

# **Biomarkers of Disease Activity in COPD and Emphysema**

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# ABSTRACT

## Background

The flaws of current methods of assessing disease *severity* in patients with COPD and emphysema are increasingly recognised, and new methods of assessing disease *activity* are urgently required. Although many potential biomarkers have been suggested to fulfil this role, few have been effectively validated, and furthermore any biomarker should be based on our current understanding of the pathophysiology the disease process. This is poorly understood, however it is apparent that neutrophil proteases (particularly neutrophil elastase (NE) and proteinase 3 (Pr3)) may represent a final common pathway leading to tissue destruction. The current thesis describes the development and validation of a new marker of NE activity ( $\text{A}\alpha\text{-Val}^{360}$ ), and the identification of a marker of Pr3 activity, as potential biomarkers of COPD and emphysema disease activity.

## Methods

Following *in vitro* validation, the performance of  $\text{A}\alpha\text{-Val}^{360}$  was assessed in a series of patient populations. Mass spectrometry was used to identify a specific marker of Pr3 activity.

## Results and Conclusion

$\text{A}\alpha\text{-Val}^{360}$  demonstrated acceptable *in vitro* and *in vivo* variability; related to physiological, radiological and patient reported outcomes in subjects with (or at risk of developing) COPD and emphysema (both with and without A1AT deficiency); increased during acute exacerbations; decreased in response to treatment; and partly related to disease progression in some populations. Also, a Pr3 specific cleavage product was identified which could be used to develop a new specific assay of Pr3 activity. These potential biomarkers of disease activity may be important in the assessment of patients with COPD and emphysema (or who are at risk of developing these conditions), particularly in early phase clinical trials.

# DEDICATION

To my family.

# **ACKNOWLEDGEMENTS AND STATEMENT OF CONTRIBUTORSHIP**

I recruited, assessed and obtained samples from a large number of patients during my PhD research, however I would like to thank all the members of the ADAPT project<sup>1</sup> (past and present) for their help arranging appointments for patients, co-ordinating studies, recruiting and assessing patients and obtaining samples which were subsequently analysed in the current thesis, but above all for their support and encouragement.

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## ABBREVIATIONS

A1AT	Alpha-1-antitrypsin
A1AT/NE	Alpha-1-antitrypsin-neutrophil elastase
ADAPT	Alpha-1-antitrypsin Assessment and Programme for Treatment
ANCA	Anti-neutrophil cytoplasmic antibody
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BMI	Body mass index
CHCA	$\alpha$ -cyano-4-hydroxycinnamic acid
COPD	Chronic obstructive pulmonary disease
CAT	COPD Assessment Test
CC-16	Clara Cell Secretory Protein 16
CRP	C-reactive protein
CT	Computed tomography
CV	Co-efficient of variation
DNA	Deoxyribonucleic acid
EBC	Exhaled Breath Condensates
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ERS	European Respiratory Society
FEV <sub>1</sub>	Forced expiratory volume in 1 second
FPA	Fibrinopeptide A
FVC	Forced Vital Capacity
LLN	Lower limit of normal
MALDI	Matrix assisted laser desorption/ionisation
GOLD	Global Initiative for chronic Obstructive Lung Disease
HPLC	High Performance Liquid Chromatography
HRCT	High Resolution Computed Tomography
IL-	Interleukin
ISWT	Incremental Shuttle Walk Test
IQR	Interquartile Range
KCO	carbon monoxide transfer co-efficient
M	Molar
MCID	Minimum clinically important difference
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
MS	Mass spectrometry
NE	Neutrophil Elastase
NICE	National Institute for Clinical Excellence
Nrf2	Nuclear erythroid-related factor
LTB4	Leukotriene B4
MRC	Medical Research Council
nm	nanometres

nM	nanomolar
NE	Neutrophil elastase
NF- $\kappa$ B	nuclear factor- $\kappa$ B
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pi	Phenotype
Pr3	Proteinase 3
SGRQ	St George's Respiratory Questionnaire
6MWD	6 minute walk distance
6MWT	6 Minute Walk Test
SE	Standard error
SLPI	Secretory Leucocyte peptidase inhibitor
SPD	Surfactant protein D
SVC	Slow vital capacity
TIMP	Tissue Inhibitor of Metalloproteinases
TLCO	Transfer Factor Lung for Carbon Monoxide
TNF- $\alpha$	Tumour necrosis factor alpha
TFA	Trifluoroacetic acid
TOF	Time of flight
$\mu$ L	Microlitre
$\mu$ M	Micromolar (es)
VEGF	vascular endothelial growth factor



# CHAPTER 1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a group of conditions that includes chronic bronchitis, airflow obstruction and emphysema and, despite the heterogeneity of this syndrome, guidelines suggest a diagnosis is only made in the presence of airflow obstruction [1, 2]. The airflow obstruction in patients with COPD is largely irreversible and defined by spirometric criteria, generally with a ratio of the Forced Expiratory Volume in 1 second ( $FEV_1$ ) and Forced Vital Capacity (FVC) of less than 0.7 [2].

The risk factors for the development of COPD are well known and include exposure to tobacco smoke (the most common risk factor in Western countries) and smoke from the combustion of biomass fuels within houses for heating or cooking (the most common risk factor worldwide) [3]. At present, alpha-1-antitrypsin (A1AT) deficiency is the only widely recognised genetic risk factor (see 1.2.4), however genetic factors are likely to be important since there is a greater prevalence of airflow obstruction amongst first degree relatives of patients with COPD [4], and they may partly account for the dramatic variation in clinical phenotype which is observed in patients with similar risk factors. Historically, it was thought there was a genetically ‘susceptible minority’ of tobacco smokers who develop COPD, based on the initial description of 13% of smokers who developed ‘airflow obstruction’ (with an  $FEV_1$  below 2.5 litres) [5]. However, more recent studies demonstrate that this is a significant underestimate and the true figure is closer to 50% [6], when COPD is defined using the Global Initiative for chronic Obstructive Lung Disease (GOLD) criteria [1]. These data are consistent with the prediction that COPD will become the 3<sup>rd</sup>

leading cause of death worldwide by 2030 [3], however it is also clear that the use of different diagnostic criteria to define COPD will predictably lead to variation in both its prevalence and incidence.

