is the production of antibodies by mature B lymphocytes. Antibodies are immunoglobulins which are composed of two identical polypeptide heavy chains and two identical light chains linked by non-covalent forces and disulphide bonds (144) (Figure 1.5). There are two light chain isotypes: kappa (κ) and lambda (λ) and each immunoglobulin molecule contain only one of these isotypes. In the process of

[5] Excerpts of this section have been previously published (126. Hampson JA, Turner AM, Stockley RA. Polyclonal free light chains: promising new biomarkers in inflammatory disease. Current Biomarker Findings. 2014;4:139-49, 143. Brebner JA, Stockley RA. Polyclonal free light chains: a biomarker of inflammatory disease or treatment target? F1000 Med Rep. 2013;5:4.)

antibody production there is an excess of free light chains (FLCs) produced daily which are secreted into the circulation before undergoing renal clearance (145).

1.4.1.1 Monoclonal versus polyclonal FLCs

FLCs produced by multiple B cell clones are termed 'polyclonal', whereas Bence Jones Proteins (BJPs) are 'monoclonal' FLCs produced by a malignant proliferation of a single clone of B cells. Much of the understanding of the structure and immunological properties of light chains originally came from the study of BJPs as they could be readily isolated from both serum and urine specimens for analysis (146).

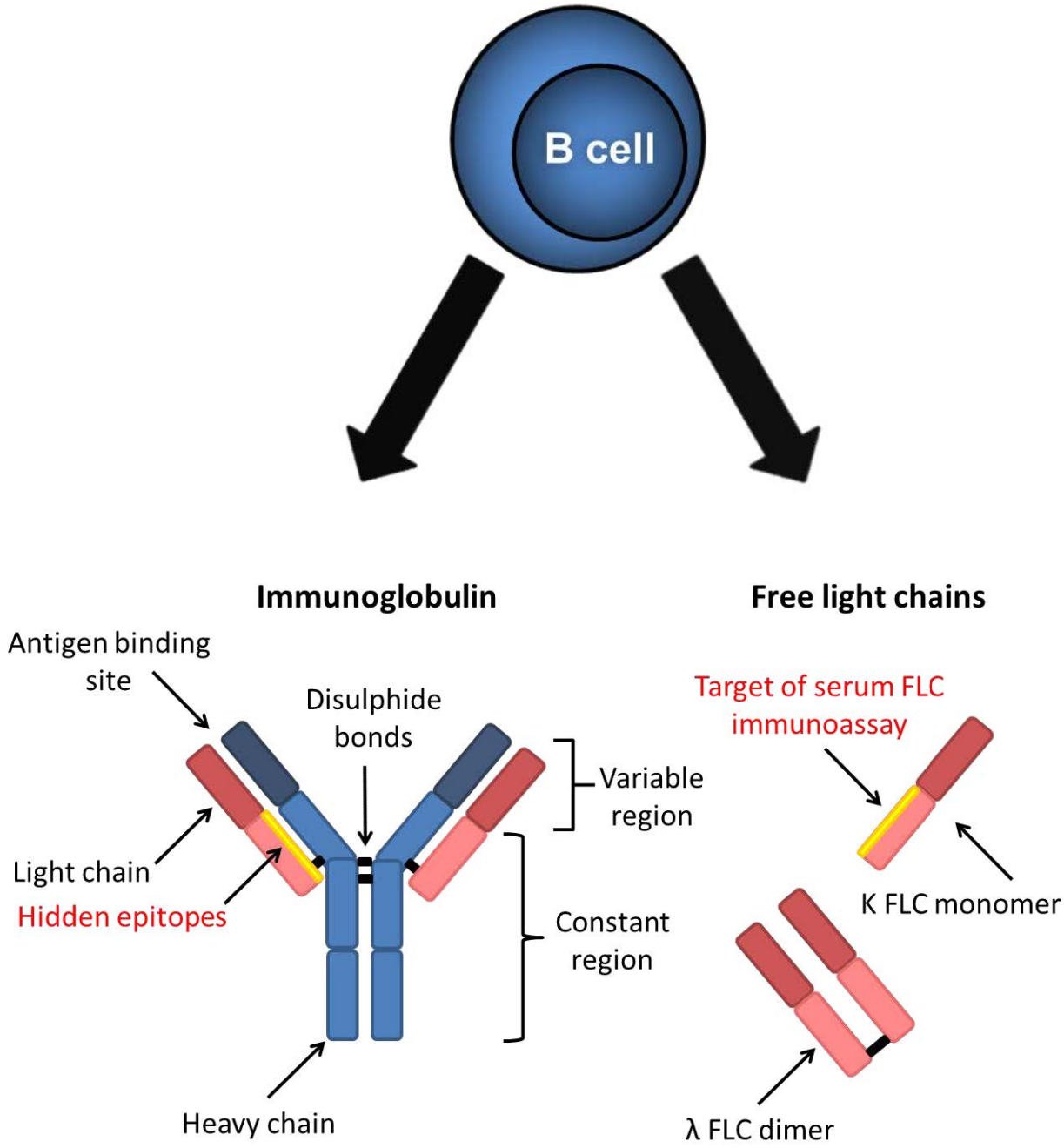

Figure 1.5: Intact immunoglobulin and free light chain structure

Immunoglobulins and free light chains are produced by mature B lymphocytes. Each immunoglobulin is composed of two heavy chains and two light chains linked by disulphide bonds. Light chains are polypeptides containing both a variable and a constant region. The serum free light chain (FLC) assay targets epitopes which are concealed between the light and heavy chains within the immunoglobulin molecule but exposed in circulating FLCs (147).

1.4.1.2 FLC structure variability

Light chains are polypeptides containing both a variable and a constant region. The amino acid sequence of the variable region of light chains is unique to each FLC and the number of amino acid residues in this region can differ (144). The complementary determining residues within the variable region of the light chains contribute to the diversity and heterogeneity of FLCs. This is likely to, at least in part, be responsible for their differing capacity to aggregate and their varying pathogenic potential (148).

"Free" light chains (i.e. those not bound to heavy chain within an immunoglobulin molecule) can exist in monomeric, dimeric or higher oligomeric and polymeric forms (144, 148-151). Classically, kappa FLCs are described as generally monomeric in form but can exist as a non-covalently linked dimer. On the contrary lambda FLCs are usually dimeric in form with covalent bonds between them (144) (Figure 1.5).

1.4.1.3 FLC synthesis

Excess FLCs secreted into the circulation are not produced by immunoglobulin breakdown (152), but are synthesised de novo by B cells which are matured beyond the pre-B cell phase (153). Immunoglobulins are produced by polyribosomes within the endoplasmic reticulum which is where the synthesis of both heavy chains and light chains occurs (154).
Interestingly, there is a conformational difference between the polypeptides of light chains that are newly synthesised compared to those secreted. The former 'precursor' light chain has up to 20 additional amino-terminal residues (155, 156). These extra residues are cleaved prior to secretion of the FLC. Initially thought to be an unimportant by-product, the immunological properties of these secreted light chains are now an area of much interest.

1.4.1.4 Production of polyclonal FLCs in inflammation

As FLCs are produced in excess as a by-product of antibody production by B cells, measuring FLCs has been proposed to be a biomarker of 'B cell activity' (145). Interest in polyclonal FLCs as a biomarker started with the observation of increased concentrations in a variety of biological fluids including blood, synovial fluid, cerebrospinal fluid (CSF) and BAL in patients suffering from different inflammatory and autoimmune conditions. Raised polyclonal FLCs have been reported in systemic lupus erythematosus (SLE) (157, 158) rheumatoid arthritis, Sjögren's syndrome (159), multiple sclerosis (160), atopic dermatitis (161), food allergy (162), inflammatory bowel disease (163), sarcoidosis (164) and a number of respiratory conditions including asthma (165), rhinitis (166), idiopathic pulmonary fibrosis, hypersensitivity pneumonitis (167), and COPD (168).

In a review by Wahren-Herlenius *et al* they describe how *"B cell differentiation and activation thresholds are disturbed, leading to skewing of the B cell pool and immunoglobulin production"* in systemic autoimmune disease (169). SLE is an example of an autoimmune disease characterised by production of multiple autoantibodies and hypergammaglobulinaemia (169). It seems plausible that the excess FLCs produced during this humoral B cell response explains the increase polyclonal FLCs seen in the disease state. However, an increase in circulating FLCs has also been observed in inflammatory conditions which are not typically associated with autoantibody production, such as asthma and idiopathic pulmonary fibrosis (165, 167). This is presumed to be due to polyclonal B cell activation. An increase in FLCs in response to certain viral infections has also been observed (170, 171), such that it is conceivable acute on chronic changes could occur in patients with chronic inflammation and coexistent viral infection.

1.4.2 Free light chain measurement

The eponymous 'Bence Jones protein' was first described in 1847 (172). Historically serum and urine protein electrophoresis and immunofixation electrophoresis have been employed to quantify monoclonal FLC overproduction and diagnose a number of haematological conditions including monoclonal gammopathy of undetermined significance, multiple myeloma, primary systemic amyloidosis and light-chain deposition disease. More recently an automated nephelometric immunoassay was developed that could quantify both kappa and lambda FLC with high sensitivity and specificity in the serum (147). The assay works by utilising antibodies that bind to epitopes on the FLCs which are concealed by the interface between the heavy chain and the light chain in an intact immunoglobulin molecule. (Figure 1.5).

The use of this serum FLC assay has been incorporated into many guidelines negating the need for urine electrophoresis in the screening of many haematological disorders (173). The quantity of circulating FLCs depends on the balance between synthesis by B cells and clearance within the kidney. A number of factors can affect the production of FLCs some of which I have already discussed (e.g. polyclonal B cell activation in autoimmune and inflammatory conditions). However, in these situations the kappa lambda ratio should remain within the normal range. In patients with monoclonal gammopathies, a monoclonal FLC overproduction will alter the balance between kappa and lambda FLC production resulting in an abnormal ratio (173). Immune suppression by either a disease process or as a result of drug therapy can cause a reduction in FLC production. Serum FLCs have a half-life of 2-6 hours and are both excreted and catabolised by the kidney thus impairment in renal function will result higher circulating FLC concentrations (174, 175) although the κ/λ ratio

should remain unaltered. Katzmann *et al* (176) established the normal ranges for serum κ and λ FLCs using the automated immunoassay (κ 3.3 – 19.4 mg/L, λ 5.7 – 26.3 mg/L, κ/λ ratio 0.26 – 1.65).

1.4.3 Biological functions and evidence of direct pathogenic role in other inflammatory diseases

In recent years our advancing knowledge of the diverse immunological functions of FLCs has also generated new interest in their potential pathogenic role in chronic inflammatory disease. Studies of the interaction between FLCs and polymorphonuclear leukocytes were prompted by the need to understand why patients with renal failure were at increased risk of bacterial infections. Due to reduced renal clearance, FLCs are increased in the serum of patients with renal failure and their ability to act as a 'uremic toxin' was therefore investigated (177). Cohen *et al* demonstrated that polyclonal FLCs isolated from the plasma of patients undergoing haemodialysis inhibited the apoptosis of neutrophils *in vitro* (178). Neutrophils are key effector cells in the innate immune response and apoptosis is essential for the resolution of inflammation, thus by inhibiting apoptosis FLCs could potentially be responsible for perpetuating chronic neutrophilic inflammation. FLCs have also been shown to influence other neutrophil functions. FLCs themselves are not chemotactic but when added to neutrophils they inhibit the chemotaxis towards FMLP (a strong neutrophil chemoattractant) (177). In addition, Cohen *et al* showed that FLCs can reduce neutrophil activation in response to FMLP as measured by a reduction in deoxyglucose uptake (177). Paradoxically Braber *et al* found that FLCs can bind to neutrophils *in vitro* and stimulate IL8

production, identifying another mechanism by which FLCs could influence inflammation (168).

Redegeld *et al* demonstrated that FLCs can elicit hapten-specific hypersensitivity reactions in sensitised mice (179). FLCs can bind to mast cells promoting activation and degranulation. This effect can be inhibited by using the FLC antagonist 'F991'. Tamm-Horsfall protein is a glycoprotein which is synthesised within the kidney and can bind to both κ and λ FLCs. Utilising knowledge of the FLC binding site within this glycoprotein, F991 a 9-mer peptide sequence of the Tamm-Horsfall protein was developed to inhibit FLCs interactions with mast cells (179, 180).

Hutchinson *et al* demonstrated that FLCs bind to a variety of cell membranes (181). They confirmed this by incubating biotinylated FLCs with different cells and using streptavidin allophycocyanin to detect bound protein. This indicated binding to a number of different cell lines as well as peripheral blood mononuclear cell subtypes. In particular, there was a high binding affinity for monocytes leading to speculation regarding the role of FLCs in antigen presentation and immune response initiation. Subsequently Thio *et al* demonstrated the ability of FLCs to bind directly to antigen and supporting the potential to initiate antigen specific cellular responses (182).

1.4.4 Polyclonal FLCs and mortality

Polyclonal FLC overproduction has been shown to be associated with an increased risk of mortality. Dispenzieri *et al* followed up over 15000 individuals aged 50 or over who had undergone FLC analysis and recorded their mortality and cause of death. All of the patients

included in the study had a normal κ/λ ratio thereby excluding any underlying monoclonal gammopathies. Correcting for age, sex and renal function they found a combined FLC level of greater than 47.2 mg/L was associated with a 2.07 hazard ratio (HR) for death (183). These results were echoed in a subsequent study where combined FLC results above a higher cut off point of 65mg/L were associated with a high risk of death within 100 days (HR 7.1, p = 0.015) (184). Forty-one % of the deaths during this period of the study were due to cardiovascular causes. A polyclonal increase of FLCs has subsequently been shown to be a risk factor for cardiovascular events in patients with type 2 diabetes (185) and an independent risk factor for mortality in patients hospitalised with decompensated heart failure (186).

Three large studies have looked at the role of polyclonal FLC concentrations in predicting mortality in patients with varying severity of chronic kidney disease (CKD). In all, combined (κ & λ) FLC levels were found to be an independent predictor of mortality (187-189). These studies highlight the potential utility of polyclonal FLCs in risk-stratification in both the general population and disease cohorts.

1.5 FLCs and the pathogenesis of COPD

In addition to the potential utility of polyclonal FLCs as a biomarker of adaptive immune activation in COPD, there is evidence that suggests FLCs could play a pathogenic role.

1.5.1 FLCs and neutrophil interaction

As previously discussed FLCs are biologically active molecules and on a mechanistic level there is evidence they have biological properties that could potentially lead to lung tissue damage through their effects on neutrophil function (177, 178). Neutrophils are key effector cells in the pathophysiology of COPD. The effects impaired neutrophil function can have on promoting inflammation in COPD is currently a topic of much interest, and methods aiming to restore normal neutrophil function are becoming an attractive prospect for novel therapeutic strategies (190).

Braber *et al* published a paper linking FLCs and neutrophils in the pathogenesis of COPD (168). The study demonstrated increased FLC levels in the serum of three murine models of emphysema and six patients with COPD compared to controls. In addition, they demonstrated that FLCs can bind to human neutrophils and activate them to produce IL8 *in vitro*. F991 (a FLC antagonist) was also shown to inhibit this binding capability and reduce the neutrophilia within the BAL fluid in a smoke exposed mouse model.

1.5.2 Light chain deposition disease

There are a growing number of case reports detailing cases of nodular and cystic lung disease associated with FLC overproduction in light chain deposition disease (LCDD) (191-198). This is a rare haematological condition which is characterised by the deposition of non-amyloid kappa or lambda light chains within the body due to overproduction by a single clone of plasma B cells (199, 200). In 2006, Colombat *et al* were the first to describe three cases of LCDD presenting with a progressive cystic lung disease, ultimately leading to

respiratory failure necessitating lung transplantation (193). In this case report, the microscopic pathological features seen in the lung were described as *"patchy deposits of an amorphous eosinophilic material in alveolar walls, small airways and vessels. Emphysematous like changes were present at the edge or at distance of the deposits"*. Immunofluorescence staining of this eosinophilic material taken from tissue specimens was positive for κ light chains. The HRCT findings of one of the patients in this case report showed a confluent cystic abnormality with a basal predominance (Image D, Figure 1.6) which bears a resemblance to the basal panlobular emphysema seen in alpha-1 antitrypsin deficiency (Figure 1.7).

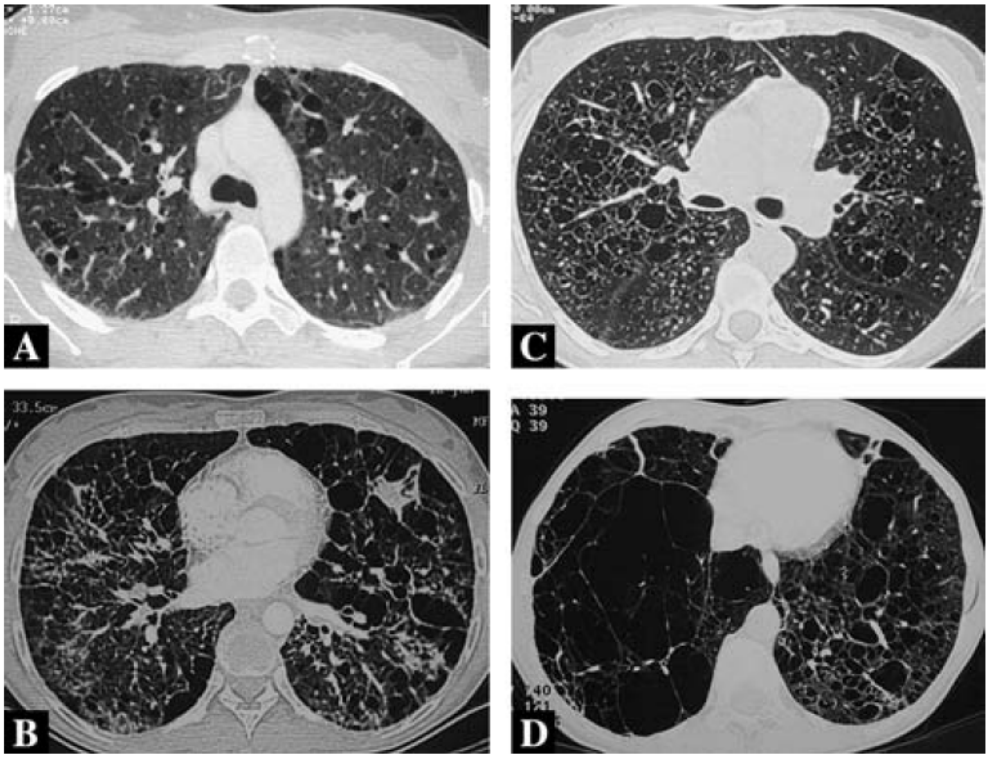

Figure 1.6: High resolution computed tomography (HRCT) scans of two patients with light chain deposition disease demonstrating progressive cystic abnormality (image reprinted with permission[6])

HRCT images show interval CT scans for two patients (patient 1 – A, B, patient 2 – C, D). Image D shows the large thin walled cysts which have become confluent with a basal predominance.

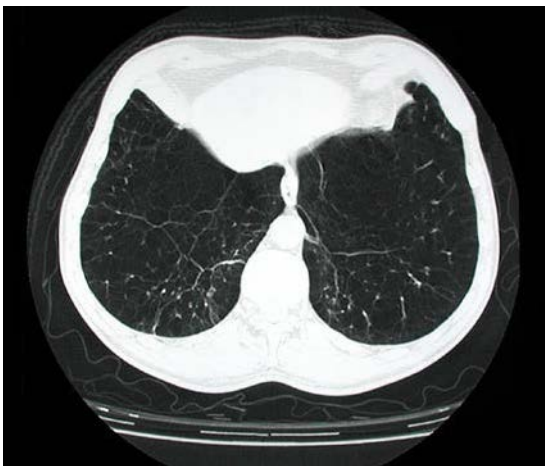

Figure 1.7: High resolution computed tomography scan showing basal panlobular emphysema in a patient with alpha-1-antitrypsin deficiency

[6] Reprinted with permission of the American Thoracic Society. Copyright © 2017 American Thoracic Society. 193. Colombat M, Stern M, Groussard O, Droz D, Brauner M, Valeyre D, et al. Pulmonary cystic disorder related to light chain deposition disease. American journal of respiratory and critical care medicine. 2006;173(7):777-80. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society.

1.6 Aims

We know that the adaptive immune system is involved in the pathogenesis of both usual and A1ATD related COPD but the exact role T cells and B cells play in the contribution to inflammation within the lung is not fully understood. Measuring the circulating FLCs in patients with COPD may provide an insight to the degree of activation of the adaptive immune system within those individuals. The primary aim of this study was to investigate the utility of polyclonal FLCs as a clinical biomarker in patients with severe alpha-1 antitrypsin deficiency (A1ATD) and usual COPD. In order to achieve this, polyclonal FLCs were measured in two large patient cohorts to establish whether they:

- Were static during stable disease
- Related to disease severity
- Distinguished clinical relevant subgroups or phenotypes
- Rose during periods of increased disease activity (i.e. during clinical exacerbations)
- Were associated with longitudinal outcomes such as mortality

These feature were chosen because they represent properties of a good biomarker (128). In addition, we examined whether levels were similar in usual COPD to A1ATD. This was undertaken, in part, to ascertain whether pulmonary immune activation is similar in the two groups, as has been shown by Baraldo *et al* (125) and in part to ensure replication of our results, which is appropriate for any biomarker study (127).

Ideally to satisfy all the criteria of a useful clinical biomarker we would also explore whether polyclonal FLCs are sensitive to effective intervention strategies (128). However, at present

there is a lack of pharmacological interventions that have been proven to explicitly impact on disease progression (201). This serves to highlight the importance of improving our understanding of the complex pathogenic mechanisms involved in the development of COPD as this has the potential to provide new avenues to develop much needed immunomodulatory treatments.

CHAPTER 2: Methods[7]

2.1 Study design and population

2.1.1 A1ATD cohort

547 patients with severe A1ATD (with a level below the putative critical threshold of 11μM) were included in this retrospective observational study and were selected according to their genotype. Only patients with a severely deficient genotype who had a stored serum sample taken at the time of their baseline assessment were included. These participants were recruited from the ADAPT programme (Antitrypsin Deficiency Assessment and Programme for Treatment) which is a large cohort of highly characterised patients with A1ATD, who undergo annual follow up in a research clinic setting. As part of this research programme these patients have detailed lung function assessments and consented to the use of their medical records and stored biological samples for research purposes. Genotyping of all patients was performed by Heredi Lab Inc (Salt Lake City, USA). ADAPT was approved by the South Birmingham Research and Ethics Committee (Ethics approval number 3359a, see Appendix for consent form). Figure 2.1 shows the flow of participants through the study and the numbers of patients included in the subgroup analyses. For the purposes of this study patients were followed up to gain information on mortality. Our centre was informed of patient deaths even if they were no longer able to attend the research clinic and hence no patients were lost to follow up for this endpoint.

[7] Excerpts of this chapter have been previously published (202. Hampson JA, Stockley RA, Turner AM. Free light chains: potential biomarker and predictor of mortality in alpha-1-antitrypsin deficiency and usual COPD. Respir Res. 2016;17:34.)

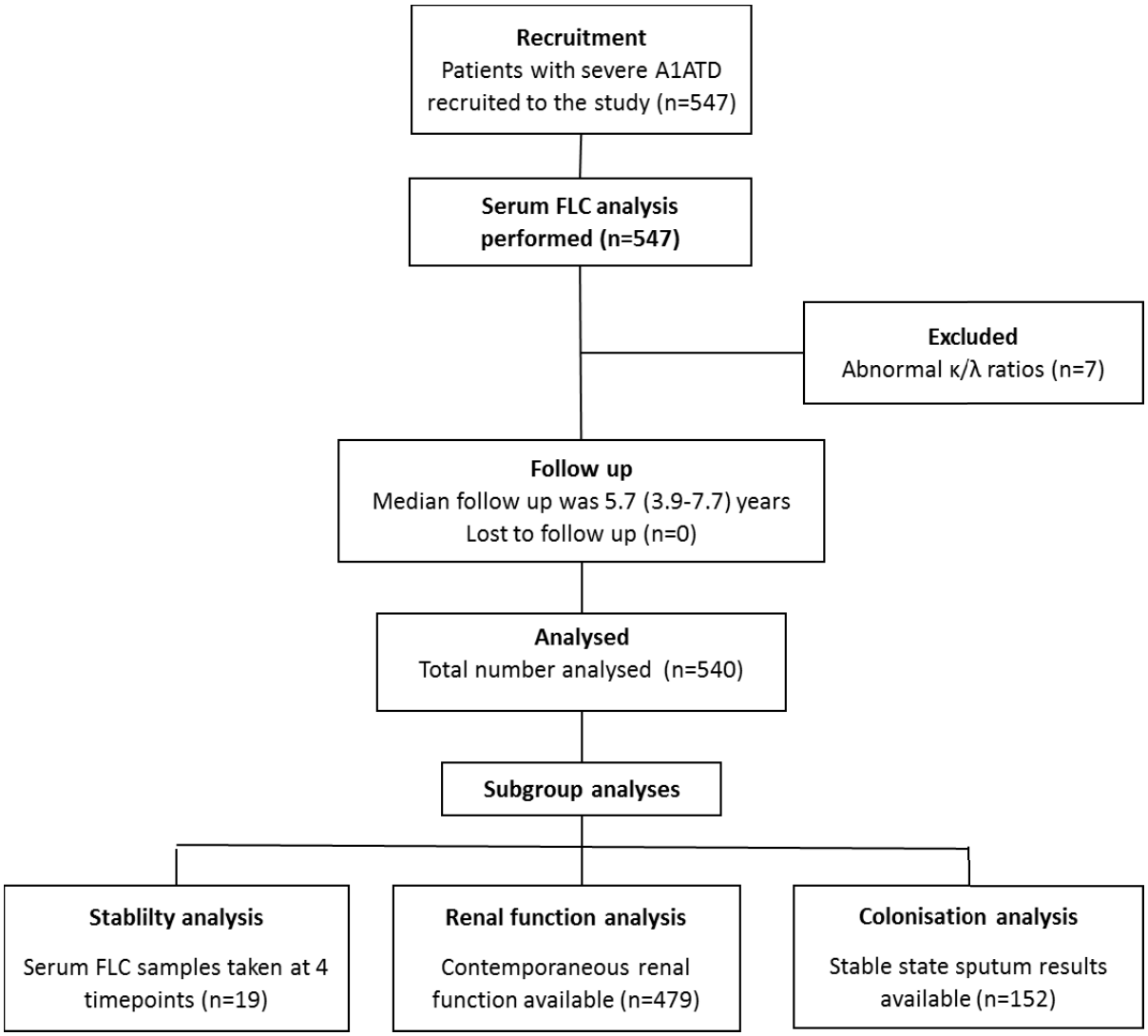

Figure 2.1 Flow of patients through the A1ATD cohort

A1ATD = alpha-1-antitrypsin deficiency, FLC = free light chain

2.1.2 Usual COPD cohort

The usual COPD patients in this study were identified from two separate established cohorts which have now merged. The West Midlands COPD Collection (WMCC) is a cohort of patients with usual COPD who have given informed consent as part of a study investigating COPD phenotypes and progression. A diagnosis of COPD on the basis of spirometry was required for recruitment to this study and patients were then offered more detailed lung function testing and a CT thorax for more detailed phenotyping. The WMCC received ethical approval from the South Birmingham National Research Ethics Service (NRES) committee (REC ref no. 07/H1207/231).

A second cohort of patients with COPD (excluding those with A1ATD) had been recruited through the Inflammatory Research Facility at Queen Elizabeth Hospital, Birmingham. This is a study examining clinical phenotypes, disease progression and epigenetics in patients with COPD and its associated co-morbidities. The study received ethical approval from the East Midlands NRES committee (REC ref no. 12/EM/0090). In both of these cohorts, patients consented to the use of their biological samples and access to their medical records for research purposes. Figure 2.2 shows the flow of patients through this cohort. Consent forms for both usual COPD cohorts are available in the Appendix.

2.1.2.1 Exacerbation cohort

Patients with an acute exacerbation of COPD were prospectively recruited into a sub-study aimed at examining the inflammatory response during these episodes. Recruitment occurred across two sites: Birmingham Heartlands Hospital and The Queen Elizabeth Hospital,

Birmingham. Patients were consented within 24 hours of admission to hospital with an exacerbation and seen at four time points – day 1, 7, 14 and 56. Serum, plasma, sputum and urine samples were collected at each visit. In addition, patients completed the validated COPD assessment test (CAT) and were asked to keep a detailed symptom diary. Ethical approval for this study was granted by the West Midlands NRES committee – Coventry and Warwickshire (REC ref no. 09/H1210/75).

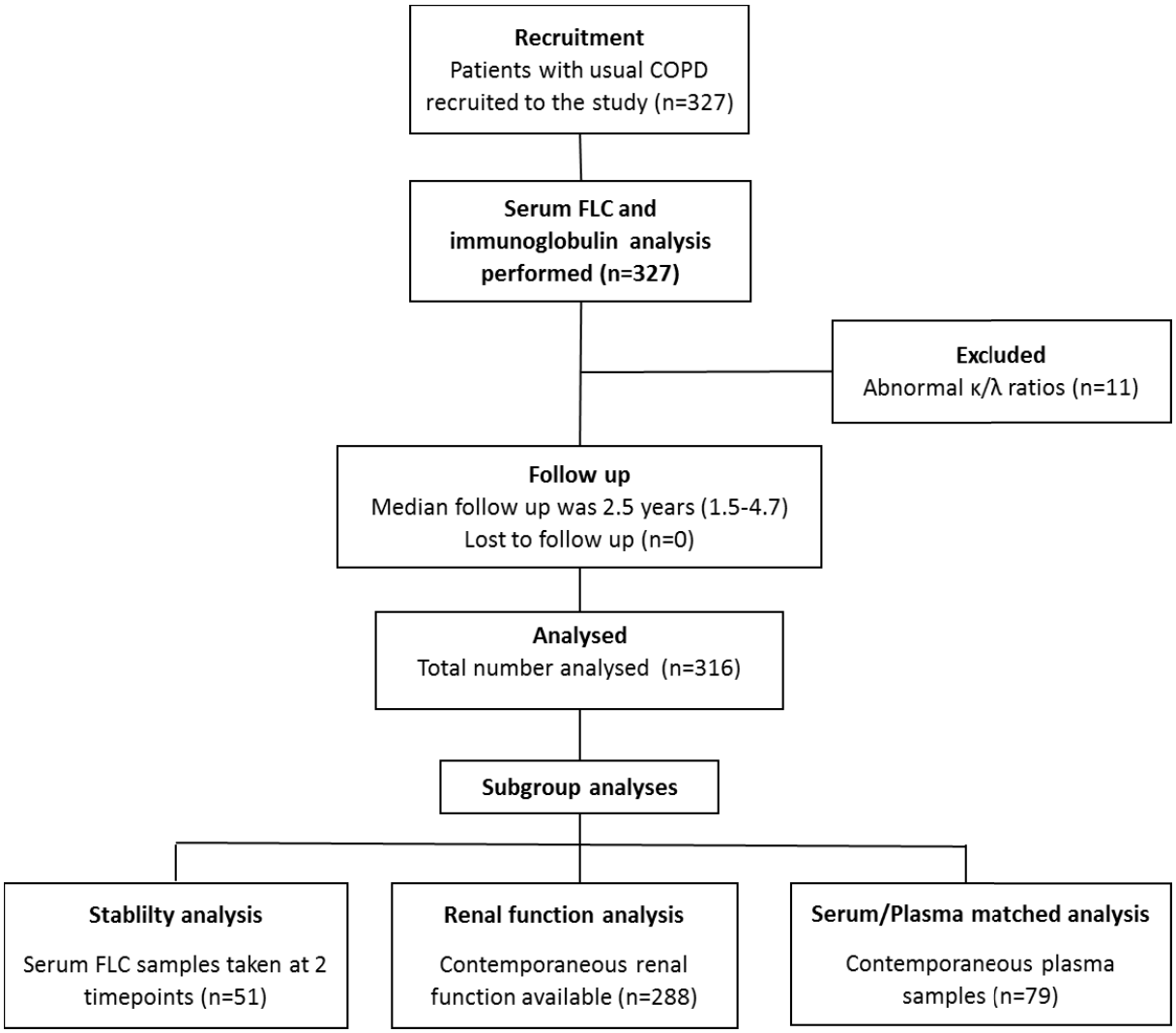

Figure 2.2 Flow of patients through the usual COPD cohort

COPD = chronic obstructive pulmonary disease, FLC = free light chain.

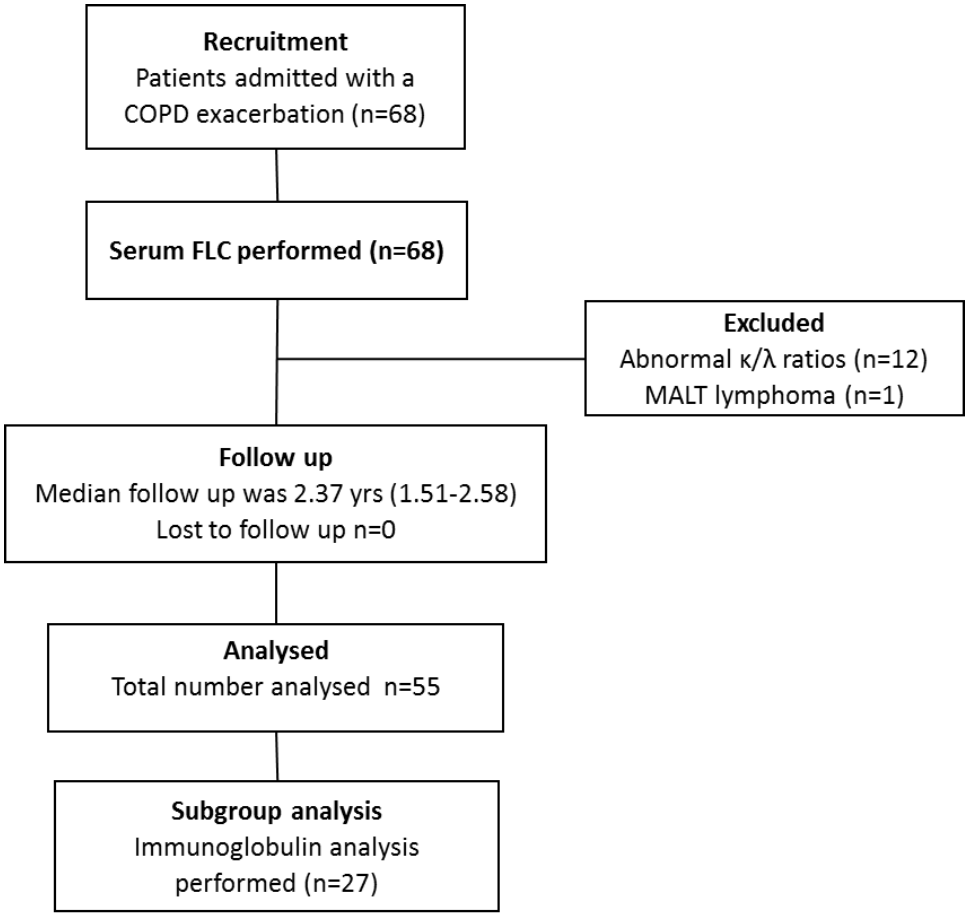

Figure 2.3 Flow of patients through exacerbation cohort

COPD= chronic obstructive pulmonary disease, FLC= free light chain, MALT= mucosa-associated lymphoid tissue.

2.1.3 Bronchiectasis cohort

Patients were recruited to the non-CF bronchiectasis cohort from a larger study entitled “A study of clinical phenotypes, disease progression and genetic susceptibility in patients with chronic obstructive pulmonary disease and bronchiectasis”. Ethical approval for this study was granted by the Birmingham Ethics Committee (code RRK3404) and Newcastle and North Tyneside Research Ethics committee (code 12/NE/0248). This was a longitudinal study to investigate symptoms, quality of life, lung function, lung radiology, blood and sputum markers of disease. Patients were recruited from hospital practice and followed up for 3 years. Baseline clinical data included demographics, medical history, current smoking status and pack years history, medications, presence of chronic bronchitis, exacerbation history and MRC and SQRQ scores. The flow of patients through this cohort is shown in Figure 2.4. Consent forms for this study population are available in the Appendix.

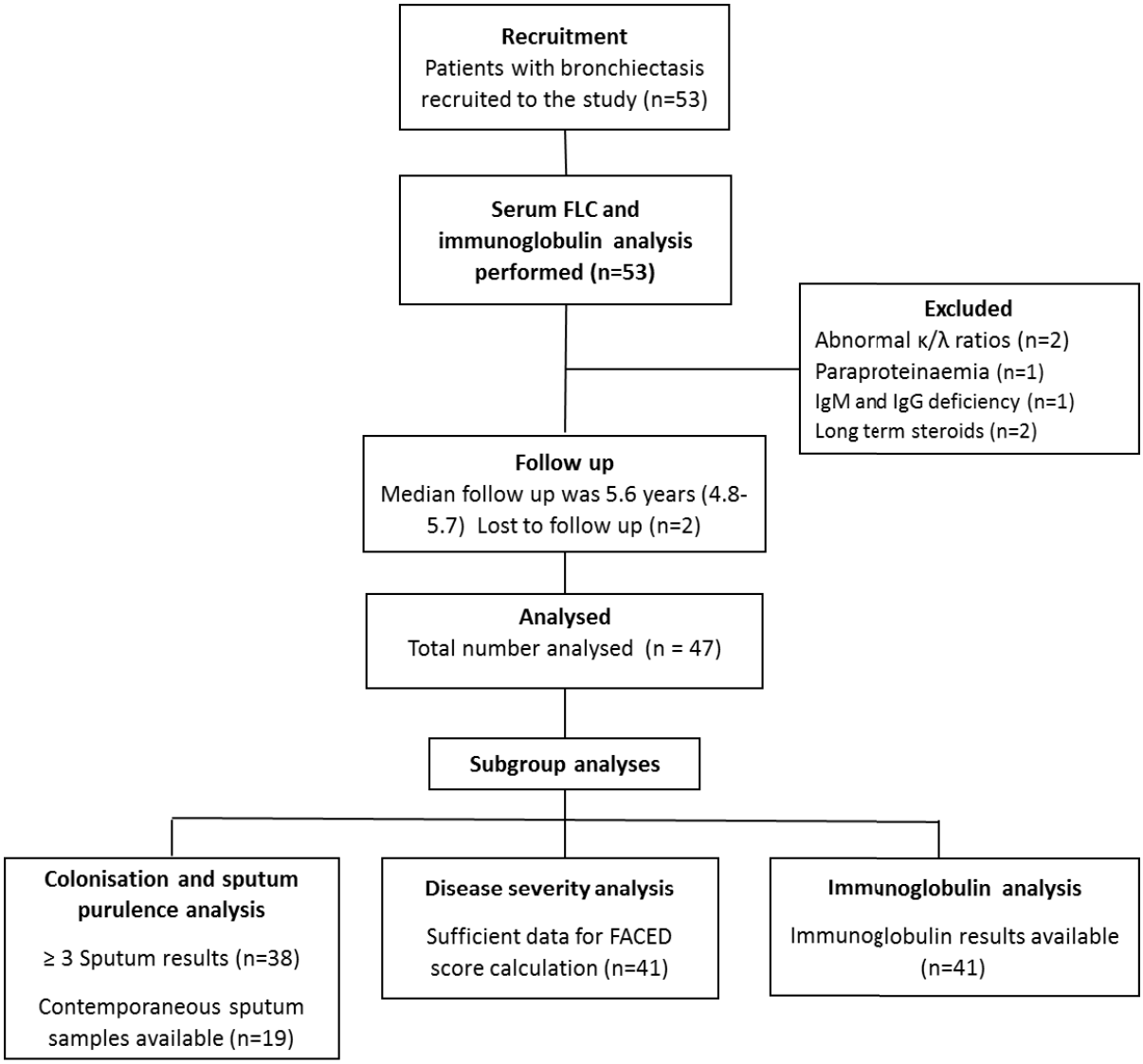

Figure 2.4 Flow of patients through the bronchiectasis cohort

FLC = free light chain. The FACED score is a severity score based on FEV_1, Age, Colonisation with pseudomonas, Extent of bronchiectasis and Dyspnoea (measured using the modified MRC score).

2.2 Clinical data

Baseline demographic data including age, sex, body mass index (BMI), current smoking status and pack years history were collated for the A1ATD, usual COPD and bronchiectasis cohorts. Research notes and medical records were used to clarify symptom history (e.g. presence of chronic bronchitis), annual exacerbation frequency and mortality. The presence of chronic bronchitis was defined using the accepted MRC definition of a cough with sputum production for at least 3 months of 2 consecutive years (10). The exacerbation history was assessed using the criteria suggested by Anthonisen et al of increased breathlessness, sputum volume and sputum purulence (203).

2.2.1 Colonisation data

2.2.1.1 Quantitative sputum culture

Patients in our A1ATD and bronchiectasis cohorts were asked to provide sputum samples for quantitative culture whilst in their stable state. This was performed by a research microbiologist. The first step in the quantitative culture process involved vortexing 1 gram of sputum sample with dithiothreitol (Sputasol, Oxoid Ltd, 100 µg/ml dithiothreitol) for 60 seconds to homogenise it. The homogenised sample was then serially diluted (concentrations 1:10 (10^{-1}) – 1:100000 (10^{-5})) using distilled water. The diluted samples were vortexed again before being plated on chocolate and blood agar plates. Figure 2.5 shows how the samples were plated. The plates were put in an incubator at 37°C and checked for bacterial growth at 24 and 48 hours. An appropriate plate was then selected to count the

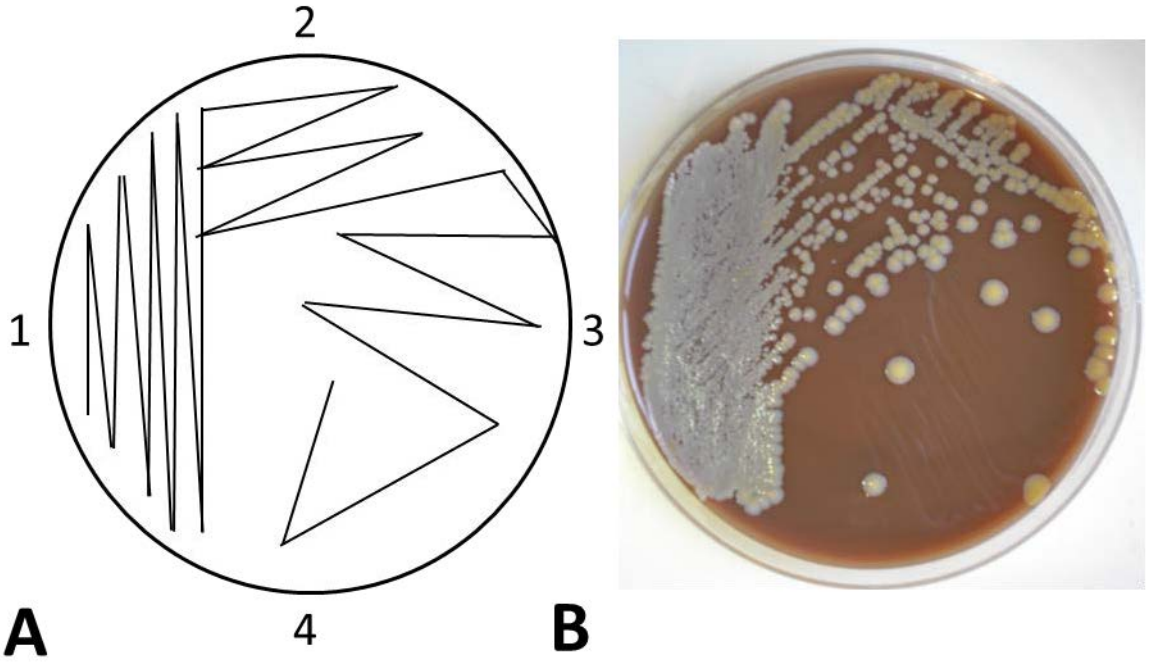

Figure 2.5 Streak plate method for preparing agar plate

Figure A demonstrates the four quadrant zigzag technique for streaking the prepared sputum sample onto the agar plate. Figure B demonstrates how a culture plate prepared using this method may look after incubation. Note there are progressively fewer colonies across the four quadrants. Individual colonies can be sampled from quadrant 4 for further testing or experiments as required.

number of colonies and this was then recorded as the number of colony forming units per millilitre (CFU/ml) of the original sputum sample as described previously (204). A number of routine techniques including microscopy, gram stain and functional tests (including oxidase and catalase tests) were then used to identify the cultured organisms.