## **1.1 DIAGNOSTIC CRITERIA FOR COPD**

Chronic bronchitis and emphysema were first described in the 19<sup>th</sup> century and a formal symptom based definition agreed by the American Thoracic Society (ATS) in 1962 [7]. However more recently, COPD has become the ‘preferred term’ for patients with airflow obstruction who were previously diagnosed with chronic bronchitis or emphysema [2]. Current guidelines from the UK National Institute for Clinical Excellence (NICE) [2], European Respiratory Society (ERS)/ATS [8] and GOLD [1] recommend that COPD is only diagnosed in the presence of symptoms with a post-bronchodilator  $FEV_1/FVC < 0.7$ . The key defining symptoms of COPD include breathlessness, a persistent cough, sputum production and wheeze, however patients may also experience ankle swelling, fatigue, and chest pain [2].

Emphysema is defined by pathological criteria, although it is increasingly recognised that the optimum method for the *in vivo* diagnosis of emphysema is computed tomography (CT) (see 1.3.3) [9]. However, CT methodology has yet to be standardised with a normal range of lung density defined, and therefore the diagnosis or quantification of emphysema is not encompassed in current diagnostic criteria for COPD. Both GOLD and NICE only recommend performing CT scans or measuring gas transfer when surgical intervention is indicated or in patients with

symptoms disproportionate to the degree of airflow obstruction [1, 2]. However, emphysema may occur in the absence of airflow obstruction and the FEV<sub>1</sub> relates poorly to the severity of emphysema quantified by CT densitometry [10], and therefore it should be recognised that diagnostic criteria based on spirometry alone will exclude a number of patients with clinically important disease. The implications of this are discussed later in the current thesis.

### **1.1.1 Using The ‘Fixed-Threshold’ to Define Airflow Obstruction**

The FEV<sub>1</sub>/FVC threshold of 0.7, to be used regardless of age, was recommended in the GOLD document for ‘simplicity’ [11], however, this has been increasingly questioned as it lacks supportive clinical evidence, statistical validity, and misclassifies some subjects at the extremes of age [12-14]. Lung function was originally accepted as impaired when below the ‘lower limit of normal’ (LLN), which is usually defined as the lower 5<sup>th</sup> percentile of a reference population (stratified by age and gender [15, 16]), a definition which is therefore used in the current thesis. More recently an ERS/ATS guideline has supported the use of these limits [17], endorsed in an editorial [18]. Reference ranges are widely used for the interpretation of clinical laboratory tests, and cross-sectional studies demonstrate that using the LLN to diagnose COPD is associated with less age and gender-related bias [19]. However, the lack of well validated post-bronchodilator reference ranges (particularly for certain ethnic groups) currently limits the widespread adoption of LLN [1], and therefore the threshold to define airflow obstruction has yet to be established. In light of this controversy, the fixed threshold is used in the current thesis, however the influence of the LLN is also considered in some of the studies.

### **1.1.2 GOLD Stage 0**

There is disagreement over the importance of chronic cough and sputum production in the presence of ‘normal’ spirometry, particularly since some of these subjects will have visible emphysema on high resolution CT (HRCT) scans of the thorax [10]. Patients with symptoms suggestive of COPD but with an FEV<sub>1</sub>/FVC above 0.7 were initially classified as ‘at risk’ (GOLD stage 0) [20] and then excluded by subsequent documents [1]. Nevertheless, some patients classified into GOLD stage 0 demonstrate progression with time [21] and have an increased mortality [22] suggesting that this is not a benign state, although this has not been a universal finding [23, 24]. However, no similar longitudinal studies have included symptomatic patients with spirometry above the LLN. Further work is therefore required in this group of patients who may have an early disease process which may be more sensitive to therapeutic intervention [25] and this is discussed in more detail in section 6.2.5.

## **1.2 THE PATHOPHYSIOLOGY OF COPD AND EMPHYSEMA**

COPD is a heterogeneous disease with individuals demonstrating a range of pathology within the large airways, small airways and parenchyma, and it is therefore unsurprising that its pathophysiology is complex and remains relatively poorly understood. Nevertheless, it is known that there are several key mechanisms related to inflammation that have been implicated in the development of COPD including oxidative stress, alveolar cell apoptosis, accelerated ageing and antiprotease-protease imbalance [26].

### **1.2.1 Oxidative Stress**

A number of studies have demonstrated evidence of greater oxidative stress in patients with COPD compared to healthy controls [27-29], indicating an association with the pathogenesis of COPD. Oxidative stress may lead directly to lung damage, and interfere with elastin synthesis and repair [26] but its detrimental effects are also potentially mediated via inactivation of anti-proteases (A1AT and secretory leucoprotease inhibitor) [30] or activation of metalloproteases [31], which is considered in more detail below (see 1.2.4). Also, patients with COPD have reduced levels of nuclear erythroid-related factor (Nrf2) which influences the expression of a number of genes, including those encoding several important antioxidants [32]. Nrf2 deficient mice have high levels of neutrophilic inflammation and are also very susceptible to the development of emphysema following exposure to cigarette smoke [33] or neutrophil elastase (NE) [34] demonstrating the pathophysiological plausibility of both oxidative stress and protease imbalance in COPD.

### **1.2.2 Alveolar Cell Apoptosis**

Apoptosis, particularly of the endothelial cells of the alveolar walls, is enhanced in patients with emphysema and may be mediated directly by proteases, including Proteinase 3 (Pr3) released by neutrophils [35]. Apoptosis is also associated with decreased expression of vascular endothelial growth factor (VEGF), a protein important in cell maintenance [36], and animal models demonstrate inactivation of VEGF leads to the early development of emphysema [37]. However,

despite VEGF inactivation, smoking induced emphysema does not occur either in the presence of antioxidant or in cathepsin-S knockout mice suggesting a complex interaction between oxidative damage, apoptosis and protease-antiprotease imbalance [38].

### **1.2.3 The Ageing Lung**

Even in the absence of pathological disease states, ageing is associated with a decline in lung function, increased pulmonary inflammation and emphysematous changes leading to the hypothesis that COPD and emphysema may represent an abnormal or premature ageing process [39]. However, the process of ageing is complex and involves a number of mechanisms which are poorly understood. Nevertheless, it is known that deoxyribonucleic acid (DNA) damage and somatic mutation increase with age, and also a relationship exists between longevity and DNA repair, suggesting this repair capacity is an important factor in ageing [40]. Also, somatic cells enter senescence after a limited number of cellular divisions due to the gradual attrition of the telomeres which protect the ends of chromosomes [41] and this may be important in subjects with COPD since telomere shortening is enhanced by oxidative stress [42]. Also, telomere length relates to pack years of tobacco smoking [43] and is reduced in subjects with COPD compared to controls [44]. Additionally, tobacco smoke, COPD and ageing (Figure 1) are associated with persistent increases in the levels of the plasma cytokines interleukin 6 (IL-6) and IL-8 which may themselves trigger inflammation, protease-antiprotease imbalance and cellular senescence [26]. Other important mechanisms in the process of ageing, which are also highly relevant to the

pathogenesis of COPD, include altered protein turnover and the accumulation of mitochondrial DNA mutations, with a corresponding increase in the production of reactive oxygen species [45].

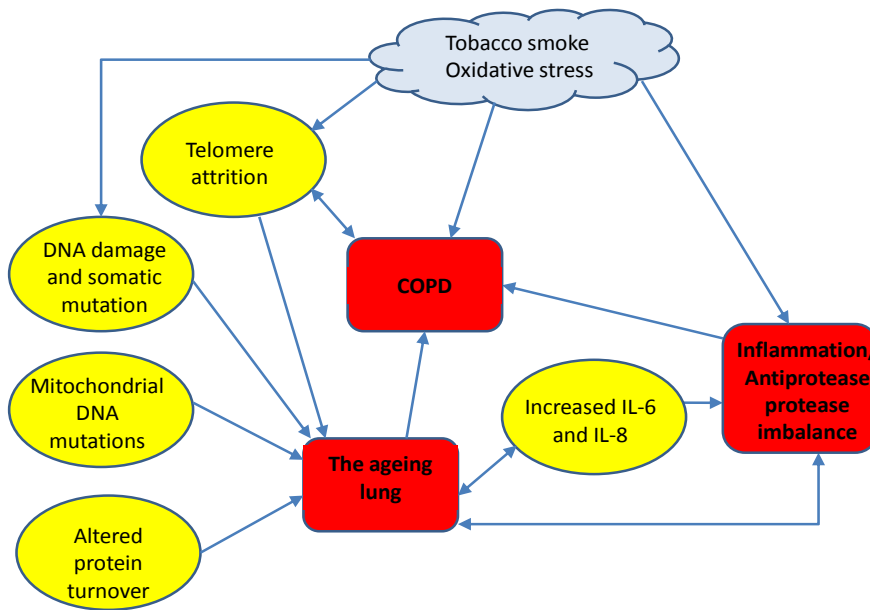


Figure 1: The complex inter-relationship of the ageing lung, antiprotease-protease imbalance, tobacco smoke and COPD which occurs over time [26].

#### 1.2.4 Antiprotease-protease Imbalance

Some work pertaining to the following sections has previously been published in Carter, R.I., Stockley, R.A. *A1AT Pathophysiology in the Lung; in Alpha-1-antitrypsin deficiency. Pathophysiology, Diagnosis and Treatment.* (eds. Bals, R. & Kohnlein, T.) 18 – 24 (Thieme, Stuttgart, 2009).

#### 1.2.4.1 *Alpha-1-Antitrypsin (A1AT)*

A1AT is the dominant human antiprotease which is synthesised by hepatocytes [46] and in smaller quantities by alveolar macrophages, circulating monocytes [47] and lung epithelial cells [48]. There are around 125 documented variants of the A1AT protein, and several are associated with deficient plasma levels. The most common variant associated with a severe A1AT deficiency occurs because of a point mutation around the mobile domain in the Z protein causing the formation of polymers which accumulate within the liver, rather than being secreted [49], and which are also less effective at inhibiting NE [50].

A1AT is the main inhibitor of free or neutrophil membrane bound NE, with which it binds irreversibly on an equimolar basis. Although NE bound to the neutrophil membrane was initially reported to be relatively resistant to inhibition, this was probably technique associated rather than a true finding [51]. A1AT is generally present in excess (even in 'deficient' states), however the elegant mathematical and *in vitro* modelling of Campbell et al [52-54] demonstrated quantum proteolytic damage occurs at the point of neutrophil degranulation even in a healthy individual, due to the relative excess of NE. Nevertheless, in healthy subjects, the destructive potential of NE is usually limited since molar equality between enzyme and inhibitor rapidly occurs as the enzyme diffuses from its point of release. However, in subjects with an A1AT concentration less than 11  $\mu\text{mol}$  (such as the homozygous Z phenotype), there is an exponential increase in the time and distance of diffusion until molar equality is reached with a corresponding increase in the potential area of proteolytic damage (Figure 2).