The research microbiology database was searched to find all stable state sputum culture results available for the patients in the cohort. A positive culture was defined as a growth ≥ 10^5 colony forming units (CFU) per ml of sputum of a "potentially pathogenic organism" (see Appendix). There is no clear consensus in the literature regarding the definitions of chronic bacterial colonisation (205). Chronic colonisation was therefore defined as ≥3 isolations of the same organism from separate sputum samples taken over a minimum of 3 months in the stable clinical state, as used previously for defining pseudomonal colonisation in patients with bronchiectasis (206).

2.2.2 Computed Tomography

In the A1ATD cohort most patients had a high-resolution CT scan performed using a quantitative protocol (see Appendix). Routine practice was to look at the formal report and record documented emphysema and bronchiectasis; in addition, I interpreted CT scans imported from other trusts. During the initial phase all scans were also checked with one of my supervisors. A random 10% of the quantitative protocol research scans were also examined to ensure consistency of reporting. Figure 1.7 (Chapter 1) demonstrates basal panlobular emphysema characteristic of severe alpha-1 antitrypsin deficiency. In the usual COPD and bronchiectasis cohorts, reports were confirmed from routine CT scans performed in clinical practice.

2.2.3 Lung function tests

See Appendix for a list of all lung function tests collated in this study (Appendix 1.4). All of the A1ATD patients undergo full lung function testing annually as part of the ADAPT research programme. Lung function data was taken from the ADAPT databases corresponding to the year the blood was taken for the baseline FLC sample.

For the usual COPD patients, medical records were examined for lung function test results.

Due to the acquisition of lung function data from several different hospitals for the usual COPD cohort, appropriate equations were used to calculate the predicted lung function values for each individual based on the age, sex and height (Appendix 1.5). This was to ensure consistency in the % predicted lung function values across different sites. Different hospitals use different lung function reference equations including the European Community of Steel and Coal (ECSC) (207) and Global Lung Initiative (GLI) equations (208) which can cause small differences in interpretation. For the purposes of the current project all data was normalised to the ECSC.

2.2.4 FACED score

In the bronchiectasis cohort a combination of clinical data, CT data and colonisation information was used to calculate the FACED severity score. The score is calculated based on FEV_1, Age, Colonisation with pseudomonas, the Extent of bronchiectasis (number of lobes of lung involved) and Dyspnoea (assessed using the modified MRC breathlessness score) (209) (See Appendix 1.6). The rationale for choosing this severity score is discussed in Chapter 5.5.2.

2.2.5 Renal function analysis

Due to the renal clearance of FLCs, the potential impact of a patient's renal function needed to be accounted for in all regression and mortality analyses. Each patient's creatinine and urea was therefore recorded. Where possible, results were taken from the same day as the sample was taken for FLC analysis. The estimated glomerular filtration rate (eGFR) was then calculated using the 4- variable abbreviated MDRD equation (= 186 x $(Creat/88.4)^{-1.154}$ x $(Age)^{-0.203}$x 0.742 (if female) x 1.210 (if black)) (210). The patients were then grouped according to their chronic kidney disease (CKD) stage. For the purposes of this study we classified patients into 5 groups (CKD stage 1 (eGFR ≥90), 2 (eGFR 60-89), 3 (eGFR 30-59), 4 (eGFR 15-29), 5 (eGFR<15)) (211). Newer versions of the guidelines now split CKD stage 3 into a (eGFR 45-69) and b (eGFR 30-44) however for the purposes of this study we grouped stage 3 patients together.

2.3 FLC analysis

FLC analysis was performed on stored serum or plasma samples using the Freelite® immunoassay (Binding site Group Ltd, Birmingham, UK) on the SPAPLUS® turbidimeter (TBS). This is an automated nephelometric assay that was developed to quantify both kappa and lambda FLC with high sensitivity and specificity in the serum (147). As discussed in the introduction, the assay works by utilising antibodies that bind to epitopes on the FLCs (which are concealed on the interface between the heavy chain and the light chain in intact immunoglobulin molecules). Figure 2.6 demonstrates how a nephelometer is used to quantify FLC.

2.3.1 Reference ranges

The previously established reference ranges used were κ FLC 3.3 – 19.4 mg/L, λ 5.71 – 26.3mg/L and κ/λ ratio 0.26 – 1.65 (176). κ and λ values were summated to give a combined FLC result (cFLC), as it is polyclonal FLC production that is of interest for this study. This combined FLC level has been utilised other studies of autoimmune and inflammatory diseases and in the general population. The accepted normal reference range for cFLC is 9.3–43.3 mg/L (183). Patients with an abnormal κ/λ ratio suggestive of a possible underlying monoclonal gammopathy were then excluded from the analysis. For patients with chronic kidney disease there is an accepted higher 'renal reference range' for the κ/λ ratio of 0.37-3.1.

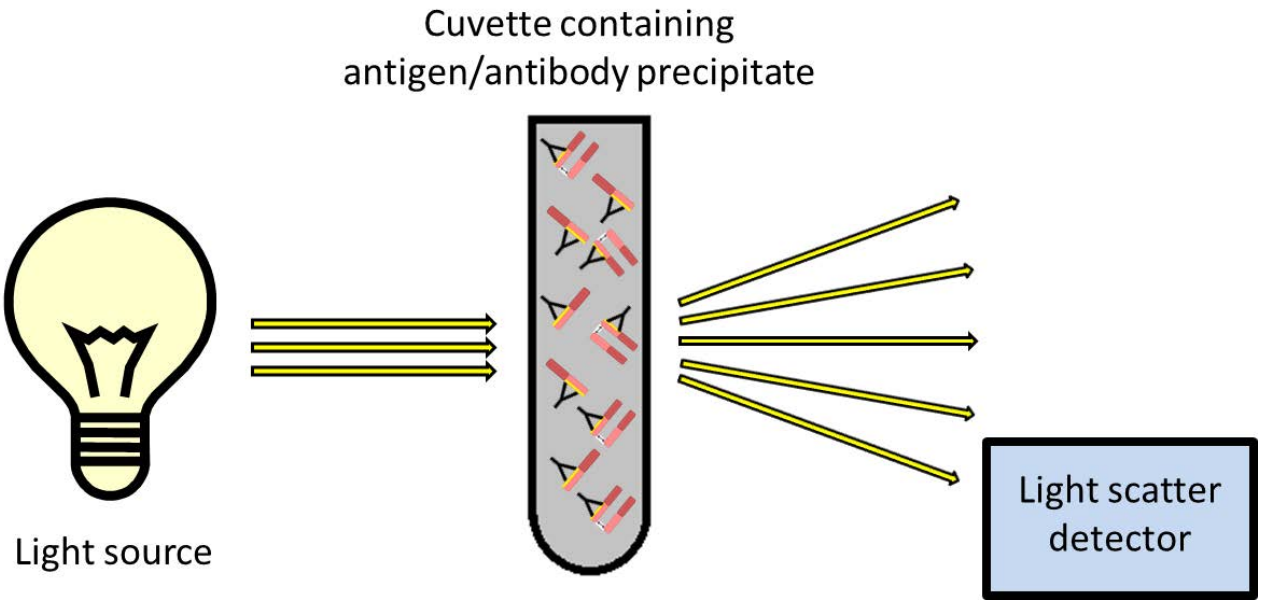

Figure 2.6 Nephelometric FLC immunoassay

The antibody and FLC antigen react to form a precipitate within a cuvette. Light is then shone through the cuvette and the precipitate causes the light to scatter. Antigen concentration is then quantified by the degree of light scatter. This method is used to quantify the number of kappa and lambda FLCs within the serum sample.

2.3.2 Usual COPD cohort –plasma and serum FLC matched samples analysis

In a randomly selected subgroup of 82 patients from the usual COPD cohort, paired serum and plasma samples were analysed. Samples were collected from patients and assayed using Freelite® by the Binding Site Group Ltd (Birmingham, UK). 3 patients were excluded from the analysis due to an abnormal κ/λ ratio.

2.3.3 Stability analysis

In 19 A1ATD patients and 59 usual COPD patients with stable disease (according to both history and serial lung function) FLC analysis was performed on ≥2 samples collected over a period ranging from 1 to 4 years to determine whether levels were static in stable patients.

2.4 Immunoglobulin analysis

To see if FLCs had any utility over and above measuring intact immunoglobulins, IgA, IgG and IgM was measured in all the patients in the usual COPD cohort (n=327). The testing for this cohort was performed by the Binding Site Group Ltd (Birmingham, UK). 11 patients were excluded from the further analysis due to an abnormal κ/λ ratio. The reference ranges used were as follow: IgA 0.845-4.99 g/L, IgG 6.103-16.16 g/L, IgM 0.35-2.42 g/L. Immunoglobulins were also measured in a subgroup of 27 patients in the usual COPD exacerbation cohort. Day 1 samples taken on admission to hospital and day 56 following resolution of their symptoms to determine any relationship to the episode.

In the bronchiectasis cohort, clinical database archives were searched to record retrospective immunoglobulin analysis results performed in usual clinical practice.

2.5 Statistical analyses

All analyses were performed using IBM SPSS statistics version 20. Use of appropriate statistical tests were determined by training and confirmed by liaising with the University statistician.

Data was explored and assessed for normality using the Shapiro-Wilk test. Medians and interquartile ranges (IQR) were reported for non-normally distributed data and means and standard deviations (SD) used for normally distributed data. P values reported are for two-tailed statistical tests.

2.5.1 Univariate analyses

2.5.1.1 Demographic factors, disease phenotypes and mortality

Combined serum κ & λ free light chain (cFLC) concentrations were compared in subgroups according to sex, smoking status, presence or absence of emphysema and bronchiectasis, chronic bronchitis, mortality status and exacerbation frequency. The FLC results were not normally distributed therefore non-parametric tests (Mann Whitney U) were utilised. Results are reported as medians with IQR.

Mann Whitney U tests were also used to compare cFLC levels in patients chronically colonised compared to those who were not in both the A1ATD and bronchiectasis cohorts.

Kruskall Wallis tests were used to determine whether cFLC levels were significantly different in patients grouped according to their CKD stage (renal function) or GOLD stage (severity of airflow obstruction). Post hoc analysis using Mann Whitney U tests were then performed to see the groups that were significantly different from each another and Bonferroni corrections were performed to calculate the significance of the statistical tests.

2.5.1.2 Stability analyses

Friedman Tests were utilised to examine cFLC levels across several time points in subgroups of the A1ATD and usual COPD cohorts. Bland-Altman plots were used to demonstrate the difference between cFLC levels at follow up compared to baseline. Linear regression analyses were also performed to exclude proportional bias of the follow up values.

2.5.2 Correlations and partial correlations

A cross-sectional analysis was performed to look for correlations between cFLC levels and demographic factors such as age as well as eGFR and lung function parameters in all three patient cohorts. Spearman's Rho correlations were performed as the cFLC levels were not normally distributed.

A Spearman's Rho correlation was also performed on contemporaneous serum and plasma FLC levels in a sub-group of 80 patients in the usual COPD cohort.

2.5.2.1 Partial correlations

Within each cohort, partial correlations were performed to look for any relationship between cFLC levels and lung function parameters controlling for other factors shown to influence cFLC levels (such as eGFR).

2.5.3 Regression analyses

Multivariate analytic techniques were used to adjust for covariates where needed; renal function (eGFR) was included in all regression analyses due to FLCs undergoing renal clearance.

2.5.3.1 Logistic regression

In the A1ATD cohort a multivariate logistic regression was performed to examine which variables predicted the presence of chronic bronchitis in the A1ATD cohort. Multiple factors are known to predict or relate to chronic bronchitis in patients with COPD including smoking status, sex, exacerbation history and severity of airflow obstruction so a multivariate analysis was performed to control for these.

Logistic regression was also used to compare cFLC levels in patient with A1ATD and COPD. This was necessary to adjust for eGFR and a number of other factors relating to cFLC levels that differed between the cohorts.

2.5.3.2 Passing-bablock regression

In addition to examining the correlation between the plasma and serum FLC levels in the subgroup of usual COPD patients, passing-bablock regression was performed to compare these analytical methods.

2.5.3.3 Survival analyses

In the A1ATD and usual COPD cohort, multivariate regression analyses were performed to examine the relationship between cFLC levels and survival. Survival time was calculated from the date the FLC sample was taken to confirmed date of death. Patients were divided into subgroups according to their serum cFLC level using 2 important threshold levels – the upper limit of normal (43.3 mg/L) and the threshold associated with death within 100 days (65 mg/L (184)) and survival probabilities were plotted using Kaplan-Meier curves. The patients were also subdivided into cFLC quartiles and Kaplan-Meier curves were plotted to demonstrate the survival of patients in each quartile. Both Log Rank and Breslow tests were performed to assess the difference in survival of these groups.

In addition, both univariate and multivariate Cox proportional hazards regression analyses were performed to estimate the hazard ratios of individual predictors of mortality in both cohorts. Multivariate regression covariates were selected if $p < 0.1$ in the univariate analysis, and there was no significant collinearity with other variables. Cox regression was conducted using cFLC as a continuous variable, and also according to the thresholds described above.

Receiver operating characteristic (ROC) curves were drawn to examine the specificity and sensitivity of cFLC for mortality prediction in the A1ATD and usual COPD cohorts.

In the usual COPD cohort, the same univariate and multivariate analyses were also performed to examine the relationship between immunoglobulin levels and demographic factors, disease severity and survival; Kaplan-Meier curves, univariate and multivariate cox regression and ROC curve analyses were conducted.

CHAPTER 3: FLCs as a biomarker in severe A1ATD[8]

3.1 Introduction

The primary aim of this chapter was to investigate the clinical utility of FLCs as a biomarker in patients with severe A1ATD. In order to be a useful biomarker there are a number of criteria that need to be fulfilled (128). Firstly, a biomarker needs to relate to the underlying disease pathology. Severe A1ATD is associated with the premature onset of COPD and immune activation may be one of the factors perpetuating inflammation in COPD (212). The immune response seen in COPD incorporates cells from both the innate and adaptive immune systems (45); an essential component of adaptive immunity is the production of antibodies by mature B lymphocytes. During antibody production there is an excess of free light chains (FLCs) produced which are secreted into the circulation before undergoing renal clearance (145). FLCs have therefore been promoted as a potential biomarker of adaptive immune activation (145). There are two FLC isotypes: kappa (κ) and lambda (λ), which can be measured independently by a highly sensitive and specific assay (147). A polyclonal increase in combined κ and λ FLC levels (cFLC) has been observed in a number of other autoimmune and inflammatory conditions (126). To date there has been one study which reported raised polyclonal FLCs in a small number of patients with usual COPD compared to controls (168). There is evidence of adaptive immune activation in patients with A1ATD. Baraldo *et al* demonstrated increased numbers of CD4+, CD8+ T cells, B cells and lymphoid follicles in

[8] Excerpts of this chapter have been previously published (202. Hampson JA, Stockley RA, Turner AM. Free light chains: potential biomarker and predictor of mortality in alpha-1-antitrypsin deficiency and usual COPD. Respir Res. 2016;17:34.)

explanted lungs from patients with severe A1ATD compared to controls (125). In addition to protease inhibition it is now recognised that A1ATD has other anti-inflammatory roles and therefore its absence can promote inflammation and oxidative stress resulting in adaptive immune activation. Measuring FLCs as a surrogate marker of adaptive immune activation in patients with A1ATD therefore seems logical.

In addition to relating to the pathophysiology of the disease, a useful biomarker should be stable, relate to disease activity and severity, identify clinically important phenotypes, respond to treatments which are known to be effective and be associated with clinically important outcomes such as disease progression and mortality (128). In order to establish whether FLCs could be a clinically useful biomarker, FLCs were measured in a large, highly characterised cohort of patients with A1ATD. This chapter gives a detailed outline of the demographics and other key patient characteristics of this cohort and also summarises the ability of FLCs to fulfil the criteria outlined above.

3.2 Patient characteristics

FLC analysis was performed on 547 patients with A1ATD. Seven patients were excluded from further analysis due to having an abnormal κ/λ ratio (repeat testing and referral to haematology for assessment was advised for these patients). The A1AT phenotypes of the remaining 540 patients were as follows: 517 PiZZ, 13 Znull, 8 $M_{malton}Z$ and 2 PiFZ. Median follow up time was 5.7 (3.9-7.7) years. The demographics of the cohort are outlined in Table 3.1. Eighty-four % of patients had airflow obstruction (defined as a post-bronchodilator FEV_1/FVC ratio <0.7); 8 of those without obstruction had emphysema on CT scan.

Variable	A1ATD cohort (n=540)
Age (years)	53.9 (45.0 – 60.9)
Sex	Male n = 311 (57.6%) Female n = 229 (42.4%)
Pack years	11.6 (0.0 – 24.0)
FEV_1 (% predicted)	50.7 (35.1 – 85.1)
KCO (% predicted)	62.3 (49.3 – 77.0)
Chronic bronchitis	185 (34.3%)
Emphysema	358 (66.3%)
Bronchiectasis	142 (26.3%)
Frequent exacerbator	129 (40.8%)
eGFR (ml/min/1.73m^2)	81.3 (70.6 – 93.9)
CKD stage	
1 eGFR ≥ 90	156(28.9%)
2 eGFR 60 - 89	279 (51.7%)
3 eGFR 30 - 59	41(7.6%)
4 eGFR 15 - 29	2 (0.4%)
5 eGFR < 15	1 (0.2%)
Unknown (no eGFR)	61 (11.3%)
cFLC (mg/L)	25.7 (21.1 – 31.7)
κ/λ	0.86 (0.71-1.08)

Table 3.1: Patient demographics in the A1ATD cohort

Continuous variables expressed as median (IQR); sex, chronic bronchitis, emphysema, bronchiectasis, exacerbators and CKD stage expressed as number in each group (%). A frequent exacerbator was defined as having 2 or more exacerbations per year. Number of patients with contemporaneous renal function = 479. BMI = body mass index, FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, eGFR = estimated glomerular filtration rate, cFLC = combined (κ + λ) free light chain level and the κ/λ ratio is shown.

3.2.1 FLCs and renal function

FLCs undergo renal clearance. It was therefore important to take into consideration renal function when analysing polyclonal FLC levels. Contemporaneous renal function was available for 479/540 patients in the ADAPT cohort as assessed by eGFR. The number of patients in each CKD stage was outlined in Table 3.1. The serum cFLC concentrations were significantly different in the different CKD stage groups (Kruskall-Wallis test p = 0.006). A post hoc analysis (using Bonferroni correction to calculate significant p value cut off ≤ 0.005) revealed that patients with CKD stage 3 had significantly higher cFLC levels compared to those with CKD stage 1 (median 29.6 (23.0-39.0) v 24.5 (20.3-30.4) mg/L, p=0.003) (Figure 3.1).

A Spearman's Rho correlation revealed a weak negative correlation between cFLC and eGFR (r_s= -0.14, p=0.003) (Figure 3.1). A significant positive correlation between age and cFLC was also demonstrated (r_s= 0.15, p=0.001), but this relationship disappeared after adjustment for eGFR (r_p= 0.08, p=0.101), suggesting this was primarily due to worsening renal function with age. Given the renal clearance of FLCs, the relationship to eGFR was expected but important to confirm.

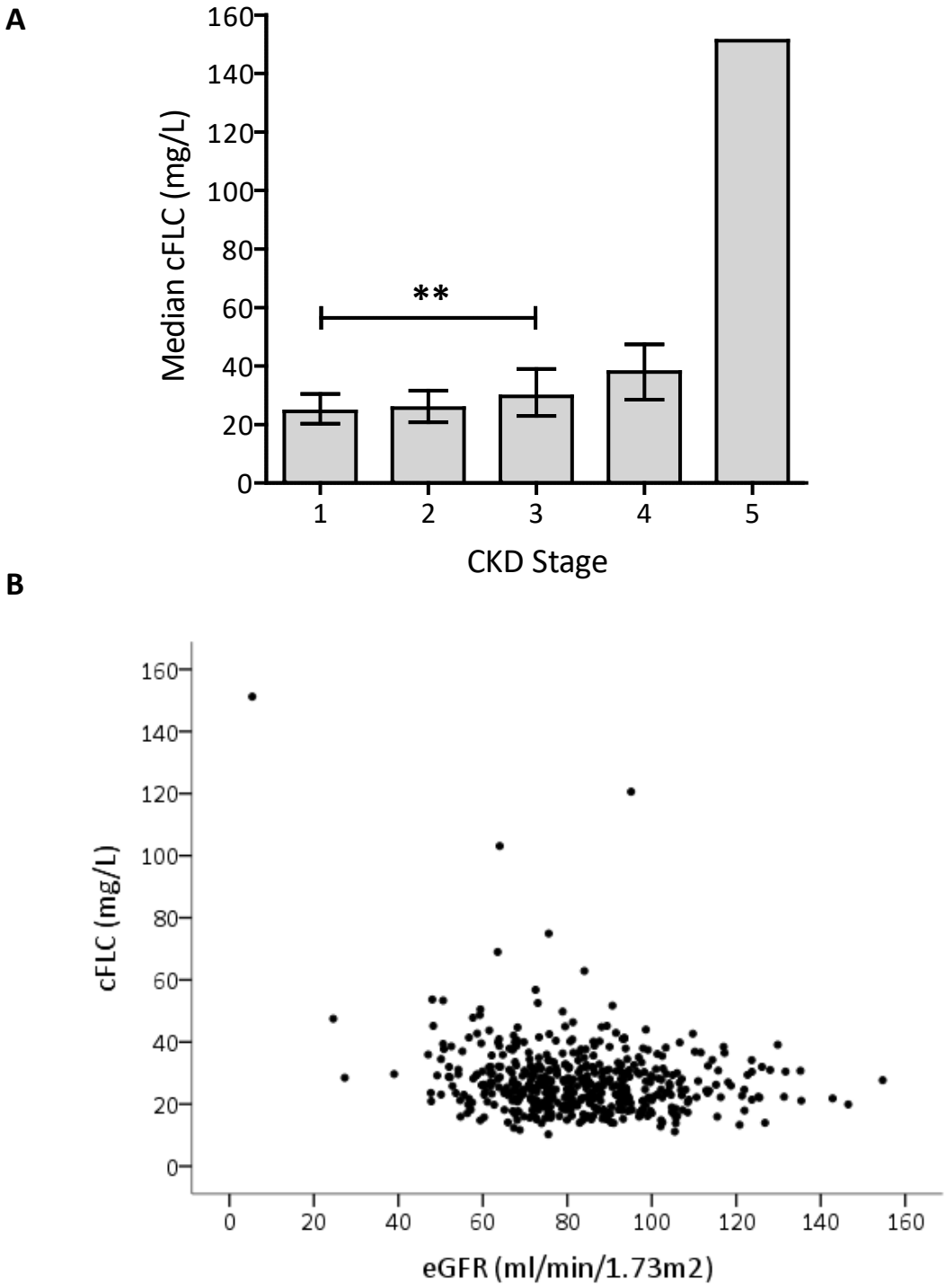

Figure 3.1: Stable state cFLC levels relationship to renal function in the A1ATD cohort

Bar chart (A) shows the relationship between combined (κ & λ) FLC (cFLC) and chronic kidney disease (CKD) stage (each bar represents the median cFLC value and error bars represent the IQR). Scatter plot (B) shows the relationship between cFLC and estimated glomerular filtration rate (eGFR). A post hoc analysis revealed that patients with CKD stage 3 had significantly higher cFLC compared to patients with CKD stage 1 (**$p \leq 0.01$). A weak negative correlation between cFLC and eGFR was also seen (r_s=-0.14, p=0.003).

3.2.2 Concurrent autoimmune disease prevalence

Increase polyclonal FLCs have been observed in a number of autoimmune diseases (as discussed in section 1.4.1.4). It was therefore important to establish the prevalence of co-existing autoimmune disease in our A1ATD cohort. The results showed that at least one autoimmune disease was present in 15.6% of patients (n=84), the most common being thyroid disease (4.1%, n=22), diabetes (3.1%, n=17), ulcerative colitis (2.1%, n=11), psoriasis (1.3%, n=7) and vasculitis (0.9%, n=5). However, cFLC levels did not differ between patients with and without a co-existing autoimmune disease (autoimmune 26.6mg/L IQR=22.3-34.4, versus no autoimmune disease 25.5mg/L IQR=20.8-31.5, p=0.125). There was no difference in the number of autoimmune conditions exhibited by the patients with cFLC levels outside the normal range (>43.3mg/L) compared to those with normal levels (Chi-squared test, p=0.320).

3.3 Stability of FLCs as a biomarker in severe A1ATD

In a subgroup of 19 patients within the A1ATD cohort, we analysed 3 further samples in addition to their baseline FLC sample with at least 1 year between sample collection time points to establish if FLC levels remained stable. All patients were clinically stable (i.e. minimum of 6 weeks post exacerbation) at the time of sample collection. There was no significant difference in cFLC levels taken at 4 time points in stable disease (Friedman test, p=0.116).

Figure 3.2 shows Bland-Altman plots demonstrating the difference between cFLC levels at follow up compared to baseline. Linear regression analyses revealed no significant proportional bias of follow up cFLC values.

In stability analyses of other biomarkers in COPD, the proportion of values within 25% of the baseline value is often quoted as a measure of stability (136). Within our A1ATD cohort 44/57 of the follow up cFLC levels were within 25% of the baseline value (77.2%). When broken down to the different time-points, the percentage of cFLC values within 25% of the baseline were as follows: 84.2% at time-point 1, 78.9% at time-point 2 and 68.4% at time-point 3.

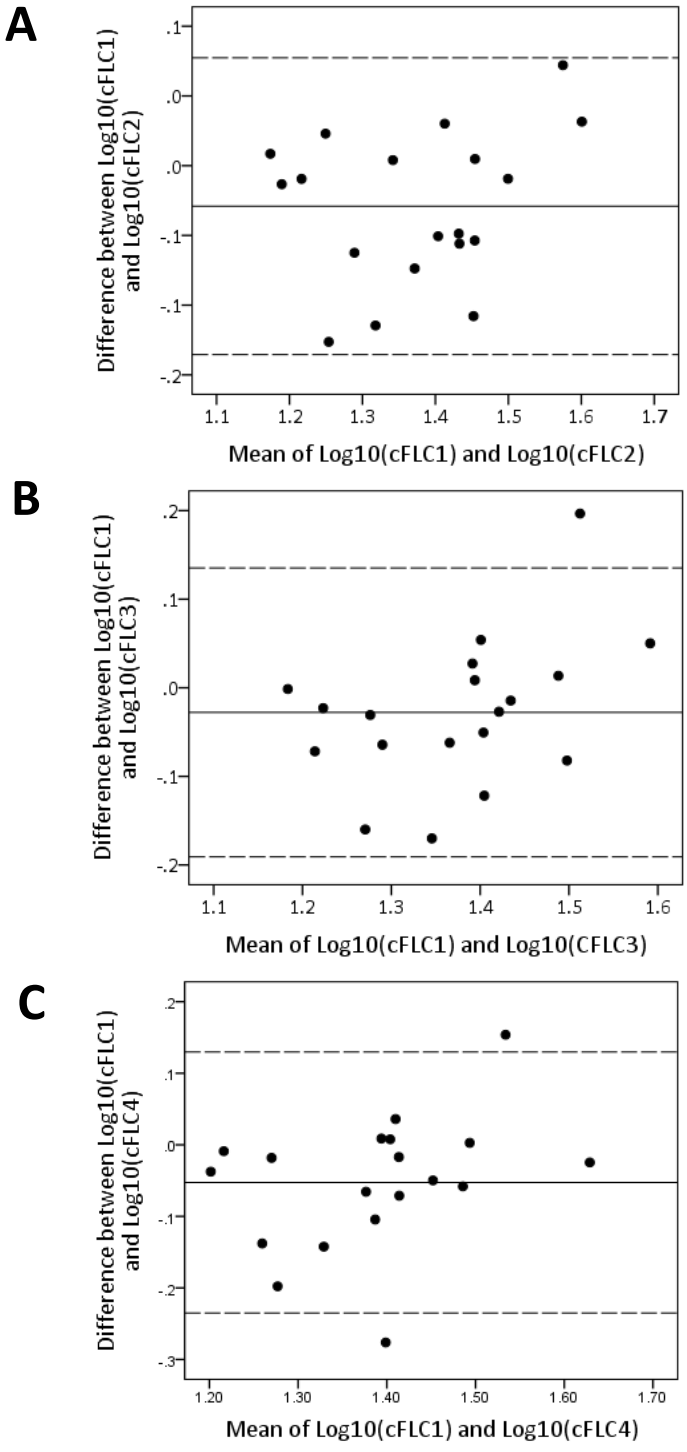

Figure 3.2: Bland Altman Plots showing the differences between cFLC between baseline (cFLC1) and three follow up time-points (cFLC2,3,4)

All cFLC values were log transformed before analysis. The solid horizontal line represents the mean difference between the Log10(cFLC) values. The dotted lines represent 95% confidence intervals. Linear regression analysis revealed no significant proportional bias between baseline and follow up cFLC values. **A.** Log10(cFLC)1 v 2, t=1.00, p=0.33 **B**. Log10(cFLC)1 v 3, t=1.94, p=0.07 **C**. Log10(cFLC)1 v 4, t=1.472, p=0.159.

3.4 FLCs and clinical phenotypes in severe A1ATD

The results from the univariate analysis of serum cFLC levels in different patient subgroups are shown in Table 3.2 for total cFLC as well as κ & λ FLC results and the κ/λ ratio.

Patients with chronic bronchitis had significantly higher cFLC levels compared to those without (median 27.0 v 25.0 mg/L, p = 0.019). This clinically important subgroup of patients with COPD suggests a greater adaptive immune response reflecting airway inflammation and/or airway colonisation. The λ FLC levels were also significantly higher (median 14.4 v 13.5 mg/L, p=0.02) and there was a trend towards the κ levels also being higher (median 12.2 v 11.4 mg/L, p=0.08). There was no significant difference in κ/λ ratio between the two groups.

No significant differences were seen with respect to presence of bronchiectasis, emphysema, or a history of frequent exacerbations. λ FLCs were, however, significantly higher in smokers compared to non-smokers (median 15.6 v 13.7 mg/L, p= 0.044).

	Group 1	Group 2	P value
Emphysema	Yes (n=358)	No (n=145)	
cFLC	25.85 (21.1-31.5)	25.41 (21.3-32.1)	0.982
κ	11.57 (9.1-15.6)	11.73 (9.2-15.0)	0.978
λ	13.70 (11.0-16.9)	13.81 (10.6-17.5)	0.872
κ/λ	0.88 (0.7-1.1)	0.85 (0.7-1.0)	0.646
Bronchiectasis	Yes (n=142)	No (n=353)	
cFLC	26.30 (21.8-31.5)	25.41 (20.9-31.9)	0.350
κ	12.33 (9.7-15.8)	11.55 (9.0-15.5)	0.136
λ	14.22 (11.2-17.0)	13.80 (10.7-17.3)	0.656
κ/λ	0.92 (0.7-1.1)	0.84 (0.7-1.1)	0.088
Chronic bronchitis	Yes (n=185)	No (n=355)	
cFLC	26.98 (21.7-33.6)	25.02 (20.8-30.8)	**0.019***
κ	12.23 (9.5-16.4)	11.41 (9.0-14.8)	0.080
λ	14.40 (11.2-17.7)	13.48 (10.6-16.8)	**0.020***
κ/λ	0.89 (0.7-1.1)	0.86 (0.7-1.1)	0.672
Current Smokers	Yes (n=45)	No (n=495)	
cFLC	27.39 (22.2-34.3)	25.56 (21.1-31.6)	0.211
κ	10.90 (9.3-17.1)	11.81 (9.1-15.4)	0.777
λ	15.64 (12.1-19.9)	13.70 (10.7-17.1)	**0.044***
κ/λ	0.82 (0.7-1.0)	0.87 (0.7-1.1)	0.070
Gender	Male (n=311)	Female (n=229)	
cFLC	25.69 (21.4-32.2)	25.75 (20.8-31.1)	0.455
κ	11.84 (9.3-15.8)	11.41 (8.9-15.0)	0.343
λ	13.88 (11.2-17.1)	13.54 (10.6-17.2)	0.425
κ/λ	0.86 (0.7-1.1)	0.86 (0.7-1.1)	0.687
Frequent exacerbations	Yes (n=129)	No (n=187)	
cFLC	24.69 (21.8-31.7)	26.36 (20.9-31.7)	0.703
κ	11.32 (9.0-14.9)	12.23 (9.6-15.4)	0.393
λ	13.46 (10.9-16.6)	13.84 (10.8-17.5)	0.865
κ/λ	0.84 (0.7-1.0)	0.91 (0.7-1.1)	0.114

Table 3.2: Comparing cFLC concentrations in different patient subgroups in the A1ATD cohort

Median individual and combined κ & λ (cFLC) levels (mg/L) reported with inter-quartile range in brackets. Mann Whitney U Tests performed to determine any statistical differences between groups 1 and 2 (*p ≤ 0.05). Where statistical differences were found the p value is highlighted in bold red text.

3.4.1 Chronic bronchitis logistic regression analysis

A logistic regression was performed to establish whether any specific variable predicts the presence of chronic bronchitis in the A1ATD cohort (Table 3.3). Chronic bronchitis has previously been shown to be more common in men and current smokers (213), associated with more severe airflow obstruction and an increased risk of exacerbations (214). A multivariate analysis was therefore necessary, to adjust for these factors. The results showed that a three variable model with the following predictor variables: serum cFLC level, frequent exacerbations (≥ 2 per annum) and FEV_1 (%predicted) was statistically significant (omnibus chi-square 16.2, df = 3, p= 0.001). However, the model only accounted for 5 – 7% of the variance, with a high negative predictive value (96.2%) but a poor positive predictive value - only successfully predicting the presence of chronic bronchitis in 9.7% of the patients. A higher cFLC and lower FEV_1 were associated with an increased likelihood of having chronic bronchitis and frequent exacerbators were 1.7 times more likely to have chronic bronchitis.

Variable	Univariate analysis P value	Multivariate model
cFLC	**0.019***	**0.032***
Current smoking status	**0.133**	
Pack years	**0.023***	
Gender	**0.055**	
Emphysema	0.553	
Bronchiectasis	0.487	
Frequent exacerbations (≥2)	**0.007***	**0.041***
FEV_1 (% predicted)	**<0.001***	**0.020***
KCO (% predicted)	**0.010***	
FEV_1/FVC	**0.007***	

Table 3.3 Predictors of chronic bronchitis in the A1ATD cohort. Univariate analysis and multivariate logistic regression model.

(*2p = ≤ 0.05). Variables with a 2p value of > 0.05 but <0.25 are highlighted in bold. Any variables with a p value <0.25 in the univariate analysis were included in the multivariate model to ensure no important variables are excluded. Only one lung function parameter was included in the multivariate models to avoid collinearity between variables. The final model demonstrated that three variables significantly predicted chronic bronchitis in the A1ATD cohort – cFLC, frequent exacerbations, FEV_1 (% predicted).

3.4.2 FLCs and chronic colonisation of the airways

The driving force behind the adaptive immune activation seen in patients with COPD is not known. It has been hypothesised that colonisation of the airways by bacteria may be an important contributing factor. Prior studies looking at the prevalence of colonisation in patients with COPD report it to be as high as 48% (215). However, this in part depends on the criteria used to define colonisation (e.g. the cut off for airway bacterial load considered clinically significant) which appears to have no published consensus. In order to examine the relationship between airway colonisation and FLC levels in the A1ATD cohort a comparison of patients known to be chronically colonised and patients that had no evidence of colonisation (i.e. no positive sputum cultures in their stable state) was performed.

Clear criteria were used to define which patients in the A1ATD cohort were chronically colonised with bacteria, based on the stored research sputum samples (2.2.1.1). Sputum specimens taken during stable disease state were available in 152/540 patients. A positive culture was defined as a growth ≥ 1×10^5 colony forming units (CFU) per ml of sputum of a "potentially pathogenic microorganism (PPM)". We compared the FLCs of 53 patients who had no positive sputum cultures in their stable state and 12 patients who were chronically colonised with ≥1 PPM. Within the chronically colonised group, two patients were colonised with pseudomonas, 4 with haemophilus influenza and 6 were chronically colonised with more than one organism. Patients who were chronically colonised had significantly higher cFLCs compared to patients with no positive cultures (Figure 3.3) (median cFLC 35.7 (26.4-42.4) versus 26.3 (22.0-31.2) mg/L, p = 0.008).

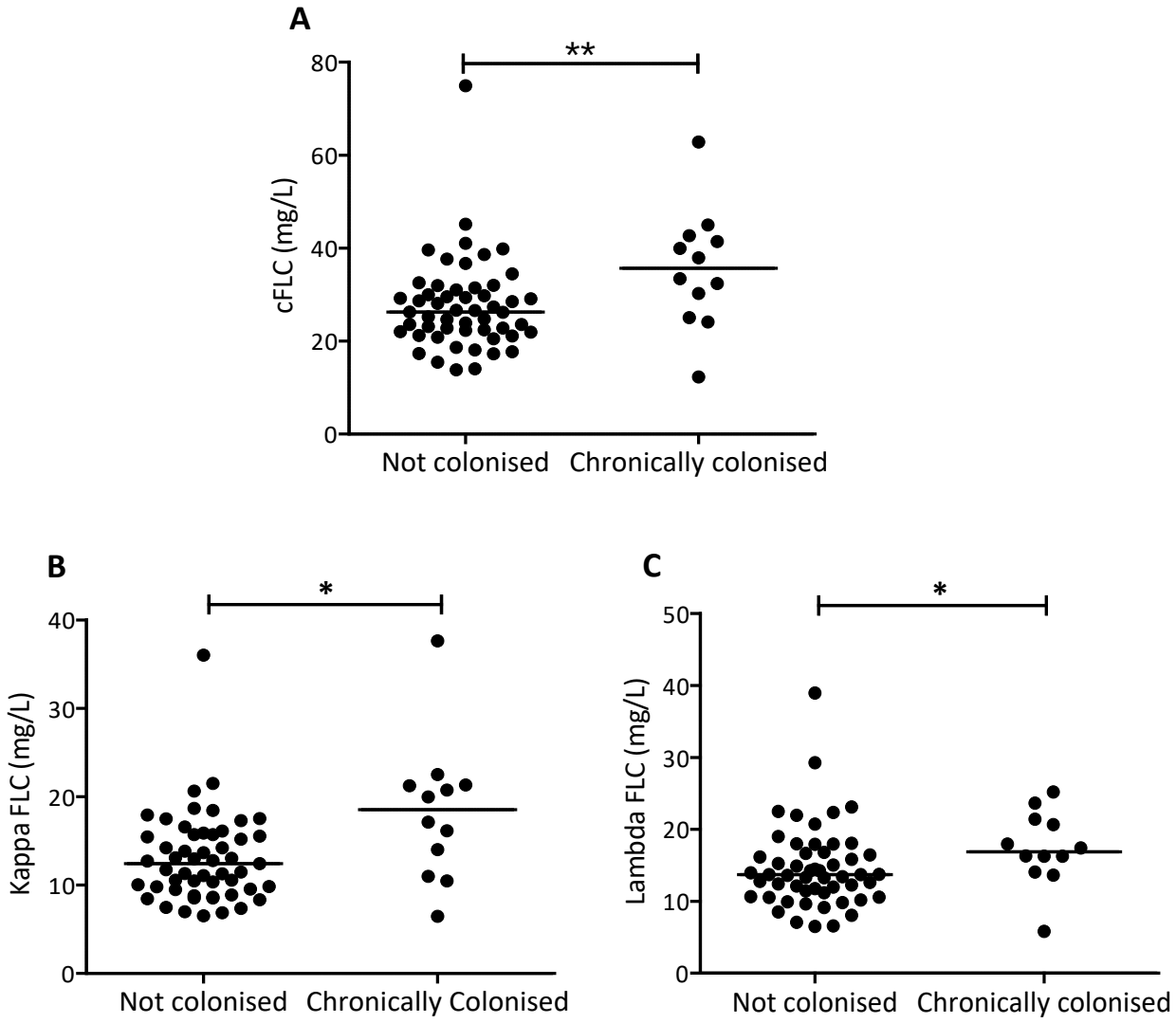

Figure 3.3 Scatterplots showing FLC levels in chronically colonised and non-colonised patients.

The figures show univariate comparisons between chronically colonised and non-colonised individuals with the A1ATD cohort. Each dot represents an individual patient and the lines represent median values per group. Mann-Whitney U tests were performed to compare the groups. **A.** Combined κ & λ FLC (cFLC) median 35.7 (26.4-42.4) v 26.3 (22.0-31.2) **B.** κ FLC median 18.5 (11.7-21.3) v 12.4 (9.5-15.7) **C.** λ FLC median 16.9 (14.6-21.2) v 13.7 (10.9-16.7). *p ≤ 0.05, **p ≤ 0.01. Graphs B and C show that both the κ and λ FLCs were significantly higher in the chronically colonised group therefore demonstrating a polyclonal difference.

3.5 FLCs relationship to COPD disease severity and activity in severe A1ATD

3.5.1 Disease severity

FEV_1 is the most commonly used marker of disease severity in COPD. Therefore, to establish if there was any relationship to COPD disease severity in the A1ATD cohort, the relationship of FLCs to baseline lung function parameters was investigated. Within the A1ATD cohort 455/540 (84.3%) had airflow obstruction as measured by a post bronchodilator FEV_1/FVC ratio of <0.7. There was no significant difference between the cFLC level in those with and without airflow obstruction (median 25.8 (21.2-31.6) v 24.9 (20.9-33.1) mg/L respectively, p = 0.937).