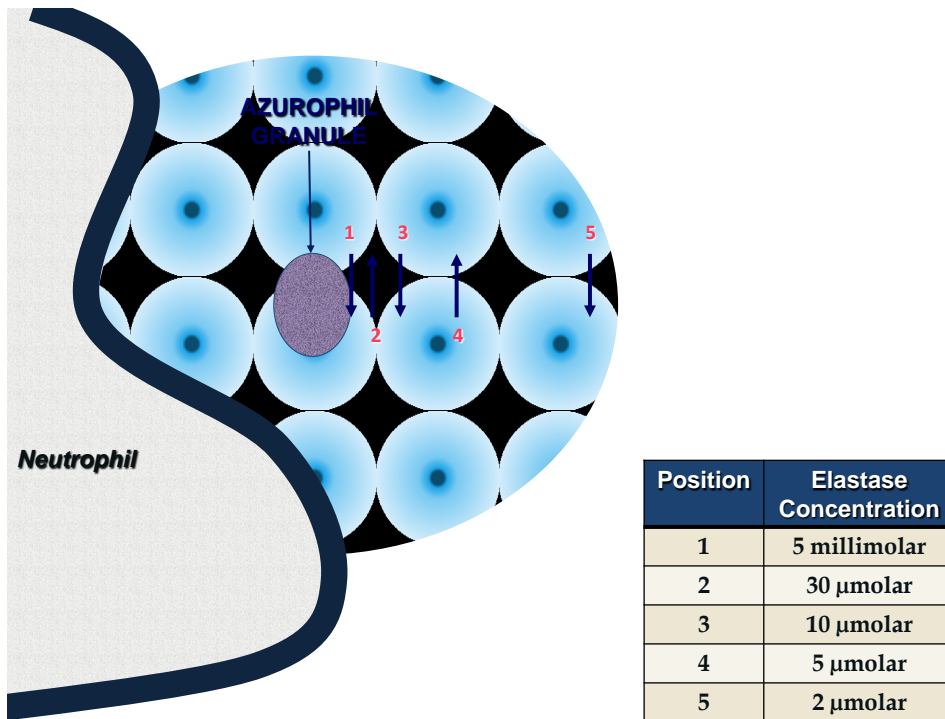


Figure 2: The relationship between the distance from the azurophil granule, the A1AT concentration and NE concentration based on the mathematical and *in vitro* modelling of Campbell et al [52-54]. The mean concentration of A1AT in the PiZ deficient patient is around 5  $\mu$ M compared to a mean concentration of 30  $\mu$ M in a healthy PiM individual. There is therefore an exponential increase in the distance until molar equality with NE is achieved in the PiZ subject, and hence the volume of potential proteolytic damage, compared to the PiM subject.

The concept of quantum proteolysis, demonstrated by mathematical and *in vitro* modelling [52-54], is also supported by the clinical observation that subjects with PiSZ A1AT deficiency (with a mean A1AT concentration of 11  $\mu$ mol) are not particularly susceptible to emphysema [55], although this remains controversial [56]. Also, there is currently no direct evidence of this excess local NE activity in PiZ patients owing to the eventual inhibition of this enzyme by the remaining A1AT, even in the deficiency state, within the neutrophil micro-environment.

#### *1.2.4.2 The Neutrophil*

Activated neutrophils with an enhanced chemotactic response are found in the circulation of individuals with COPD [57] and these cells also demonstrate abnormal destructive potential [58] and increased adhesion and spontaneous migration under flow [59]. Neutrophils migrate into the bronchial submucosa under the influence of a variety of chemokines and chemoattractants including tumour necrosis factor alpha (TNF- $\alpha$ ), IL-8, leukotriene B4 (LTB4), NF- $\kappa$ B and matrix metalloproteinase 12 (MMP-12) [60, 61] via post-capillary venules of the bronchial circulation [59], while (uniquely) neutrophils enter the lung parenchyma via the pulmonary circulation at the capillary level [62]. Integrins are crucial for neutrophil passage through the endothelium [63], however, NE is localised to the leading edge of migrating neutrophils [64] and inhibition of NE prevents migration [65] suggesting the importance of this enzyme for transmigration, particularly through the perivascular basement membrane.

Upon activation, neutrophils generate reactive oxygen species and degranulate releasing proteases, particularly NE and Pr3, which are capable of damaging or degrading a wide range of proteins including elastin, collagen, fibronectin and Tissue Inhibitor of Metalloproteinases (TIMP). Neutrophil degranulation may therefore lead to damage to the airway epithelium and cilia [66] and increased mucosal permeability [67].

#### *1.2.4.3 NE and the implications of antiprotease-protease imbalance*

It is increasingly accepted that inflammation is central to the development of COPD and an antiprotease-protease imbalance may represent a final common pathophysiological pathway, although there may be a number of underlying mechanisms. Our understanding of this process has been greatly enhanced by the observation in the 1960s that severe early onset emphysema was associated with A1AT deficiency [68], with subsequent animal models demonstrating human NE could induce emphysematous changes [69]. Other serine proteinases, known to be co-localised within the azurophil granule, are also important since cathepsin G may produce mucous gland hyperplasia [69] while Pr3 is also capable of inducing emphysematous changes [70] (which is discussed further in section 1.2.4.4). Since A1AT is the main inhibitor of all these enzymes it was hypothesised that emphysema and COPD developed from a relative excess of enzyme versus inhibitor at the site of disease. However, in the absence of an acute exacerbation [71], enzyme activity has not been demonstrated in the airways of patients with COPD and even in A1AT deficiency, only low levels of enzyme activity are found in sputum in the stable clinical state [71] and therefore conclusive support of this mechanism is currently lacking.

Studies have subsequently shown that enzymes belonging to 2 other groups (the cysteine proteases [72] and the MMPs [73]) are also capable of elastin degradation, while MMP-12 knockout mice are protected against emphysema [74] which raised the possibility that MMP-12 could also cause emphysema directly. However, it is far more complex than this and Churg and colleagues have demonstrated that MMP-12 is required for TNF- $\alpha$  receptor processing, and in TNF- $\alpha$  and TNF- $\alpha$  receptor knockout mice, emphysema also does not occur on exposure to

cigarette smoke [75]. Furthermore, A1AT treatment prevents TNF- $\alpha$  release in smoking animals [76] and thus also prevents the development of emphysema [55]. In addition, NE can activate cathepsin B [77] (a cysteine protease which can also produce emphysema [72]) and MMPs [78], and is capable of inhibiting TIMP, the main inhibitor of MMPs [79]. Also, in A1AT deficient subjects, A1AT augmentation therapy decreases cathepsin B and MMP-2 activity, even though these enzymes are not inhibited by A1AT [80]. Furthermore ageing, cell senescence, tobacco smoke and COPD are also linked via a complex interaction with antiprotease-protease imbalance (Figure 1) [26]; in the absence of proteases, alveolar cell apoptosis does not lead to emphysema [38]; and oxidative stress may particularly lead to local inactivation of A1AT [30] or activation of MMPs [31]. These data support the theory of the central role of proteases, particularly NE, in the pathogenesis of COPD (Figure 3), indicating that a marker of NE activity may be an effective marker of COPD disease activity (see 1.5.2)

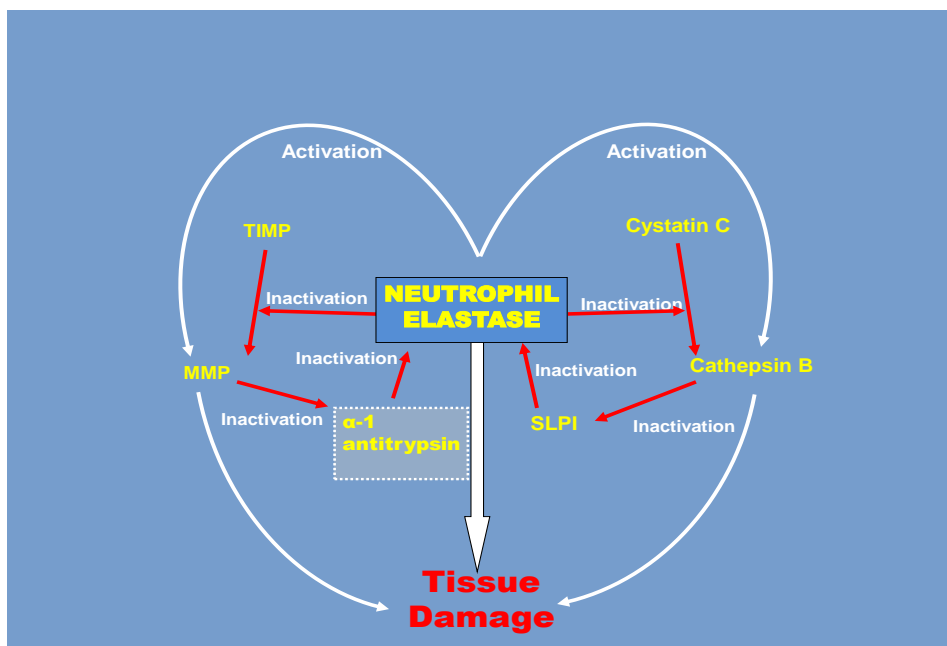


Figure 3: The complex relationships between cysteine, serine and metalloproteases. SLPI is secretory Leucocyte peptidase inhibitor.

#### *1.2.4.4 Pr3 and the implications of antiprotease-protease imbalance*

Although NE is central to the pathogenesis of COPD and emphysema, Pr3 may be of equal or even greater importance since it is co-localised with NE within the azurophil granule of the neutrophil, and it is also capable of cleaving the matrix proteins elastin, fibronectin and type IV collagen [70, 81] and instillation into the lungs of hamsters induces an emphysematous like process [70]. Pr3 is potentially of greater importance than NE in the pathogenesis of COPD, since the concentration of Pr3 within each neutrophil is about 3 times higher than the concentration of NE [82] and thus upon activation greater quantities of Pr3 are released [83]. Furthermore, although Pr3 is inhibited by both the local lung inhibitor elafin [84] and A1AT (though not oxidised A1AT) [85], there is disagreement over the efficacy of A1AT inhibition when Pr3 is bound to elastin [85, 86] and, in contrast to NE, Pr3 is not inhibited by the major local serine proteinase inhibitor (SLPI) [81] which is found primarily within airway secretions. Also, the association rate constants for A1AT and elafin (the mutual inhibitors of these enzymes) are greater for NE than for Pr3 and the inhibitors will therefore preferentially bind NE. This is primarily of importance at the point of degranulation when the enzymes are present in a relative excess over their inhibitors, and Pr3 may therefore contribute disproportionately to the area of quantum proteolysis observed around neutrophils even in healthy individuals, which would still be exponentially greater in those with A1AT deficiency [54].

Pr3 is one of the less well studied of the serine proteases because of difficulties in isolating the protein and the absence of specific substrates and inhibitors [82]. Also, N-terminal sequencing of Pr3 was initially difficult [87] leading to the early description of 4 independent proteins, all located within the azurophil granule but associated with separate pathological and physiological processes [85] namely:

- Pr3
- p29b (with microbiocidal properties)
- myeloblastin (a regulator of growth and differentiation in leukaemic cells)
- the target antigen for cytoplasmic anti-neutrophil cytoplasmic antibody (cANCA) in people with granulomatosis with polyangitis (formerly Wegener's Granulomatosis)).

However rather than 4 separate entities, DNA sequencing confirmed a single hybridising gene and therefore a single protein with a high sequence homology to the other serine proteases [87]. Initial research focussed on the role of Pr3 as the target for auto-antibodies in the pathogenesis of granulomatosis with polyangitis, however since it is likely to be central to the pathophysiology of COPD and emphysema, the current thesis also describes the identification of Pr3 cleavage products which could be used to generate a specific assay of pre-inhibition Pr3 activity (2.11 and Chapter 8).