As the FLC results were not normally distributed (Kolmogorov-Smirnov test p <0.001) Spearman's Rho correlations with lung function parameters were performed to look for any significant relationships (Table 3.4). Weak positive correlations were seen between κ, κ/λ, cFLC and FEV_1. A weak positive correlation was also seen between κ FLC levels and FEV_1/FVC ratio and weak negative correlations between κ FLC levels and RV (residual volume % predicted) and κ/λ ratio and RV. Scatterplots (Figure 3.4, 3.5, 3.6) revealed 3 potential outliers with very high κ FLC levels (>60mg/L normal range = 3.3-19.4) and cFLC levels (>100mg/L normal range = 9.3-43.3). The clinical notes of these patients were examined to identify the possible cause for these high cFLC results. One patient had CKD stage 5 so partial correlations controlling for eGFR were then performed (see below). The two other patients had significant liver cirrhosis. Consideration as to whether these potential outliers should be

removed was given. The number of patients outside the 3 median absolute deviations from the median was calculated but given the broad range of cFLC results this approach resulted in many patients being excluded from analysis. Liver cirrhosis is a complication of A1ATD and therefore the condition potentially causing their increased polyclonal FLC level is also relevant to the disease cohort and on this basis and the lack of a robust statistical way of identifying outliers the decision was made not to exclude them from the analysis.

As discussed in section 3.2.1, weak negative correlations between cFLC and age and renal function (as measured by eGFR) were present. Partial correlations controlling for these factors were then performed to determine the effect on the relationship between FLC levels and lung function measurements independent of age and eGFR (Table 3.5) Only cases where all the appropriate clinical information was available were therefore included in this correlation (n = 387). The results show that despite controlling for age and renal function the weak correlations between κ FLC levels and lung function remain.

		FEV_1 %p (n=539)	FVC %p (n=539)	FEV_1/FVC (n=539)	TLC %p (n=449)	RV %p (n=449)	KCO %p (n=455)	TLCO %p (n=454)
κ	r_s	0.105	0.063	0.087	-0.055	-0.115	0.034	0.032
	p	**0.015***	0.147	**0.043***	0.241	**0.015***	0.467	0.492
λ	r_s	0.064	0.025	0.052	0.023	-0.020	-0.008	0.028
	p	0.140	0.557	0.227	0.633	0.679	0.866	0.553
κ/λ	r_s	0.087	0.070	0.068	-0.067	-0.108	0.036	0.015
	p	**0.044***	0.102	0.116	0.154	**0.022***	0.444	0.755
cFLC	r_s	0.086	0.042	0.073	-0.019	-0.067	0.010	0.026
	p	**0.046***	0.330	0.092	0.682	0.154	0.825	0.582

Table 3.4 Spearman's Rho correlations between FLC levels and lung function parameters

FEV_1 = Forced expiratory volume in 1 second, FVC = Forced vital capacity, TLC = Total lung capacity, RV = Residual volume, KCO = Corrected gas transfer, TLCO = Transfer factor of the lung for carbon monoxide, cFLC = combined κ & λ FLC (mg/L), r_s= correlation coefficient, %p = percentage predicted. (*Statistically significant p values are highlighted)

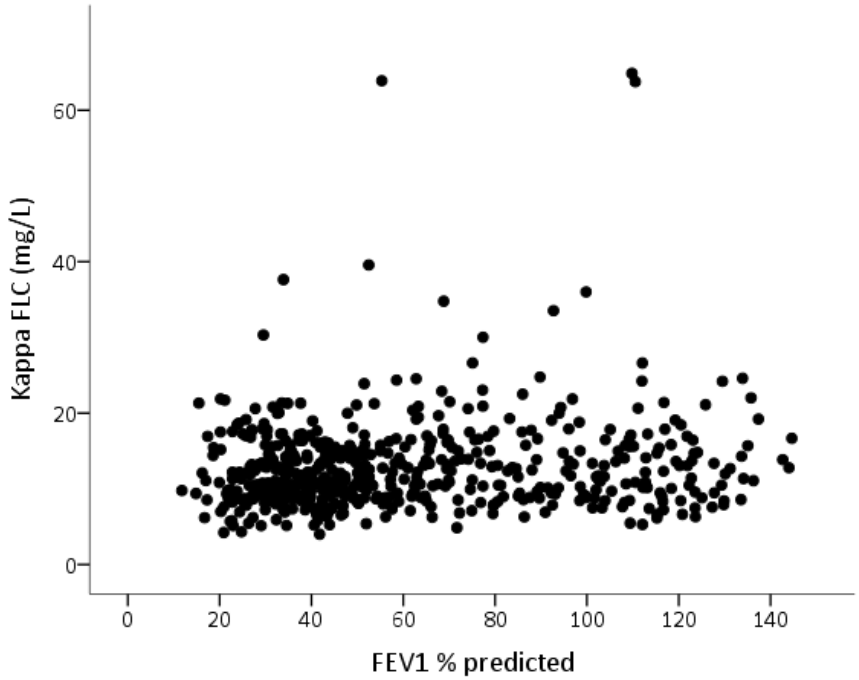

Figure 3.4 Scatterplot of κ FLC against FEV_1 (% predicted) in the A1ATD cohort

FLC = free light chain, FEV_1 = forced expiratory volume in 1 second. Each dot represents a single patient. (Spearman's Rho r_s=0.105, p=0.015)

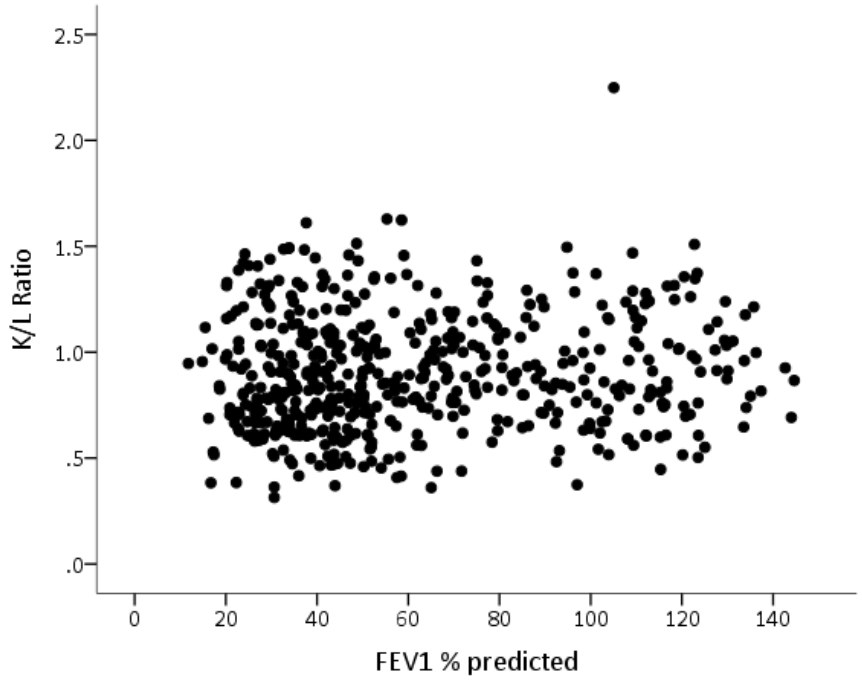

Figure 3.5 Scatterplot of κ/λ ratio against FEV_1 (% predicted) in the A1ATD cohort

FEV_1 = forced expiratory volume in 1 second. Each dot represents a single patient. (Spearman's Rho r_s=0.087, p=0.044)

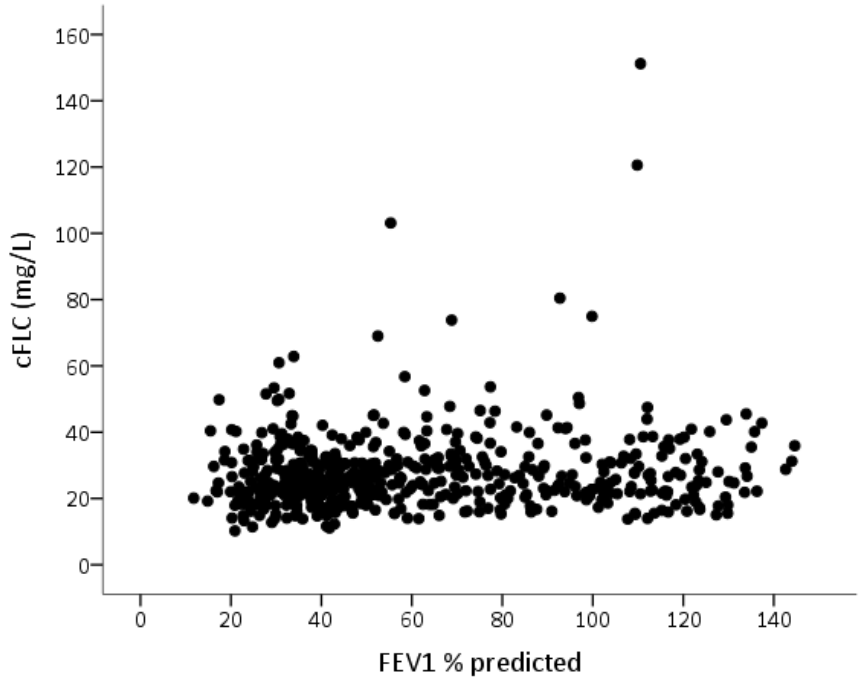

Figure 3.6 Scatterplot of cFLC (mg/L) against FEV_1 (% predicted) in the A1ATD cohort

cFLC = combined κ & λ free light chain (mg/L), FEV_1 = forced expiratory volume in 1 second. Each dot represents a single patient. (Spearman's Rho r_s=0.086, p=0.046)

Cont. Age & eGFR		FEV1 %p (n=389)	FVC %p (n=389)	FEV1/FVC (n=389)	TLC %p (n=389)	RV %p (n=389)	KCO %p (n=389)	TLCO %p (n=389)
Kappa	r_p	0.130	0.040	0.130	-0.101	-0.129	0.102	0.100
	p	**0.010***	0.427	**0.010***	**0.045***	**0.011***	**0.043***	**0.049***
Lambda	r_p	0.058	-0.008	0.078	-0.058	-0.058	0.057	0.061
	p	0.255	0.873	0.123	0.253	0.254	0.258	0.230
κ/λ ratio	r_p	0.116	0.050	0.103	-0.087	-0.108	0.082	0.058
	p	**0.022***	0.325	**0.041***	0.084	**0.033***	0.104	0.249
cFLC	r_p	0.099	0.016	0.111	-0.085	-0.099	0.085	0.085
	p	**0.050***	0.750	**0.029***	0.094	0.051	0.094	0.092

Table 3.5 Partial correlation (controlling for age and eGFR) between FLC levels and lung function parameters

FEV_1 = Forced expiratory volume in 1 second, FVC = Forced vital capacity, TLC = Total lung capacity, RV = Residual volume, KCO = Corrected gas transfer, TLCO = Transfer of the lung for carbon monoxide, eGFR = estimated glomerular filtration rate, cFLC = combined (κ & λ) free light chain (mg/L), r_p = correlation coefficient, %p = percentage predicted.

3.5.1.1 FLCs according to GOLD guidelines classification of airflow obstruction in A1ATD

Although no strong correlations between cFLC and FEV_1 were demonstrated, when patients were sub-grouped according to the severity of their airflow obstruction (GOLD guidelines: mild FEV_1 >80% predicted, moderate 50-80%, severe 30-50% and very severe <30%) significant differences between the groups were identified (Kruskall Wallis test ($\chi^2(3) = 11.26$, $p = 0.01$). The median cFLC within the groups were as follows: mild 26.5, moderate 27.8, severe 25.4, and very severe 23.2. A post-hoc analysis revealed the groups that were significantly different from one another (Figure 3.7). A Bonferroni calculation was used to calculate the cut off for significance ($p \leq 0.05/10 = 0.005$). The only significant difference between individual groups was a higher cFLC in patients with moderate airflow obstruction compared to those with very severe airflow obstruction ($p = 0.002$).

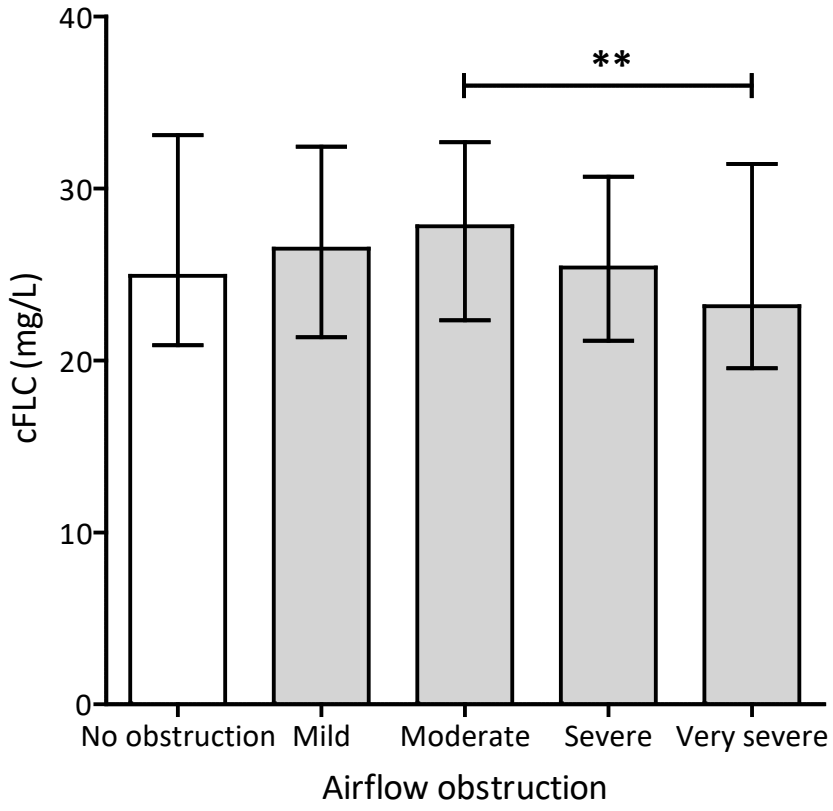

Figure 3.7 Bar chart showing median cFLC levels in patients sub-grouped according to the severity of their airflow obstruction

Bars represent median combined (κ & λ) FLC (mg/L) in each group. Error bars represent interquartile range. **p ≤ 0.005.

3.5.2 Disease activity

In addition to relating to disease severity, an ideal biomarker should reflect 'disease activity' as a surrogate marker of the underlying pathophysiological process which ultimately causes end-organ damage (216). FEV_1 decline (i.e. the degree of reduction in FEV_1 per year) has previously been utilised as a marker of disease activity in COPD. It does have limitations however as it doesn't directly reflect the underlying pathophysiology of the disease and a reduction in FEV_1 is also reflective of the preceding disease process rather than suggestive of current disease activity. However, in the absence of another validated marker of disease activity the relationship of FLCs to lung function decline was explored.

3.5.2.1 Lung function decline

The relationship between cFLC levels and disease severity as measured by lung function parameters in the A1ATD cohort was weak. However, when patients were sub-grouped according to the severity of their airflow obstruction a significantly higher cFLC level was seen in the moderate versus the very severe group. It has been previously documented that FEV_1 decline is faster in the earlier stages of COPD particularly GOLD stage 2 (217). To establish if this was true within the A1ATD cohort, the decline in FEV_1 and KCO were calculated for all patients that had a minimum of 4 recorded lung function tests. There was adequate physiological data available to calculate FEV_1 % decline in 373/540 (69%) and KCO % decline in 338/540 (62.6%) of patients.

Figure 3.8 shows the median FEV_1 and KCO % decline per year in the A1ATD cohort subdivided according to their degree of airflow obstruction. A significant difference between

the FEV_1 decline in the different GOLD groups was found (Kruskall Wallis test $\chi^2(4) = 11.7$, $p = 0.02$). A post-hoc analysis demonstrated that patients with GOLD stage 2 (moderate) airflow obstruction had significantly greater FEV_1 decline compared to those with no airflow obstruction p=0.002 (Bonferroni calculation was used to calculate the cut off for significance $p \leq 0.05/10 = 0.005$). A different pattern was seen in KCO decline (Figure 3.8 B) whereby the decline appeared to increase with worsening airflow obstruction. A significant difference in KCO decline between the different groups was also demonstrated (Kruskall Wallis test $\chi^2(4) = 12.4$, $p = 0.015$) and post-hoc analysis revealed the patients with GOLD stage 4 (very severe obstruction) had significantly faster decline in KCO than those with GOLD stage 1 (mild obstruction) p= 0.004.

Despite cFLC being the highest in gold stage 2 patients who also have the greatest rate of FEV_1 decline there was no significant correlation between FEV_1 decline and cFLC (Spearman's Rho r_s= 0.087, p=0.095).

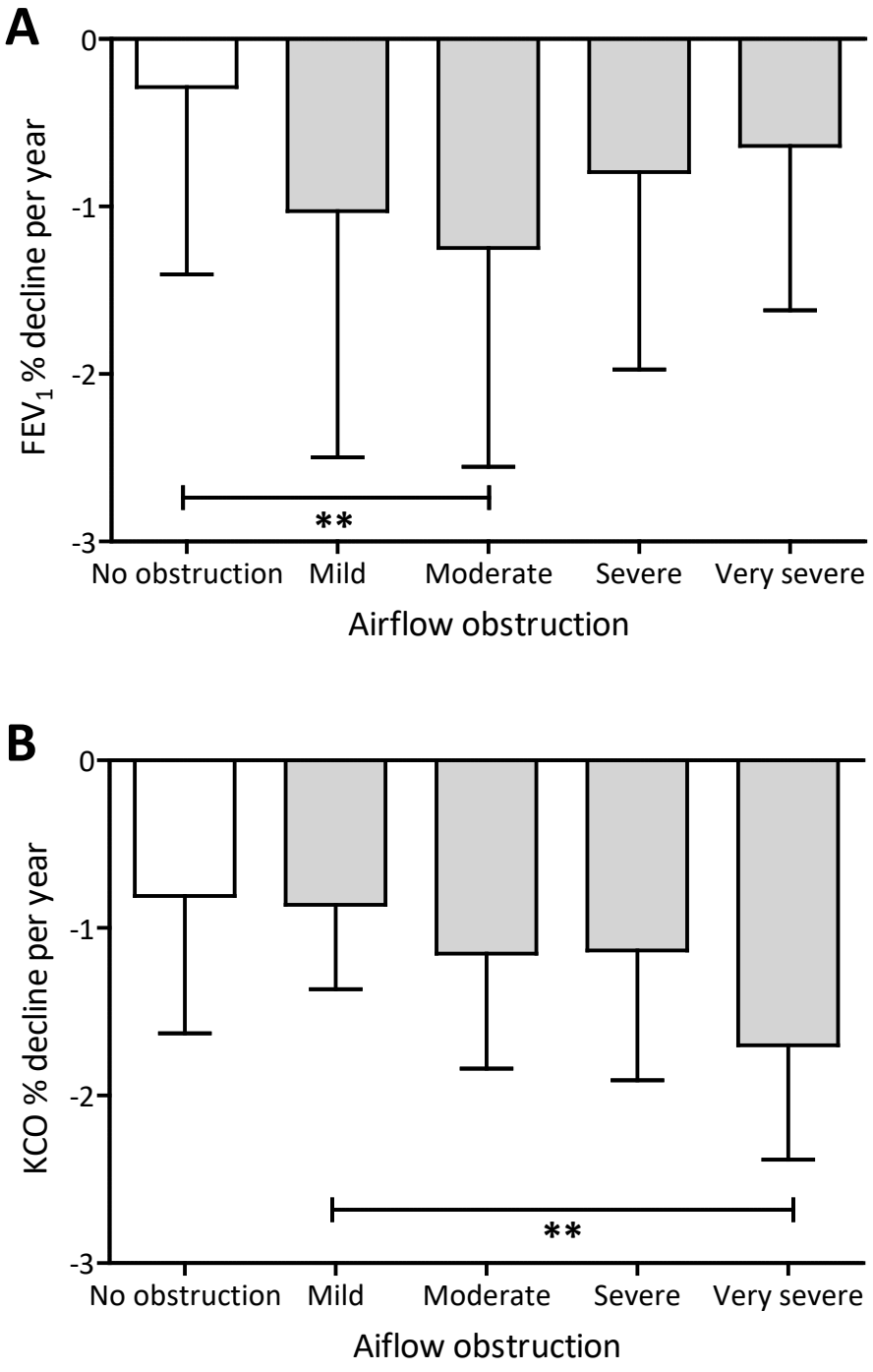

Figure 3.8 Bar charts showing lung function decline in the A1ATD cohort subdivided by GOLD stage of airflow obstruction

Bars represent median FEV_1 % decline per year (A) and KCO % decline per year (B). Error bars represent interquartile range. FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer. **p ≤ 0.005.

3.6 FLCs and longitudinal outcomes in A1ATD

3.6.1 Mortality

Sixty-nine (12.8%) patients died during the follow up period. Patients who died had significantly higher baseline cFLC levels compared to those still alive (median 29.18 (22.7-39.9) v 25.17 (21.0-31.0) mg/L, p = 0.001). Both κ and λ levels were significantly higher but there was no significant difference in the κ/λ ratio between the two groups suggesting that polyclonal FLC production was higher in those that died (Table 3.6).

A univariate cox proportional hazards regression analysis identified important mortality predictor variables in the A1ATD cohort (Table 3.7). A multivariate analysis was then performed and 3 variables were found to significantly predict mortality in this population: serum cFLC, age and FEV_1 (hazard ratios 1.04 p=0.001, 1.07 p= <0.001, and 0.97 p = <0.001 respectively). Only one lung function parameter was included in the multivariate analysis to avoid issues due to collinearity between variables.

Mortality	Dead (n = 69)	Alive (n=471)	P value
cFLC	29.18 (22.7-39.9)	25.17 (21.0-31.0)	**0.001***
κ	14.07 (9.8-17.7)	11.55 (9.0-14.8)	**0.003***
λ	15.40 (11.8-20.1)	13.6 (10.7-16.9)	**0.005***
κ/λ	0.94 (0.7-1.2)	0.85 (0.7-1.1)	0.259

Table 3.6 FLCs and mortality in the A1ATD cohort

Median individual and combined κ & λ (cFLC) levels (mg/L) reported with inter-quartile range in brackets. Mann Whitney U Tests performed to determine any statistical differences between the groups (*p ≤ 0.05). Where statistical differences were found the p value is highlighted in bold red text.

Variable	Univariate HR (CI)	P value	Multivariate HR (CI)	P value
Serum cFLC	1.03 (1.01 - 1.04)	**<0.001***	1.04 (1.01 - 1.06)	**0.001***
Pack years	1.02 (1.00 - 1.03)	0.056	1.01 (0.99 - 1.03)	0.518
Current smoker	1.02 (0.47 - 2.24)	0.957		
Gender	1.22 (0.74 - 2.01)	0.431		
Emphysema	1.85 (1.01 - 3.39)	**0.045***	1.03 (0.47 - 2.25)	0.947
Bronchiectasis	1.26 (0.75 - 2.13)	0.389		
Frequent exacerbator	1.72 (0.87 - 3.42)	0.120		
Age	1.07 (1.04 - 1.09)	**<0.001***	1.07 (1.04 - 1.11)	**<0.001***
Chronic bronchitis	1.90 (1.17 - 3.08)	**0.010***	1.21 (0.67 - 2.17)	0.531
BMI	0.91 (0.86 - 0.97)	**0.002***	0.94 (0.88 - 1.02)	0.137
eGFR	0.98 (0.97 - 1.00)	**0.020***	0.99 (0.98 - 1.00)	0.104
FEV_1 (% predicted)	0.97 (0.96 - 0.99)	**<0.001***	0.97 (0.96 - 0.99)	**<0.001***
KCO (% predicted)	0.97 (0.95 - 0.98)	**<0.001***		
FEV1/FVC	0.04 (0.01 - 0.18)	**<0.001***		

Table 3.7 Predictors of mortality in the A1ATD cohort: univariate and multivariate cox proportional hazards regression analysis

HR = hazard ratio, CI = 95% confidence intervals, cFLC = combined κ & λ free light chain level mg/L, BMI = body mass index, eGFR = estimated glomerular filtration rate, FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, FVC = forced vital capacity. A frequent exacerbator was defined as having 2 or more exacerbations per year. (*p ≤ 0.05).

3.6.1.1 Mortality in cFLC subgroups

Multivariate cox regression analysis was repeated using 2 important threshold cFLC levels – the upper limit of normal (43.3mg/L) and the threshold previously associated with death within 100 days (65mg/L) (184) (Table 3.8). The hazard ratio (HR) for death for patients with a cFLC level greater than the upper limit of normal in the A1ATD cohort was 2.89 (95% CI 1.47-5.70, p=0.002). The multivariate analysis demonstrates that this increased risk is independent of age and FEV_1. When the higher cut of 65mg/L was utilised the HR for death increased to 14.97 (95% CI 4.25 – 52.72, p<0.001 Table 3.8). The Kaplan-Meier plots showed significant differences in the survival curves according to both these levels (Figure 3.9).

	Model 1		Model 2	
Variable	Multivariate HR (95%CI)	P value	Multivariate HR (95%CI)	P value
cFLC ≥ 43.3 mg/L	2.89 (1.47-5.70)	**0.002***		
cFLC ≥ 65 mg/L			14.97 (4.25-52.72)	**<0.001***
Age	1.07 (1.04-1.10)	**<0.001***	1.07 (1.040-1.10)	**<0.001***
FEV_1 (% predicted)	0.97 (0.96-0.98)	**<0.001***	0.97 (0.96-0.98)	**<0.001***

Table 3.8 Predictors of mortality in the A1ATD cohort: cox proportional hazards regression analysis models

Model 1 shows that a combined κ & λ free light chain level (cFLC) of greater than 43.3mg/L, age and forced expiratory volume in 1 second (FEV_1) are independent risk factors for mortality in the A1ATD cohort. Model 2 uses a higher cFLC cut off of 65 mg/L. Hazard ratios (HR) reported with 95% confidence intervals (CI) in brackets. *p≤0.05 highlighted in red.

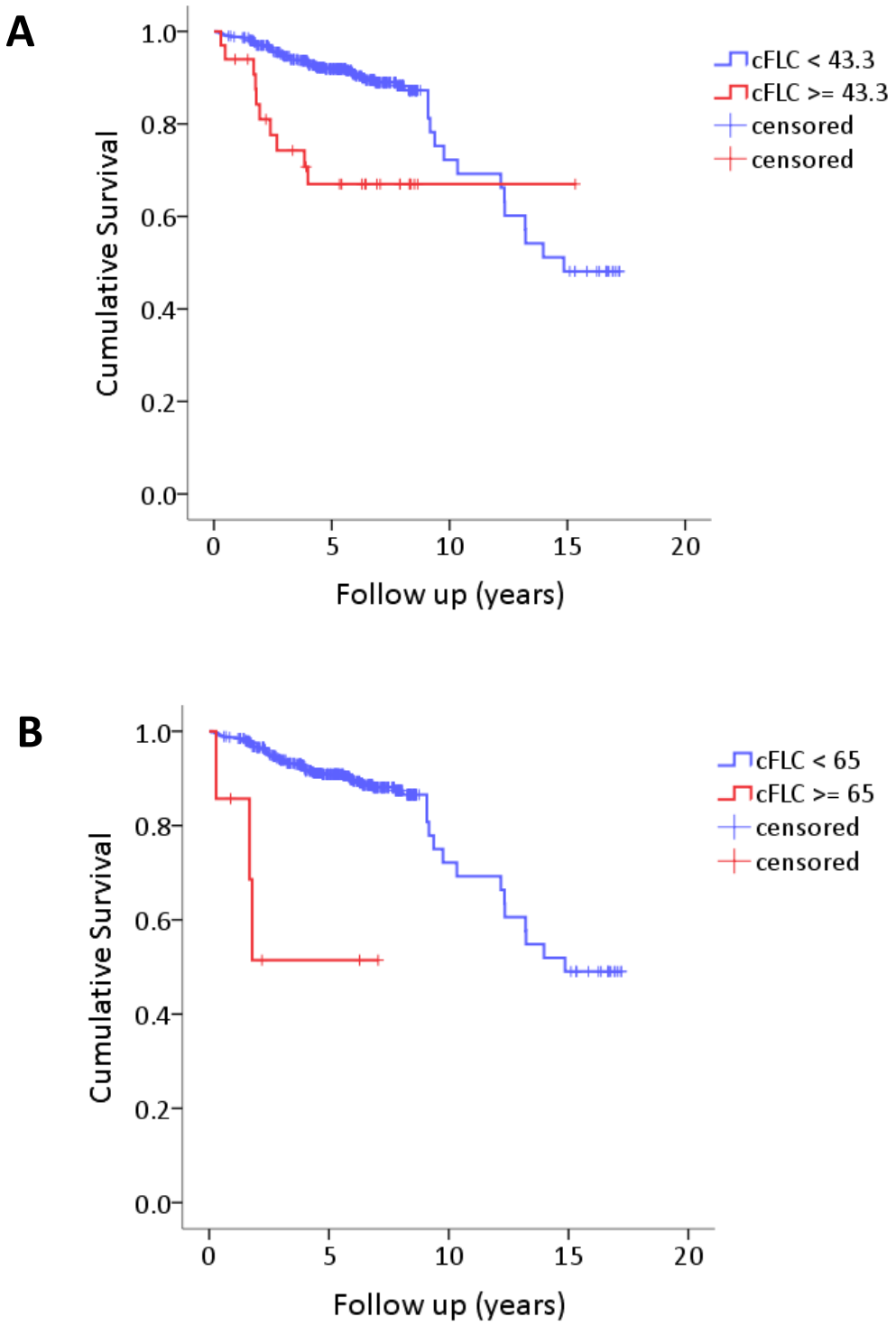

Figure 3.9 Kaplan-Meier curves according to cFLC thresholds in the A1ATD cohort

Blue line represents patients with a combined κ & λ free light chain (cFLC) of less than **A.** 43.3mg/L (the upper limit of normal), n=507, **B.** 65mg/L, n=533. Red line represents those with a cFLC level above these thresholds, n= 33 and n =7 and respectively. The Kaplan-Meier plots showed significant differences in the survival curves according to both these levels, **A.** p = 0.001, **B.** p<0.001 by Log Rank test.

3.6.1.2 Mortality according to cFLC quartiles

The patients were subdivided into cFLC quartiles. The Kaplan-Meier plot demonstrating survival of patients in each quartile (Figure 3.10) shows that during the initial period of follow up an increase in cFLC quartile was associated with a higher mortality (Breslow test p = 0.003). However, as the survival curves cross at later time points the log rank test was not statistically significant (p= 0.115).

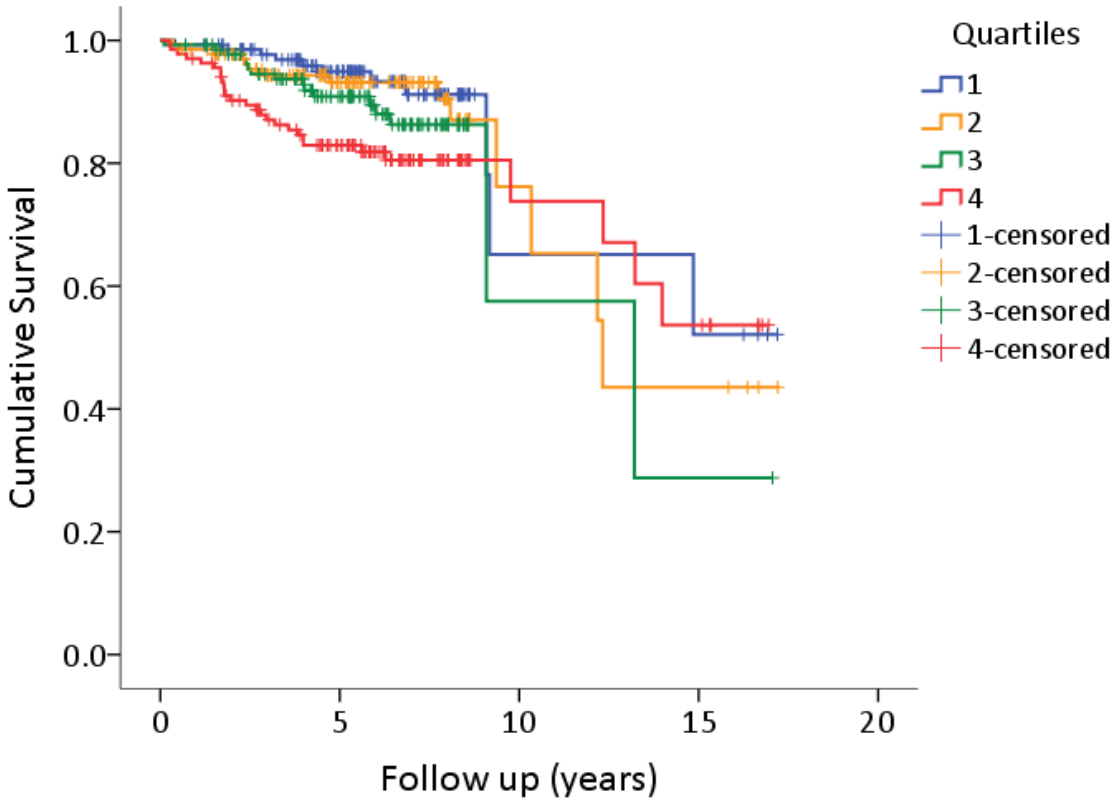

Figure 3.10 Kaplan-Meier curves according to cFLC quartiles in the A1ATD cohort

Patients were subdivided into combined κ & λ free light chain (cFLC) quartiles. The coloured lines represent each quartile: blue represents quartile 1 (cFLC 10.2 - 21.2 mg/L), orange represents quartile 2 (cFLC 21.12 - 25.7mg/L), green represents quartile 3 (cFLC 25.7 - 31.7mg/L) and red represents quartile 4 (cFLC 31.7 – 151.2mg/L), n = 135 in each group. Breslow p=0.003, Log Rank p = 0.115.

3.6.2 ROC curve analysis

Figure 3.11 shows a receiver operating characteristic (ROC) curve which examines the specificity and sensitivity of cFLC for mortality prediction in the A1ATD cohort. The area under the curve was 0.62 (95% CI 0.5-0.69, p=0.001). The overall accuracy of cFLC in identifying patients who die subsequently is therefore poor.

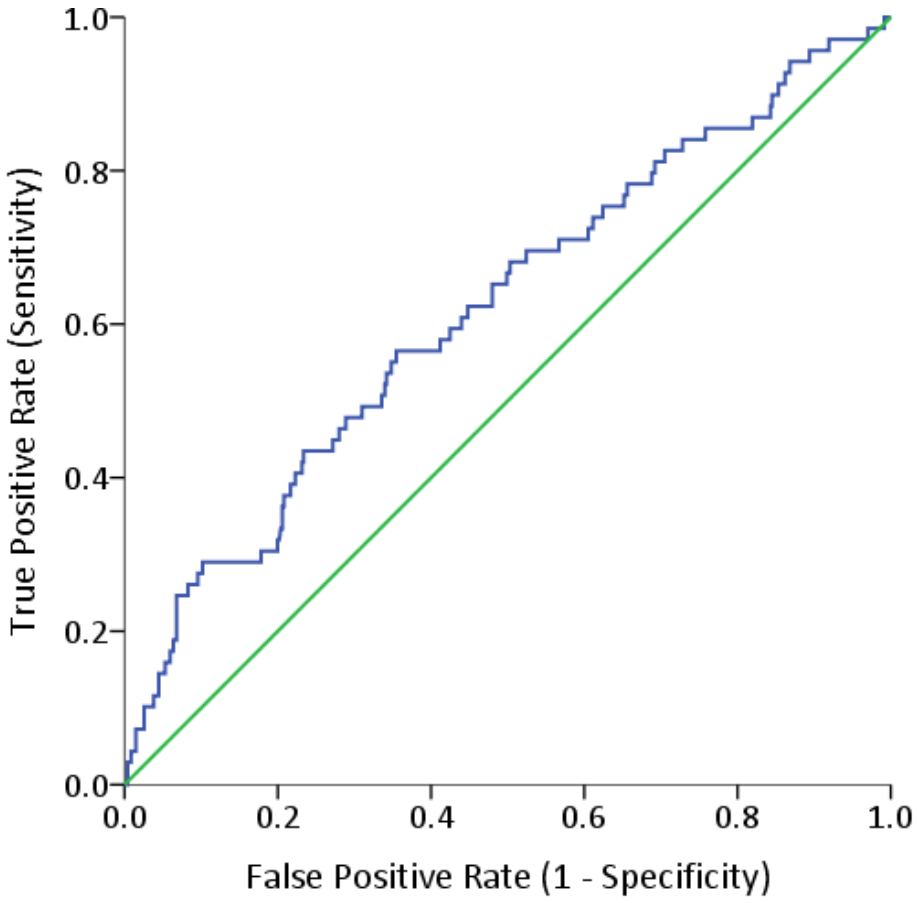

Figure 3.11 Receiver operating characteristics (ROC) curve assessing the sensitivity and specificity of combined κ & λ FLC for mortality prediction in severe A1ATD

Area under the curve = 0.62 (95% CI 0.5-0.69, p=0.001).

3.7 Discussion

The results presented in this chapter report the ability of FLCs to fulfil the criteria of a useful clinical biomarker in A1ATD related COPD.

3.7.1.1 FLC Stability

No significant difference was seen in cFLCs taken from patients with stable disease at different time points over several years, suggesting that cFLCs are reproducible in stable disease. FLC stability compares well to other biomarkers in COPD. For example Dickens *et al* looked at the repeatability of biomarkers in the ECLIPSE cohort (136) including the percentage of subjects in which the 3-month follow up sample was within 25% of the baseline for 16 biomarkers. CC-16 and fibrinogen were the only biomarkers with superior stability to FLCs (with 90% and 89% within 25 % of baseline respectively compared to 84.2% for FLC at the first follow up). However, the results are not entirely comparable as their follow up time frame was shorter in the ECLIPSE cohort and therefore less variability may be expected. Nevertheless, overall the stability of FLCs is similar to that of other promising COPD biomarkers.

3.7.1.2 FLC relationship to disease phenotypes

Useful biomarkers should help identify disease phenotypes which may help stratify treatment strategies. For example, a prior study demonstrated that AαVal360 (a NE specific fibrinogen cleavage product) was higher in patients with severe A1ATD and visible emphysema compared to those without (218). Within the A1ATD cohort a number of differences in cFLC between various patient subgroups were identified.

Patients with chronic bronchitis were found to have significantly higher cFLC levels compared to those without. Chronic bronchitis is recognised to be a clinically relevant subgroup within airways disease being associated with more rapid FEV_1 decline (219), increased exacerbation frequency (220) and a greater risk of death (221). However, the difference between cFLCs in patients with chronic bronchitis compared to those without was relatively small, thus the result must be interpreted with clinical caution.

The prevalence of other autoimmune diseases was low in our A1ATD cohort, and no relationships were seen between cFLC levels and autoimmune disease burden. However, prior studies suggest that cFLCs change during periods of disease 'activity' (e.g. in rheumatoid arthritis (159) and systemic lupus erythematosus (157)) such that presence of well controlled (inactive) autoimmune conditions might explain the lack of association observed.

Many questions regarding the role B cells play in the development of COPD remain unanswered. For example, which antigens drive the B cell response? Is the response specific to the lung or not? Commonly hypothesised antigen sources are microbes colonising the airways, smoke constituents and breakdown products of the extracellular matrix (222). In the A1ATD cohort we found that chronically colonised patients had significantly higher cFLC levels, supporting the hypothesis that colonisation itself may be an important factor in adaptive immune activation. Another theory is that infection or colonisation with bacteria leads to a breakdown in self – tolerance, promoting an immune reaction to self-antigens. This concept is well established in a number of autoimmune diseases (223). In support of this theory, Calabrese et al have shown that the novel cytokine Interleukin-32 (IL-32) is expressed within the lungs of patients with COPD and may play a role in amplifying the

adaptive immune response to antigens by promoting the production of other pro-inflammatory cytokines such as TNFα (224). IL-32 has been shown to be upregulated in other autoimmune conditions such as rheumatoid arthritis and inflammatory bowel disease (225, 226).

The results also showed that current smokers had significantly higher λ FLC levels compared to non-smokers or ex-smokers. Smoking is known to affect the adaptive immune response within the lung. Interestingly several studies have shown smoking to be associated with a systemic reduction in immunoglobulin levels (227-231) however the mechanism by which this occurs is unknown. Mili *et al* observed lower immunoglobulin levels but 37% higher B cell counts in smokers versus non-smokers in a large cohort of middle aged men (230). However, higher autoantibody levels in smokers have also been reported (232).

3.7.1.3 FLC relationship to disease severity

There was no difference in cFLC in patients with and without airflow obstruction in the A1ATD cohort. From previous studies the median cFLC in a healthy population is ≈ 20mg/L (176) which is lower than that of our A1ATD cohort even in the absence of airflow obstruction. This may suggest that patients with A1ATD show evidence of a greater adaptive immune response even when they have not developed airflow obstruction. However, to confirm this and determine its course we would need to measure FLCs in an age and renal function matched healthy population.

The fact that A1ATD patients with normal lung function have a cFLC comparable to those with airflow obstruction may reflect the likelihood that some of these patients may have

early lung disease with lung function still within the normal range. Indeed, eight patients who did not have airflow obstruction still had emphysema seen on their CT scan. Within the group with normal spirometry there was a trend toward those with emphysema having a higher cFLC level although this did not meet statistical significance (median 30.1 v 24.6, p=0.063). An alternative explanation is that an increase in FLC production may occur as a result of inflammation caused by the unopposed action of proteases, Z A1AT polymerisation within the liver or lung (ER stress) and other immune modulatory effects resulting from the lack of A1AT (125).

Other biomarkers show a relationship to disease severity however there was not a strong relationship between cFLC and FEV_1 in A1ATD. When patients were sub-grouped according to the severity of their airflow obstruction, a significantly higher cFLC level was, nevertheless, seen in the moderate (GOLD stage 2) versus the very severe group (GOLD stage 4). This is somewhat counterintuitive since the number of B cell follicles in the lung of patients with COPD increases with worsening airflow obstruction however (49). A contemporaneous increase in excess FLC production with increasing lymphoid follicle number would therefore seem more likely and a clear relationship (at least with severity) might be expected. However, FLC was assessed in the blood and not the lung. The concept of systemic inflammation and circulating inflammatory markers resulting from an 'overspill' from the lung remains an area of debate (233). If this is true of FLCs in COPD then measuring FLCs as a marker of B cell activation within the lung may be expected to be a measure of current local disease 'activity' rather than 'severity'. The data may therefore suggest that although there are a greater number of B cells within the lung of A1ATD patients with more severe COPD (125) it does not necessarily reflect the degree of B cell activation in terms of

immunoglobulin and FLC production. This may explain the lack of relationship between cFLC and FEV_1. It is also possible that patients with more severe COPD may have been more likely to be taking immunomodulatory drugs such as high dose inhaled or oral steroids. Treatment with corticosteroids has been shown to reduce the lymphocytic infiltration of the small airways in COPD (234) and reduce the number of lymphoid follicles in severe COPD (235).

3.7.1.4 FLC relationship to disease activity

In other autoimmune and inflammatory conditions such as rheumatoid arthritis and systemic lupus erythematosus, FLCs have been shown to relate to disease activity as measured by disease activity scores such as the Disease Activity Score S28 (DAS28) and the SLE disease activity index (SLEDAI) (157, 159).

Disease activity in COPD is generally regarded as low with slow progression. FEV_1 decline is often used as a surrogate marker for disease activity in patients with COPD.

It is recognised that average FEV_1 decline is faster in the earlier stages of COPD particularly GOLD stage 2 (217). Within the A1ATD cohort, patients with GOLD stage 2 (moderate) airflow obstruction had significantly faster annual FEV_1 decline compared to those without airflow obstruction. However, despite GOLD stage 2 patients also having a significantly higher cFLC level compared to GOLD stage 4 patients no significant correlation between cFLC and FEV_1 decline was evident.

FLCs have a short half-life of around 2-3 hrs for κ FLCs and 5-6hrs for λ FLCS (236). In a study examining the use of FLCs as a biomarker in RA and primary Sjogren's syndrome this was promoted as a potential benefit as they were deemed to reflect short term disease activity better than other markers of B cell activity with a longer half-life such as total

gammaglobulin or IgG levels (159). FEV_1 decline has disadvantages as a marker of disease activity in COPD as it could be considered as more reflective of rate of end organ damage rather than short term disease activity. The relationship between circulating FLCs and disease activity in A1ATD related COPD may be better assessed by looking at FLCs during COPD 'exacerbations' where there is a temporary worsening of symptoms requiring additional treatment. This was not possible within the A1ATD cohort but was performed within a usual COPD cohort (See Chapter 4.6.2).

3.7.1.5 FLC relationship to longitudinal outcomes

Patients who subsequently died during the period of follow up were found to have a significantly higher baseline FLC level compared to those who remained alive. This is consistent with the finding that a high FLC level is a risk factor for mortality within the general population (183). Multivariate analysis by Cox regression, to assess whether cFLC associated with mortality, showed that cFLC, increasing age and lower FEV_1 (all $p<0.001$) significantly predicted death, with a cFLC level above the normal range conferring an odds ratio for death of 2.89 (1.47 - 5.70) p=0.002. The Kaplan-Meier plots showed significant differences in the survival curves according to both this level and the higher figure of 65mg/L.

The fact that the ROC curve c-statistic was 0.62 does not preclude cFLC as a useful prognostic test in severe A1ATD. For mortality prediction, a number of combined variables is often utilised and it is recognised that the use of single c-statistics could cause important risk factors to be excluded from cumulative risk prediction scores (237). A large study investigating the ability of inflammatory biomarkers to improve mortality prediction in COPD

found the best predictive model utilised the following variables: age, BODE and hospitalisation history (C statistic of 0.686, $p<0.001$) but the addition of a panel of inflammatory biomarkers increased that to 0.726 ($p=0.003$) (133). The ability of cFLC to improve mortality prediction as part of a model of both clinical risk factors and blood biomarkers is therefore worthy of further investigation.

3.7.1.6 Conclusions

Overall FLCs do fulfil some criteria of a useful biomarker demonstrated by their stability during the stable disease state, relationship to certain phenotypes (chronic bronchitis and chronic colonisation) and mortality. The strongest feature is the ability to predict mortality and this could be useful in risk stratifying patients with the aim of early intervention to improve outcomes.

CHAPTER 4: FLCs as a biomarker in usual COPD[9]

4.1 Introduction

In Chapter 3, the ability of polyclonal free light chains to fulfil the criteria of a useful clinical biomarker in severe A1ATD was examined. In order to establish whether the associations found were also true in "usual" (non A1ATD) COPD and therefore applicable to a larger patient population, FLC analysis was performed in a cohort of 327 patients and the following criteria were again assessed:

1. Relationship to underlying disease pathophysiology
2. Stability
3. Ability to identify clinically important phenotypes
4. Relationship to disease severity and activity
5. Ability to predict longitudinal outcomes

In addition to the criteria listed above an ideal biomarker should also be sensitive to treatments that are known to be effective however (as discussed in Chapter 1) this is difficult as at present there is a lack of pharmacological interventions that have been unequivocally proven to impact on disease progression in COPD (201).

[9] Excerpts of this chapter have been previously published (202. Hampson JA, Stockley RA, Turner AM. Free light chains: potential biomarker and predictor of mortality in alpha-1-antitrypsin deficiency and usual COPD. Respir Res. 2016;17:34.)

Braber et al first linked FLCs and their association with neutrophils to the underlying pathogenesis of usual COPD (168). They found increased serum FLC levels in three smoke-exposed murine models and in a small number of usual COPD patients (n=6) compared to controls. Immunohistochemical staining using κ and λ specific antibodies was performed on lung specimens from patients with COPD and an increase in FLC expression around large airways and within follicles was observed compared to specimens from healthy control subjects. In addition, in vitro experiments demonstrated that FLCs could bind to neutrophils and promote IL8 production and treating mice with F991 (an FLC antagonist) reduced the BAL neutrophil counts of the smoke exposed murine lungs (168). This highlighted the potential pathogenic role FLCs may have in COPD as well as being a surrogate marker of adaptive immune activation within the lung.

Baraldo et al found a similar degree of adaptive immune activation in the lungs of patients with A1ATD related and usual COPD as evidenced by the number of lymphocytes and lymphoid follicles (125). Similar levels of polyclonal FLCs might therefore be expected. In addition to examining the clinical utility of FLCs as a biomarker in usual COPD in this chapter, a comparison with the A1ATD cohort was also undertaken.

4.2 Patient Characteristics

FLC analysis was performed in 327 patients with usual COPD. Eleven patients were excluded from final analysis due to an abnormal κ/λ ratio (repeat testing and referral to haematology for assessment was advised for these patients). The median follow up time was 2.5 (1.5-4.7) years. Clinical features and demographic data are shown in Table 4.1.

Variable	Usual COPD cohort (n=316)
Age	68.8 (61.5 – 75.1)
Sex	Male n = 182 (57.6%) Female n = 134 (42.4%)
Pack years	44.1 (29.5 – 62.2)
FEV_1 (% predicted)	46.4 (35.0 – 61.0)
KCO (% predicted)	59 (47.5 – 77.0)
Chronic bronchitis	198 (62.7%)
Emphysema	257 (81.3%)
Bronchiectasis	96 (30.3%)
Frequent exacerbator	193 (61.1%)
eGFR	85.8 (69.7 – 101.1)
CKD stage	
1 eGFR ≥ 90	119 (37.6%)
2 eGFR 60 - 89	130 (41.1%)
3 eGFR 30 - 59	35 (11.1%)
4 eGFR 15 - 29	4 (1.3%)
5 eGFR < 15	0 (0%)
Unknown (no eGFR)	28 (8.9%)
cFLC (mg/L)	31.9 (24.0 – 43.3)
κ/λ	0.86 (0.72-1.06)

Table 4.1: Patient demographics in the usual COPD cohort

Continuous variables expressed as median (IQR); sex, chronic bronchitis, emphysema, bronchiectasis, exacerbators and chronic kidney disease (CKD) stage expressed as number in each group (%). A frequent exacerbator was defined as having 2 or more treated exacerbations per year. Number of patients with contemporaneous renal function = 288. BMI = body mass index, FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, eGFR = estimated glomerular filtration rate, cFLC = combined (κ + λ) free light chain level and the κ/λ ratio is shown.

4.2.1 FLCs and renal function

Contemporaneous renal function was available for 288/316 patients within the usual COPD cohort. The number of patients at each CKD stage is outlined in Table 4.1. There was a similar relationship between cFLC level, eGFR and CKD stages (Figure 4.1) to that observed in A1ATD. Significant differences in cFLC were seen between the different CKD groups (Kruskall Wallis test $\chi^2(3) = 17.81$, $p<0.001$). A post hoc analysis (Bonferroni correction to calculate significant p value cut off 0.05/5=0.008) revealed that patients with CKD stage 4 had significantly higher cFLC levels than those with CKD stage 1 ($p=0.006$). FLC levels correlated negatively with eGFR ($r_s=-0.24$, $p<0.001$). The strength of this correlation was reduced by adjustment for age, but remained statistically significant ($r_p=-0.13$, $p=0.021$).

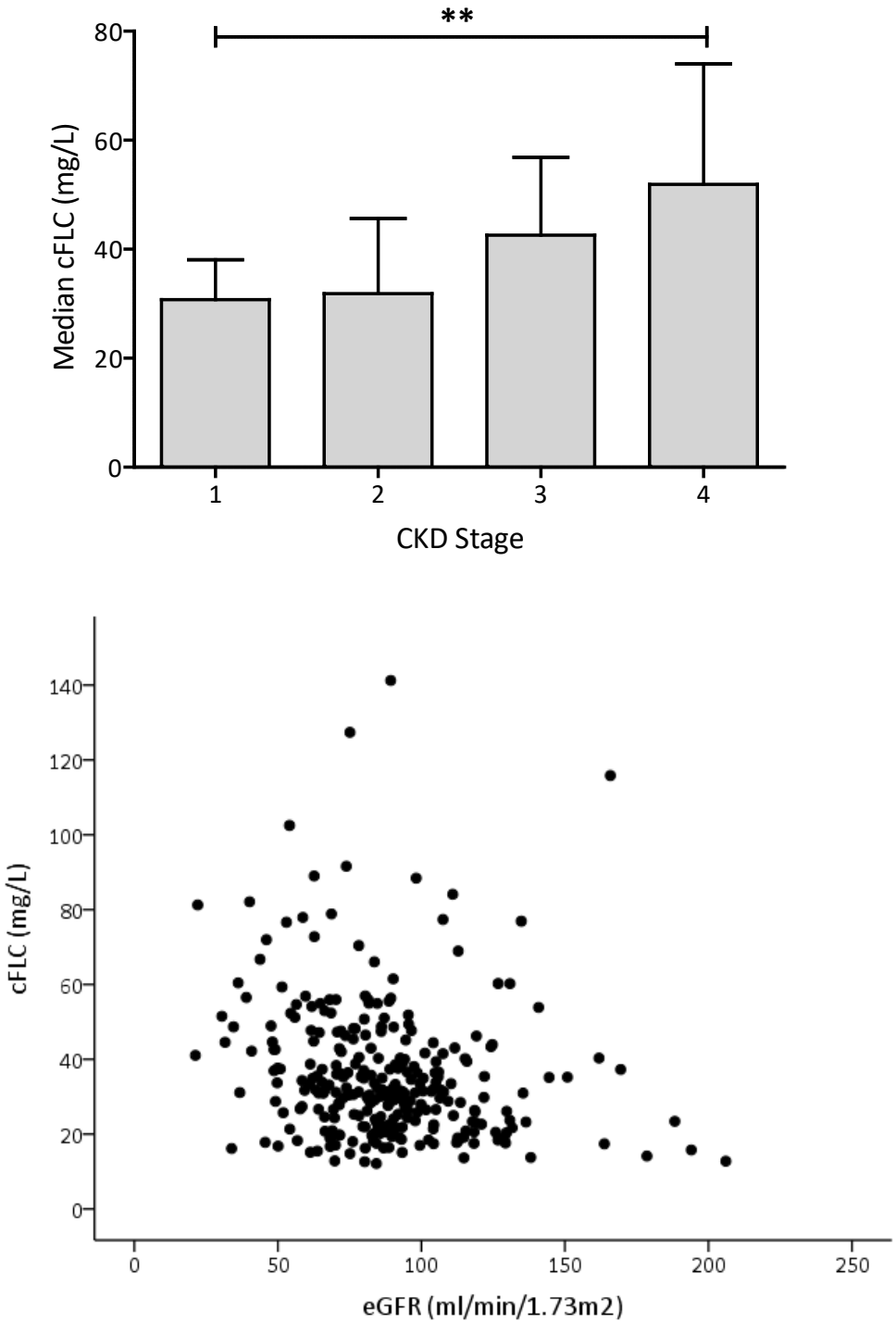

Figure 4.1: Stable state cFLC levels relationship to renal function in the usual COPD cohort

Bar chart (A) shows the relationship between combined (κ & λ) FLC (cFLC) and chronic kidney disease (CKD) stage (each bar represents the median cFLC value and error bars represent the IQR). Patients with CKD stage 4 had significantly higher cFLC compared to patients with CKD stage 1 (**p ≤ 0.01). Scatter plot (B) shows the negative correlation between cFLC and estimated glomerular filtration rate (eGFR) (r_s=-0.24, p<0.001).

4.3 Plasma and serum FLC matched samples analysis

The Freelite assay was developed for use with serum samples. However, for the purposes of this study there was a requirement to determine levels in plasma, when serum was unavailable. There is limited data for the use of plasma samples (238) with the Freelite assay. Therefore, matched serum and plasma samples were analysed in a subgroup of 82 patients from the usual COPD cohort to determine whether the results were equivalent. Three patients were then excluded from the analysis due to an abnormal κ/λ ratio.

The FLC results were not normally distributed therefore non-parametric statistical tests were utilised. There was a significant correlation between serum and plasma κ and λ values (Spearman's Rho: 0.96 and 0.98 respectively, p = <0.001) (Figure 4.2). The results from both matrices were also equivalent using Passing-Bablok analysis (κ: y=0.94x -0.32 and λ: y=0.92x - 0.05) and linear regression analysis (κ: y=0.90x - 0.5 and λ y=0.92x - 0.11).

Figure 4.3 shows a Bland-Altman plot demonstrating the difference between the serum and plasma cFLC values. The assay showed good agreement whether plasma or serum samples were tested: κ: positive predictive value (PPV): 89%, negative predictive value (NPV): 93% and λ: PPV: 100%, NPV: 93%. The results therefore showed there was good analytical agreement between FLC values in plasma and serum samples. All FLC results were therefore grouped together in the usual COPD cohort (whichever sample was used) for subsequent statistical analyses

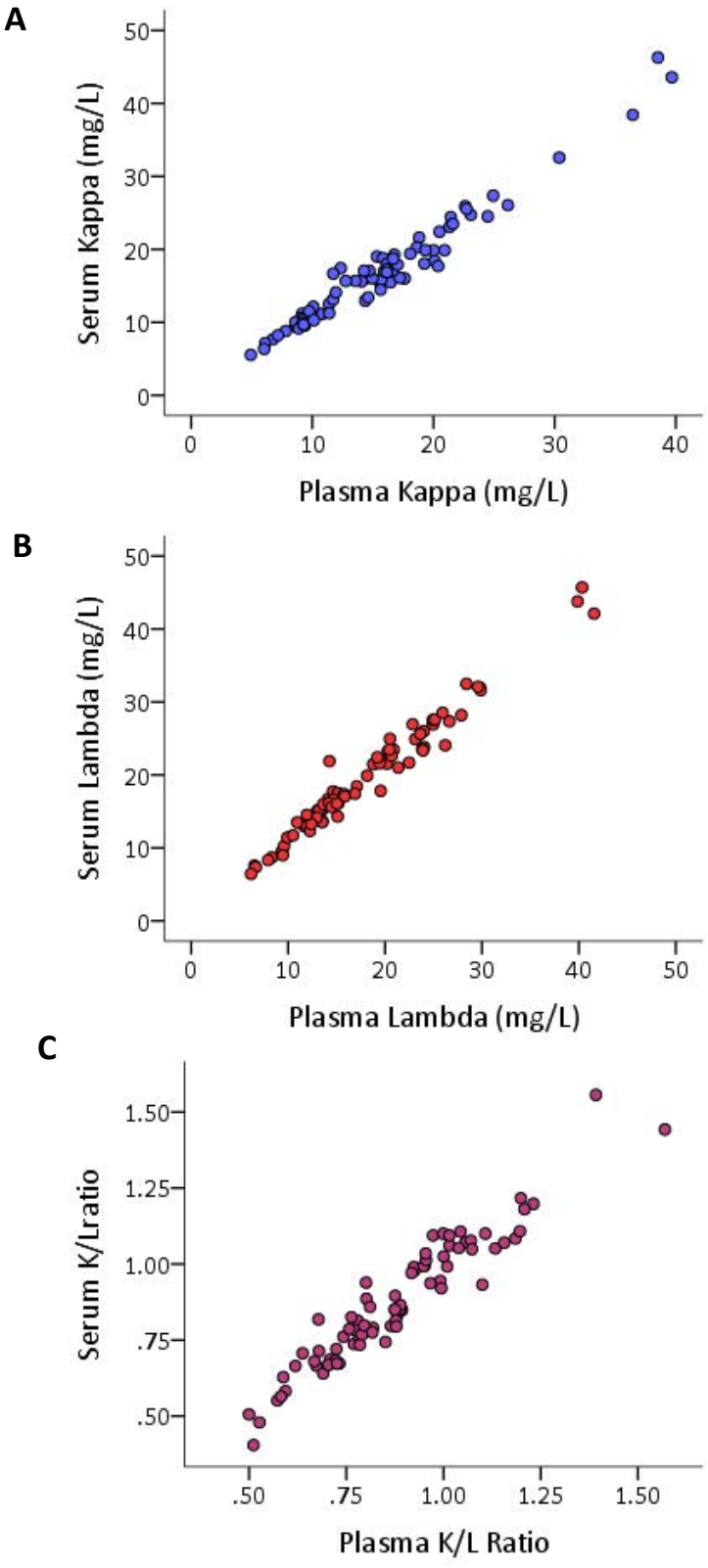

Figure 4.2: Scatterplots demonstrating the correlation between serum and plasma FLC values in n=79 usual COPD patients

A. Serum κ versus plasma κ (r_s =0.96, p<0.001), **B.** Serum λ versus plasma λ (r_s=0.98, p<0.001), **C**. Serum κ/λ ratio versus plasma κ/λ ratio (r_s=0.95, p<0.001).

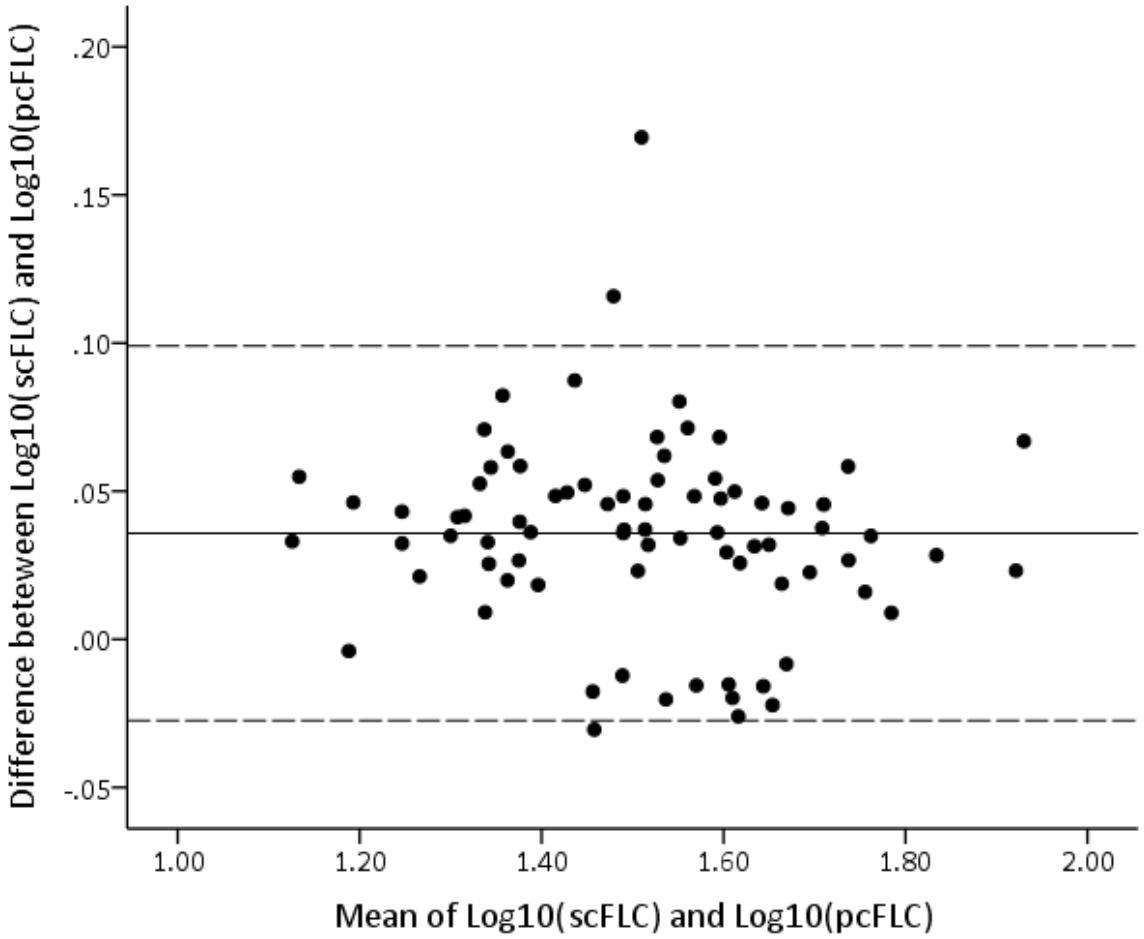

Figure 4.3 Bland Altman plot demonstrating the variability between serum and plasma cFLC in the matched analyses

All cFLC values were log transformed before analysis. The solid horizontal line represents the mean difference between the Log10(cFLC) values. The dotted lines represent 95% confidence intervals. Linear regression analysis revealed no significant proportional bias between matched serum and plasma cFLC values (t=-1.065, p=0.29).

4.4 Stability of FLCs as a biomarker in usual COPD

In a subgroup of 51 patients with usual COPD a repeat FLC analysis was performed on samples obtained after a 1 year interval to establish the stability of FLC within this patient population. There was no significant difference between baseline and follow up cFLC values (Wilcoxon signed rank test Z=-1.91, p=0.056). Seventy one percent of follow up cFLC values were within 25% of the baseline value. Figure 4.4 shows a Bland-Altman plot demonstrating the agreement between the baselines and follow up cFLC levels.

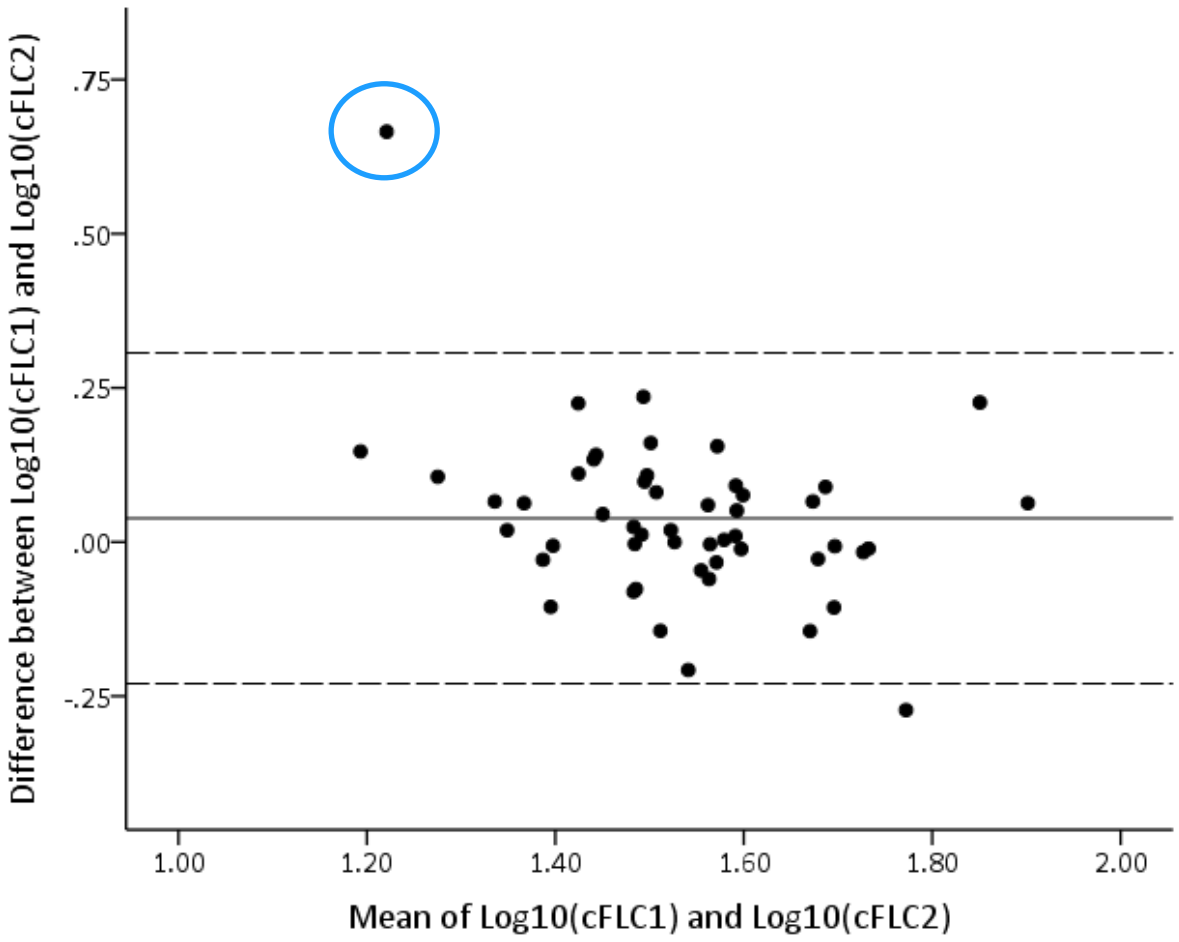

Figure 4.4 Bland Altman plot showing difference between cFLC at baseline and 1 year follow up in n=51 usual COPD patients.

All cFLC values were log transformed before analysis. The solid horizontal line represents the mean difference between the Log10(cFLC) values. The dotted lines represent 95% confidence intervals. After removal of one influential observation (circled in blue) linear regression provided no evidence of proportional bias (t =-1.52, p=0.136).

4.5 FLCs and clinical phenotypes in usual COPD

Table 4.2 shows the univariate analysis of FLCs in different subgroups within the usual COPD cohort. The men were found to have significantly higher FLC levels than the women (cFLC 35.35 v 29.51 mg/L $p<0.001$). This finding could not be explained by a difference in demographic factors such as age and eGFR (median men v women: age 69.4 v 68.1, $p=0.129$, eGFR 87.2 v 83.8, $p=0.354$) or smoking habit.

Current smokers were found to have a significantly lower κ/λ ratio with a trend towards higher κ levels than non-smokers. A weak positive correlation between λ FLC levels and pack years was identified ($r_s=0.13$, $p=0.027$).

Although patients with stable COPD were asked to provide sputum samples, as for the A1ATD group, there were too few for meaningful analysis therefore the relationship of FLCs to chronic colonisation in usual COPD was not investigated.

No significant differences in cFLC were seen with respect to presence of chronic bronchitis, bronchiectasis, emphysema, or a history of frequent exacerbations (defined as ≥2 treated episodes per year).

	Group 1	Group 2	P value
Emphysema	**Yes (206)**	**No (47)**	
cFLC	31.92	36.42	0.131
κ	14.73 (10.8-19.7)	17.23(11.6-23.6)	0.063
λ	17.43 (12.6-24.7)	19.54 (13.5-25.0)	0.331
κ/λ	0.85 (0.7-1.0)	0.93 (0.7-1.2)	0.086
Bronchiectasis	**Yes (73)**	**No (169)**	
cFLC	33.33 (25.1-47.5)	32.36 (23.7-44.5)	0.643
κ	15.99 (10.7-20.8)	14.92 (10.8-20.1)	0.565
λ	18.52 (12.5-24.7)	17.71 (12.8-24.3)	0.830
κ/λ	0.86 (0.7-1.1)	0.87 (0.7-1.0)	0.387
Chronic bronchitis	**Yes (189)**	**No (116)**	
cFLC	33.29 (25.0-45.0)	30.89 (22.2-39.7)	0.111
κ	15.40 (11.3-21.3)	14.66 (10.2-18.3)	0.119
λ	18.06 (12.9-24.5)	16.04 (12.3-22.1)	0.163
κ/λ	0.88 (0.7-1.1)	0.84 (0.7-1.0)	0.419
Current Smokers	**Yes (121)**	**No (188)**	
cFLC	31.5 (22.9-42.4)	33.66 (24.7-44.5)	0.271
κ	14.25 (10.2-18.3)	15.58 (11.6-20.5)	0.066
λ	16.86 (12.9-23.7)	18.01 (12.2-23.8)	0.777
κ/λ	0.82 (0.7-1.0)	0.90 (0.7-1.1)	**0.031**
Gender	**Male (182)**	**Female (134)**	
cFLC	35.35 (27.4-47.2)	29.51 (21.6-38.8)	**<0.001**
κ	16.40 (12.3-21.6)	13.50 (9.6-16.8)	**<0.001**
λ	19.15 (13.6-25.4)	15.05 (11.5-21.5)	**<0.001**
κ/λ	0.88 (0.8-1.1)	0.87 (0.7-1.0)	0.363
Frequent exacerbations	**Yes (193)**	**No (82)**	
cFLC	31.15 (22.9-39.9)	32.52 (26.6-45.8)	0.081
κ	14.26 (10.0-17.8)	15.5 (11.7-20.8)	0.085
λ	16.32 (12.1-22.7)	18.16 (13.6-24.2)	0.070
κ/λ	0.86 (0.7-1.1)	0.85 (0.7-1.0)	0.942

Table 4.2: Comparison of cFLC in 2 patient subgroups within the usual COPD cohort

Median individual and combined κ & λ (cFLC) levels (mg/L) reported with inter-quartile range in brackets. Mann Whitney U Tests performed to determine any statistical differences between groups 1 and 2 (*p ≤ 0.05). Where statistical differences were found the p value is highlighted in bold red text.

4.6 FLCs relationship to COPD disease severity and activity

4.6.1 Disease severity

To establish if there was any correlation between FLCs and disease severity in usual COPD, a cross sectional analysis with lung function parameters was undertaken. FEV_1 percentage predicted is the current standard way of classifying severity of airflow obstruction in COPD. As the FLC results were not normally distributed non-parametric Spearman's Rho correlations with lung function parameters was undertaken (Table 4.3). Within this cohort 6 patients had radiological emphysema but no evidence of airflow obstruction. Weak positive correlations were found between κ FLC and FEV_1 and both κ and cFLC levels and the FEV_1/FVC ratio.

FLC levels correlated positively with age (κ r_s=0.3, p<0.001, λ r_s=0.25 p<0.001, cFLC r_s=0.28 p<0.001). Correlations were therefore performed controlling for age and eGFR (Table 4.4). Only patients with all the appropriate information were included (n=131) and no significant correlations remained between lung function parameters and FLCs.

There are a number of validated symptom scores which are utilised assessing the symptomatic burden of COPD as a measure of disease severity. The medical research council (MRC) breathlessness score was recorded for 283/316 (69%) of the patients in the usual COPD cohort at the time the sample for FLC was taken. When the patients were grouped according to their MRC score no significant difference in cFLC between the groups was seen (Kruskall Wallis test $\chi^2(2) = 2.81$, p=0.590, Figure 4.6).

		FEV_1 %p (n=308)	FVC %p (n=306)	FEV_1/FVC (n=309)	TLC %p (n=153)	RV %p (n=152)	KCO %p (n=167)	TLCO %p (n=157)
K	r_s	0.131	-0.010	0.132	0.010	-0.031	0.055	-0.005
	p	**0.022***	0.856	**0.020***	0.903	0.700	0.482	0.952
λ	r_s	0.073	-0.057	0.095	-0.009	0.002	0.018	-0.019
	p	0.204	0.317	0.094	0.914	0.985	0.815	0.810
κ/λ	r_s	0.105	0.046	0.108	-0.010	-0.056	0.039	0.011
	p	0.067	0.422	0.059	0.902	0.494	0.617	0.891
cFLC	r_s	0.100	-0.038	0.113	0.006	-0.007	0.033	-0.012
	p	0.080	0.511	**0.047***	0.940	0.929	0.675	0.885

Table 4.3 Spearman's' Rho correlations between FLC levels and lung function parameters in the usual COPD cohort

FEV_1 = Forced expiratory volume in 1 second, FVC = Forced vital capacity, TLC = Total lung capacity, RV = Residual volume, KCO = Corrected gas transfer, TLCO = Transfer factor of the lung for carbon monoxide, cFLC = combined κ & λ FLC (mg/L), r_s= correlation coefficient, %p = percentage predicted. (*Statistically significant 2p values are highlighted)

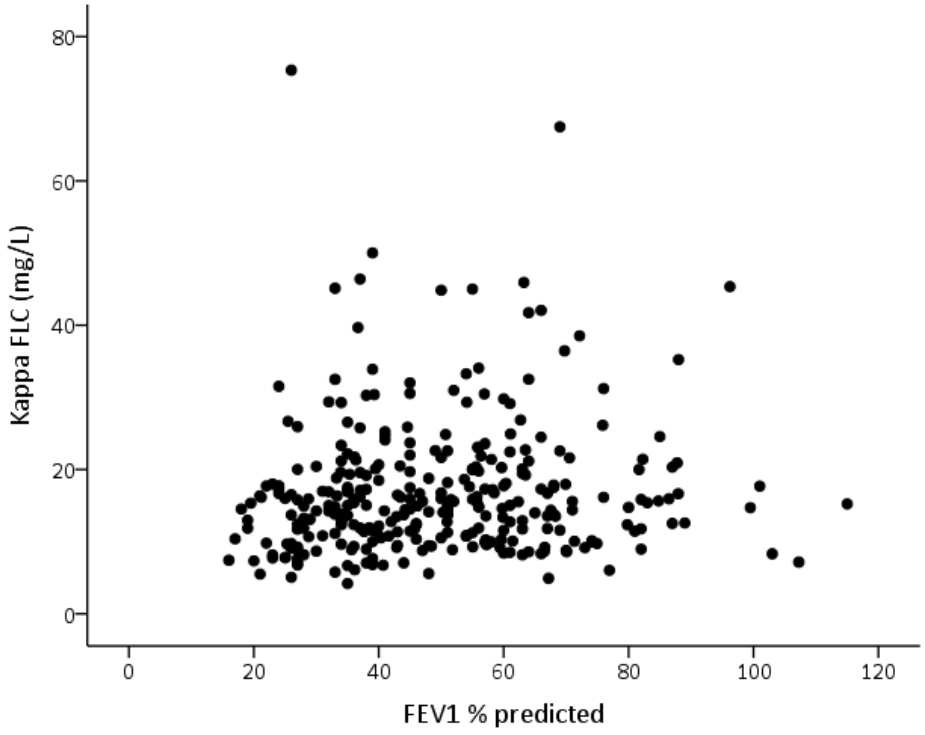

Figure 4.5 Scatterplot of κ FLC against FEV1 (% predicted) in the usual COPD cohort

FLC = free light chain, FEV_1 = forced expiratory volume in 1 second. Each dot represents a single patient. (Spearman's Rho r_s= 1.31, p=0.02)

Cont. Age & eGFR		FEV1 %p (n=131)	FVC %p (n=131)	FEV1/FVC (n=131)	TLC %p (n=131)	RV %p (n=131)	KCO %p (n=131)	TLCO %p (n=131)
Kappa	r_p	0.073	-0.025	0.097	0.056	0.076	-0.088	-0.110
	p	0.406	0.776	0.268	0.522	0.387	0.313	0.208
Lambda	r_p	0.061	-0.048	0.107	0.034	0.072	-0.056	-0.078
	p	0.487	0.583	0.221	0.694	0.407	0.521	0.373
κ/λ ratio	r_p	0.009	-0.012	0.033	-0.041	-0.044	0.047	-0.034
	p	0.920	0.894	0.705	0.637	0.615	0.590	0.694
cFLC	r_p	0.069	-0.039	0.107	0.046	0.077	-0.074	-0.097
	p	0.428	0.655	0.221	0.597	0.377	0.397	0.268

Table 4.4 Partial correlation (controlling for age and eGFR) between FLC levels and lung function parameters in the usual COPD cohort

FEV_1 = Forced expiratory volume in 1 second, FVC = Forced vital capacity, TLC = Total lung capacity, RV = Residual volume, KCO = Corrected gas transfer, TLCO = Transfer of the lung for carbon monoxide, eGFR = estimated glomerular filtration rate, cFLC = combined (κ & λ) free light chain (mg/L), r_p = correlation coefficient, %p = percentage predicted.

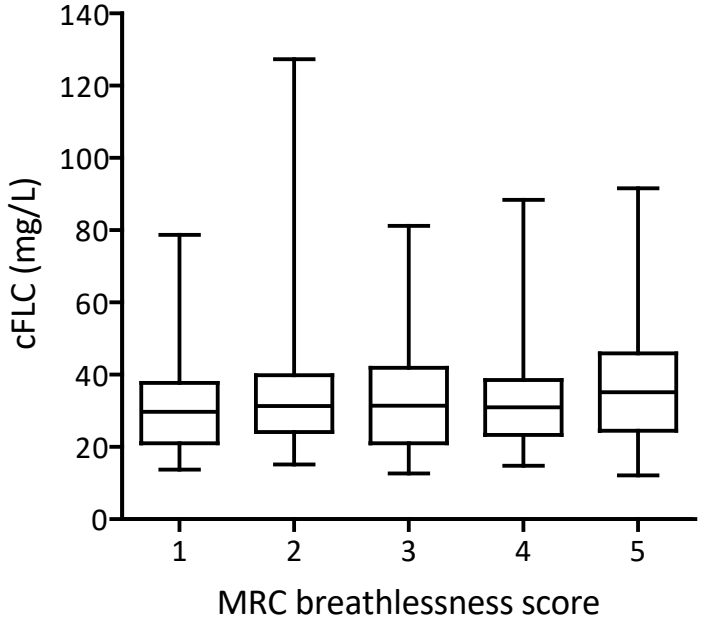

Figure 4.6: Box plot showing cFLC levels according to MRC breathlessness score in the usual COPD cohort

Horizontal lines represent medians, boxes represent interquartile ranges and whiskers represent minimum to maximum values. MRC = Medical Research Council, cFLC = combined κ & λ free light chain levels (mg/L).

4.6.1.1 FLCs according to severity of airflow obstruction in usual COPD

When patients were sub-grouped according to their severity of airflow obstruction (GOLD stages: mild FEV_1 >80% predicted, moderate 50-80%, severe 30-50% and very severe <30%) significant differences between the groups were identified (Kruskall Wallis test ($\chi^2(4)$ = 12.35, p=0.015). The median cFLC (mg/L) within the groups were: no airflow obstruction 28.52, mild 32.8, moderate 34.8, severe 33.3, and very severe 26.5. A post-hoc analysis found cFLC levels were significantly higher in patients with moderate airflow obstruction compared to those with very severe airflow obstruction (Mann Whitney U test p = 0.002). A Bonferroni calculation was used to calculate the level for significance ($p \leq 0.05/10 = 0.005$) (Figure 4.7). The same relationship was seen between cFLCs and GOLD group of airflow obstruction in the A1ATD cohort (Figure 3.7).

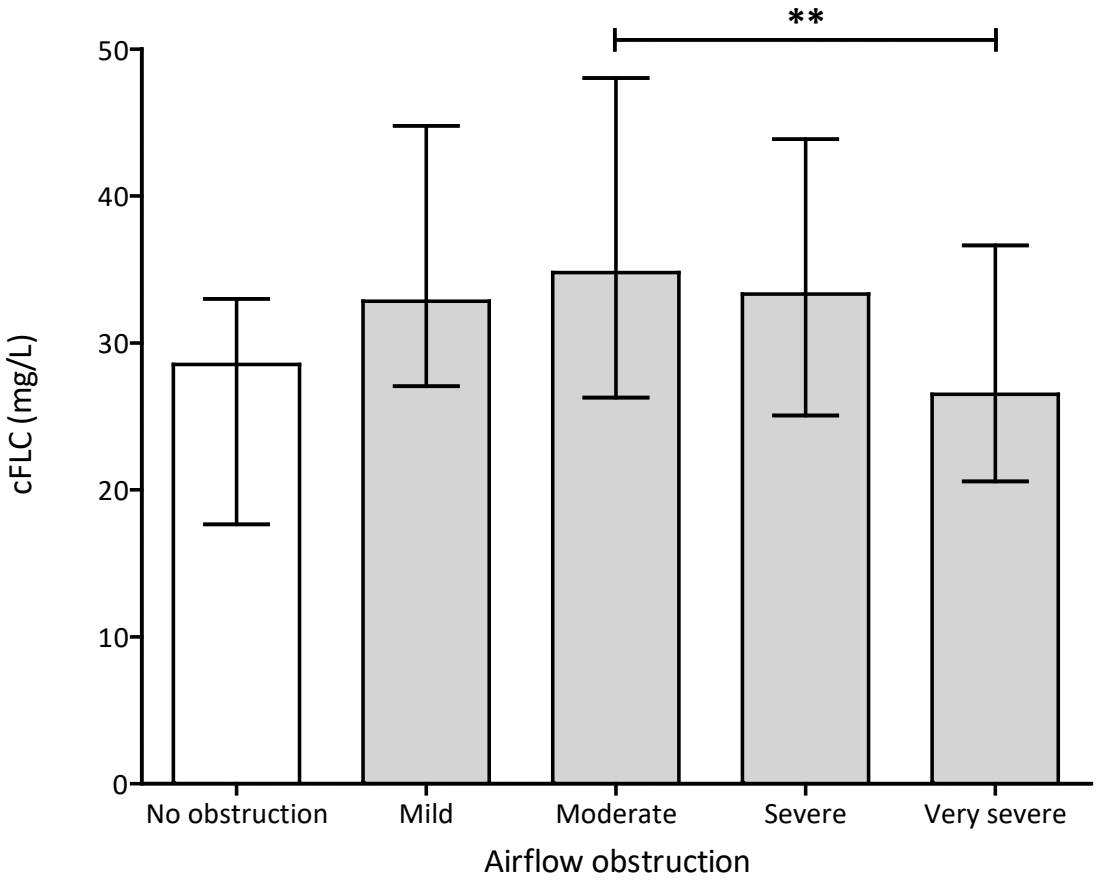

Figure 4.7 Bar chart showing median cFLC levels in usual COPD patients sub-grouped according to the severity of their airflow obstruction

Bars represent median combined (κ & λ) FLC (mg/L) in each group. Error bars represent interquartile range. **p ≤ 0.005.

4.6.2 Disease activity

To establish if polyclonal FLCs increased during periods of disease activity in usual COPD, they were measured in a separate cohort of 68 patients admitted to hospital with an exacerbation of COPD. FLC levels were measure on day 1 of the admission and at day 56 following resolution of their symptoms. One patient was excluded from analysis due to a diagnosis of MALT lymphoma and 12 other patients were excluded due to an abnormal κ/λ ratio (these patients were recalled for repeat testing and referral to haematology for investigation as appropriate). The demographics and other patient characteristics on admission are outlined in Table 4.5. There was a significant polyclonal increase in FLCs from day 1 to day 56. There were concurrent decreases in white cell count (WCC), COPD assessment test (CAT) score and respiratory rate (Table 4.6).

As expected there was a negative correlation between cFLC levels and eGFR taken on day 1 (Figure 4.8). At day 1 cFLC levels were also found to correlate positively with CRP and age and there was a weak negative correlation between κ/λ ratio and pack year history (Figure 4.9).

The positive correlation between cFLC and CRP was also evident at day 56 but interestingly, a negative correlation with WCC was seen at this timepoint (r_s=-0.51, p=0.009, Figure 4.10).