### **1.3 MARKERS OF DISEASE ACTIVITY, SEVERITY AND PROGRESSION IN COPD AND EMPHYSEMA**

The pathophysiology of COPD and emphysema is complex however, it is important that biomarkers of progression and response to treatment measure processes which are central to this disease. At present, in the absence of an effective marker of disease activity, markers of end-organ damage (disease severity) are widely used although it is increasingly recognised that they are insensitive to change and cannot predict future progression, since an initial assumption that those with the most severe disease progress at the greatest rate is flawed [88]. There is therefore renewed interest in identifying biomarkers of disease activity [89] which may allow the identification of patients who are at risk of disease progression, before the development of irreversible lung disease, and which could also be used as early measures of treatment efficacy, particularly in stage II and III clinical trials. This section reviews the important measures of disease severity and also explores other potential biomarkers of disease severity and activity in COPD.

#### **1.3.1 Spirometry**

The FEV<sub>1</sub> is a widely used measure of outcome in clinical trials and disease severity in routine clinical practice, and relates to all cause, respiratory and cardiovascular mortality [90]. The relationship to all cause mortality is interesting and is probably explained by either a common

inflammatory phenotype linking a number of distinct disease processes, or indicates the role of  $FEV_1$  as a surrogate for overall health status.

<b>GOLD Grade</b>	<b>Severity of Airflow Obstruction</b>	<b><math>FEV_1</math> (% predicted)</b>
<b>1</b>	Mild	$\geq 80$
<b>2</b>	Moderate	50 to 80
<b>3</b>	Severe	30 to 50
<b>4</b>	Very Severe	$<30$

*Table 1: The GOLD spirometric severity classification of COPD (in subjects with an  $FEV_1/FVC < 0.7$ ) [1]. The 2010 NICE classification is similar, although patients with an  $FEV_1 < 50\%$  and respiratory failure are also considered to have very severe disease [2].*

Clinical guidelines suggest the use of spirometry to classify disease severity in patients with COPD (Table 1), although it is increasingly recognised that a multidimensional approach or test is required to assess patients accurately [1]. Additionally, although short term improvements in  $FEV_1$  may relate to improvements in patient symptomatology, this physiological measure also has important flaws. Firstly although there is a highly significant relationship between health care status and  $FEV_1$ , this relationship is relatively weak (Figure 4) [91], and a similarly weak relationship between spirometry and exercise capacity has also been reported [92]. Secondly there is significant day-to-day variability of  $FEV_1$  which may be greater than the progression observed in patients with COPD over many years [93], and therefore prolonged follow-up is required to determine whether deterioration has truly occurred. For these reasons,  $FEV_1$  is a poor surrogate marker for early phase studies of potential disease modifying agents. Thirdly, around half of patients with COPD have significant increases in  $FEV_1$  following the administration of a bronchodilator [94], although there is considerable intra-individual variability of this measure



with repeated testing, and furthermore the degree of reversibility does not relate to response to current treatments [95]. Nevertheless, bronchodilator reversibility may relate to disease progression [96-99], although this has not been demonstrated in all studies [95, 100]. Fourthly, pharmacological interventions may significantly increase FEV<sub>1</sub> yet fail to influence mortality [101]. Finally and importantly, it is recognised that COPD is a group of distinct pathological processes and the FEV<sub>1</sub> relates poorly to the presence of emphysema and its severity as quantified by CT densitometry [10] indicating that assessment using FEV<sub>1</sub> alone will not provide sufficient information about overall disease severity.

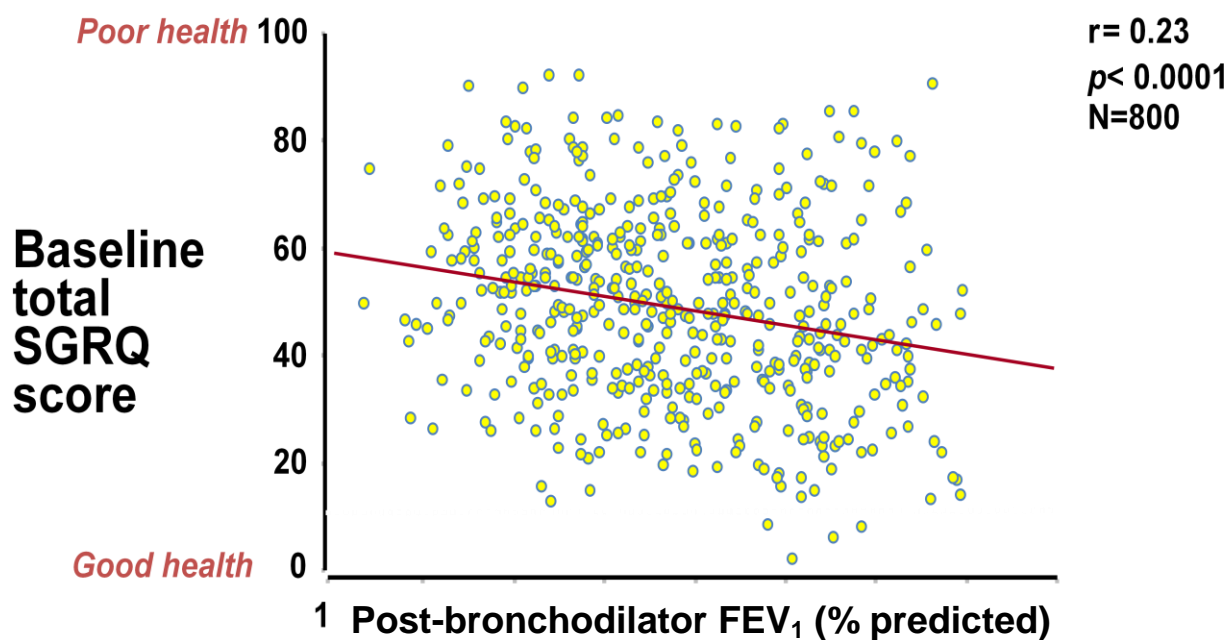


Figure 4: The relationship between FEV<sub>1</sub> (% predicted) and the baseline St George's Respiratory Questionnaire (SGRQ) (section 1.3.4.2) is highly significant, however there is a wide spread of values within this population. Reprinted with permission from British Medical Journal publishing group limited [91].