Variable	Exacerbation cohort (n=55)
Age	68.2 (62.4-75.2)
Sex	Male n=31 (56.4%) Female n=24 (43.6%)
Pack years	42.5 (29.3-60.0)
FEV_1 (% predicted)	40.0 (29.5-55.5)
KCO (% predicted)	64.0 (49.5-75.5)
BMI (kg/m^2)	24.1 (20.6-31.3)
Chronic bronchitis	25/51 (49.0%)
Emphysema	44/53 (83.0%)
Bronchiectasis	14/53 (26.4%)
Frequent exacerbator	55/55 (100%)
Annual exacerbation frequency	3 (2.0-5.0)
eGFR	82.1 (65.0-101.5)
CKD stage	
1 eGFR ≥ 90	25 (45.5%)
2 eGFR 60 - 89	18 (32.7%)
3 eGFR 30 - 59	11 (20.0%)
4 eGFR 15 - 29	1 (1.8%)
5 eGFR < 15	0 (0%)
cFLC (mg/L)	33.1 (23.8-52.7)
κ/λ ratio	1.13 (0.88-1.37)
CRP	29 (9.0-69.0)
WCC	12.3 (8.6-15.2)
CAT score	31 (25-35)

Table 4.5: Exacerbation cohort patient demographics and baseline characteristics

Continuous variables expressed as median (IQR); sex, chronic bronchitis, emphysema, bronchiectasis, exacerbators and chronic kidney disease (CKD) stage expressed as number in each group (%). A frequent exacerbator was defined as having 2 or more exacerbations per year. FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, BMI = body mass index, eGFR = estimated glomerular filtration rate, cFLC = combined (κ + λ) FLC, CRP =C reactive protein, WCC = white cell count, CAT = COPD assessment test.

Variable	Day 1	Day 56	Z	P value
κ	17.63 (11.4-28.2)	17.87 (11.50-30.5)	-1.97	**0.048***
λ	15.23 (11.5-24.3)	18.31 (12.3-25.1)	-2.77	**0.006****
cFLC	33.09 (23.8-52.7)	34.32 (25.7-52.1)	-2.44	**0.015***
κ/λ	1.13 (0.88-1.37)	1.10 (0.84-1.36)	-1.52	0.129
CRP	29.0 (9.0-69.0)	11.5 (4.8-39.0)	-1.62	0.104
WCC	12.25 (8.6-15.2)	8.8 (8.0-11.2)	-2.06	**0.040***
CAT score	31 (25-35)	25 (19-31)	-3.57	**<0.001****
Pulse	94.3 (84.0-102.5)	82.5 (72.0-92.3)	-1.46	0.145
Systolic BP	134.0 (117.0-143.3)	130.5 (114.0-142.8)	-0.526	0.599
Diastolic BP	72.5 (67.5-82.3)	75.5 (70.3-988.8)	-1.411	0.158
RR	20 (18-22)	18 (16-20)	-2.53	**0.011***

Table 4.6: Clinical variables at day 1 and day 56 in the exacerbation cohort

Variables highlighted in yellow increased and variables highlighted in blue decreased. Median values quoted with interquartile range in brackets. Wilcoxon signed rank test used to identify significant differences in variables between day 1 and day 56. Significant p valued highlighted in red with an asterisk. cFLC= combined (κ & λ) FLC level, CRP = c-reactive protein, WCC= white cell count, BP = blood pressure, RR = respiratory rate.

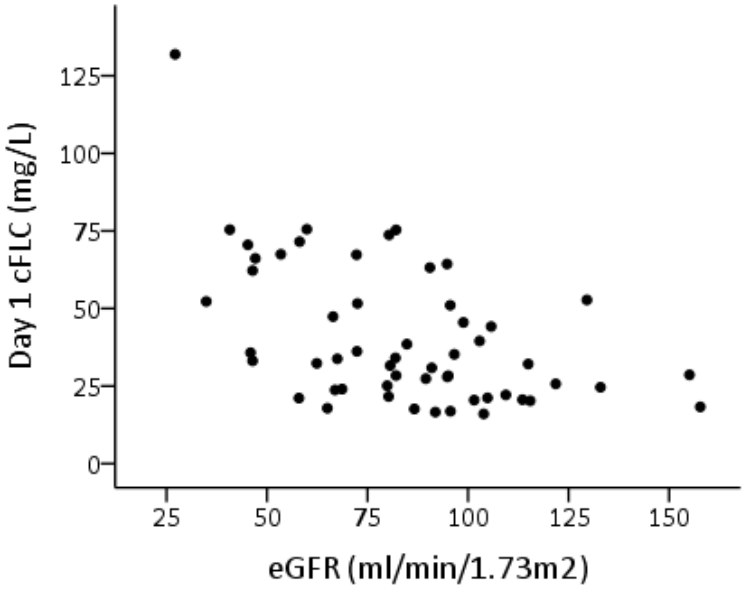

Figure 4.8 Scatterplot showing relationship between day 1 cFLCs and renal function in the exacerbation cohort

Spearman's Rho r_p=-0.48 p=<0.001. cFLC= combined κ & λ (mg/L), eGFR = estimated glomerular filtration rate.

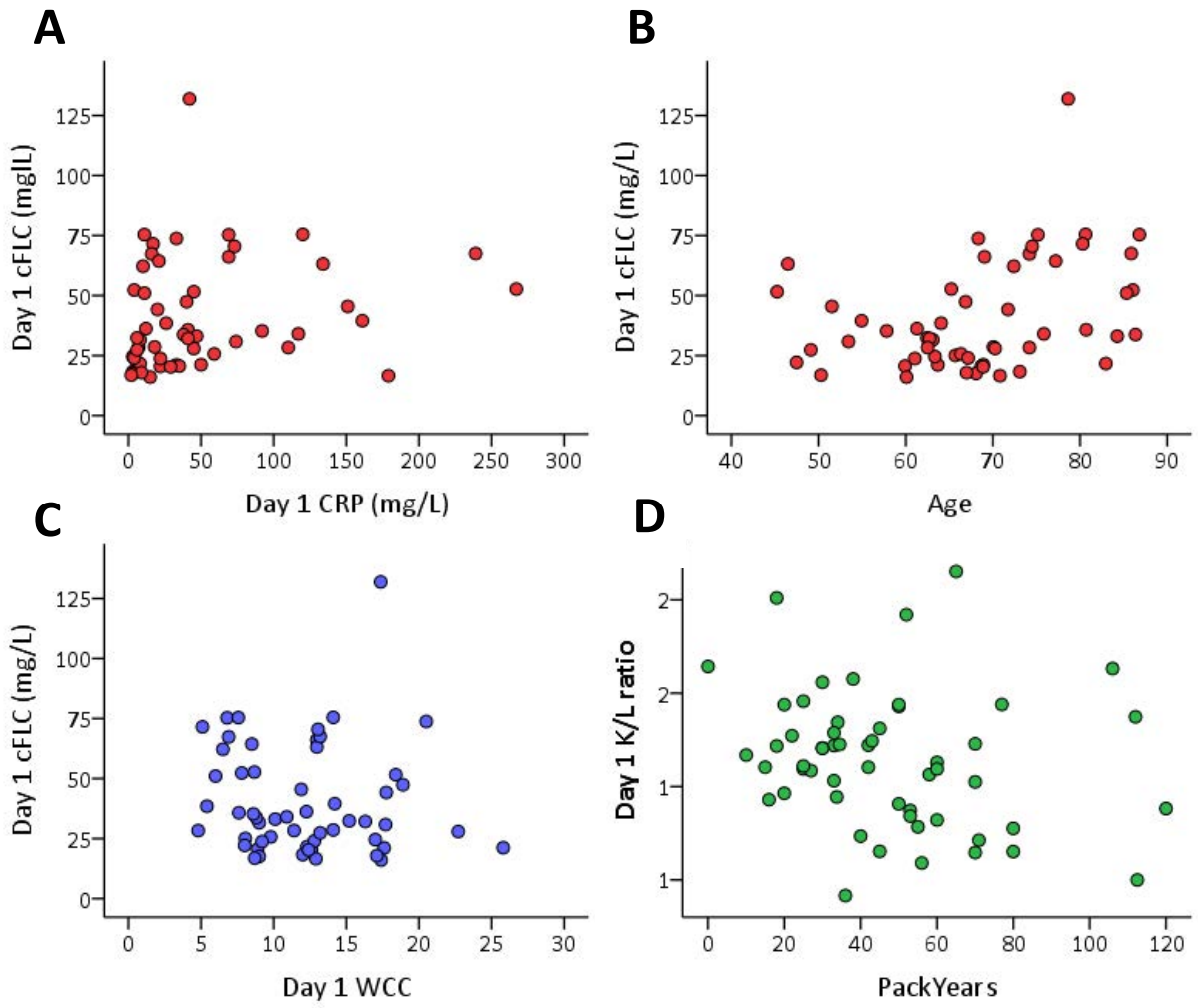

Figure 4.9: Scatterplots demonstrating relationship of FLCs to CRP, WCC, age and smoking in usual COPD patients on day 1 of an exacerbation

Spearman's Rho correlations- A: r_p= 0.37 p=0.005**, B: r_p=0.37. p=0.005** C: r_p=-0.147, p=0.283 D: r_p=-0.29, p=0.036*. cFLC = combined κ & λ FLC level (mg/L), CRP = C reactive protein, WCC= white cell count.

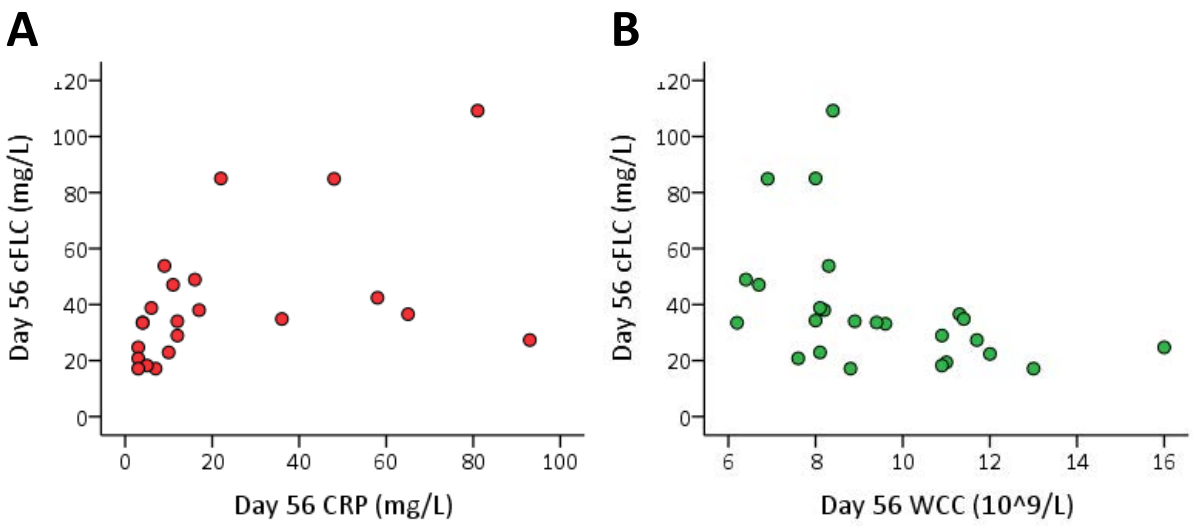

Figure 4.10 Scatterplots showing relationship of cFLC to CRP and WCC day 56 post exacerbation

Spearman's rho correlation A: r_p= 0.59, p=0.004 B: r_p=-0.51, p=0.009. cFLC = combined κ & λ FLC level (mg/L), CRP= C reactive protein, WCC= white cell count.

4.6.2.1 Immunoglobulin analysis

To explore the relationship between FLCs and immunoglobulin production during exacerbations, 3 major classes: IgA, IgG and IGM were measured in a subgroup of 27 patients at day 1 and day 56. No significant changes in immunoglobulin levels were seen during the course of the exacerbation (Figure 4.11). At day 1, cFLC levels correlated positively with IgG levels but not IgA or IgM but at day 56 cFLC correlated positively with both IgA and IgG levels (r_p=0.45, p=0.022* and r_p=0.56, p=0.003** respectively, Figure 4.12).

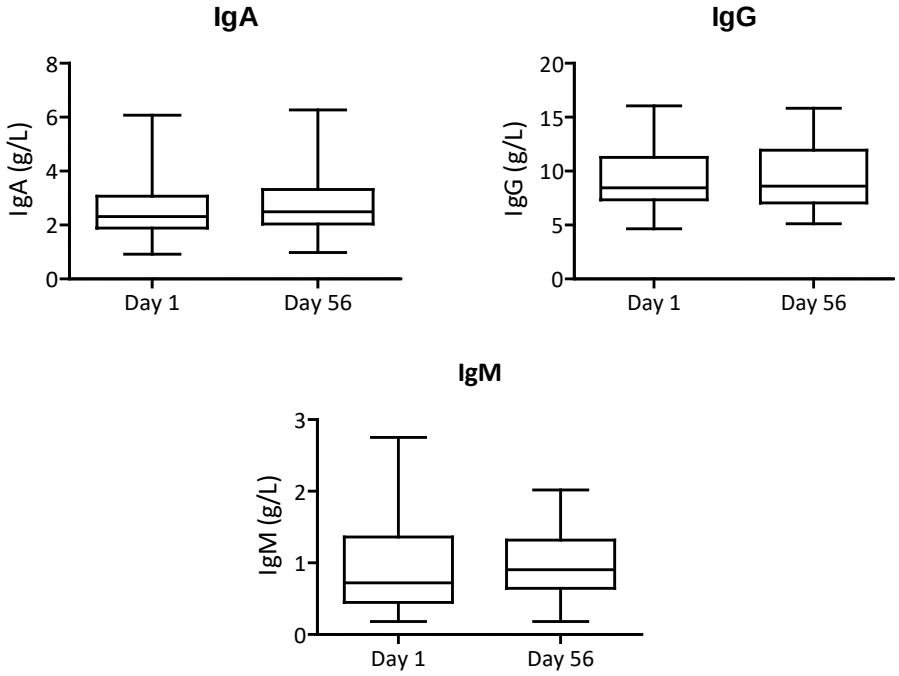

Figure 4.11 Box plots showing immunoglobulin levels at day 1 and 56 in n=27.

Horizontal line represents median value, box represents interquartile range and whiskers represent minimum and maximum values. No significant difference change in levels was seen (Wilcoxon signed rank test IgA p=0.657, IgG p=0.501, IgM p=0.581)

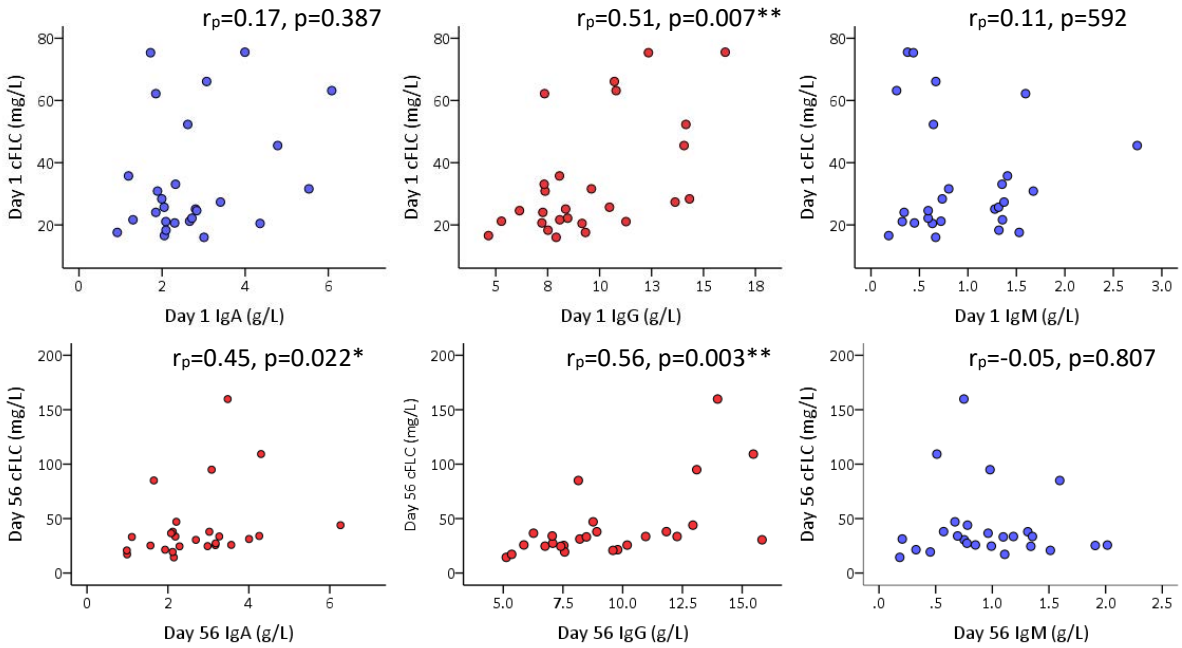

Figure 4.12: Scatterplots showing correlations between cFLC and immunoglobulin levels at day 1 and 56 in n=27

Significant positive correlations highlighted with red dots. Blue dots represent parameters that did not correlate.

4.6.2.2 FLCs and longitudinal outcomes in the exacerbation cohort

The median follow up time for this cohort was 2.37 years (1.51-2.58). During this time 13 patients died (23.6%). However, there was no significant difference in day 1 or day 56 cFLC levels in the patients who died compared to those who survived (median 34.1 v 31.9, p= 0.80 and 34.9 v 34.2, p=0.91 respectively).

4.7 FLCs and longitudinal outcomes in usual COPD

4.7.1 Mortality

Within the stable usual COPD cohort ninety-one (28.8%) patients died during the follow up period. The patients who died had significantly higher baseline cFLC levels compared to those who remained alive (36.4 (26.0-51.5) v 31.5 (23.3-41.3) mg/L, p=0.014, Table 4.7.

Univariate cox regression analysis was performed to identify any variables that significantly predicted mortality within this cohort. All variables with a p value ≤ 0.1 in the univariate analyses were included in the initial multivariate analyses. Non-significant variables (with a p value > 0.05) were then removed to build a final multivariate model. In the final model, the variables that were found to independently predict mortality were cFLC and FEV_1 % predicted (hazard ratios: 1.02 p<0.001, 0.97 p<0.001 respectively). These tables are summarised below (Table 4.7 and Table 4.8)

Mortality	Dead (n = 91)	Alive (n = 225)	P value
cFLC	36.42 (26.0-51.5)	31.47 (23.3-41.3)	**0.014***
κ	15.97 (11.6-23.3)	14.81 (10.50-19.0)	**0.046***
λ	19.22 (13.3-27.9)	16.3 (12.4-22.8)	**0.008***
κ/λ	0.82 (0.7-1.0)	0.88 (0.7-1.0)	0.117

Table 4.7 FLCs and mortality in the usual COPD cohort

Median individual and combined κ & λ (cFLC) levels (mg/L) reported with inter-quartile range in brackets. Mann Whitney U Tests performed to determine any statistical differences between the groups (*p ≤ 0.05). Where statistical differences were found the p value is highlighted in bold red text.

Variable	Univariate HR (CI)	P value	Multivariate HR (CI)	P value
cFLC	1.02 (1.01 - 1.03)	**<0.001***	1.02 (1.01-1.02)	**<0.001***
Pack years	1.01 (1.00 - 1.01)	0.161		
Current smoker	0.97 (0.63 - 1.49)	0.872		
Gender	1.46 (0.95 - 2.24)	0.087		
Emphysema	1.05 (0.60 - 1.85)	0.861		
Bronchiectasis	0.83 (0.51 - 1.38)	0.478		
Frequent exacerbator	1.20 (0.71 - 2.02)	0.503		
Age	1.02 (1.00 - 1.05)	0.065		
Chronic bronchitis	1.22 (0.77 - 1.92)	0.403		
BMI	0.95 (0.91 - 0.98)	**0.002***		
eGFR	1.01 (1.00 - 1.01)	0.113		
FEV_1 (% predicted)	0.98 (0.96 - 0.99)	**<0.001***	0.97 (0.96-0.99)	**<0.001***
KCO (% predicted)	0.98 (0.97 - 1.00)	**0.010***		
FEV1/FVC	0.04 (0.01 - 0.19)	**<0.001***		

Table 4.8: Predictors of mortality in the usual COPD cohort: univariate and multivariate cox proportional hazards regression analysis

HR = hazard ratio, CI = 95% confidence intervals, cFLC = combined κ & λ free light chain level mg/L, BMI = body mass index, eGFR = estimated glomerular filtration rate, FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, FVC = forced vital capacity. A frequent exacerbator was defined as having 2 or more exacerbations per year. (*p ≤ 0.05)

4.7.1.1 Mortality in cFLC subgroups

The patients were sub-grouped according to two cFLC thresholds – 43.3mg/L (the upper limit of normal) and 65mg/L (previously shown to be a risk factor for mortality within 100 days (184)) and multivariate cox regression analyses were then repeated. Within the usual COPD cohort, the hazard ratio for mortality associated with a cFLC greater than 43.3mg/L was 1.8 (p=0.009) and increased to 2.39 for patients with a cFLC of greater than 65mg/L (Table 4.9). Significant differences between the survival curves were seen at both of these thresholds (Figure 4.13).

	Model 1		Model 2	
Variable	Multivariate HR (95%CI)	P value	Multivariate HR (95%CI)	P value
cFLC ≥ 43.3 mg/L	1.80 (1.16-2.80)	**0.009***		
cFLC ≥ 65 mg/L			2.39 (1.29-4.40)	**0.005***
FEV_1 (% predicted)	0.98 (0.96-0.99)	**<0.001***	0.98 (0.96-0.99)	**<0.001***

Table 4.9: Predictors of mortality in the usual COPD cohort: cox proportional hazards regression analysis models

Model 1 shows that a combined κ & λ free light chain level (cFLC) of greater than 43.3mg/L and forced expiratory volume in 1 second (FEV_1) are independent risk factors for mortality in the A1ATD cohort. Model 2 uses a higher cFLC cut off of 65 mg/L. Hazard ratios (HR) reported with 95% confidence intervals (CI) in brackets. *p≤0.05 highlighted in red

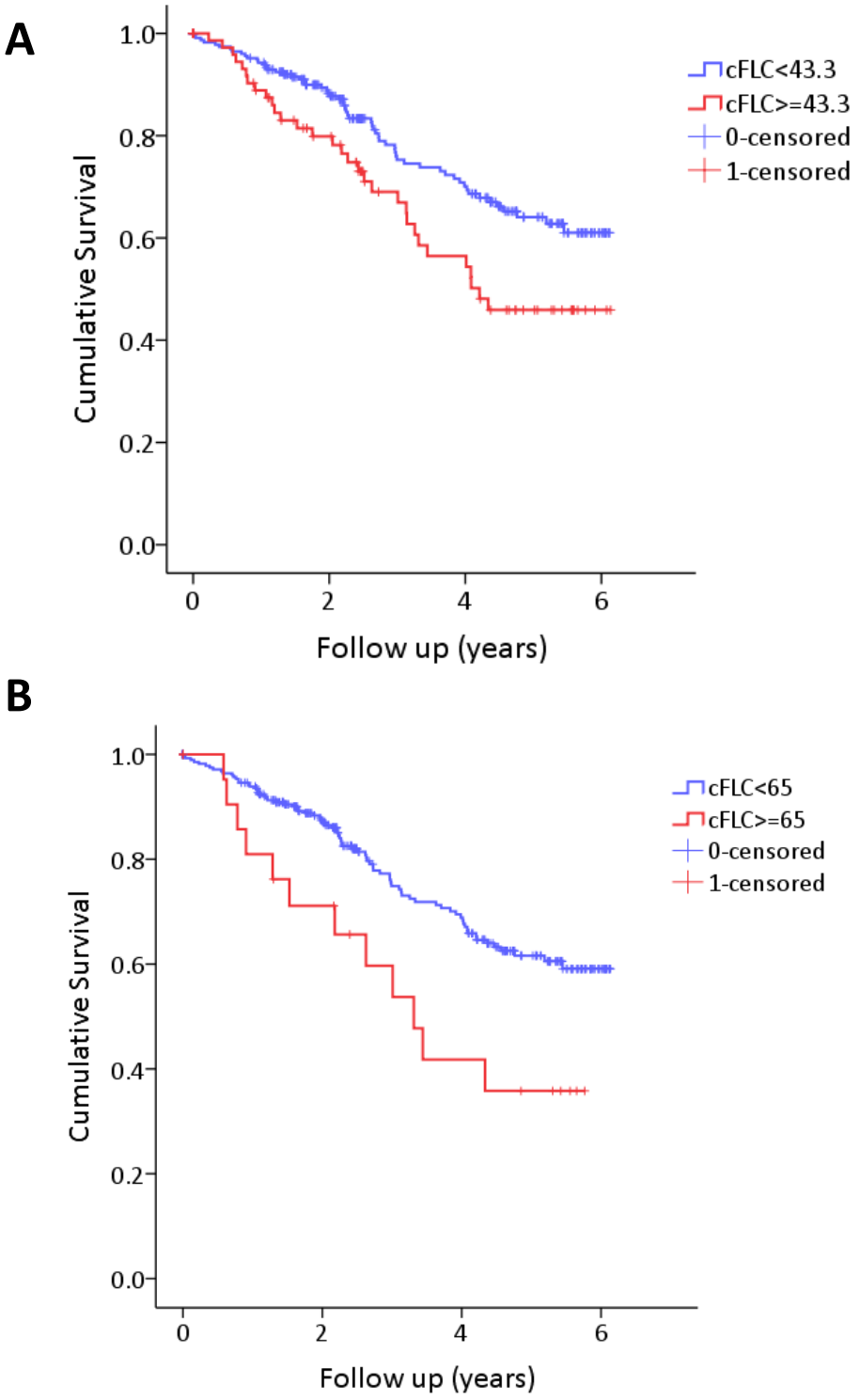

Figure 4.13: Kaplan-Meier curves according to cFLC thresholds in the usual COPD cohort

The blue line represents patients with a combined κ & λ free light chain (cFLC) of less than A. 43.3mg/L (the upper limit of normal), n=237, B. 65mg/L, n=292. Red line represents those with a cFLC level above these thresholds, n= 79 and 24 respectively. The Kaplan-Meier plots showed significant differences in the survival curves according to both these levels, A. p = 0.013, B. p=0.012 by Log Rank test.

4.7.2 ROC curve analysis

ROC curve analysis was performed to assess the sensitivity and specificity of cFLC in predicting mortality within the usual COPD cohort. The area under the curve was 0.59 (95% CI 0.52-0.66, p=0.015 Figure 4.14).

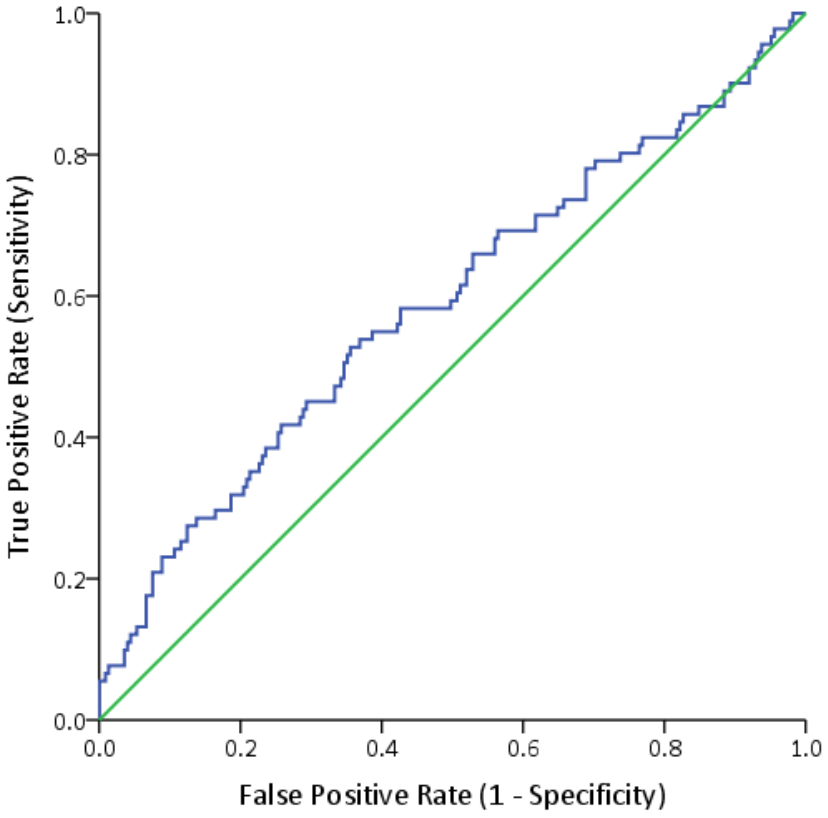

Figure 4.14 Receiver operating characteristics (ROC) curve assessing the sensitivity and specificity of combined κ & λ FLC for mortality prediction in usual COPD.

Area under the curve = 0.59 (95% CI 0.52-0.66, p=0.015).

4.8 Immunoglobulin analysis

4.8.1 Introduction

In order to establish if measuring FLCs has any benefit over performing immunoglobulin analysis – IgA, IgG and IgM was measured in all 316 patients in the usual COPD cohort. The immunoglobulin results were not normally distributed therefore non-parametric statistical tests were utilised.

4.8.2 Results

Positive correlations were found between FLC levels and IgA and IgG but not IgM (Table 4.10). The strongest correlation was between cFLC and IgA levels (r_s = 0.492, p<0.001, Figure 4.15). A partial correlation controlling for eGFR (n=283) strengthened this relationship further (r_p= 0.545, p<0.001).

Significant differences in immunoglobulin levels according to gender were seen. Women were found to have lower median IgA and IgG levels (2.14 v 2.57 g/L, p<0.001 and 8.48 v 9.29 g/L, p=0.029 respectively) and higher median IgM levels compared to men (0.8 v 0.67 g/L, p=0.007). Scatterplots were drawn to identify any outliers contributing to the differences seen (Figure 4.16). One outlier with a significantly raised IgM level was identified but removal of this patient did not influence the overall results.

IgM levels were also found to have a weak negative correlation with both age (r_s= -0.12, p=0.037) and BMI (r_s=-0.14, p=0.017).

		κ	λ	κ/λ	cFLC
IgA	r_s	0.445	0.493	-0.078	0.492
	p	**<0.001****	**<0.001****	0.168	**<0.001****
IgG	r_s	0.484	0.408	0.130	0.458
	p	**<0.001****	**<0.001****	**0.021***	**<0.001****
IgM	r_s	0.056	0.098	-0.094	0.087
	p	0.324	0.082	0.097	0.122

Table 4.10: Spearman's Rho correlations between FLC levels and immunoglobulin levels

r_s = correlation coefficient. (*Statistically significant 2p values are highlighted in red)

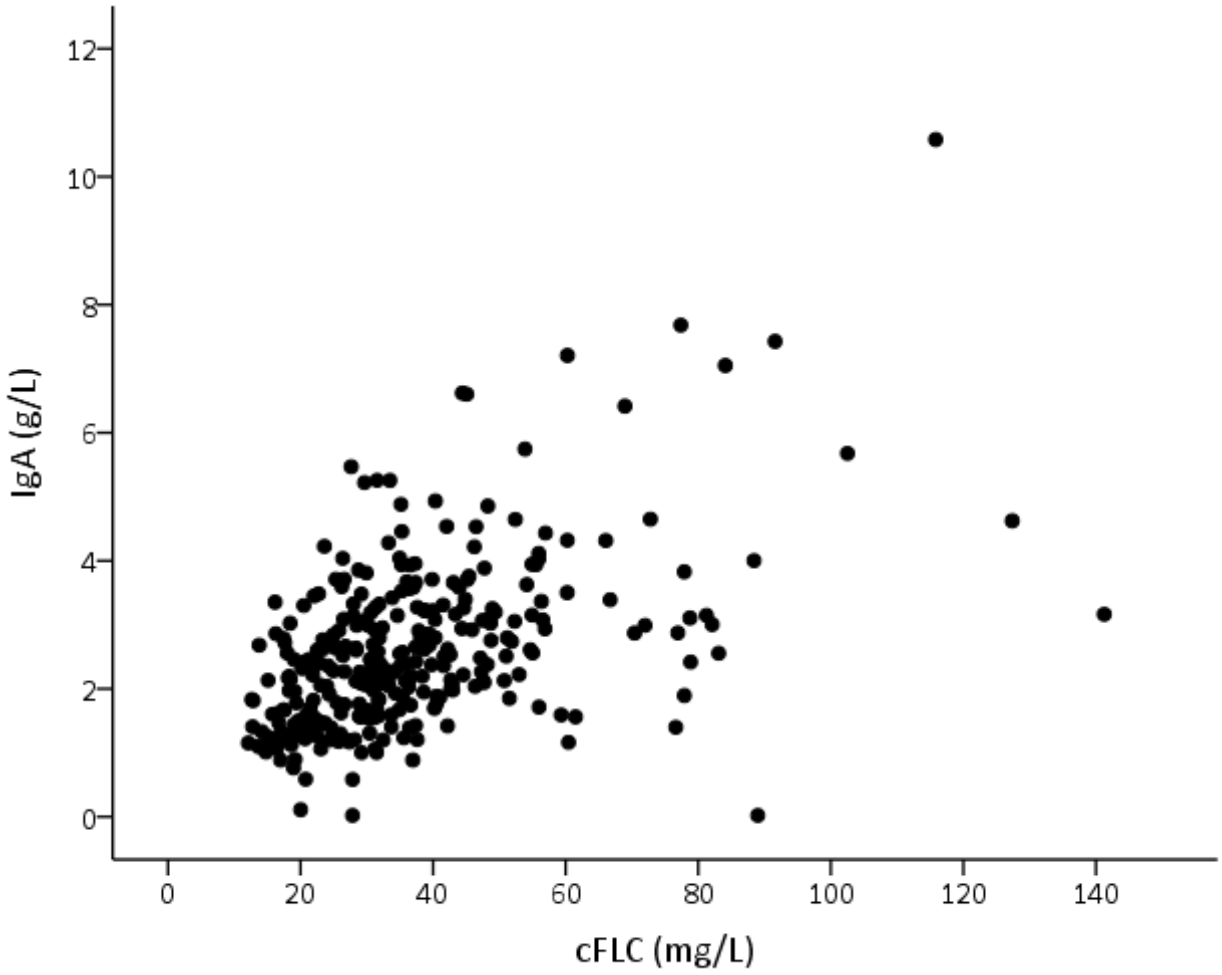

Figure 4.15: Scatterplot showing correlation between IgA and cFLC levels in the usual COPD cohort

IgA = Immunoglobulin A, cFLC = combined κ & λ FLC level (mg/L). Spearmen's Rho r_s=0.49, p<0.001.

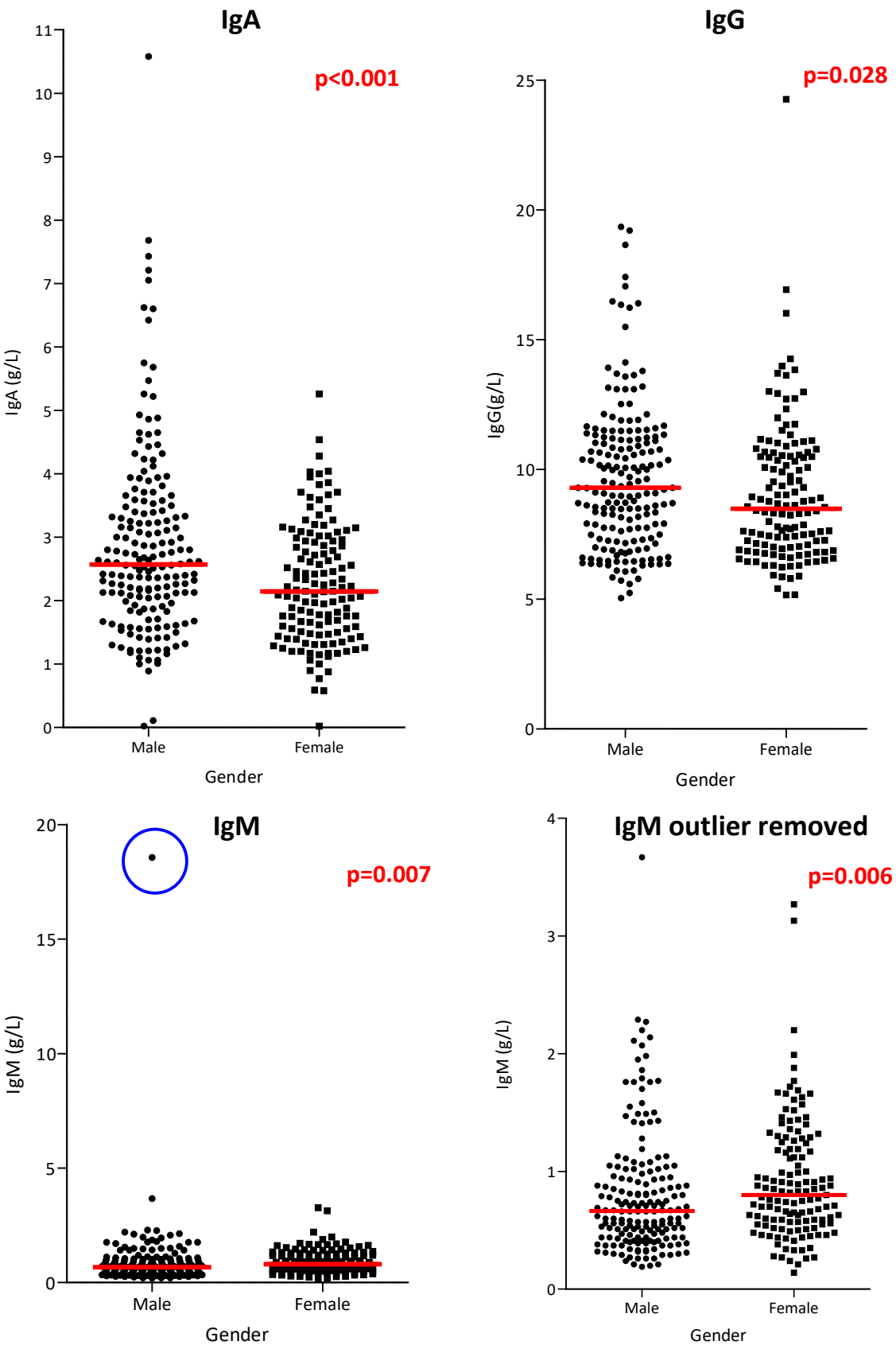

Figure 4.16: Scatterplots of immunoglobulin levels according to gender

Horizontal red lines represent median values. IgM outlier highlighted with blue circle.

4.8.2.1 Immunoglobulin and disease phenotypes in usual COPD

Previous studies have found smoking to cause a systemic reduction in immunoglobulin levels (227-231) however that was not evident within this cohort. There was no significant difference in IgA, IgG or IgM levels between current smokers and non-smokers (median IgA 2.36 v 2.44 g/L, p=0.344, IgG 8.73 v 9.03 g/L, p=0.628, IgM 0.74 v 0.70 g/L, p=0.344).

There was a trend of patients having ≥ 2 exacerbations annually having lower IgA levels (median 2.27 v 2.56 g/L, p=0.056) and there was a weak but statistically significant negative correlation between IgG and the number of annual exacerbations (r_s=-0.112, p=0.041). There were no significant differences in any of the immunoglobulin results with respect to the presence of emphysema, bronchiectasis or chronic bronchitis.

4.8.2.2 Immunoglobulin relationship to disease severity in usual COPD

There was a weak positive correlation between IgG levels and FEV_1 (% predicted) (r_s =0.138, p=0.015). No other significant correlations between immunoglobulin levels and lung function parameters were found.

4.8.2.3 Immunoglobulin relationship to longitudinal outcomes in usual COPD

IgA levels were significantly higher in patients who died compared to those remaining alive (median 2.66 v 2.33 g/L, p=0.014, Table 4.11). Univariate cox regression analysis demonstrated that only IgA levels significantly predicted mortality (HR 1.32, p<0.001).

The whole cohort (n=316) was therefore subdivided into IgA quartiles and Figure 4.17 shows the associated difference in survival curves. There is some overlap of quartile 1 and 2 but the increased risk of mortality associated with IgA results in the top 2 quartiles is apparent.

Univariate cox regression analysis shows the hazard ratio for death for patients with IgA results in the top quartile (≥3.18g/L) was 1.9 (1.24-2.92), p=0.003). Figure 4.18 shows the significant difference in the survival curves above and below this threshold. Multivariate cox regression was then undertaken to determine whether this relationship was independent of other factors that predicted mortality within this cohort. Table 4.12 outlines the results which show that IgA is an independent risk factor for mortality, which is not influenced by gender despite men having higher IgA levels than women (corrected HR in the final 3 variable model (IgA>3.18g/L, BMI and FEV_1% predicted) of 1.78 (p=0.014)). A ROC curve analysis was performed to assess the sensitivity and specificity of IgA in the prediction of mortality within this cohort. The area under the curve was 0.59 (p=0.014, Figure 4.19)

	Dead (n =91)	Alive (n=225)	P value	Univariate HR (CI)	P value
IgA	2.66 (1.71-3.17)	2.33 (1.65-3.04)	**0.014***	1.32 (1.16-1.51)	**<0.001***
IgG	8.68 (7.14-11.39)	8.94 (7.25-10.68)	0.685	1.04 (0.98-1.11)	0.234
IgM	0.74 (0.51-1.03)	0.70 (0.5-1.07)	0.992	0.94 (0.76-1.17)	0.595

Table 4.11: Immunoglobulin levels and mortality in the usual COPD cohort

Median immunoglobulin (g/L) reported with inter-quartile range in brackets. Mann Whitney U Tests performed to determine any statistical differences between the groups (*p ≤ 0.05). Univariate cox regression analysis performed to establish if immunoglobulin levels predict mortality. Where statistical differences were found the p value is highlighted in bold red text. HR= hazard ratio, CI= confidence interval.

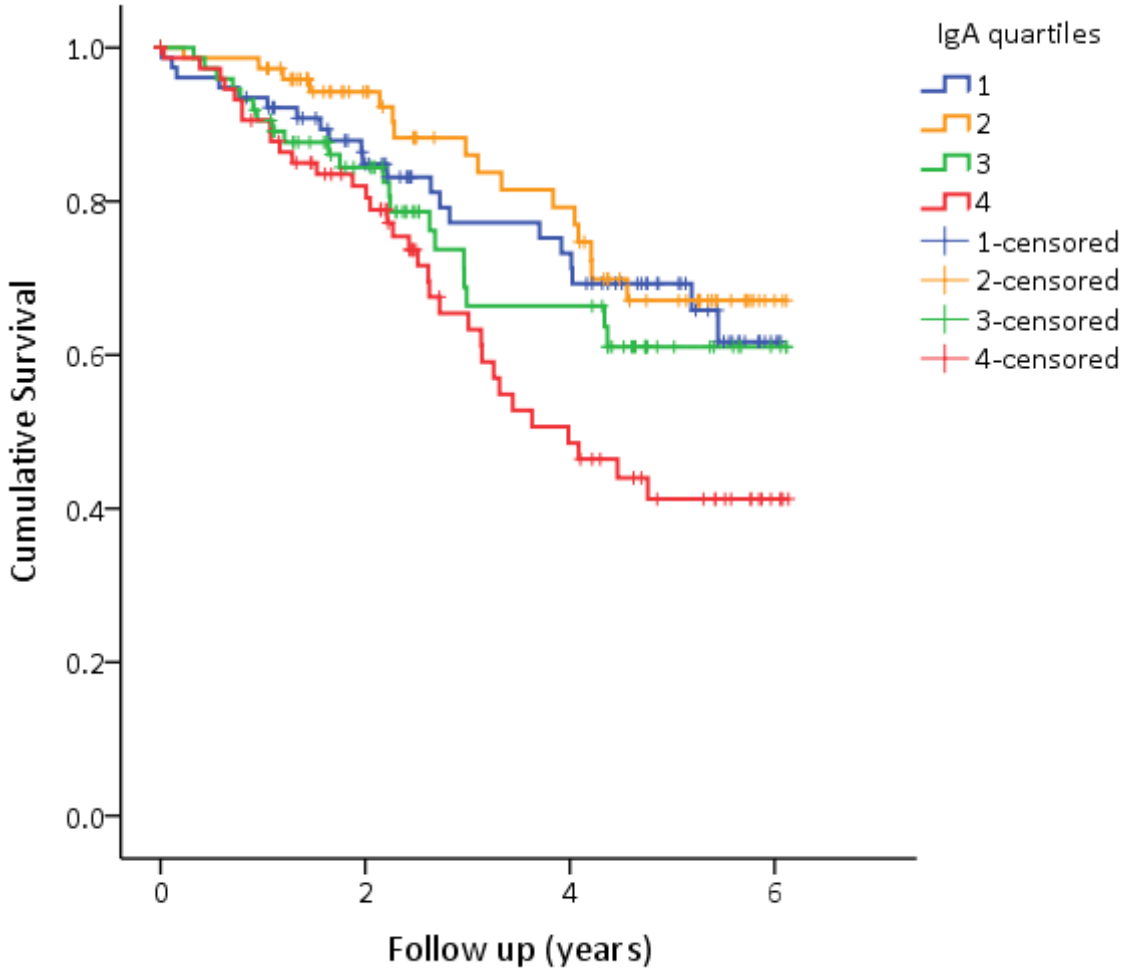

Figure 4.17: Kaplan-Meier graph according to IgA quartiles in the usual COPD cohort

Patients were subdivided into IgA quartiles. The coloured lines represent each quartile: blue represents quartile 1 (IgA <1.67g/L), orange represents quartile 2 (IgA 1.67-2.40g/L), green represents quartile 3 (IgA 2.40-3.18g/L) and red represents quartile 4 (≥3.18), n = 79 in each group. Breslow p=0024, Log Rank p =0.016.

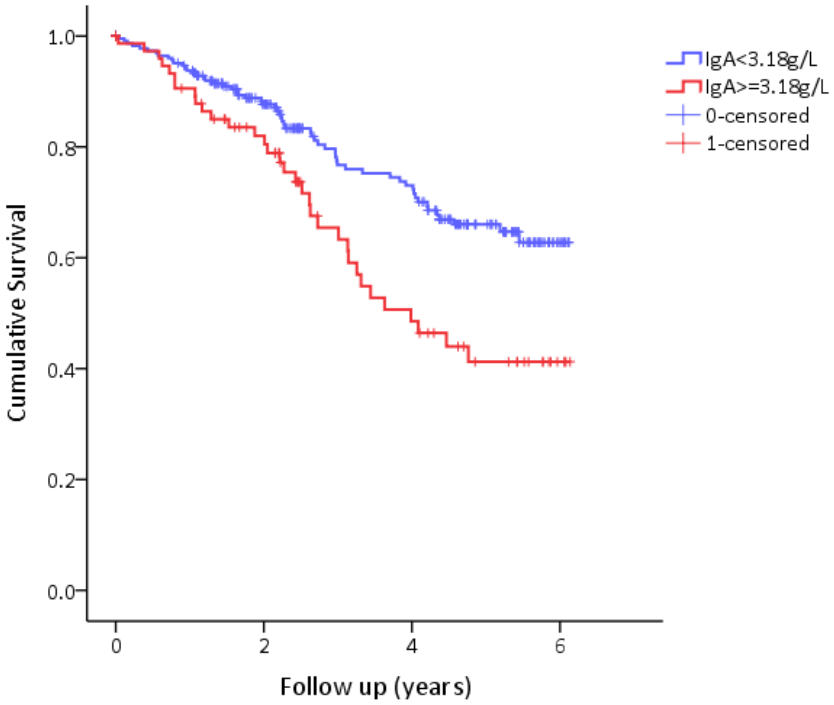

Figure 4.18: Kaplan-Meier plot according to IgA levels in the usual COPD cohort

Blue line represents patients with an IgA level <3.18g/L (the threshold for the upper quartile within the usual COPD cohort, n=237). Red line represents those with an IgA level ≥3.18g/L (n= 79). Log Rank p = 0.003.

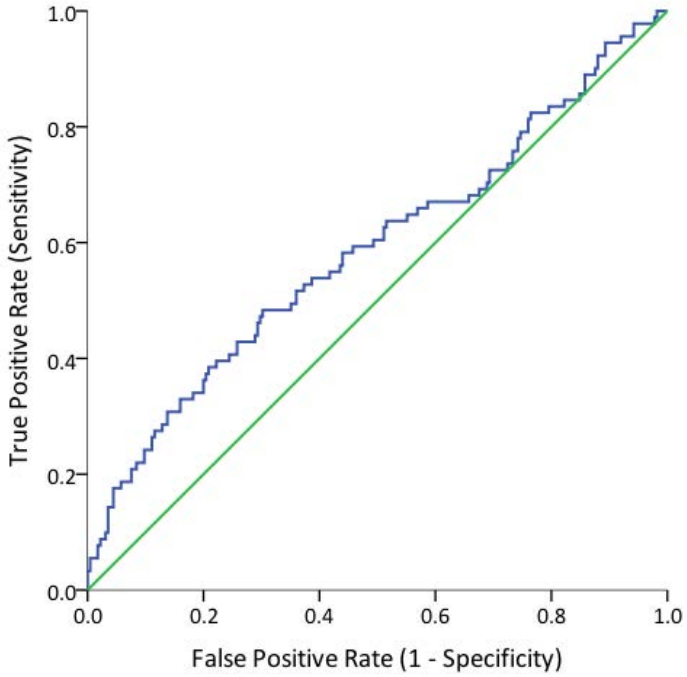

Figure 4.19 Receiver operating characteristics (ROC) curve assessing the sensitivity and specificity of combined IgA for mortality prediction in usual COPD.

Area under the curve = 0.59 (95% CI 0.52-0.66, p=0.014).

Variable	Univariate HR (CI)	P value	Multivariate HR (CI)	P value
IgA >3.18g/L	1.9 (1.24-2.92)	**0.003**	1.71 (1.07-2.72)	**0.024***
Pack years	1.01 (1.00 - 1.01)	0.161		
Current smoker	0.97 (0.63 - 1.49)	0.872		
Gender	1.46 (0.95 - 2.24)	0.087	1.19 (0.74-1.90)	0.474
Emphysema	1.05 (0.60 - 1.85)	0.861		
Bronchiectasis	0.83 (0.51 - 1.38)	0.478		
Frequent exacerbator	1.20 (0.71 - 2.02)	0.503		
Age	1.02 (1.00 - 1.05)	0.065	1.01 (0.98-1.04)	0.400
Chronic bronchitis	1.22 (0.77 - 1.92)	0.403		
BMI	0.95 (0.91 - 0.98)	**0.002***	0.96 (0.92-0.99)	**0.009***
eGFR	1.01 (1.00 - 1.01)	0.113		
FEV_1 (% predicted)	0.98 (0.96 - 0.99)	**<0.001***	0.98 (0.96-0.99)	**0.001***
KCO (% predicted)	0.98 (0.97 - 1.00)	**0.010***		
FEV1/FVC	0.04 (0.01 - 0.19)	**<0.001***		

Table 4.12: Predictors of mortality in the usual COPD cohort: univariate and multivariate cox proportional hazards regression analysis

HR = hazard ratio, CI = 95% confidence intervals, cFLC = combined κ & λ free light chain level mg/L, BMI = body mass index, eGFR = estimated glomerular filtration rate, FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, FVC = forced vital capacity. A frequent exacerbator was defined as having 2 or more exacerbations per year. (*$p \leq 0.05$)

4.9 Comparison with severe A1ATD cohort

The primary purpose of this analysis was to compare the cFLC levels between the severe A1ATD and usual COPD groups, rather than their clinical features, as cohorts were recruited in different settings, and differences between A1ATD and usual COPD are well known.

The patient demographics and significant differences between the two cohorts are outlined in Table 4.13. In comparison to the severe A1ATD cohort, the usual COPD patients were significantly older, had a greater number of pack years, more severe COPD as measured by FEV_1 (% predicted) but better renal function. The presence of chronic bronchitis and a history of frequent exacerbations (defined as ≥ 2 per year) were also more common in the usual COPD cohort. In the univariate analysis, usual COPD patients were found to have significantly higher cFLC levels (31.9 v 25.7 mg/L, $p<0.001$). However, since cFLC related to several clinical features in both A1ATD and usual COPD it was necessary to adjust by regression analysis for these features prior to comparing cFLC levels; this was particularly important because most of these also differed between A1ATD and our usual COPD cohort as outlined above (Table 4.13).

This was achieved by forced entry of eGFR, FEV_1 % predicted, presence of chronic bronchitis and age as covariates in a linear regression seeking associations of cFLC levels, to which the presence of A1ATD was then added as a final variable. Since cFLCs were non-normally distributed the values were logged prior to regression analysis, which resulted in normal distribution of the subsequent standard residuals. A significant model was created ($F(5,543) = 14.67$, $p<0.001$) although it only counts for 11.1% of the variance seen (adjusted $R^2 = 0.11$).

It did however show that A1ATD was a significant predictor of a lower cFLC level independent of eGFR, FEV_1, chronic bronchitis and age ($p<0.001$, Table 4.14).

Variable	A1ATD cohort (n=540)	Usual COPD cohort (n=316)	P value
Age	53.9 (45.0 – 60.9)	68.8 (61.5 – 75.1)	**<0.001***
Sex	Male n = 311 (57.6%) Female n = 229 (42.4%)	Male n = 182 (57.6%) Female n = 134 (42.4%)	0.999
Pack years	11.6 (0.0 – 24.0)	44.1 (29.5 – 62.2)	**<0.001***
FEV_1 (% predicted)	50.7 (35.1 – 85.1)	46.4 (35.0 – 61.0)	**<0.001***
KCO (% predicted)	62.3 (49.3 – 77.0)	59 (47.5 – 77.0)	0.533
Chronic bronchitis	185 (34.3%)	198 (62.7%)	**<0.001***
Emphysema	358 (66.3%)	257 (81.3%)	0.002
Bronchiectasis	142 (26.3%)	96 (30.3%)	0.678
Frequent exacerbator	129 (40.8%)	193 (61.1%)	**<0.001***
eGFR	81.3 (70.6 – 93.9)	85.8 (69.7 – 101.1)	0.063
CKD stage			**0.002***
1 eGFR ≥ 90	156(28.9%)	119 (37.6%)	
2 eGFR 60 - 89	279 (51.7%)	130 (41.1%)	
3 eGFR 30 - 59	41(7.6%)	35 (11.1%)	
4 eGFR 15 - 29	2 (0.4%)	4 (1.3%)	
5 eGFR < 15	1 (0.2%)	0 (0%)	
Unknown (no eGFR)	61 (11.3%)	28 (8.9%)	
cFLC (mg/L)	25.7 (21.1 – 31.7)	31.9 (24.0 – 43.3)	**<0.001***
κ/λ	0.86 (0.71-1.08)	0.86 (0.72-1.06)	0.967

Table 4.13: Comparison of patient demographics in the severe A1ATD and usual COPD cohorts

Number of patients with contemporaneous renal function = 479 A1ATD, 288 usual COPD. Continuous variables expressed as median (IQR); sex, chronic bronchitis, emphysema, bronchiectasis, exacerbators and CKD stage expressed as number in each group (%). A frequent exacerbator was defined as having 2 or more exacerbations per year. BMI = body mass index, FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, eGFR = estimated glomerular filtration rate, cFLC = combined (κ + λ) free light chain level (mg/L).

Variable	B (95% CI)	Std error B	P value
eGFR	-0.001 (-0.002- -0.001)	<0.001	**<0.001**
Age	0.001 (0 – 0.003)	0.001	**0.023**
Chronic bronchitis	0.033 (0.007 - 0.06)	0.013	**0.013**
FEV_1% predicted	4.8×10^{-4} (0 – 0.001)	<0.001	**0.021**
A1ATD	-0.09 (-0.133 - -0.053)	0.020	**<0.001**

Table 4.14: Linear regression analysing log cFLC in all stable patients in the severe A1ATD and usual COPD cohorts

The table shows the regression coefficients (B) and significance of variables. The two most important variables in the model were eGFR and A1ATD.

4.10 Discussion

4.10.1.1 FLC Stability

The results show that polyclonal FLCs are static in the stable disease state in usual COPD. There was no significant difference between the baseline and one year follow up samples however only 71% were within 25% of the baseline value compared to 84.2% in the A1ATD cohort. The usual COPD patients are older and are likely to have a greater burden of other comorbidities, which may have had an influence on the stability of their FLC levels (239).

4.10.1.2 FLC and immunoglobulin relationships to disease phenotypes in usual COPD

Similarly to the A1ATD cohort, the patients with chronic bronchitis had a higher median cFLC value compared to those without (33.29 v 30.89 mg/L) although this did not achieve statistical significance (p=0.11) in usual COPD which may perhaps reflect the greater degree of airways inflammation in the former group (240) and a potential immune modulatory role of AAT

(241).

Current smokers were found to have a significantly lower κ/λ ratio with a trend towards higher κ levels than non-smokers. This differs from the A1ATD cohort where the current smokers (though few) had significantly higher λ FLC levels. Baraldo et al assessed the clonality of B-cells found within lymphoid follicles within lungs of A1ATD and usual COPD patients (125). Oligoclonal and monoclonal B-cell populations were seen in 3/3 patients with severe A1ATD and 2/3 patients with usual COPD. They therefore felt that the immune activation was to 'specific' antigens. As B cells only produce κ or λ FLCs it is possible that an adaptive immune response to smoking could promote an oligoclonal/monoclonal B cell

proliferation resulting in excess κ/λ FLCs. Thus, the findings in both cohorts are potentially reflective of a smoking effect on FLC production, although a reason for the difference in clonality between A1ATD and COPD will require further investigation.

Males were found to have significantly higher cFLC levels compared to females, which was an unexpected finding and could not be accounted for by any documented demographic differences between them. There is known to be a male predominance of haematological conditions associated with monoclonal overproduction of FLCs such as monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (242). However, any patient with an abnormal κ/λ was excluded from the analysis, hence undetected gammopathies should not have influenced our results. A difference in immunoglobulin levels was also demonstrated, with the males having significantly higher IgA and IgG levels but significantly lower IgM levels. A previous study investigating the effects of demographic factors on immunoglobulin levels found male sex to be positively associated with IgA levels and negatively associated with IgG and IgM levels (243). The reason for the differences seen was thought to relate to the effects of sex hormones, which have been shown to influence immunoglobulin production in vitro (244, 245) and in animal models (246) and hence remains the most likely explanation.

As in the A1ATD cohort, no significant difference in cFLC levels was seen between frequent and non-frequent exacerbators. However, there was a trend towards patients with a lower IgA having more exacerbations and a weak negative correlation between IgG levels and annual exacerbation frequency. IgG subclass deficiency is relatively common in patients with COPD (247) and has been shown to increase the susceptibility of patients to respiratory tract infections (248) which may explain the finding that patients with a lower IgG level had a

higher number of annual exacerbations although subclass levels have yet to be measured. IgA is essential in the mucosal defence of the body to infective insults with the ability to inhibit bacteria from adhering to epithelial cells (249). It is tempting therefore to hypothesise that the trend towards frequent exacerbators also having lower IgA levels may also have reduced respiratory tract mucosal defence resulting in an increased susceptibility to infections. However, there are two IgA isotypes within the body: IgA1 and IgA2 with the former predominating in the serum and the latter in mucosal secretions (secretory IgA). Despite there being lower levels of IgA in serum than IgG, more IgA is produced within the body compared to all other antibody isotypes combined emphasising its importance in the humoral immune defence (250). It is important to recognise however the distinct differences in the production, structure and function of secretory and serum IgA. Serum IgA is produced in the bone marrow whereas secretory IgA is synthesised by plasma cells within mucosal membranes. Serum IgA, which was measured in this study, is predominantly monomeric IgA1 and its immune functions are not fully understood. It has been shown to bind other serum proteins including A1AT and albumin (251) as well as monocytes and granulocytes and is thought to have an anti-inflammatory role (252). Secretory IgA is predominantly dimeric in form linked by a secretory component and provides an important defence against pathogens by protecting mucosal surfaces within the body (250). In terms of what is already known about IgA production in COPD, Burnett et al found an increase in the number of cells containing IgA (both IgA1 and IgA2 subclasses) in lung tissue specimens from patients who had died from 'chronic bronchitis' and bronchiectasis compared to controls. Interestingly, the proportion of IgA1 and IgA2 was the same in patients with bronchiectasis and controls but patients with chronic bronchitis had significantly higher IgA1 levels (253). In a small study

of patients with COPD infected sputum samples were shown to contain more IgA than non-infected samples but no significant difference in serum IgA levels (254). More recently Ladjemi *et al* demonstrated that incubating bronchial epithelium from COPD patients with B cells from healthy donors promotes IgA1 production. However, IgA was also shown to accumulate in sub-epithelial layers in COPD lung tissue and did not necessarily result in an increase in secretory levels (255).

4.10.1.3 FLC relationship to disease severity

FLC levels did not correlate with disease severity. However, when patients were sub-grouped according to the severity of their airflow obstruction, patients with moderate (GOLD stage 2) obstruction were found to have significantly higher FLC levels than those with severe (GOLD stage 4) obstruction. The same pattern was also seen in the A1ATD cohort. As discussed in Chapter 3.7.1.3, FEV_1 decline is known to be greatest in patients with moderate airflow obstruction and this is sometimes viewed as a surrogate marker of disease 'activity'. It is possible that cFLC levels therefore relate better to disease activity than disease severity. Alternatively, it is possible that this reflects a treatment effect as a greater proportion of GOLD stage 4 patients are likely to be treated with high dose inhaled steroids which have been shown to modulate the adaptive immune system response within the lung in COPD (234, 235).

4.10.1.4 FLC and disease activity in usual COPD

In order to establish whether FLCs varied according to episodes of increased disease activity in COPD they were measured in a separate cohort of patients at day 1 and day 56 of an exacerbation. A significant polyclonal increase in cFLC levels from day 1 to day 56 was

observed although the absolute difference in median values was small. This could suggest that FLCs alter with acute changes in disease activity which more likely represents an immune stimulus reflecting an infective nature of the exacerbation episodes associated with bacteria or viruses. It was not possible as part of this study to obtain sufficient samples to determine the infective nature of the episodes but would clearly be important in a future project.

There was no significant difference in the day 1 or day 56 cFLC levels in the patients who subsequently died compared to those who survived although the numbers were small. Previous studies have shown that exacerbations are associated with a worse quality of life, greater speed of FEV_1 decline and an increased risk of mortality (15-17) and this could be explored in more detail within a follow up study powered for such an outcome.

In order to establish whether FLCs could be a clinically useful biomarker of disease activity in COPD they would also need to be measured in a larger cohort with a longer period of follow up. Overall there was a significant increase in group cFLC levels from day 1 to day 56 however not all patients had an increase. It would be important to determine whether a rise in FLC level during such episodes was associated with a worse short-term prognosis given the association between FLC levels and mortality within the larger usual COPD cohort demonstrated here. It may be that a high-risk subgroup of patients who have unresolved inflammation resulting in chronic adaptive immune activation and a greater risk of mortality could be predetermined however further work is needed to investigate this possibility.

4.10.1.5 FLC and immunoglobulin relationships to longitudinal outcomes

The results show that cFLC levels are an independent risk factor for mortality in patients with usual COPD with a level above the upper limit of normal conferring a hazard ratio of 1.8 (corrected for severity of COPD as measured by FEV_1 in a multivariate analysis). Polyclonal FLC levels have previously been shown to be a risk factor for mortality within both the general population and in patients with CKD (183, 187-189).

Serum IgA levels were also found to significantly predict mortality in this patient population supporting the concept that adaptive immune activation is an important identifying factor for mortality in patients with COPD. Hurme et al found IgA levels to significantly predict mortality within a cohort of 285 nonagenarians (256). This is consistent with an 'immune risk phenotype' where specific parameters including: CD4:CD8 ratio of <1, poor T-cell proliferative responses to mitogens, increased CD8+, CD28−, CD57+ cells, low B cells and cytomegalovirus (CMV) seropositivity which predicts mortality in a healthy aged population (257, 258). The authors hypothesised that the chronic antigenic stimulation caused by viruses such as CMV and other organisms lead to an increase in senescent T cells and other immune system changes which result in immunoparesis and an increased risk of death. A significant proportion of COPD patients suffer from recurrent infective exacerbations or are colonised with specific bacteria which may similarly cause chronic antigenic stimulation of the adaptive immune system resulting in dysregulation and thereby increase the risk of mortality. To investigate this further we could establish a similar cohort including the bacterial colonisation status and follow their immune response and prognosis.

Other inflammatory biomarkers have previously been shown to be risk factors for mortality in patients with COPD. For example baseline C reactive protein is a risk factor for mortality in

patients with mild to moderate COPD (259, 260) but not in patients with more severe COPD (261). The ECLIPSE study found several other inflammatory biomarkers to be independent risk factors for mortality including: white cell count, neutrophil count, interleukin 6, CCL-18, PARC and SP-D and showed that adding a panel of biomarkers to clinical predictive models improved their predictive value (133). The increased risk of death associated with having a high polyclonal FLC level or IgA is a novel finding as no recognised mortality prediction tool currently used in usual COPD includes markers of adaptive immune activation. Further work is therefore needed to establish whether adding cFLC and/or IgA levels to existing mortality prediction tools could increase their prognostic accuracy. Within the current cohort a positive correlation was seen between IgA and cFLC levels. Having a cFLC level >43.3mg/L (the upper limit of normal) or and IgA level or >3.18g/L (the cut off of the upper quartile) conferred similar hazard ratios for death (1.80, p=0.009 and 1.71, p=0.024 respectively). Neither test was superior at ROC curve analysis as the area under the curve was 0.59 for both. Measuring FLCs alone may therefore have no benefit over measuring IgA levels for this specific utility as Immunoglobulin testing is available in all hospitals and less expensive to perform.

4.10.1.6 Conclusions

FLCs show some characteristics consistent with the criteria for a useful biomarker in usual COPD.

1. Relationship to underlying disease pathophysiology: FLCs have been shown to be increased in murine models of emphysema and in a small number of usual COPD patients compared to controls. They can interact with neutrophils to promote IL8

production and prevent their apoptosis which could potentiate inflammation within the lung.

2. Stability: FLCs are static in the stable disease state in usual COPD

3. Ability to identify clinically important phenotypes: cFLCs were found to be higher in males within this cohort and a significant difference in the κ/λ ratio was observed in smokers but cFLCs were unable to differentiate important clinical phenotypes such as chronic bronchitis.

4. Relationship to disease severity and activity: cFLCs do not have a linear relationship with disease severity as measured by FEV_1 however when patients were sub-grouped according to their GOLD severity, patients with moderate airflow obstruction were found to have significantly higher FLC levels than those with very severe impairment. The same relationship was seen within the A1ATD cohort. FLCs significantly rise during COPD exacerbations but the influence of this on longitudinal outcomes is currently unknown.

5. Ability to predict longitudinal outcomes: cFLC levels measured in the stable state are an independent risk factor for mortality in patients with usual COPD.

In addition to the findings above we found that cFLC levels correlate positively with IgA and IgG levels and that IgA levels are similarly associated with mortality within this cohort. This supports the findings of Chapter 3 regarding the importance of adaptive immune activation as a risk factor for mortality in patients with COPD. The utility of adding FLCs as a biomarker to mortality prediction models is worthy of further investigation.

CHAPTER 5: FLCs and colonisation in a bronchiectasis cohort

5.1 Introduction

Bronchiectasis is the permanent dilatation of the airways, which may or may not be associated with airway thickening. It is diagnosed radiologically by high-resolution computed tomography scanning. The hallmark clinical features of bronchiectasis are chronic cough with sputum production, bacterial airway colonisation and frequent lower respiratory tract infections (262).

A significant proportion of patients with A1ATD related and usual COPD have co-existent bronchiectasis. Earlier reports of the incidence of bronchiectasis in A1AT deficient individuals were as high as 43% although based on small study populations (66, 71). In a larger study of 74 (PiZ) subjects, Parr et al reported the incidence of "clinically relevant" bronchiectasis to be 27%, which is similar to the reported incidence in usual COPD (72). The incidence of bronchiectasis in our larger A1ATD and usual COPD cohorts reported in Chapter 3 and 4 were 28.6% and 30.2% respectively and therefore in line with previous studies.

A recent Meta-Analysis found the presence of bronchiectasis in patients with COPD to be associated with a higher exacerbation frequency, isolation of pathogenic organisms, more severe airflow obstruction and an increased risk of mortality (263).

In Chapter 3, the potential utility of polyclonal FLCs as a biomarker in severe A1ATD was examined. In addition to predicting mortality within this patient population, patients who were known to have chronically colonised airways were found to have higher FLC levels than those without evidence of colonisation. This raised the issue of whether colonisation could be driving a chronic adaptive immune response in certain individuals. To explore this relationship further, FLCs were measured in a small cohort of patients with bronchiectasis.

5.2 Patient Characteristics

FLC analysis was performed in 53 patients with non-cystic fibrosis (CF) related bronchiectasis. Six patients were then excluded from analysis for the following reasons: 2 patients had abnormal ratios, 1 patient had a known paraproteinaemia, 1 patient had an IgG and IgM deficiency and 2 patients were on long-term oral steroids. Demographic data for the remainder is shown in Table 5.1. Median follow up time was 5.6 years (IQR 4.8-5.7) and the underlying aetiology of bronchiectasis of patients within this cohort is shown in Table 5.2.

Variable	Bronchiectasis cohort (n=47)
Age (years)	68 (61.8-72.4)
Sex	Male n = 9 (19.1%) Female n = 38 (80.9%)
Pack years	0 (0-2)
FEV_1 (% predicted)	68.8 (24.9)
KCO (% predicted)	101.0 (94.0-107.0)
Chronic bronchitis	36/41 (87.8 %)
Emphysema	2/43 (0.05%)
Frequent exacerbator	26/41 (63.4%)
eGFR (ml/min/1.73m^2)	85.0 (72.8 – 91.1)
CKD stage	
1 eGFR ≥ 90	12 (25.5%)
2 eGFR 60 - 89	29 (61.7%)
3 eGFR 30 - 59	3 (6.4%)
4 eGFR 15 - 29	1 (2.1%)
5 eGFR < 15	0 (0%)
Unknown (no eGFR)	2 (4.3%)
cFLC (mg/L)	29.9 (25.6-39.4)
κ/λ	0.89 (0.29)

Table 5.1: Patient demographics in the bronchiectasis cohort

Continuous variables are expressed as median (IQR); sex, chronic bronchitis, emphysema, exacerbators and chronic kidney disease (CKD) stage expressed as number in each group (%). A frequent exacerbator was defined as having 2 or more treated exacerbations per year. Number of patients with contemporaneous renal function = 45. FEV_1 = forced expiratory volume in 1 second, KCO = corrected gas transfer, eGFR = estimated glomerular filtration rate, cFLC = combined (κ + λ) free light chain level and the κ/λ ratio is shown.

Bronchiectasis aetiology	(n=47)
Idiopathic	15 (31.9%)
Sequelae to childhood infection	22 (46.8%)
Primary ciliary dyskinesia	2 (4.3%)
ABPA	1 (2.1%)
Aspiration pneumonia	2 (4.3%)
Pink disease	2 (4.3%)
Airways disease	3 (6.4%)

Table 5.2 Bronchiectasis aetiology

Aetiology of bronchiectasis within the cohort expressed as number in each group (%). ABPA = Allergic Broncho Pulmonary Aspergillosis

5.3 Concurrent autoimmune disease

Thirteen patients (27.7%) had at least one concurrent autoimmune disease (Positive ANCA (n=5), Thyroid disease (n=4) rheumatoid arthritis (n=2), vitiligo (n=1), polymyalgia rheumatica (n=1), ulcerative colitis (n=1), one patient had 3 conditions). There was no significant difference in cFLC levels between patients with and without a concurrent autoimmune disease (median 28.4 v 31.2 mg/L, p=0.20).

Twenty-five patients have been tested for antinuclear antibodies (ANA). There were no significant differences in cFLC levels in patients with and without a positive ANA (median 28.43 v 40.32 mg/L, p=0.095).

5.4 FLCs and clinical phenotypes

Table 5.3 shows the results of the univariate analysis of FLC levels (κ, λ and cFLC) in the bronchiectasis cohort grouped according to their gender, smoking history and clinical phenotype. No significant differences between the groups were seen.

	Group 1	Group 2	P value
Current Smokers	**Yes (n=11)**	**No (n=33)**	
cFLC	30.5 (27.5-38.6)	29.9 (25.5-41.3)	0.728
κ	14.3 (12.5-15.3)	14.9 (11.4-18.0)	0.847
λ	15.4 (14.5-21.8)	15.4 (12.9-20.7)	0.417
κ/λ	0.95 (0.68-0.98)	0.94 (0.750-1.11)	0.545
Gender	**Male (n=9)**	**Female (n=38)**	
cFLC	31.3 (26.6-44.2)	29.8 (25.5-39.8)	0.570
κ	15.1 (11.4-18.6)	14.4 (11.8-17.7)	0.766
λ	15.4 (13.7-25.8)	15.7 (13.2-20.5)	0.665
κ/λ	0.97 (0.62-1.02)	0.93 (0.78-1.05)	0.746
Frequent exacerbations	**Yes (n = 26)**	**No (n = 15)**	
cFLC	31.9 (27.0-46.1)	27.7 (24.0-34.6)	0.144
κ	15.6 (12.7-19.4)	14.3 (10.3-15.1)	0.137
λ	16.1 (13.6-25.1)	14.4 (12.0-19.5)	0.151
κ/λ	0.90 (0.69-1.14)	0.94 (0.77-0.98)	0.871
Chronic bronchitis	**Yes (n=36)**	**No (n=5)**	
cFLC	30.2(24.9-41.8)	25.9 (22.2-33.5)	0.202
κ	14.8 (11.6-17.6)	13.1 (9.3-16.8)	0.339
λ	15.4 (13.3-21.8)	14.1 (11.6-17.3)	0.202
κ/λ	0.94 (0.72-1.03)	0.91 (0.71-1.10)	0.905

Table 5.3 Comparison of FLCs in subgroups according to phenotype and gender in the bronchiectasis cohort

Median individual and combined κ & λ (cFLC) levels (mg/L) reported with inter-quartile range in brackets. Mann Whitney U Tests performed to determine any statistical differences between groups 1 and 2 (*$p \leq 0.05$).

5.4.1 FLC relationship to colonisation status and sputum purulence

5.4.1.1 FLC relationship to airway colonisation

The prevalence of bacterial colonisation in patients with non-cystic fibrosis related bronchiectasis has been reported to be as high as 64% (264). All the patients within the current bronchiectasis cohort were asked to provide sputum samples during their stable state to establish whether they were colonised with any potentially pathogenic microorganisms (PPMs – see Appendix 1.3). For the purposes of this study a positive culture was defined as a growth ≥ 1 x 10^5 colony forming units (CFU) per ml of sputum of a PPM. Chronic colonisation was defined as ≥3 isolations of same organism from separate sputum samples over minimum of ≥3 months (206). Patients were then sub-grouped according to the colonising organism (pseudomonas, haemophilus influenzae, other/mixed growth of multiple PPMs). Figure 5.1 A shows the breakdown of the cohort according to their colonisation status. Nine patients did not provide an adequate number of sputum samples for colonisation status to be confirmed. Figure 5.1 B shows the cFLC levels in patients sub-grouped according to their colonising organism. No significant difference between the groups was seen (Kruskall Wallis test $\chi^2(4) = 3.79$, p=0.435).

Additionally, when patients were simply grouped according to whether they were colonised or not, no significant difference in FLC levels was seen (median cFLC mg/L 31.3 (26.6-42.5) versus 29.2 (22.9-35.6) mg/L respectively, p = 0.29) Figure 5.2.

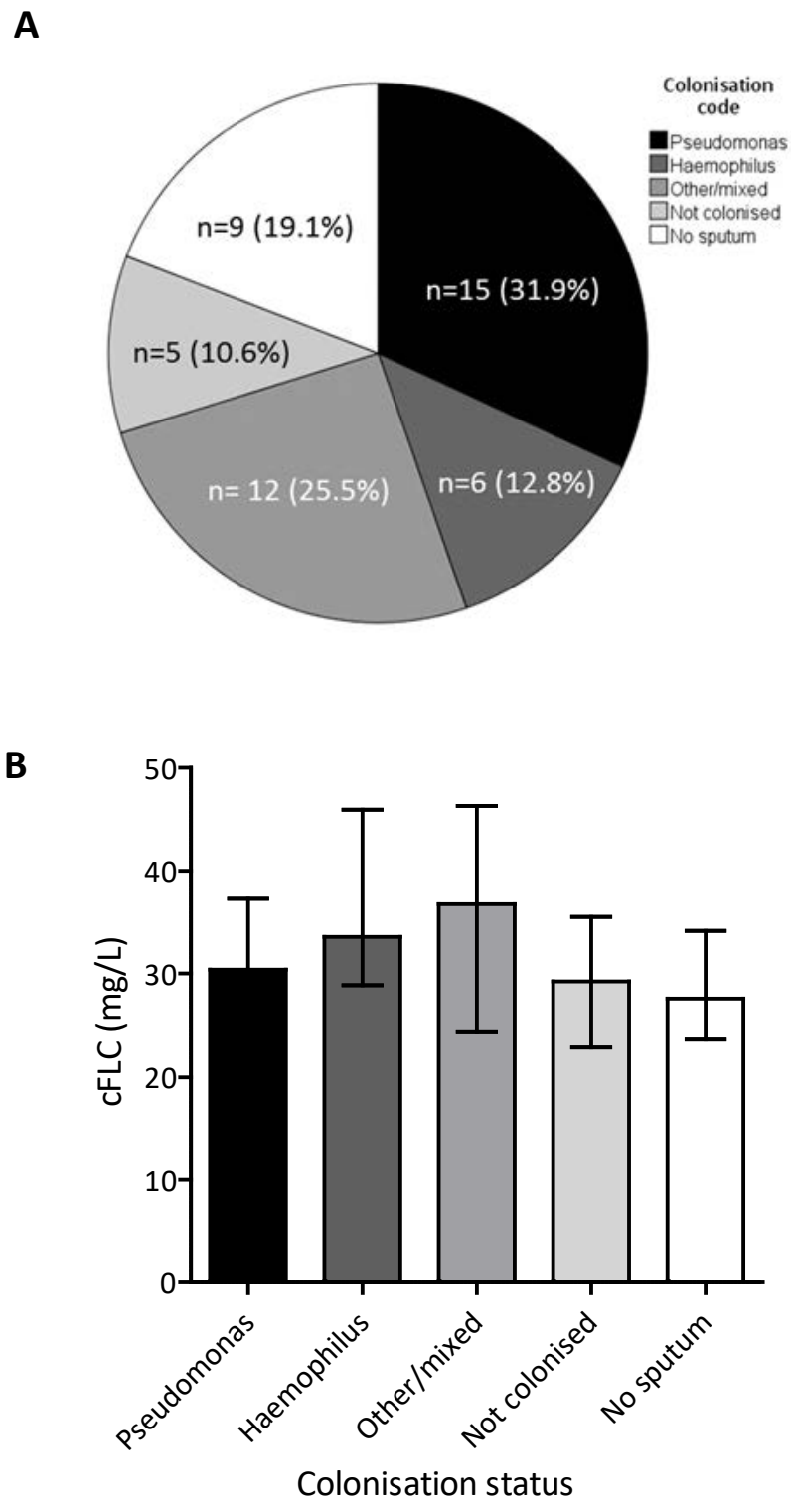

Figure 5.1 The colonisation status of the patients in the bronchiectasis cohort and relationship to cFLCs

A. Pie chart showing the proportion of patients according to colonisation status. B. Bar chart showing median FLC levels (error bars represent interquartile range) in the different groups. There was no significant relationship to colonisation status (Kruskall Wallis test p=0.435).

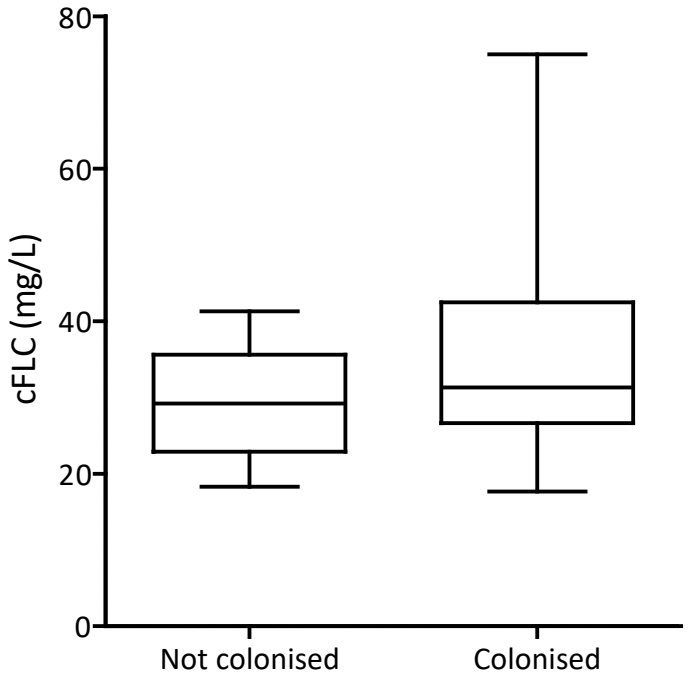

Figure 5.2: Box plot showing cFLC levels in bronchiectasis patients who are chronically colonised or not with potentially pathogenic organisms

Univariate comparison of the combined κ & λ free light chain (cFLC) levels between the non-colonised (n=5) versus the chronically colonised (n= 33) patients in the bronchiectasis cohort. Horizontal lines represent medians, boxes represent interquartile ranges and whiskers represent minimum to maximum values. There was no significant difference between the combined κ & λ free light chain (cFLC) levels (median cFLC mg/L 31.3 (26.6-42.5) versus 29.2 (22.9-35.6), p = 0.29).

5.4.1.2 FLC relationship to sputum purulence

Sputum purulence has previously been shown to correlate with markers of airway inflammation in patients with bronchiectasis (265). Nineteen patients had sputum cultures taken at the same time as their FLC sampling. The sputum samples were categorised by a research microbiologist as mucoid, mucopurulent or purulent according to their appearance. There was a trend towards patients with purulent sputum having higher serum FLC levels compared those with mucopurulent sputum although this did not achieve conventional statistical significance (median cFLC 36.0 (29.5-38.4) versus 29.8 (23.2-30.6) mg/L respectively p=0.065, Figure 5.3).

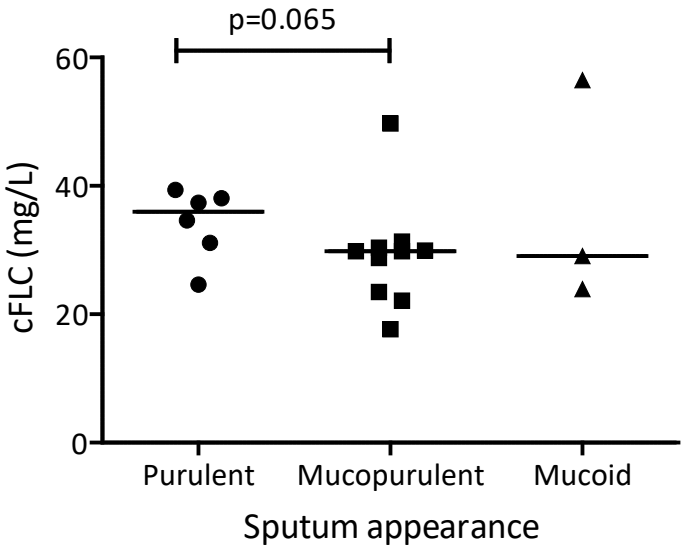

Figure 5.3 Scatter plot showing the relationship of cFLC to sputum purulence in patients with bronchiectasis

Comparison between combined κ & λ free light chain (cFLC) levels of patients sub-grouped according to their sputum purulence within the bronchiectasis cohort. Each dot/square/triangle represents an individual patient and the lines represent median values per group. The groups were compared using the Mann-Whitney U test. There was a trend towards patients with purulent sputum having higher cFLC levels compared to those with mucopurulent sputum although this did not achieve statistical significance (median cFLC (mg/L) 36.0 (29.5-38.4) versus 29.8 (23.2-30.6) respectively, p=0.065).

5.5 FLCs relationship to bronchiectasis disease severity

5.5.1 FLC relationship to airflow obstruction

In patients with bronchiectasis, chronic airway inflammation results in structural damage of the airways and mucus plugging which can be associated with an accelerated decline and FEV_1 resulting in airflow obstruction (266, 267). Greater systemic inflammation, colonisation with pseudomonas and a high exacerbation frequency have been shown to be risk factors for lung function decline in patients with non-CF bronchiectasis (266).

The relationship of FLCs to lung function parameters within this cohort was therefore examined. However, no correlation with FEV_1 (r_s=-0.10, p=0.49), FEV_1/FVC (r_s=-0.10, p=0.49) or gas transfer (KCO r_s=-0.17, p=0.33) was identified (Figure 5.4). In addition, when patients were sub-grouped according to the severity of their airflow obstruction there was no significant differences between the groups (Kruskall Wallis test χ^2 (4) =1.60, p=0.81, Figure 5.5).

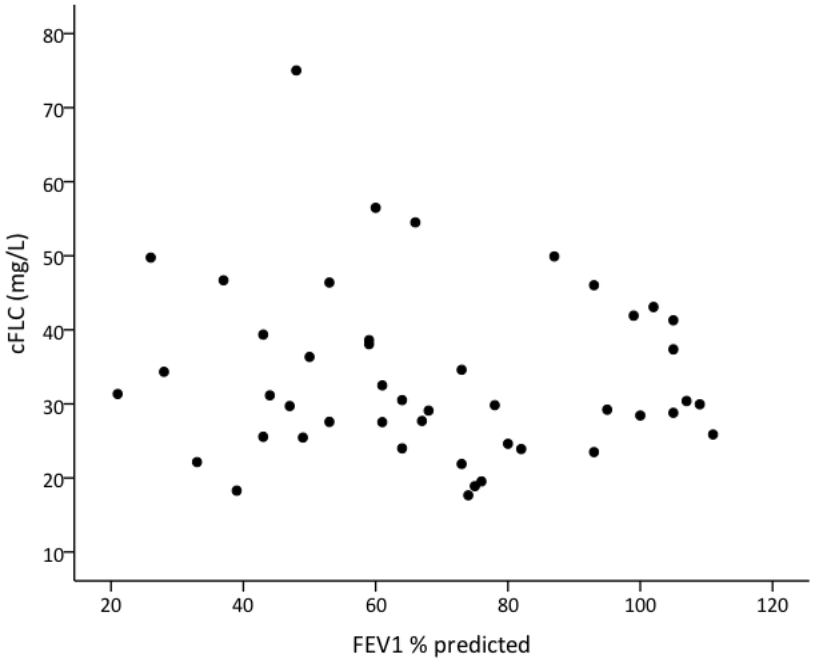

Figure 5.4 Scatterplot showing the relationship between cFLC and airflow obstruction in the bronchiectasis cohort.

cFLC = combined (κ & λ) free light chain, FEV_1 = forced expiratory volume in 1 second. Each dot represents a single patient. (Spearman's Rho r_s=-0.10, p=0.49).