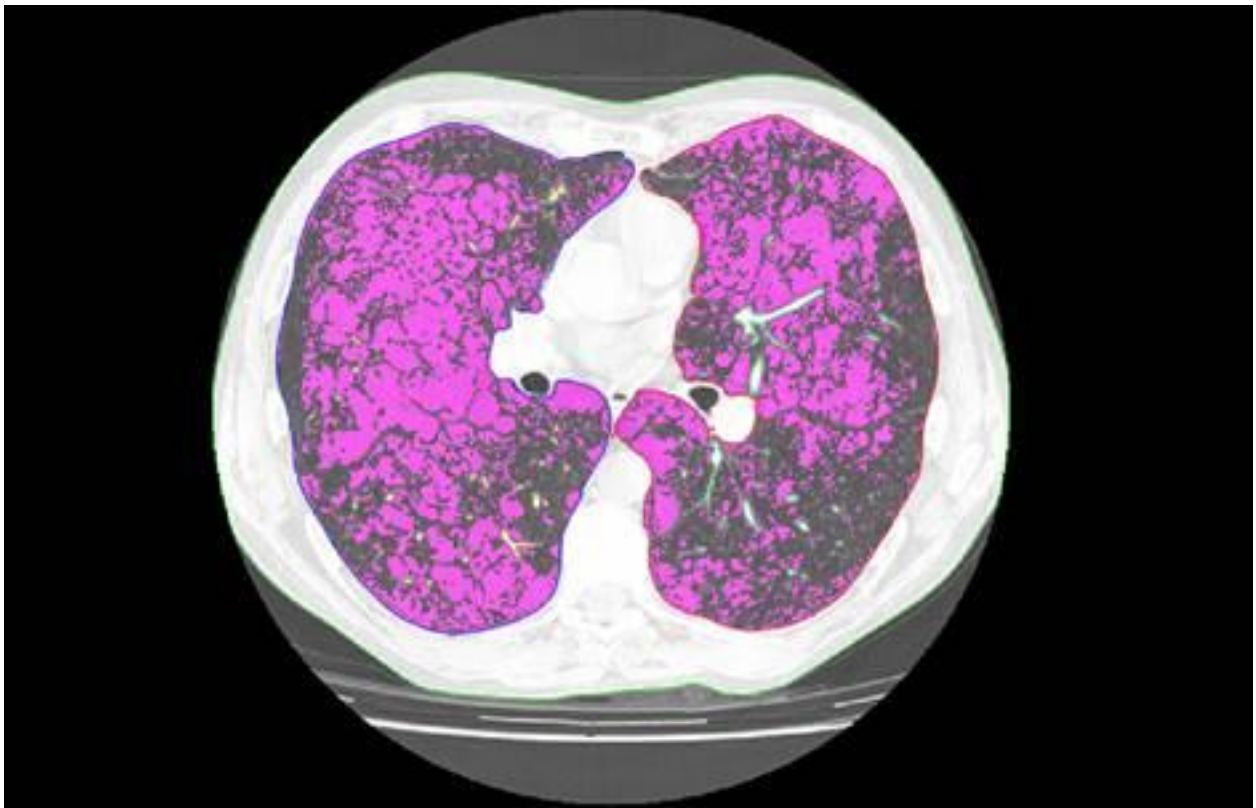
### **1.3.2 Gas Transfer**

The measurement of the transfer factor of the lung for carbon monoxide (TLCO) or the carbon monoxide transfer co-efficient (KCO) are well established measures of alveolar function [102], and cross-sectional studies demonstrate that abnormalities of KCO relate better to upper zone emphysema while FEV<sub>1</sub> relates better to lower zone predominant emphysema [103]. Also, KCO relates to pathological measures of emphysema severity [104] and even isolated KCO abnormalities are associated with a worse SGRQ (see 2.1.5.1) [103]. Few longitudinal studies have validated KCO, although one small study conducted in subjects with spirometry predominantly in the normal range, demonstrated there was no significant progression of KCO over a 22 year period and no difference in the rate of progression of KCO in smokers versus ex-smokers, indicating the stability of this marker in ‘healthy’ controls. In contrast, studies in subjects with COPD [21] and with PiZ A1AT deficiency [9] demonstrated a decline in KCO, although this did not achieve conventional statistical significance in the PiZ population in whom CT densitometry was a more sensitive measure of progression [9]. Gas transfer alone is also therefore not an effective measure of overall COPD/emphysema disease severity and progression.

### **1.3.3 HRCT Scans of the Thorax**

The use of HRCT now enables *in vivo* assessment of the amount and distribution of emphysema and may detect disease prior to physiological abnormalities [10], which previously could only be achieved by pathological analysis of lung resection or post-mortem specimens [104]. Quantitative

measures of lung density on HRCT (Figure 5) have advantages over visual severity scales since they are objective and relate well to exercise tolerance and health status [105], and are more sensitive measures of emphysema progression and better predictors of mortality than FEV<sub>1</sub> [106]. Also, low dose, multi-slice CT protocols enable multiple scan repetition with radiation exposures below the annual background radiation dose [107]. CT densitometry has therefore been approved by the United States Federal Drug Administration for use as a clinically meaningful endpoint in pharmaceutical trials [108].



*Figure 5: CT densitometry, demonstrating areas of low attenuation which are highlighted by automated software.*

The voxel index and the 15<sup>th</sup> percentile point are the two most widely used and validated densitometric scores, and may be calculated using semi-automated or automated computer protocols (Figure 5). The voxel index refers to the percentage of the lung which is below a set density threshold (in Hounsfield Units) while the 15<sup>th</sup> percentile point is a density threshold in Hounsfield Units which encompasses the least dense 15% of the lung (similarly the 5<sup>th</sup> percentile point refers to a 5% density threshold). Although the voxel index correlates well with the severity of emphysema in pathological specimens [109], it is threshold dependent and progression varies with disease severity [110]. Conversely, while the 15<sup>th</sup> percentile point is threshold independent [110], studies are yet to correlate this densitometric measure with pathological specimens, and to date this analysis has been restricted to the 5<sup>th</sup> percentile point. Furthermore, while the voxel index may correlate well with pathological severity of emphysema [109], a normal range for lung densitometry has yet to be defined and therefore densitometry cannot yet be reliably used to discriminate between normal and emphysematous lung.