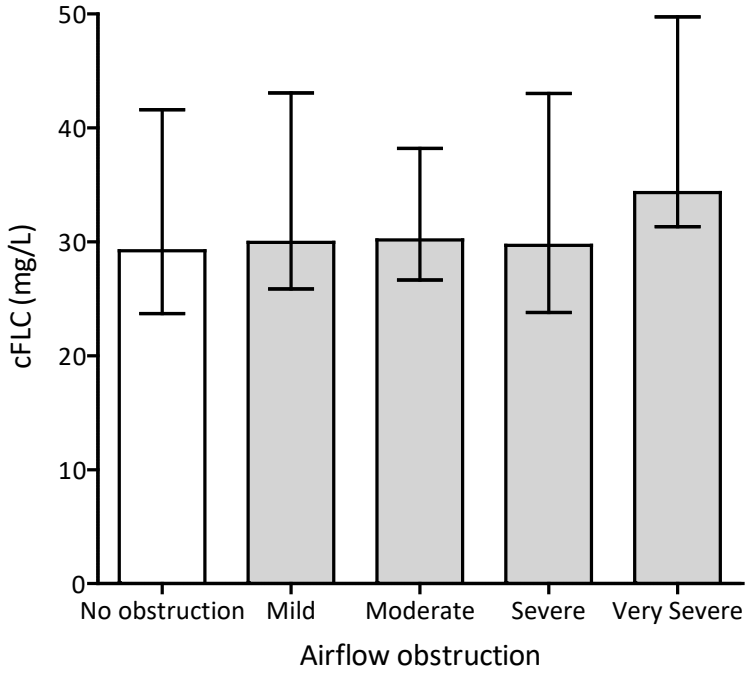

Figure 5.5 Bar chart showing cFLC levels in bronchiectasis patients grouped according to the severity of their airflow obstruction

Bars represent median combined (κ & λ) FLC (mg/L) in each group. Error bars represent interquartile range.

5.5.2 FLCs relationship to the FACED severity score in bronchiectasis

There is no agreed severity index used routinely in the assessment of bronchiectasis, however in recent years a number of scoring systems to assist with prognostication have been proposed. HRCT scan changes correlate poorly with lung function impairment limiting the utility of HRCT severity scores alone (268). This prompted the need for more detailed validated scoring systems taking into account other clinical parameters to help assess severity in bronchiectasis. Martinez-Garcia et al described the 'FACED' tool to predict mortality in a cohort of patients with bronchiectasis based on a score calculated from the following variables: **F**EV_1, **A**ge, **C**olonisation with pseudomonas, the **E**xtent of bronchiectasis (number of lobes of lung involved) and **D**yspnoea (assessed using the modified MRC breathlessness score) (209). Another widely used severity scoring system is the 'Bronchiectasis Severity Index' which contains many of the same variables as the FACED tool but also includes BMI, previous hospital admissions and the number of exacerbations within the last year (269). However this data was not available for our retrospective cohort analysis and the FACED score has been shown to be superior in predicting 15 year mortality (270) so this severity score was chosen for the purposes of the current study.

The FACED score was calculated for 39/47 of the patients within the bronchiectasis cohort (see Appendix 1.6); eight patients did not have all the data available to calculate the score so were excluded from the analysis (MRC scores not recorded at the time of sample collection). No significant difference between cFLC levels was seen between the patients grouped according to their FACED scores (Kruskall Wallis ($\chi^2(6) = 5.77$, $p=0.450$) Figure 5.6.

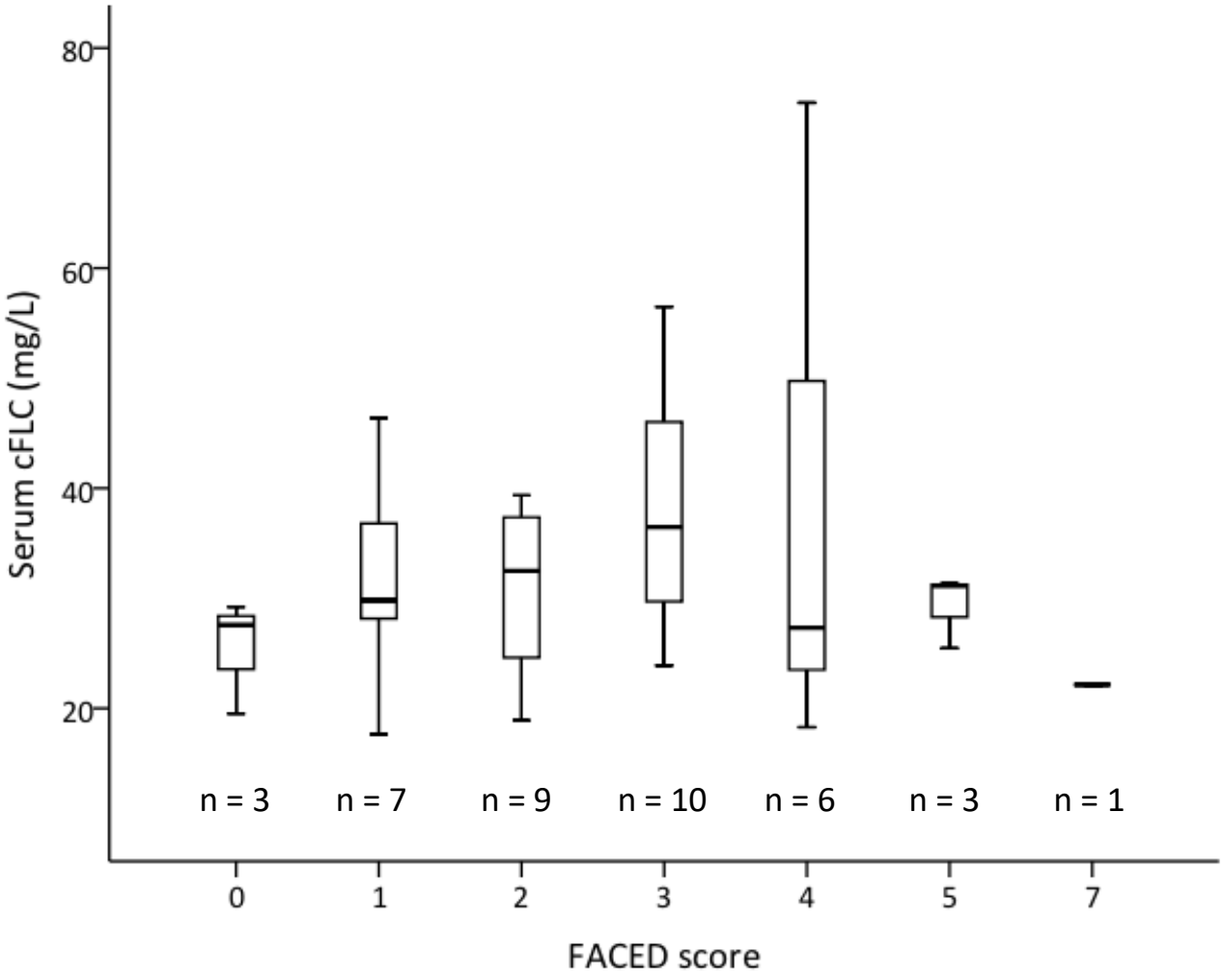

Figure 5.6 Box plot showing the relationship of cFLC levels to FACED severity score in patients with bronchiectasis

The FACED score was calculated for n=39 patients based on their **F**EV$_1$, **A**ge, **C**olonisation with pseudomonas, **E**xtent of bronchiectasis and **D**yspnoea (measured using the modified MRC score). Horizontal lines represent medians, boxes represent interquartile ranges and whiskers represent minimum to maximum values. FACED score of 0 = least severe, 7 = most severe. cFLC = combined (κ & λ) free light chain.

5.6 FLCs and longitudinal outcomes

Four patients died during the follow up period. There was no significant difference between the cFLC levels in the patients who died compared to those still alive (median cFLC 23.9 (27.1-39.8) versus 30.5 (19.7-41.2) mg/L, p=0.214). A survival analysis was not conducted within this cohort as there were too few deaths for this to be statistically robust (271).

5.7 Immunoglobulin analysis

Immunoglobulin results were recorded for 41/47 patients (87.2%) within the bronchiectasis cohort. Positive correlations were identified between FLC levels (κ, λ and cFLC) and IgA and IgG levels. The strongest correlation was between cFLC and IgA levels (r_s= 0.702, p<0.001** Figure 5.7) There was no significant correlation between FLC levels and IgM.

A positive correlation between IgA levels and BMI was also seen (r_s = 0.42, p=0.006*). No significant differences in immunoglobulin levels was seen between current smokers and non-smokers (IgG median 10.91 v 10.74 g/L, p=0.914, IgA median 3.02 v 2.49 g/L, p=0.30, IgM 0.72 v 1.03 g/L, p=0.106). No significant differences in immunoglobulin levels were seen with respect to gender, frequent exacerbator status (≥ 2 per year) or chronic bronchitis.

Patient who subsequently died were found to have significantly lower IgM levels than those still alive (Median 0.39 v 0.99 g/L, p=0.038*), however as only four patients died during the follow up period the clinical significance of this result is uncertain.

		κ	λ	κ/λ	cFLC
IgA	r_s	0.685	0.697	0.014	0.702
	p	**<0.001****	**<0.001****	0.929	**<0.001****
IgG	r_s	0.411	0.313	0.17	0.373
	p	**0.008***	**0.046***	0.287	**0.016***
IgM	r_s	-0.189	0.012	-0.29	-0.093
	p	0.237	0.938	0.066	0.563

Table 5.4 Spearman's Rho correlation of FLC levels and Immunoglobulin levels in the bronchiectasis cohort

r_s =correlation coefficient. (*Statistically significant 2p values are highlighted in red)

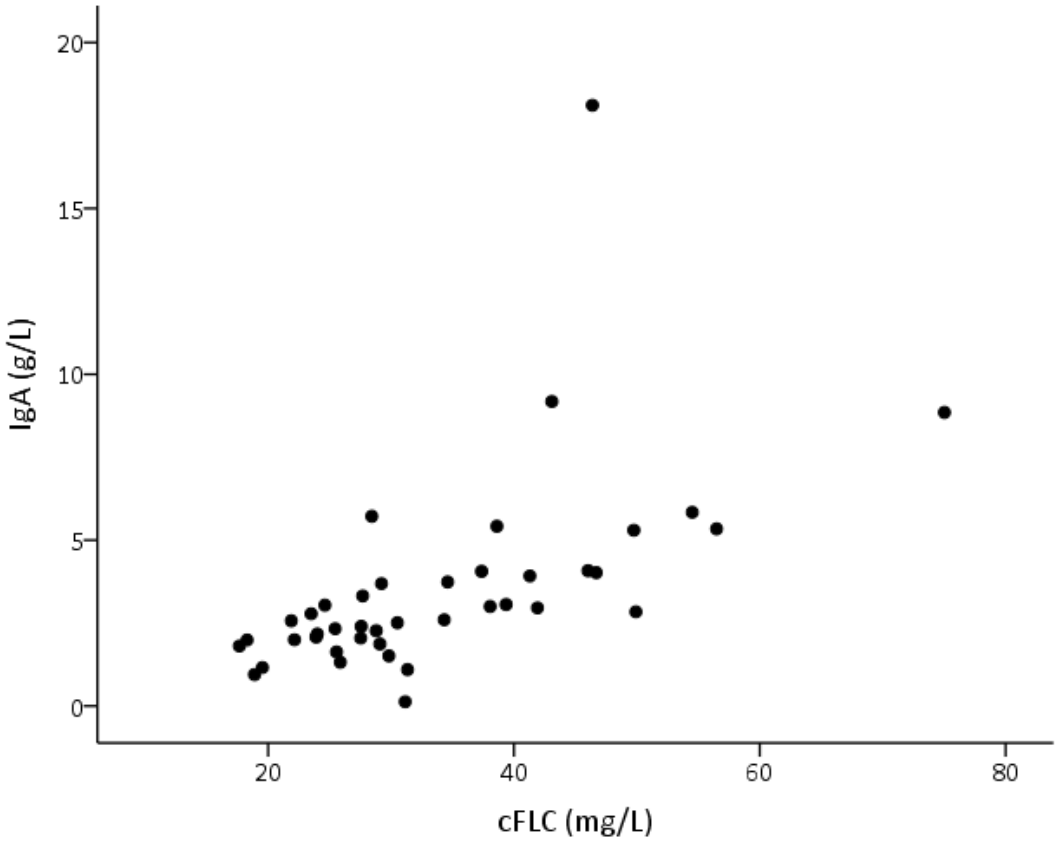

Figure 5.7 Scatterplot showing correlation between IgA and cFLC levels in the bronchiectasis cohort.

IgA = Immunoglobulin A, cFLC = combined κ & λ FLC level (mg/L). Spearmen's Rho r_s=0.70, **p<0.001****

5.8 Discussion

The main aim of this chapter was to explore the relationship of FLC levels and bacterial colonisation of the airways. This small bronchiectasis cohort was utilised due to the detailed information on their colonisation status. However, no significant difference was seen between FLC levels of bronchiectasis patients colonised with different organisms. In addition, there was no significant difference in FLC levels between those who were chronically colonised compared to those patients who were not.

Although no direct relationship of FLC to colonisation status was seen there was a trend towards patient with purulent sputum having higher cFLC levels. Purulent sputum occurs as a result of neutrophil recruitment to the airways indicating activation of the adaptive immune response (265). In addition to being a marker of colonisation (215) studies have demonstrated that sputum colour (measured using a sputum colour chart) is associated with active proteolytic enzymes and increased inflammatory cytokine levels within the airways (265, 272). As sputum purulence is therefore a marker of airway inflammation it seems logical that patients with purulent sputum may have a greater adaptive immune response reflected in higher serum FLC levels.

There are a number of potential reasons why we did not see the same relationship of FLCs to chronic colonisation within this cohort. As previously discussed in Chapter 4, it has been hypothesised that FLCs could play a role in the pathogenesis of COPD and it is possible that this is not the case for non-CF bronchiectasis. Bronchiectasis is a very heterogeneous condition with many different potential aetiologies. Ideally a sub-group analysis should be performed according to the underlying cause of the bronchiectasis as it is possible that FLCs

may be relevant to some aetiologies and not others. Unfortunately, the number in this cohort was too small to draw conclusions from subgroup analyses according to aetiology. Although some pathological processes are common to both bronchiectasis and COPD, there are also some differences. In both, the airways are infiltrated with neutrophils, macrophages and lymphocytes and there is a vicious cycle of infection and inflammation (43, 273). As previously described in Chapter 1 lymphoid follicles are seen in the lungs of patients with COPD (49) and this has also been reported in a subgroup of patients with 'follicular' bronchiectasis (274). This was first described by Whitwell in 1952 and was so named due to the prominent pathological feature being excessive lymphoid tissue formation within the walls of the affected airways and surrounding alveoli. This pathological subtype corresponds to the commonest form of bronchiectasis whereby 'cylindrical' or 'tubular' bronchiectasis is seen radiologically. It would also be interesting to look at FLC levels according to pathological and radiological subtype however when reviewing the CT reports of the patients within this cohort the radiologists had not consistently reported the type of bronchiectasis that was seen, so it was not possible to perform this analysis robustly and would clearly be an important issue for any future studies.

In COPD, it is recognised that chronic inflammation and disease progression occurs despite smoking cessation, and may be driven by periods of exacerbation, which can also be non-infective in nature. This was the reason for the emergence of the autoimmune hypothesis in the pathogenesis of COPD. It is possible that FLC production could in part be driven by an autoimmune phenomenon and exacerbated by infection. This prompted me to look at the antinuclear antibody (ANA) titres within this cohort but no relationship to FLC levels was seen. ANA testing was inconsistently performed within this cohort and the number of

patients with ANA sampled was likely too small to enable robust conclusions. Interestingly the proportion of patients with concurrent autoimmune disease was higher in this cohort than in the A1ATD and COPD cohorts.

5.8.1 Immunoglobulin analysis

The British Thoracic Society guidelines on non-CF bronchiectasis advocate that all patients with bronchiectasis have serum immunoglobulin levels checked due to the possibility of an underlying antibody deficiency as the cause of their bronchiectasis (262). Similar to the findings in the usual COPD cohort, the FLC levels in this cohort of bronchiectasis patients were found to correlate positively with both IgA and IgG levels but not IgM.

In this cohort patients who died were found to have significantly lower IgM levels than those still alive. However only 4 died during the follow up period so the importance of this finding remains uncertain. IgM is the first antibody response when foreign antigen is encountered. Circulating serum IgM also contains so-called 'natural' antibodies that are considered important modulators of immune homeostasis (275) in addition to IgM antibody produced in response to antigen stimuli. Selective IgM deficiency is associated with increased risk of potentially life-threatening infections including upper and lower respiratory tract infections and bronchiectasis. IgM deficiency has also been linked to a number of autoimmune diseases but the cause and effect relationship has not been differentiated (276).

Given that immunoglobulins are routinely tested in all patient with bronchiectasis it would be important to examine immunoglobulin levels in a larger cohort of bronchiectasis patients with respect to longitudinal outcomes. The main purpose of measuring immunoglobulin levels is to rule out an abnormally low value, however, in view of our findings in usual COPD

that high cFLC levels and IgA levels significantly predict mortality it would be important to determine whether the same relationship exists in bronchiectasis or whether low IgM levels predict mortality as this could be easily added to existing mortality prediction scores such as the FACED tool.

5.8.2 Limitations

There were a number of limitations that may have influenced the results within this chapter. Patients were recruited from secondary care bronchiectasis clinics. This was a strength, as patients under routine follow up are more likely to be chronically colonised and have a high exacerbation frequency and hence more likely to determine the relevance of colonisation to FLC levels. However, it could also be a weakness as this would add an element of selection bias such that results may not be generally representative of patients with bronchiectasis. The primary aim of assessing FLCs in this cohort was to look for a relationship to colonisation rather than examining the utility of FLCs as a biomarker in patients with bronchiectasis. However, the data was far from clear and a much larger cohort would need to be studied in order to determine such a utility.

Only one sample of serum was taken from the patients so it was not possible to determine the stability of FLCs over time within this cohort. There was also a relatively small number of patients compared to the A1ATD and usual COPD cohorts. It is likely that larger cohorts of patients will be needed to assess such phenotypic trends. As this was a retrospective cohort analysis there were also some gaps in the data which reduced the sample size further for analyses such as the relationship of cFLC to the FACED bronchiectasis severity score. It was

also not possible to undertake survival analyses due to the small number of patients who died during the follow up period.

CHAPTER 6: Conclusions and future work[10]

6.1 Conclusions

The main objective of this study was to investigate the utility of polyclonal FLCs as a clinical biomarker in severe A1ATD and usual COPD. As previously discussed, key properties of a clinically useful biomarker are that it is reproducible in stable disease, relates to disease severity and relates to outcome. The results demonstrate that cFLCs meet many of these criteria, notably being associated with subsequent mortality in both cohorts. No significant difference was seen in cFLCs taken from patients with stable disease at different time points, suggesting that cFLCs are reproducible in stable disease. A strong relationship between cFLC levels and disease severity was not seen, although there was a difference observed between patients with and without chronic bronchitis, which is recognised to be a clinically relevant subgroup within airways disease (277). Chronic bronchitis is associated with more rapid FEV_1 decline (219), increased exacerbation frequency (278) and a greater risk of mortality (279). However, the difference between cFLCs in patients with chronic bronchitis compared to those without was relatively small, thus the result must be interpreted with caution in terms of clinical relevance.

The most significant finding in this study was that in both the A1ATD and usual COPD cohorts, cFLC levels were a predictor of mortality, independent of age and severity of renal impairment with cFLC levels above the normal range conferring a hazard ratio of 2.89 (1.47-5.70, p=0.002) and 1.8 (1.16-2.8, p=0.009) respectively. In addition, cFLC levels were found

[10] Excerpts of this chapter have been previously published (202. Hampson JA, Stockley RA, Turner AM. Free light chains: potential biomarker and predictor of mortality in alpha-1-antitrypsin deficiency and usual COPD. Respir Res. 2016;17:34.)

to positively correlate with IgA and IgG levels in the usual COPD cohort and IgA levels similarly predicted mortality. Several studies have shown a link between immune system activity, inflammation and risk of death: an increase in polyclonal cFLCs predicted mortality in the general population (183) and cFLC >65 mg/L was a risk factor for death within 100 days (184). The association between inflammation and cardiovascular death is well recognised (280), and 41 % of the deaths in those with cFLC>65 mg/L was from cardiovascular disease (183). A recent systematic review supported the concept that the relationship between cardiovascular disease and COPD goes beyond common aetiological factors such as smoking (281); cFLCs could be partly a factor in this association.

Furthermore, many important questions regarding the role B cells play in the development of COPD remain unanswered. As previously discussed it is not known what drives the B cell response in COPD or whether this response is specific to the lung or not. If it were lung specific, then this might account for the lack of relationship to co-morbid systemic diseases linked to immune activation. Commonly hypothesised antigen sources are microbes colonising the airways, smoke constituents and breakdown products of the extracellular matrix (222). In the A1ATD cohort we found that chronically colonised patients had significantly higher cFLC levels, supporting the hypothesis that colonisation may be an important driving factor of adaptive immune activation. Alternatively there has been much research about the interaction of microorganisms and the associated immune response triggering a response to self-antigens resulting in autoimmunity through a number of different mechanisms (223). These include so-called “molecular mimicry” (where the microorganism has a peptide epitope similar to a self-protein), through the production of

superantigens by microorganisms themselves or are due to other modifications of self-antigens brought about by infection related tissue injury and oxidative stress (223).

The difference in cFLC observed between usual COPD and A1ATD imply that this is a more important pathogenic theme in usual COPD, although this does not exclude immune activation also contributing to the disease process in A1ATD. This result is contrary to the recent report of equivalent levels of lymphoid follicles in lung tissue from a small cohort of A1ATD patients with very severe lung disease, compared to usual COPD (125). It is possible that immune activation represents a feature of advanced disease in both conditions, as most of our patients had severe disease, thus further studies are indicated.

cFLC levels were also examined in a cohort of patients with bronchiectasis to examine the relationship between cFLC and colonisation further. Within this cohort, no meaningful relationship was seen. However, this may be due the heterogeneous nature of patients with bronchiectasis in compared to the more homogenous nature of disease in A1ATD. The variation in aetiology, differing severity and clinical phenotypes means that a larger cohort was needed to identify any clear relationships with colonisation and to examine the relationship between FLC, immunoglobulin levels and longitudinal outcomes in this bronchiectatic population.

In summary polyclonal FLCs are a feature of severe A1ATD related and usual COPD. Levels relate to chronic bronchitis and the presence of colonisation in A1ATD, rise during periods of

exacerbation in usual COPD and predict mortality in both. Suggesting an association of these features with disease activity, colonised disease states and late stage disease. However, these preliminary results raise many issues and further questions that need to be answered namely:

1. Cause and effect: are FLCs simply a marker of adaptive immune activation or are they pathogenic in COPD?
2. Is it only bacterial colonisation driving the adaptive immune response or are other factors involved?
3. Relationship to mortality: are polyclonal FLCs simply a marker of mortality risk or a cause of increased mortality?
4. Are FLCs present within the airways of patients with COPD and how does this relate to their serum level and other factors including bacterial colonisation?

In order to clarify the studies reported here further areas of research need to be followed or consolidated.

6.2 Future epidemiological work

It is important to establish whether the link to mortality seen within these cohorts is a reproducible finding. Further work is warranted to investigate the role of adding biomarkers of adaptive immune function, such as cFLC and IgA, to mortality prediction tools in usual COPD. The Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) study was a prospective study designed to identify novel biomarkers that may help

phenotype patients or predict disease progression. As previously discussed in Chapter 3, the group found that the addition of a panel of inflammatory biomarkers to a number of clinical variables (age, BODE and hospitalisation history) increased the C statistic from 0.686 ($p<0.001$) to 0.726 ($p=0.003$) (133). If samples have been stored from this cohort of more than 1800 patients, FLC and IgA levels could be measured in these patients to determine whether their addition added further to the predictive value of the model.

In healthy aging populations, other adaptive immune system markers of the so called 'immune risk phenotype' (CD4:CD8 ratio <1, inadequate T cell responses, high numbers of CD8+, CD28- and CD57+ T cells, low B cell number) have been shown to increase risk of mortality (256, 258). More recently IgA levels were also found to predict mortality in a cohort of Finnish nonagenarians (256). It has been hypothesised that the cause of this immune risk phenotype in the elderly may be due to chronic stimulation of the immune system by infections such as CMV resulting in immunosenescence (257). It may also be of value to examine these other parameters of adaptive immune function in the ageing COPD patient population. Of particular interest would be the effects of chronic bacterial colonisation of the lung and whether this relates to or is the cause of the change to these markers (including IgA) and hence the association/cause of longitudinal outcomes.

It is important that the relationship of polyclonal FLCs to bronchiectasis and colonisation is further elucidated. As discussed in Chapter 1, a significant proportion of patients with COPD have co-existent bronchiectasis. As immunoglobulin levels are routinely assessed in patients with bronchiectasis in the diagnostic pathway advised by the British Thoracic Society, it

should be possible to do this retrospectively in a further and larger cohort of patients with bronchiectasis thereby consolidating the association (262). One way to facilitate this would be to utilise an established bronchiectasis cohort such as the EMBARC (a European Bronchiectasis Registry) (282). If a biomarker of adaptive immune function was shown to predict poor outcome in chronically colonised patients with bronchiectasis, it would be important to see if targeting these patients with prophylactic antibiotics affected outcome by reducing the bacterial load within the lungs and the subsequent persistent systemic inflammatory response.

The A1ATD cohort is similar in disease severity to the American A1ATD registry (283), thus results are likely to be generalisable to other A1ATD populations. However, the usual COPD group generally had severe COPD, and exhibited a high prevalence of emphysema, thus our results may be less generalisable to milder usual COPD cohorts and those without emphysema. This is in part due to the inclusion of family screened, non-index cases in A1ATD, not undertaken in usual COPD. Consequently, future work in milder COPD populations, perhaps recruited from primary care, could be undertaken to ensure wider generalisability and a better comparison to the A1ATD group. For this purpose, the local BLISS cohort (a prospectively recruited primary care cohort composed of patients diagnosed with COPD by their GP and identified through a linked case finding trial) could be a suitable source to widen work (284).

6.3 Future work to examine the effects of FLCs on neutrophil function

In order to investigate a more direct relevance of FLCs to the pathogenesis of COPD, further functional laboratory work is necessary. The first question to answer is to confirm whether FLCs have a pathogenic effect in COPD through polymorphonuclear (PMN) activation. Braber et al previously demonstrated that in vitro, FLCs can bind to human neutrophils causing their activation and production of IL8 (168). Antagonising FLCs (using F991) was also shown to inhibit this binding capability and reduce neutrophilia within the BAL fluid in a smoke exposed mouse model (168). Mechanistically cFLCs have biological properties that could therefore potentially damage lung tissue through interaction with neutrophils (168, 177, 178). It has also been shown previously that migratory accuracy of neutrophils is lower in COPD than A1ATD (285); it is possible that cFLCs might be partly responsible since cFLCs were significantly higher in COPD in this study and have been previously shown to inhibit migration of PMNs towards chemoattractants in vitro (177). Furthermore, several case reports detail nodular and cystic lung disease associated with cFLC overproduction in light chain deposition disease (LCDD) (191-194). This condition is characterised by the deposition of non-amyloid κ or λ light chains, and presents with progressive cystic lung disease ultimately leading to respiratory failure necessitating lung transplantation (193). It is possible to therapeutically antagonise cFLCs using the compound F991 in animals (168, 286, 287). Thus, it remains important to clarify whether the pro-inflammatory effects of cFLCs play a role in COPD, and represent a suitable target for an anti-inflammatory strategy.

6.3.1 FLC neutrophil stimulation assays

In order to confirm the work of Braber et al, neutrophils (extracted from patient samples using Percol density centrifugation) (288) would be stimulated with physiological concentrations of FLCs in vitro, and IL8 release measured by ELISA. Inter patient variability will be measured to validate this further. For this purpose, neutrophils will be isolated from the peripheral blood of 5 healthy controls, 5 usual COPD and 5 A1ATD patients on 3 different days and IL8 release in response to FLCs measured. These results will provide evidence that it represents a stable process.

To characterise the neutrophil response to FLCs, additional markers of neutrophil function, specifically phagocytosis, the oxidative burst, degranulation and chemotaxis should be measured following stimulation with FLCs. The effect of FLCs on ROS production and phagocytosis can be measured using the Phagoburst and Phagotest assays respectively (BD Biosciences) (289). The Phagoburst assay measures neutrophil ROS production in response to stimulation with E.coli via the reduction of dihydrorhodamine (DHR) which is measured using flow cytometry. Similarly, the Phagotest assay uses flow cytometry to measures the phagocytosis of fluorescently labelled E. coli. ROS production and phagocytosis will be measured in response to stimulation with either vehicle control or varying concentrations of FLCs. In addition, the effect of FLCs on neutrophil degranulation will be measured using an MPO release assay which utilises TMB as a chromogenic substrate in order to measure the release of enzymatically-active MPO from activated phagocytes.

Finally, in order to examine whether FLCs are chemoattractant in their own right experiments will be performed to see their effects on neutrophil migration using an Insall chamber. All the above assays are well established in our department (289, 290).

6.4 Develop a FLC sputum assay

The next question to answer is whether FLCs are present in the airways of patients with COPD and examine how this relates to their serum levels. Do they play a local role in inflammation and how does this reflect response to bacterial colonisation?

One of the advantages of measuring FLCs in serum is that this is a routinely performed test in hospital laboratories for the diagnosis of haematological conditions. However, sputum biomarkers in COPD directly assess airway inflammation. In addition to cell counts such as neutrophil, macrophage and eosinophil counts other molecules such as cytokines, proteases, anti-proteases and markers of oxidative stress have been measured in the sputum of patients with COPD (291). As with all biomarkers, validation is key to ensuring that sputum markers are clinically useful (see Chapter 1.3).

To examine whether FLCs can be measured accurately in sputum as well as serum and plasma, a study has been planned in collaboration with the Binding Site to validate a sputum FLC assay.

So far, testing has been performed on 5 samples of sputum sol taken from patients with usual COPD. Linearity was confirmed and all 5 showed doubling dilutions, starting with an initial dilution of 1 in 50 into assay sample diluent remained as predicted. Six independent

assays were then performed over several days using fresh sample dilutions, which by the final assay involved testing samples that had been frozen and thawed from -80°C on 4 occasions, also giving an indication of sample stability.

The inter-assay reproducibility was comparable to that obtained with the internal reference thus indicating a 1/50 sputum dilution gives an acceptable level of reproducibility (κ sample mean coefficient of variance (CV) 7.22 versus internal reference (IR) CV 5.72, lambda mean sample CV 7.34 versus IR CV 7.47). Interestingly, the mean kappa FLC value was higher than lambda for all 5 samples, which is different to that seen in respective serum. No marked lowering of values occurred as the assays were performed, suggesting that stability to freeze thaw cycles was good.

Spike recovery was then performed using 10µL of nephelometric Kappa Free or Lambda Free kit calibrator added to 700µL of 1/50 dilution of 2 sputum samples. Samples were assayed and the recovery of the added free light chain assessed (mean recovery 100% and 95.8% respectively). A 1/50 sputum sol dilution therefore appears to provide a suitable matrix for the quantitation of free light chains.

Having established that FLCs can be accurately assayed in sputum, further work will aim to quantify the levels of FLC in larger cohorts of patient with both A1ATD and usual COPD and not only look at correlation with serum values but also clinical parameters such as lung function, inflammatory markers, chronic bronchitis, bacterial colonisation and outcomes. Sample collection is currently ongoing. It would also be important to measure sputum FLCs during periods of exacerbations. The studies in this thesis found that in usual COPD that there is a significant rise in serum cFLCs from day 1 to day 56 during exacerbations but did

not assess local changes within the lung during this time. This would be of particular importance when considering targeted therapy, such as FLC inhibition (section 6.3) which could prove to be of value either in chronic care or as part of exacerbations only.

In summary polyclonal FLC predict mortality in patient with severe A1ATD and usual COPD and may be an important biomarker for risk stratification. Further work is needed to establish if they are pathogenic in these conditions and could be responsible in part for the aberrant neutrophil functions previously described in COPD and hence a useful drug target.

APPENDIX 1: Methods supplements

1.1 Consent forms

Consent forms are included for the following patient cohorts who were utilised for the purposes of this research

1. Assessment for Alpha-1 Antitrypsin Deficiency
2. A study of clinical phenotypes, disease progression and epigenetics in patients with chronic obstructive pulmonary disease and its associated co morbidities.
3. A study of clinical phenotypes, disease progression and genetic susceptibility in patients with chronic obstructive pulmonary disease
4. An open study to investigate the measurement and variability of inflammatory cells, mediators and erythrocyte abnormalities in spontaneously produced sputum, blood and urine from patients with COPD who present with acute exacerbations
5. An open study to investigate the measurement and variability of inflammatory cells, mediators and erythrocyte abnormalities in spontaneously produced sputum, blood and urine from patients with COPD who present with acute exacerbations
6. A study of clinical phenotypes, disease progression and genetic susceptibility in patients with chronic obstructive pulmonary disease, cystic fibrosis bronchiectasis and non-CF bronchiectasis

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PATIENT INFORMATION SHEET

Assessment for Alpha$_1$ Antitrypsin Deficiency

What is the study about?

You or a member of your family has been identified as having alpha$_1$ antitrypsin deficiency. This is an inherited condition that is believed to increase the risk of development of lung health problems. However very little detailed information has been collected on the way this deficiency affects patients and some studies have suggested that lung disease may run in families even without the deficiency. It is likely however that the deficiency highlights the tendency to develop these diseases and when present will make them worse.

We wish to learn as much as possible about the deficiency and its relationship to lung disease and for this reason invite you to participate in our alpha$_1$ antitrypsin deficiency assessment programme.

Sub-study: As part of a recently awarded National Institute for Health Research Rare Diseases Translational Research Collaboration it is important to be able to perform more detailed tests and assessments on a group of patients that includes both those with milder disease and those at the more severe end of the spectrum. This project will enable a critical comprehensive assessment of the health economic burden of alpha-1-antitrypsin deficiency which has never been undertaken. It will also specifically develop and apply new laboratory tests to measure enzymes in blood and secretions which can contribute to damage to the lung. This will help to identify those individuals at risk of progression and hence most likely to benefit from alpha-1-antitrypsin replacement therapy.

What will I have to do?

In broad terms you will undergo all the routine questioning, examination and tests that we normally undertake when assessing somebody who presents with lung disease. However we hope to do this more carefully and in more detail than is routinely carried out by your own doctor or specialist.

We will ask many questions about your past and present symptoms, health and wellbeing. In addition you will be examined thoroughly to determine the presence of signs related to lung disease. You will be asked to perform some lung function testing which assesses how your lungs work and their ability to take oxygen in and out of your body. We may also perform a specialised CT scan of your lungs (if you have not had one) which is a very sensitive technique of detecting damage that has occurred. Finally we will ask you to provide blood samples and if you have a cough productive of sputum we will arrange for you to collect this over several hours on one day before coming to see us.

Once all these tests have been performed we will be able to determine whether you have lung disease related to alpha$_1$ antitrypsin deficiency. This will be explained to you and any modifications in your treatment that are indicated will be communicated to both yourself and your own doctor.

It is our general clinical routine to follow patients with established lung disease on a long-term basis. Patients are usually seen annually to assess their wellbeing and follow any progress in the condition. If you have alpha$_1$ antitrypsin deficiency we would wish to see you once a year to assess your symptoms,

clinical signs and repeat the extensive lung function tests. After the first year the lung function may be repeated less frequently (2 or 3 yearly) depending on whether these are changing or are stable.

Sub-study: An abdominal ultrasound scan to assess any liver abnormalities may also be performed.

NOTE The CT scan exposes you to a small degree of radiation – about the equivalent of 6 months background radiation in the UK. Although this dose is safe (it is the same as a single x-ray of the abdomen), it is important that you inform us if you are likely to be pregnant as we will not carry out the test in these circumstances.

Study Flow Chart

PROCEDURE	Consultation Screening Visit	Baseline Visit	Follow-up Visits (annually)
Written informed consent	X		
Demographic and medical history	X		
Fingerprick blood test to confirm AAT level, genotype and phenotype	X[a]		
Routine haematology and chemistry		X	X
Serum and plasma for inflammatory markers		X	X
Optional DNA blood sample		X[b]	
Sputum sample for microbiology, cell count and inflammatory markers [c]		X	X
Abdominal ultrasound scan		X[d]	
Health Status Questionnaires		X	X
Pre-bronchodilator lung function tests		X	
Post-bronchodilator lung function tests		X	X
Oxygen saturation		X	X
Vicorder measurements		X	X
CT scan of chest [e]		X	
Physical examination		X	X
Record of exacerbation history		X	X
Record of current medication		X	X

[a] Only if this has not been previously confirmed
[b] Can be performed at either baseline or follow-up visit
[c] Only if produces spontaneous sputum
[d] Sub-study only and performed within the first 12 months
[e] To confirm presence and distribution of emphysema and bronchiectasis if patient has not previously had a CT scan of the chest

What are the benefits?

The major purpose of the study is to find out as much as possible about the lung disease and other health problems associated with alpha$_1$ antitrypsin deficiency. This will provide the background information that enables us to manage the disease appropriately and design studies to assess the role of alpha$_1$ antitrypsin replacement therapy in both the short and long term. The investigations that we undertake will allow us to advise upon the degree of lung disease that you have and simple measures that you can undertake with your current treatment in order to try and stabilize the lung disease. In addition the lung function tests that we will perform will help us to optimize your current treatment in order to improve your breathlessness where possible.

What are the risks?

All the investigations that are taking place are entirely routine in the assessment of patients with lung disease. As such they are repeated on many occasions in the same patient without any adverse effects. The only minor problem that is likely to occur is a slight degree of bruising in some patients when they have their blood taken.

What are the alternatives?

There are currently no alternatives to finding the information that is required other than the assessment programme outlined above.

What happens if I do not wish to take part?

If you do not wish to participate in the assessment programme this will be fully understood. Your own general practitioner, or the consultant chest physician who normally looks after you, will be informed of the diagnosis and provided with advice on how to assess and manage your follow up along the lines outlined above. If for any reason they or you require further advice from us in the future we will be only too pleased to see you. It is important to emphasize that your overall management by your doctor will not be affected by your decision.

What happens to the samples?

All biological samples will be processed and stored locally using a unique ID number known only to research staff at the local site.

Sub-study: Anonymised samples will be sent to the main site in Birmingham and used in their entirety for subsequent biomarker analysis.

What happens to the information?

The important information that we collect will help in the understanding of variation and progression of health in alpha$_1$ antitrypsin deficiency. The research information obtained from the samples collected will be the basis for future studies on the role of alpha$_1$ antitrypsin replacement therapy or use of newer treatments to help the disease. Neither your name or any details relating to you personally will be released to any other person outside the research programme.

Your anonymised clinical information will be entered onto the Alpha One International Registry (A.I.R). The worldwide database contains valuable information regarding the incidence and distribution of alpha$_1$ antitrypsin deficiency between countries, helping to advance knowledge and understanding of the condition. Access to the database is strictly limited and password protected.

Sub-study: CT scans performed in connection with the National Institute for Health Research Rare Diseases Translational Research Collaboration will be anonymised and sent to the main site in Birmingham for data analysis.

Who is taking part?

All subjects that we identify with alpha$_1$ antitrypsin deficiency and in some cases members of their family will be asked to take part. At present we know of over 800 such patients as yourself and it is likely that there are several thousand similar people yet to be identified.

Sub-study: The study is being conducted at up to 10 centres in the UK where leading consultants have an interest in alpha-1-antitrypsin deficiency. They include major research centres at hospitals based in Birmingham, London, Cambridge, Leicester, Manchester, Nottingham and Southampton. A total of 200 patients with either mild or severe disease will be recruited to the sub-study.

What if something goes wrong?

Since the study involves only simple tests that could form part of your routine care and is not a clinical trial, we do not expect any harm to come to you. Whatever part of the study you choose or decide not to take part in will not affect your future care. If you are harmed by taking part in this research project there are no special compensation arrangements. In between visits to the assessment centre your own doctor will be largely responsible for your care but (depending on where you live) we may collaborate with your doctor and help by seeing you if you become unwell.

What happens at the end of a study?

There is no fixed end point to the assessment programme and it will provide a unique opportunity to learn a great deal about alpha$_1$ antitrypsin deficiency. We will keep you informed of your own progress how the assessment programme is going and how our understanding is developing by means of patient meetings and newsletters.

What if I have more questions or do not understand something?

The doctors, co-ordinator and nurse involved in the alpha$_1$ antitrypsin assessment programme will happily answer your questions on any occasion when you visit. If questions arise between visits you will be able to contact the centre and either speak to somebody at that time or arrange to do so.

What happens now if I decide to take part?

If you decide to take part in the programme now we will arrange an appointment in the not too distant future for you to come to the assessment centre for the investigations outlined above. This will be arranged to suit everybody's convenience and all the assessment will be completed where possible on a single visit.

What happens if I change my mind during the study?

If you change your mind during the study, it is important that you notify the assessment centre. This will enable any investigations that have been organized or visits to be cancelled. Your decision will be passed on to both your own doctor and where appropriate your own specialist in order that they can arrange for appropriate appointments to monitor your progress. Providing you are agreeable we would like to contact your own doctor or specialist from time to time in order to find out how you are progressing. However if in the future you once again decide to join the programme, we would be only too pleased to see you.

ADAPT (Antitrypsin Deficiency Assessment and Programme for Treatment), Lung Function and Sleep Department, ADAPT Office (office 4), Queen Elizabeth Hospital, Mindelsohn Way, Edgbaston, Birmingham. B15 2WB

Professor R. A. Stockley, Director - ADAPT Project

Mrs. Rebecca Bray, Co-ordinator, Registry Office

Research Team

University Hospital Birmingham NHS
NHS Foundation Trust
Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham
B15 2WB

CONSENT FORM FOR PARTICIPATION IN RESEARCH
Assessment for Alpha$_1$ Antitrypsin Deficiency

Version and date of Patient Information Leaflet**: V 4.1, 5 February 2014**

	Please Initial	
	Yes	**No**
I have been given a full explanation of the programme and read the patient information sheet and have had all my questions answered and voluntarily agree to participate in the alpha$_1$ antitrypsin deficiency assessment programme.		
	Yes	**No**
I understand that that if I suffer from any unexpected problems that it may be important to contact both my own doctor and the research staff at the alpha-1-antitrypsin study centre.		
	Yes	**No**
I understand fully that I am free to withdraw from the programme at any time without giving a reason and that this will not adversely affect my future management.		
	Yes	**No**
I understand that my medical records will be examined by study team and possibly regulatory authorities, however my records will remain confidential		
	Yes	**No**
I agree to have my anonymised CT data and biological samples transferred to the main study site in Birmingham for subsequent analysis.		
	Yes	**No**
I agree to have my name and clinical information added to the National registry for alpha-1-antitrypsin deficiency.		
	Yes	**No**
I agree to have my anonymised clinical information added to the International registry for alpha$_1$ antitrypsin deficiency.		
	Yes	**No**
I understand that my General Practitioner and any local hospital consultants involved with my care will be informed of my participation in the assessment programme		

……..
Patient's signature **Date**

Name in BLOCK CAPITALS……

Responsible investigator: I have explained the nature and purpose of this study

……
Responsible investigator/representative signature **Date**

Name in BLOCK CAPITALS……..

University Hospital Birmingham NHS
NHS Foundation Trust
Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham
B15 2WB

Title: A study of clinical phenotypes, disease progression and epigenetics in patients with chronic obstructive pulmonary disease and its associated co morbidities.

You have been asked to participate in a clinical study for research purposes. Before you decide to take part it is important that you understand why the study is being performed, what it involves, and any possible risks and benefits for you. Take your time reading the following information and discuss it with others if you wish.

Introduction to the research & invitation to take part

You have been diagnosed as having the symptoms or signs of chronic obstructive pulmonary disease (COPD) which is a chronic disease that usually progresses slowly. At present there is no specific therapy for COPD and it is also being appreciated that having COPD means it is more likely that you will develop other diseases such as heart disease, diabetes, kidney disease, gum disease and osteoporosis (thinning of the bones). The purpose of the study is to learn as much about COPD and how it affects you and your general health so that we can develop new understanding of the disease and hence develop new treatment strategies.

This study is being carried out by the research department in the Chronic Diseases Resource Centre, which is part of University Hospitals Birmingham NHS Foundation Trust, and is led by Professor RA Stockley. The department has a special interest in understanding and treating chronic lung disease, in particular COPD. You have been contacted because you already have evidence of COPD and we believe that you are suitable to take part in the study.

In summary

- The research aims to determine your symptoms and how they affect you. This includes a full general examination, and all the routine breathing tests, blood sputum tests (if you produce some) and scans (only if you have not had one before as part of your routine assessment and if clinically indicated) that we normally undertake when assessing patients with COPD as well as tests to detect other health problems known to be associated with COPD including but not limited to heart disease, diabetes, and osteoporosis.
- If you take part, you will be seen once a year in this special clinic (which will replace one of your routine appointments if you regularly attend outpatients) asked to provide routine blood, sputum and urine samples, undergo breathing tests and scans (if you have not already had them), a limited dental examination by a dentist and a hygienist will be performed with collection of plaque and saliva samples and answer questions about yourself, your quality of life, work experience and your chest problems
- All information will be stored in such a way that you cannot be identified by anyone without Professor Stockley's permission

What is the study about?

The main purpose of the study is to learn as much as possible about your lung disease. We wish to study in detail how chest infections, smoking and various other factors influence your lung condition and how this progresses over time. Recent research suggests that COPD also has effects elsewhere in the body, chiefly but not limited to heart disease, diabetes, joint problems etc. and we wish to study this in more detail. This knowledge will help in developing newer forms of treatment. All the information obtained from you will be stored on a secure database and will be used in this study and in future research involving COPD.

What will I have to do?

In broad terms you will undergo all the routine questioning, examination and tests that we normally undertake when assessing somebody who presents with lung disease. However we hope to do this in more detail than is routinely carried out by your own doctor or specialist.

- We will ask many questions about your past and present symptoms, health and wellbeing.
- you will be examined thoroughly
    - to determine the presence of signs associated with lung disease.
    - for presence of other health problems known to be associated with COPD.
- you will be asked to perform routine lung function testing which assesses how your lungs work and their ability to take oxygen in and out of your body.
- we will ask you to provide 50mls blood (approximately 3 tablespoons) and urine samples and if you have a cough productive of sputum we will arrange for you to collect this over several hours on the day before coming to see us.
- You will undergo a limited dental examination by a dentist and a hygienist and both plaque and saliva samples will be collected.
- We may also perform a specialised CT scan of your lungs (only if it is clinically indicated and if you have not had one already as part of your routine assessment) which is a very sensitive technique of detecting lung damage that has occurred.
- We may perform a DEXA scan (only if it is clinically indicated and if you have not had one already) which is a technique to detect the extent of thinning of your bones.

We know that COPD is influenced by your genes which have made you susceptible to developing the disease. To determine the influence of genes, (if you agree), we will collect and store a sample of your blood from which we can extract your DNA. No information about the genetic data on you, can or will be released as we will not be able to link this with your name as the sample will be coded once it is processed and separated from any information that identifies it as yours. As medical science progresses new ideas and new genes related to lung disease are likely to become known and for this reason we will store the sample for such future studies. It is important to emphasise that the whole study will be confidential and specific procedures have been put in place to separate all confidential information that could help identify you from the results of genetic DNA analysis.

It is our general clinical routine to follow patients with established lung disease on a long-term basis. We would wish to see you at least once but thereafter we would also wish to review you once year in the CDRC, which will replace any routine clinic appointment, to assess your symptoms, clinical signs and repeat the comprehensive tests and lung function

tests so that we can identify any factors that cause progression of the COPD. After the third year the lung function may be repeated less frequently (2 or 3 yearly) depending on whether these are changing or are stable. After 4 years we will also re assess the presence or progression of any of the complicating conditions associated with COPD and ensure you are on the right treatment.

NOTE The routine CT and DEXA scans expose you to a small amount of radiation. The total dose will be equivalent to about 6 months (to 2 years) of background radiation in the UK. Although this dose is safe, it is important that you inform us if you are likely to be pregnant as we will not carry out the CT scan in these circumstances.

What are the benefits?
The major purpose of the study is to find out as much as possible about COPD. The investigations that we undertake will allow us to advise upon the degree of lung disease that you have and simple measures that you can undertake with your current treatment in order to try and stabilise the lung disease. In addition the breathing tests that we will perform will help us to optimise your current treatment in order to improve your breathlessness where possible. Finally we will help you and your doctor to identify and manage any associated health problems affecting other parts of your body that we detect.

What are the risks?
All the investigations that are taking place are entirely routine, used in the assessment of patients with lung disease. As such they are repeated on many occasions in the patients without any adverse effects. The only minor problem that is likely to occur is a slight degree of bruising in some patients when they have their blood taken.

What are the alternatives?
There are currently no alternatives to finding the information that is required other than the assessment programme outlined above.
There are many ways of looking at blood and sputum samples, but relating them to lung scans, breathing tests, symptoms and genes has not been done together before. It is therefore a new area of research, and there are no similar studies being done in the UK at present. Also the association of COPD with heart disease, diabetes, osteoporosis and other health care issues is a new area of research and there is a lot to be learnt from this study and how they should be managed.

What happens if I do not wish to take part?
The study is entirely voluntary and if you do not wish to participate it will not affect your current or future care. It is important to emphasize that your overall management by your doctor will not be affected if you do not wish to take part.

What happens to the information?
If you decide to take part you will need to allow access to your medical records. They may be looked at by the research team, by the hospital Research and Development department and by regulatory authorities who check that the study is being carried out properly. By signing this form you are giving permission for this to be done and all information will be kept confidential. However the results of any tests we carry out will be provided to your own doctors if it influences your health or management together with any suggestions about changing your treatment

The information collected will be stored on a secure computer, but your name will not. This is known as linked anonymised data, meaning that only Professor Stockley or a

delegated deputy will be able to link any of the information to your name. He or a delegated deputy will have sole access to a written record of your information, stored in a secure facility at University Hospitals Birmingham. All the data collected, samples you provide, and their results, including any information about your genes, will be coded with a number. The results of tests on your samples and about your genes will not be available to anyone outside of the research team and our collaborators. The link to your name will be destroyed 15 years after the study ends according to national guidelines.

Once the data is collected it will be the property of the research department. The data will be used for future research into COPD. Research undertaken will involve observational assessment, quantification of impact on health care and studies of factors that affect the outcome in COPD.

Future Research

If you agree, your hospital number will be held on a registry of COPD patients that are involved in research. Only the research team involved in this study will have access to this registry and it will be held on a secure NHS computer. If you agree, we will contact you about other research projects or studies that we think you might be suitable for. You can choose not to have your name on this registry. If you agree to be on the registry, you do not have to take part in any studies we tell you about, that would be your choice. You can also choose to have your name removed from the registry at any time, and without giving a reason, by telephoning or in writing to the address given at the end of this information sheet.

Who will have access to the data?

Members of the research team led by Prof Stockley will have full access to the database. Access will be built on a role based model, with registered users having graded levels of access to the data. They will have unique user names and passwords and there will be a record of anyone logging in to the database ensuring we have a record of users accessing the system.

The results of the study may be published in a medical journal, but your identity will not be revealed. The results may be used in statistical tests, in the development of new treatments and diagnostic tests.

Who is taking part?

About 1000 other patients with COPD will be asked to take part at the University Hospital Birmingham and Heart of England hospitals.

What if something goes wrong?

Since the study is not a clinical trial and involves only simple tests that could form part of your routine care, we do not expect any harm to come to you. Whatever part of the study you choose or decide not to take part in will not affect your future routine care.

What happens at the end of a study?

Throughout the study, and when it ends, your hospital doctor and general practitioner will continue to treat your chest problems and be kept informed of the results of our tests, so they will not need to repeat them unnecessarily.

What if I have more questions or do not understand something?
The doctors and nurses involved in the research study will happily answer your questions on any occasion when you visit. If questions arise between visits you will be able to contact the centre and either speak to somebody at that time or arrange to do so if for any reason if it is inconvenient.

What happens now if I decide to take part?
If you decide to take part in the programme now we will arrange an appointment in the not too distant future for you to come to the assessment centre for the investigations outlined above. This will be arranged to suit everybody's convenience and all the assessment will be completed where possible on a single visit.

Will my General Practitioner (GP) be informed?
If you give your permission, your GP and usual hospital doctor will be informed of your participation in the study.

What happens if I change my mind during the study?
You are free to withdraw your participation at any time, and it will not affect your future care. If you withdraw your consent after your samples have been analysed it will be the responsibility of the research team to ensure that the samples are destroyed if you so wish.

Who can I contact about the study?
In the first instance any concerns or questions should be addressed to either your GP or hospital doctor. If you have further concerns you can contact:
Professor R. A. Stockley
Dr Simon Gompertz
Dr Elizabeth Sapey
Dr Alice Turner
Dr Adam Usher
Dr Anita Pye

Address for correspondence:
Dr Anita Pye
Office 4, Lung Function & Sleep Dept
Queen Elizabeth Hospital Birmingham
Mindelsohn way
Edgbaston
Birmingham, B15 2WB

Thank you for taking time to read this information leaflet

University Hospital Birmingham NHS
NHS Foundation Trust
Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham
B15 2WB

CONSENT FORM

Title: A study of clinical phenotypes, disease progression and epigenetics in patients with chronic obstructive pulmonary disease and its associated co-morbidities.

I……(Name in BLOCK CAPITALS)

	Initials
Have read the attached information concerning my participation in this study and have had the opportunity to discuss it and ask questions. All my questions have been answered in a satisfactory way.	☐
I voluntarily consent to take part in this study.	☐
I know that at any time, and without giving a reason, withdraw my participation in the study and that my future care and management will not be affected.	☐
I understand that I will have a copy of this Patient Information Leaflet and Written Consent to keep.	☐
I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
I hereby give permission for samples taken as part of this study to be stored for future use by the research team.	☐
I understand that data collected during the study will be stored on the database for use in future studies.	☐
I hereby give permission for my GP and hospital consultant to be informed about my participation in this research study.	☐
I hereby give my permission for my hospital number to be held on a COPD register held on a secure NHS computer and for the research team to contact me about future research projects	Yes ☐ No ☐

……..
Patient's signature Date

Name in BLOCK CAPITALS …….

Responsible investigator
I have explained the nature and purpose of this study for the person named above

……
Responsible investigator/representative signature Date

Name in BLOCK CAPITALS …….

Patient information leaflet and Written consent form

Patient information leaflet

Study code: RRK3404

Title: A study of clinical phenotypes, disease progression and genetic susceptibility in patients with chronic obstructive pulmonary disease

You have been asked to participate in a clinical study for research purposes. Before you decide to take part it is important that you understand why the study is being performed, what it involves, and the possible risks and benefits for you. Take your time reading the following information and discuss it with others if you wish.

Introduction to the research & invitation to take part

This study is being carried out by the research department in the Lung Investigation Unit at the Queen Elizabeth Hospital, which is part of University Hospitals Birmingham NHS Foundation Trust, and is led by Professor RA Stockley. Several hospitals in the West Midlands are working closely with Professor Stockley in order to help with the study. The department has a special interest in understanding and treating chronic lung disease, in particular chronic obstructive pulmonary disease, or COPD for short. You have been chosen because your hospital doctor has identified you as suffering from COPD and believes that you are suitable to take part in the study.

What is the research about?

The main purpose of the study is to find patterns of symptoms, breathing tests, blood and sputum tests that identify particular groups of patients with COPD. We also hope to establish the importance of environmental influences, such as chest infections, in the development of COPD and how it progresses over time. Other research suggests there may be genetic influences in COPD, which may affect particular groups of patients, or affect how their breathing tests change over time. We plan to study this in more detail to find out which genes are important in the types of COPD and its progression.

What will I have to do?

<u>Please read this section carefully as it details the procedures that are specific to research and not part of usual clinical care</u>

If you agree to take part you will be seen by a research doctor at the time of your usual out-patient clinic appointment. They will explain more about the study and what it would mean for you. The study will take place over 5 years, and will involve seeing a research doctor after one of your out-patient appointments each year for at least 3 years. There will be no extra visits to the hospital other than

those for your usual out-patient appointments. If you would rather participate for only one visit, rather than several, please inform the research doctor.

If you have brought a sample of sputum with you, you will be asked if some could be used for the research. If you have not brought any sputum with you, and usually cough it up every day, you will be given a container in which you may bring a sample on your next visit.

A blood sample will be collected from you. This can be done at the same time as any other blood tests your hospital doctor has asked for. We will collect 4 small tubes of blood for the study, which is the equivalent of about 2 tablespoons.

We will process the blood and sputum samples so that they can be stored safely in secure freezers at the University of Birmingham. For this study they will be used to examine markers relevant to COPD. They may also be valuable for future research in other areas, but will not be used for other studies without prior ethical approval.

We will also extract your DNA from your blood, which will be stored in the same way. Your DNA will be used to look for genes that may influence the development of COPD. Your DNA will not be used for other studies.

As well as collecting these samples we will also collect information about you by talking to you and by looking at your medical notes. This will include your date of birth, gender, height, weight, date you were diagnosed with COPD and whether you smoke now, or have done so in the past. We will also ask some questions about symptoms that people with COPD often experience, and ask you to fill in a questionnaire about the way COPD affects you. We will also look in your medical notes to see what medicines you take, the results of any breathing tests you have had done recently, and scans (if you have had one). All of this information will be recorded in written form only.

All of the above procedures should take no more than half an hour on the first occasion, and be a little shorter on later visits. This will be in addition to seeing your usual hospital doctor.

What are the benefits?
You will have no personal benefit from the study. Your participation and donation of samples may benefit patients with COPD in the future by helping us to understand the disease better, and helping us to develop new treatments.

What are the risks?
We do not expect any harm to come to you as a result of providing samples or talking to the researchers. Sometimes blood tests can be uncomfortable, or leave bruising, but this will be temporary.

What are the alternatives?
There are many ways of looking at blood and sputum samples, but relating them to breathing tests, symptoms and genes has not been done together before. It is therefore a new area of research, and there are no similar studies being done in the UK at present.

What if I do not want to take part?
The study is entirely voluntary and if you do not wish to participate it will not affect your future care.

What happens to the information?
If you decide to take part you will need to allow access to your medical records. They may be looked at by the research team, by the hospital research and development department and by regulatory authorities who check that the study is being carried out properly. By signing this form you are giving permission for this to be done.

The information collected will be stored on a secure computer, but your name will not. This is known as linked anonymised data, meaning that only Professor Stockley will be able to link any of your information to your name. He will have sole access to a written record of your information, stored in a secure facility at University Hospitals Birmingham. All the data collected, samples you provide, and their results, including any information about your genes, will be coded with a number. The results of tests on your samples and about your genes will not be available to anyone outside of the research team and our collaborators. The link to your name will be destroyed after 15 years. Once the data is collected it will be the property of the research department.

The results of the study may be published in a medical journal, but your identity will not be revealed. The results may be used in statistical tests, research and development of new treatments, diagnostic tests and medical aids.

Who else is taking part?
About 500 other patients with COPD will be asked to take part.

What if something goes wrong?
Since the study involves only simple tests that could form part of your routine care, we do not expect any harm to come to you. Whatever part of the study you choose or decide not to take part in will not affect your future care. If you are harmed by taking part in this research project there are no special compensation arrangements.

What happens at the end of the study?
Throughout the study, and when it ends, your hospital doctor and general practitioner will continue to treat your chest problems.

What happens if I have more questions?
If you do not understand something in this leaflet, or have further questions you may ask the researcher now, or your hospital doctor.

What happens now if I decide to take part?
If you decide to take part you will be asked to read, sign and date the Written Consent Form attached to this sheet. By signing it you acknowledge that you have understood the aims of the research, and what you are being asked to do.

In summary

- The research aims to find out patterns of symptoms, breathing tests, blood and sputum tests and scans in patients with COPD
- If you take part you will be asked to provide blood and sputum samples, and answer questions about yourself and your chest problems at one out-patient appointment per year for 3 years, or as many as you can manage
- All information will be stored in such a way that you cannot be identified by anyone without Professor Stockley's permission

Will my General Practitioner (GP) be informed?
If you give your permission, your GP will be told about your participation in the study.

What happens if I change my mind during the study?
You are free to withdraw your participation at any time, and it will not affect your future care. If you withdraw your consent after your samples have been analysed it will be the responsibility of your hospital doctor to ensure that the samples are destroyed if you so wish.

Who can I contact about the study?
In the first instance any concerns or questions should be addressed to either your GP or hospital doctor. If you have further concerns you can contact

Professor Robert Stockley

Thank-you for reading this information leaflet

Written consent

Study code:

Title: A study of clinical phenotypes, disease progression and genetic susceptibility in patients with chronic obstructive pulmonary disease

Enrolment number:

I..... (Name in BLOCK CAPITALS)

	Initials
Have read the attached information concerning my participation in this study and have had the opportunity to discuss it and ask questions. All my questions have been answered in a satisfactory way.	☐
I voluntarily consent to take part in this study.	☐
I know that at any time, and without giving a reason, withdraw my participation in the study and that my future care and management will not be affected.	☐
I understand that I will have a copy of this Patient Information Leaflet and Written Consent to keep.	☐
I hereby give my permission to authorise personnel from the research team to examine my medical records.	☐
I hereby give permission for samples taken as part of this study to be stored for future use by the research team.	☐
I understand that samples taken as part of this study will not be used in other studies without prior ethical approval.	☐
I hereby give permission for my GP and hospital consultant to be informed about my participation in this research study.	☐

....
Patient's signature Date

.....
Name in BLOCK CAPITALS

Responsible investigator

I have explained the nature and purpose of this study for the person named above

....
Responsible investigator/representative signature Date

.....
Name in BLOCK CAPITALS

University Hospital Birmingham NHS
NHS Foundation Trust

Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham
B15 2WB

Participant Information Sheet and Written Informed Consent Form

Subject initials: ……. **Enrolment No:** ……..

Title: An open study to investigate the measurement and variability of inflammatory cells, mediators and erythrocyte abnormalities in spontaneously produced sputum, blood and urine from patients with COPD who present with acute exacerbations.

You are being asked to take part in a simple research study. Before you decide to participate it is of importance that you understand why the study is performed, what it involves and possible benefits, risks and discomfort. Take your time to read the following information and discuss it with your hospital doctor, if you wish.

Introduction to the research and invitation to take part

This study is being carried out by the research department in the Lung Investigation Unit at the Queen Elizabeth Hospital, which is part of University Hospitals Birmingham NHS Foundation Trust and is led by Professor RA Stockley.

You have been chosen because you have been identified as suffering from COPD and periodically you experience an increase in your symptoms of cough, sputum and breathlessness (an exacerbation).

What is the research study about?

It is believed that patients with COPD have high levels of lung damage and higher levels of certain body cells and substances that cause the lung damage compared with people without COPD. These cells and substances can be detected in the sputum and blood of patients with COPD. However less is known about how these cells and substances change over time when the disease is stable or during an increase in your symptoms of cough, sputum production and breathlessness (called an exacerbation). It is also unclear how these cells and substances relate to your symptoms of cough, sputum production and breathlessness.

This is an exploratory research study to investigate how the cells and substances change in sputum, blood and urine over an 8 week period in patients who present with an exacerbation of COPD.

What will I have to do?

If you are not experiencing an exacerbation now, we will ask you to complete a symptom diary, which asks about your daily symptoms. If your symptoms deteriorate for 2 or more consecutive days, you should contact us and you will be seen by a member of the research team. At this time, we will take a blood sample and collect your sputum and urine. If any treatment is required, this will be prescribed by your usual health care worker, although we may provide advice about the most appropriate treatment.

If you have just been admitted to hospital with an exacerbation, we will ask about your symptoms, and take a blood sample from you today, with a sample of urine and a sample of your sputum, if possible.

Follow up visits will occur at day 7, 14 and 56, where blood, sputum and urine samples will be collected again.

Who analyses the samples?

The samples will only be analysed by the Queen Elizabeth Hospital, Birmingham. Test results will be kept confidential in keeping with recent regulations.

What are the benefits?

You will have no personal benefit from the study, other than advice. The participation and donation of samples may provide benefit to future patients with COPD and related diseases by helping doctors to understand the diseases better and by increasing the possibility of developing new treatments.

What are the risks?

Nothing experimental will be done beyond the collection of samples, which is routine. The risks from giving a sample of blood are a slight discomfort and the possibility of a bruise developing where the blood was taken.

What are the alternatives?

The measurement of specific substances in sputum and blood samples to investigate mechanisms in lung diseases is a new area of research. There are no other alternatives to this project which is unique to our department.

As smoking can worsen your condition, stopping smoking is one of the most important things you can do to help your symptoms. If you are currently smoking your doctor will talk to you about the risks of continuing to smoke and the benefits of stopping.

What if I do not want to take part?

The study is entirely voluntary and if you do not wish to participate it will not affect your present or future treatment and management.

What happens to the information?

If you consent to taking part in the study you need to allow us access to your medical records. These may be inspected by the University Hospital Birmingham, the trust that is sponsoring the study and may be inspected by regulatory authorities and/or the Independent Ethics Committee to check that the study is being carried out correctly. By signing the Written Informed Consent Form you are giving permission for this to be done.

With your permission, we will contact both your General Practitioner and Hospital Consultant and inform them that you are taking part in the study, and provide them with any results (such as blood or sputum measurements) which may be helpful for your medical management.

The information collected will be stored on a computer but your name will not. All the data collected and samples that you provide will be coded with a number, with only your doctor having the link for this to your name. The link will be destroyed after 15 years.

The results from the study may be published in a medical journal, but your identity will not be revealed.

These data will be used:

- For statistical analysis.
- Understanding the nature of exacerbations.

Who else is taking part?

Approximately 250 people will be asked to take part in the study.

What if something goes wrong?

This is not a study with a new drug and all tests and collections have been used before in routine clinical management of patients with COPD and do not cause any ill health.

We do not expect any harm to come to you and there are no special compensation arrangements.

What happens at the end of the research study?

When you have finished taking part in this study, your doctors will continue to manage your COPD in the usual way.

What if I have more questions or do not understand something?

If you do not understand any of the information in this leaflet or have any other questions please feel free to ask your hospital Doctor at any time.

What happens now if I decide to take part?

If you decide to take part you will be asked to read and then sign and date the Written Informed Consent Form attached to this information leaflet. By signing the consent form you acknowledge that you have understood the requirements for participating in the study.

What happens if I change my mind during the research study?

You are free to withdraw your participation in this study at any time. If you withdraw your future treatment and your level of care will not be affected. If you change your mind about participation the link between your name and number also enables samples that have not been analysed to be traced and destroyed. If you withdraw your consent after the samples have been sent for analysis it will be the responsibility of the research team to ensure that the samples are destroyed.

Contact name and number:

In the first instance if you have any concerns or questions you should contact:

Professor Stockley:

Di Griffiths

If you have any other concerns about the conduct of this study you can contact:

Dr Christopher Counsell, Research and Development Manager

Birmingham Clinical Research Office
Room 17, Education Centre, 1st Floor
Queen Elizabeth Hospital Birmingham
Birmingham, B15 2WB

Or
Patient Advice and Liaison Services
Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham, B15 2WB

Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham
B15 2WB

WRITTEN CONSENT

Subject initials: …… **Enrolment No:** ……..

Title: An open study to investigate the measurement and variability of inflammatory cells, mediators and erythrocyte abnormalities in spontaneously produced sputum, blood and urine from patients with COPD who present with acute exacerbations.

I, ……
(Subject name, block letters) Initials

Have read the attached information concerning my participation in this study and have had the possibility to discuss it and put questions. ☐

All my questions have been answered in a satisfactory way and I give my consent voluntarily to participate in this study. ☐

I know that I can, at any time and without giving a reason, withdraw my participation in the study and that my future care and management will not be affected. ☐

I understand that I shall keep a copy of this Subject Information & Written Informed Consent. ☐

I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS trust where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

I agree that my GP and other medically qualified people who may assist in my care during the study may be informed of my participation in the study. ☐

……
Subject's signature Date

……
Name, block letters

RESPONSIBLE INVESTIGATOR
I have explained the nature and purpose of the study for the person mentioned above.

……
Responsible investigator/representative signature Date

……
Name, block letters

Heartlands Hospital
Bordesley Green
Birmingham
B9 5SS

Participant Information Sheet and Written Informed Consent Form

Subject initials: ……. **Enrolment No:** ……..

Title: An open study to investigate the measurement and variability of inflammatory cells, mediators and erythrocyte abnormalities in spontaneously produced sputum, blood and urine from patients with COPD who present with acute exacerbations.

You are being asked to take part in a simple research study. Before you decide to participate it is of importance that you understand why the study is performed, what it involves and possible benefits, risks and discomfort. Take your time to read the following information and discuss it with your hospital doctor, if you wish.

Introduction to the research and invitation to take part

This study is being carried out by the research department in the Lung Investigation Unit at the Queen Elizabeth Hospital, which is part of University Hospitals Birmingham NHS Foundation Trust and is led by Professor RA Stockley.

You have been chosen because you have been identified as suffering from COPD and periodically you experience an increase in your symptoms of cough, sputum and breathlessness (an exacerbation).

What is the research study about?

It is believed that patients with COPD have high levels of lung damage and higher levels of certain body cells and substances that cause the lung damage compared with people without COPD. These cells and substances can be detected in the sputum and blood of patients with COPD. However less is known about how these cells and substances change over time when the disease is stable or during an increase in your symptoms of cough, sputum production and breathlessness (called an exacerbation). It is also unclear how these cells and substances relate to your symptoms of cough, sputum production and breathlessness.

This is an exploratory research study to investigate how the cells and substances change in sputum, blood and urine over an 8 week period in patients who present with an exacerbation of COPD.

What will I have to do?

If you are not experiencing an exacerbation now, we will ask you to complete a symptom diary, which asks about your daily symptoms. If your symptoms deteriorate for 2 or more consecutive days, you should contact us and you will be seen by a member of the research team. At this time, we will take a blood sample and collect your sputum and urine. If any treatment is required, this will be prescribed by your usual health care worker, although we may provide advice about the most appropriate treatment.

If you have just been admitted to hospital with an exacerbation, we will ask about your symptoms, and take a blood sample from you today, with a sample of urine and a sample of your sputum, if possible.

Follow up visits will occur at day 7, 14 and 56, where blood, sputum and urine samples will be collected again.

Who analyses the samples?

The samples will only be analysed by the Queen Elizabeth Hospital, Birmingham. Test results will be kept confidential in keeping with recent regulations.

What are the benefits?

You will have no personal benefit from the study, other than advice. The participation and donation of samples may provide benefit to future patients with COPD and related diseases by helping doctors to understand the diseases better and by increasing the possibility of developing new treatments.

What are the risks?

Nothing experimental will be done beyond the collection of samples, which is routine. The risks from giving a sample of blood are a slight discomfort and the possibility of a bruise developing where the blood was taken.

What are the alternatives?

The measurement of specific substances in sputum and blood samples to investigate mechanisms in lung diseases is a new area of research. There are no other alternatives to this project which is unique to our department.

As smoking can worsen your condition, stopping smoking is one of the most important things you can do to help your symptoms. If you are currently smoking your doctor will talk to you about the risks of continuing to smoke and the benefits of stopping.

What if I do not want to take part?

The study is entirely voluntary and if you do not wish to participate it will not affect your present or future treatment and management.

What happens to the information?

If you consent to taking part in the study you need to allow us access to your medical records. These may be inspected by the University Hospital Birmingham, the trust that is sponsoring the study and may be inspected by regulatory authorities and/or the Independent Ethics Committee to check that the study is being carried out correctly. By signing the Written Informed Consent Form you are giving permission for this to be done.

With your permission, we will contact both your General Practitioner and Hospital Consultant and inform them that you are taking part in the study, and provide them with any results (such as blood or sputum measurements) which may be helpful for your medical management.

The information collected will be stored on a computer but your name will not. All the data collected and samples that you provide will be coded with a number, with only your doctor having the link for this to your name. The link will be destroyed after 15 years.

The results from the study may be published in a medical journal, but your identity will not be revealed.

These data will be used:

- For statistical analysis.
- Understanding the nature of exacerbations.

Who else is taking part?

Approximately 250 people will be asked to take part in the study.

What if something goes wrong?

This is not a study with a new drug and all tests and collections have been used before in routine clinical management of patients with COPD and do not cause any ill health.

We do not expect any harm to come to you and there are no special compensation arrangements.

What happens at the end of the research study?

When you have finished taking part in this study, your doctors will continue to manage your COPD in the usual way.

What if I have more questions or do not understand something?

If you do not understand any of the information in this leaflet or have any other questions please feel free to ask your hospital Doctor at any time.

What happens now if I decide to take part?

If you decide to take part you will be asked to read and then sign and date the Written Informed Consent Form attached to this information leaflet. By signing the consent form you acknowledge that you have understood the requirements for participating in the study.

What happens if I change my mind during the research study?

You are free to withdraw your participation in this study at any time. If you withdraw your future treatment and your level of care will not be affected. If you change your mind about participation the link between your name and number also enables samples that have not been analysed to be traced and destroyed. If you withdraw your consent after the samples have been sent for analysis it will be the responsibility of the research team to ensure that the samples are destroyed.

Contact name and number:

Dr Alice Turner: Telephone

If you have any other concerns about the conduct of this study you can contact:

Dr Christopher Counsell, Research and Development Manager

Birmingham Clinical Research Office
Room 17, Education Centre, 1st Floor
Queen Elizabeth Hospital Birmingham
Birmingham, B15 2WB

Or
Patient Advice and Liaison Services
Queen Elizabeth Hospital Birmingham
Edgbaston
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Heartlands Hospital

Bordesley Green

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B9 5SS

WRITTEN CONSENT

Subject initials: …….. **Enrolment No:** ……..

Title: An open study to investigate the measurement and variability of inflammatory cells, mediators and erythrocyte abnormalities in spontaneously produced sputum, blood and urine from patients with COPD who present with acute exacerbations.

I, ...

(Subject name, block letters) Initials

Have read the attached information concerning my participation in this study and have had the possibility to discuss it and put questions. ☐

All my questions have been answered in a satisfactory way and I give my consent voluntarily to participate in this study. ☐

I know that I can, at any time and without giving a reason, withdraw my participation in the study and that my future care and management will not be affected. ☐

I understand that I shall keep a copy of this Subject Information & Written Informed Consent. ☐

I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS trust where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

I agree that my GP and other medically qualified people who may assist in my care during the study may be informed of my participation in the study. ☐

.....

Subject's signature Date

.....

Name, block letters

RESPONSIBLE INVESTIGATOR

I have explained the nature and purpose of the study for the person mentioned above.

.....

Responsible investigator/representative signature Date

.....

Name, block letters

University Hospital Birmingham NHS
NHS Foundation Trust

Queen Elizabeth Hospital Birmingham
Edgbaston
Birmingham
B15 2WB

Patient information leaflet and Written consent form

Patient information leaflet

Study code: RRK3404

Title: A study of clinical phenotypes, disease progression and genetic susceptibility in patients with chronic obstructive pulmonary disease, cystic fibrosis bronchiectasis and non-CF bronchiectasis

You have been asked to participate in a clinical study for research purposes. Before you decide to take part it is important that you understand why the study is being performed, what it involves, and the possible risks and benefits for you. Take your time reading the following information and discuss it with others if you wish.

Introduction to the research & invitation to take part

This study is being carried out by the research department in the Lung Function & Sleep Department at the Queen Elizabeth Hospital, which is part of University Hospitals Birmingham NHS Foundation Trust, and is led by Professor RA Stockley. Several hospitals in the West Midlands are working closely with Professor Stockley in order to help with the study. The department has a special interest in understanding and treating chronic lung disease, in particular chronic obstructive pulmonary disease, or COPD for short, cystic fibrosis bronchiectasis and non-CF bronchiectasis. You have been chosen because your hospital doctor has identified you as suffering from one or more of these conditions and believes that you are suitable to take part in the study.

What is the research about?

The main purpose of the study is to find patterns of symptoms, breathing tests, blood and sputum tests that identify particular groups of patients with COPD, cystic fibrosis bronchiectasis and non-CF bronchiectasis. We also hope to establish the importance of environmental influences, such as chest infections, in the development of COPD, cystic fibrosis bronchiectasis and non-CF bronchiectasis and how it progresses over time. Other research suggests there may be genetic influences in COPD, cystic fibrosis bronchiectasis and non-CF bronchiectasis, which may affect particular groups of patients, or affect how their breathing tests change over time. We plan to study this in more detail to find out which genes are important in these conditions and their progression.

What will I have to do?

<u>Please read this section carefully as it details the procedures that are specific to research and not part of usual clinical care</u>

If you agree to take part you will be seen by a member of the research team at the time of your usual out-patient clinic appointment. They will explain more about the study and what it would mean for you. The study will take place over 5 years,

and will involve seeing a member of the research team after one of your out-patient appointments each year for at least 3 years. There will be no extra visits to the hospital other than those for your usual out-patient appointments. If you would rather participate for only one visit, rather than several, please inform the research team.

If you have brought a sample of sputum with you, you will be asked if some could be used for the research. If you have not brought any sputum with you, and usually cough it up every day, you will be given a container in which you may bring a sample on your next visit.

A blood sample will be collected from you. This can be done at the same time as any other blood tests your hospital doctor has asked for. We will collect 4 small tubes of blood for the study, which is the equivalent of about 2 tablespoons.

We will process the blood and sputum samples so that they can be stored safely in secure freezers at the University of Birmingham. For this study they will be used to examine markers relevant to COPD, cystic fibrosis bronchiectasis and non-CF bronchiectasis. They may also be valuable for future research in other areas, but will not be used for other studies without prior ethical approval.

We will also extract your DNA from your blood, which will be stored in the same way. Your DNA will be used to look for genes that may influence the development of COPD, cystic fibrosis bronchiectasis and non-CF bronchiectasis. Your DNA will not be used for other studies.

As well as collecting these samples we will also collect information about you by talking to you and by looking at your medical notes. This will include your date of birth, gender, height, weight, date you were diagnosed and whether you smoke now, or have done so in the past. We will also ask some questions about symptoms that people with lung disease often experience, and ask you to fill in a questionnaire about the way your illness affects you. We will also look in your medical notes to see what medicines you take, the results of any breathing tests you have had done recently, and scans (if you have had one). All of this information will be recorded in written form only.

All of the above procedures should take no more than half an hour on the first occasion, and be a little shorter on later visits. This will be in addition to seeing your usual hospital doctor.

What are the benefits?

You will have no personal benefit from the study. Your participation and donation of samples may benefit patients with COPD, cystic fibrosis

bronchiectasis and non-CF bronchiectasis in the future by helping us to understand the disease better, and helping us to develop new treatments.

What are the risks?

We do not expect any harm to come to you as a result of providing samples or talking to the researchers. Sometimes blood tests can be uncomfortable, or leave bruising, but this will be temporary.

What are the alternatives?

There are many ways of looking at blood and sputum samples, but relating them to breathing tests, symptoms and genes has not been done together before. It is therefore a new area of research, and there are no similar studies being done in the UK at present.

What if I do not want to take part?

The study is entirely voluntary and if you do not wish to participate it will not affect your future care.

What happens to the information?

If you decide to take part you will need to allow access to your medical records. They may be looked at by the research team, by the hospital research and development department and by regulatory authorities who check that the study is being carried out properly. By signing this form you are giving permission for this to be done.

The information collected will be stored on a secure computer, but your name will not. This is known as linked anonymised data, meaning that only Professor Stockley will be able to link any of your information to your name. He will have sole access to a written record of your information, stored in a secure facility at University Hospitals Birmingham. All the data collected, samples you provide, and their results, including any information about your genes, will be coded with a number. The results of tests on your samples and about your genes will not be available to anyone outside of the research team and our collaborators. The link to your name will be destroyed after 15 years. Once the data is collected it will be the property of the research department.

The results of the study may be published in a medical journal, but your identity will not be revealed. The results may be used in statistical tests, research and development of new treatments, diagnostic tests and medical aids.

Who else is taking part?

About 500 other patients with COPD, cystic fibrosis bronchiectasis and non-CF bronchiectasis will be asked to take part.

What if something goes wrong?

Since the study involves only simple tests that could form part of your routine care, we do not expect any harm to come to you. Whatever part of the study you choose or decide not to take part in will not affect your future care. If you are harmed by taking part in this research project there are no special compensation arrangements.

What happens at the end of the study?

Throughout the study, and when it ends, your hospital doctor and general practitioner will continue to treat your chest problems.

What happens if I have more questions?

If you do not understand something in this leaflet, or have further questions you may ask the researcher now, or your hospital doctor.

What happens now if I decide to take part?

If you decide to take part you will be asked to read, sign and date the Written Consent Form attached to this sheet. By signing it you acknowledge that you have understood the aims of the research, and what you are being asked to do.

In summary

- The research aims to find out patterns of symptoms, breathing tests, blood and sputum tests and scans in patients with COPD, cystic fibrosis bronchiectasis and non-CF bronchiectasis
- If you take part you will be asked to provide blood and sputum samples, and answer questions about yourself and your chest problems at one out-patient appointment per year for 3 years, or as many as you can manage
- All information will be stored in such a way that you cannot be identified by anyone without Professor Stockley's permission

Will my General Practitioner (GP) be informed?

If you give your permission, your GP will be told about your participation in the study.

What happens if I change my mind during the study?

You are free to withdraw your participation at any time, and it will not affect your future care. If you withdraw your consent after your samples have been analysed it will be the responsibility of your hospital doctor to ensure that the samples are destroyed if you so wish.

Who can I contact about the study?

In the first instance any concerns or questions should be addressed to either your GP or hospital doctor. If you have further concerns you can contact

Professor Robert Stockley

Thank-you for reading this information leaflet

University Hospital Birmingham NHS
NHS Foundation Trust
Queen Elizabeth Hospital Birmingham, B15 2WB

Study code: RRK3404 **Enrolment No:**

Title: A study of clinical phenotypes, disease progression and genetic susceptibility in patients with chronic obstructive pulmonary disease, cystic fibrosis bronchiectasis and non-CF bronchiectasis

I..... (Name in BLOCK CAPITALS)

	Initials
Have read the attached information concerning my participation in this study and have had the opportunity to discuss it and ask questions. All my questions have been answered in a satisfactory way.	☐
I voluntarily consent to take part in this study.	☐
I know that at any time, and without giving a reason, withdraw my participation in the study and that my future care and management will not be affected.	☐
I understand that I will have a copy of this Patient Information Leaflet and Written Consent to keep.	☐
I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
I hereby give permission for samples taken as part of this study to be stored for future use by the research team.	☐
I understand that samples taken as part of this study will not be used in other studies without prior ethical approval.	☐
I hereby give permission for my GP and hospital consultant to be informed about my participation in this research study.	☐

....
Patient's signature Date

.....
Name in BLOCK CAPITALS

Responsible investigator

I have explained the nature and purpose of this study for the person named above

....
Responsible investigator/representative signature Date

.....
Name in BLOCK CAPITALS

1.2 Severe A1ATD CT scanning protocol

All subjects will be scanned within 4 hours after administration of a short-acting bronchodilator by spiral multi-slice CT of the chest in the supine position. Following 3 deep breaths the patient will be asked to take another deep breath and to hold it for the duration of the scan (full inspiration for approximately 10 seconds). This procedure can be repeated if applicable. No contrast medium will be injected.

The scan will be taken from the diaphragm in the direction of the neck, to minimize inhalation artefacts in the images.

Preferred scanning parameters will be 140 kVp, 40 mA, and 10 second scanning time, pitch 1.5 (4 times 5mm collimation), with reconstructed slice thickness of 5mm and 1mm. Field of view 500mm.

Image reconstruction algorithms will be performed according to a standardized protocol of each CT scanner, using a smooth filter (defined by the type of the CT scanner).

1.3 Potentially pathogenic organism (PPM) classification (205).

PPMs	Non PPMS
Haemophilus influenza	Streptococcus viridans
Haemophilus parainfluenzae	Neisseria species
Streptococcus pneumoniae	Candida species
Moraxella Catarrhalis	Corynebacterium species
Pseudomonas	Enterococcus species
Staphylococcus aureus	Coagulase negative staphylococcus aureus
Enterobactacteriaceae • Escherichia coli • Proteus mirabilis • Klebsiella pneumonia • Serratia marcescens • Enterobacter cloacae	

1.4 Lung function parameters recorded

Lung function parameter	Unit	Abbreviation
Forced expiratory volume in 1 second	L	FEV_1
Forced expiratory volume in 1 second % predicted	%	FEV_1
Forced vital capacity	L	FVC
Forced vital capacity % predicted	%	FVC
Forced expiratory volume in 1 second/ forced vital capacity ratio		FEV_1/FVC
Total lung capacity	L	TLC
Residual Volume	L	RV
Transfer factor of lung for carbon monoxide (or 'gas transfer')	mmol/min/kPa	TLCO
Gas transfer corrected for volume	mmol/min/kPa/L	KCO

1.5 Lung function recommended normal regression equations

Variable	Men	Women
FEV_1	= 4.30H - 0.029A - 2.49	= 3.95H - 0.025A - 2.60
FVC	= 5.76H - 0.026A - 4.34	= 4.43H - 0.026A - 2.89
TLC	= 7.99H - 7.08	= 6.60H - 5.79
RV	= 1.31H - 0.022A - 1.23	= 1.81H + 0.16A - 2.00
TLCO	= 11.11H - 0.066A - 6.03	= 8.18H - 0.049A - 2.74
KCO	= - 0.013A + 2.2	= - 0.007A + 2.07

H = Height (in metres), A = Age (years). FEV1: Forced expiratory volume in 1 second, Forced vital capacity, TLC: Total lung capacity, RV: Residual volume, TLCO: Transfer factor of the lung for carbon monoxide, KCO: gas transfer corrected for volume.

1.6 The FACED bronchiectasis severity score (209)

Maximum score = 7. FEV_1 = Forced Expiratory Volume in 1 second. mMRC= Modified Medical Research Council score

FACED	**Points**
FEV_1 % predicted	
≥ 50%	0
< 50%	2
Age	
<70	0
≥70	2
Chronic colonisation with pseudomonas	
No	0
Yes	1
Extent of bronchiectasis (number of lobes)	
1-2	0
>2	1
Dyspnoea (mMRC score)	
0 - 2	0
3 - 4	1

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