Scan reproducibility is also difficult to assure, particularly in longitudinal studies, due to a number of factors including ageing or replacement of x-ray tubes [110]. Although these can partly be controlled for, the level of inspiration of the study subject also influences the results [111] and further mathematical adjustment is required to account for this. Finally, particularly in multi-centre studies, differences in scanners and acquisition protocols influence densitometric readings and therefore it is recommended that serial examinations of a single study subject be performed on the same scanner [107]. Although the use of techniques that enable adjustment for variation in the level of inspiration and calibration protocols (using materials of a known density such as water, air or lung “phantoms”) reduce longitudinal variation, uncertainty remains over the

most appropriate methodology [111]. Consequently, further research is required before CT densitometry is considered a universally accepted method of severity assessment and for long term monitoring of patients in trials or routine clinical practice. Nevertheless, and despite its current flaws, it remains the best current measure of emphysema *in vivo* and therefore new biomarkers for COPD should relate (at least in part) to CT densitometry.

### **1.3.4 Patient Reported Outcomes**

#### *1.3.4.1 Medical Research Council Breathlessness Scale*

Dyspnoea, related to exertion or exacerbations, is both common and distressing in patients with COPD and can impact on social and recreational activities and, in those with more severe symptoms, can limit the performance of activities of daily living [112]. Although the principle physiological process leading to the perception of dyspnoea is likely to relate to changes in lung mechanics, volumes and activation of respiratory muscles, there are also important psychological and social influences on this complex symptom [112]. The degree of dyspnoea can be assessed using the Medical Research Council (MRC) dyspnoea scale (Table 2) which was first described in 1960 [113] and is widely used because it relates to important clinical outcomes, including 5 year mortality [114]. A modified version of the scale also exists (mMRC) which includes comparable descriptors, but has a scale which ranges from 0 to 4 rather than one to 5 [1]. There is a partial correlation between the MRC scale and more objective shuttle walk testing [115], however there is a poor relationship between MRC and FEV<sub>1</sub> which indicates the inadequacy of FEV<sub>1</sub> as a measure of symptomatology in patients with COPD. However, while the MRC scale is

helpful in assessing dyspnoea, it remains subjective and this is particularly important in longitudinal studies where half of patients reporting an improvement, have actually deteriorated using objective assessment [116]. The MRC and mMRC can therefore only form part of a global assessment of patients with COPD.

Grade	Degree of Breathlessness
1	Not troubled by breathlessness except on strenuous exercise
2	Short of breath when hurrying or walking up a slight hill
3	Walks slower than contemporaries on level ground because of breathlessness, or has to stop for breath when walking at own pace
4	Stops for breath after walking about 100 metres or after a few minutes on level ground
5	Too breathless to leave the house, or breathless when dressing or undressing

*Table 2: The MRC dyspnoea scale has been widely used since 1960 to assess patients with COPD [2].*

#### 1.3.4.2 SGRQ

The SGRQ is a subjective quality of life score which measures the influence of health on an individual's overall satisfaction with life. The score is therefore a measure of both disease severity and impact on everyday social, occupational and domestic activities.

The SGRQ questionnaire comprises 50 items and is divided into 2 parts; part one encompasses the symptoms score, and part 2 the activity and impacts (psychosocial) scores. A total weighted score is also calculated and each score ranges from 0 (no impairment) to 100 (maximum impairment). Although patients with a lower FEV<sub>1</sub> have a worse SGRQ, the overall relationship

between FEV<sub>1</sub> and SGRQ is not strong (Figure 4) [91]. SGRQ scores are also worse in subjects with COPD who experience greater numbers of exacerbations [117], which is consistent with the greater rate of disease progression in those patients [118], and relates to other measures of disease severity including the 6 minute walk test, and the short form 36 [119].

The SGRQ is frequently used as an outcome measure in clinical trials and the TORCH study, for example, demonstrated a mean reduction of 3.0 units in subjects receiving combination therapy over the 3 year study [120]. Although this may not be clinically significant for the group, since the suggested minimum clinically important difference (MCID) is 4.0 units [91], it still suggests many individual patients had a significant improvement. Furthermore, determining the MCID is complex since there is no gold standard measure of health status, and also there is controversy over the meaning of clinical significance. Most studies consider the MCID should relate to a threshold that is 'just-noticeable' to a patient, or is considered to be important to a clinician (although there is clear disparity between what is considered important by a patient or by a clinician) [121]. Alternatively, the MCID may be determined by exploring the relationship between the score and a major health event (e.g. admission to hospital, or death) [122], although this may set too great a threshold.

The SGRQ is however well validated and its clinical significance is reasonably accepted, although its sensitivity for detecting improvement (treatment effect) or deterioration (disease progression) in a longitudinal trial is more questionable. However, while the SGRQ is self-administered, its analysis is labour intensive and it is therefore not suitable for routine clinical

practice. Consequently the COPD Assessment Test (CAT) has been developed with the expectation that it will be applied in more routine practice.

#### *1.3.4.3 The COPD Assessment Tool (CAT)*

The CAT (see 2.1.5.2) is a validated measure of quality of life in patients with COPD which has been shown to be higher (i.e. a more symptomatic patient) in a population of subjects experiencing an acute exacerbation of COPD compared to a population in the stable state [123]. Additionally, the CAT correlates with SGRQ, MRC score, age and FEV<sub>1</sub> when performed in the stable state [119]. The tool consists of 8 questions, rated on a severity scale from 1 to 5, giving a maximum severity (worse symptoms) of 40. A statistically significant decrease in the CAT score (from baseline) is described in subjects following pulmonary rehabilitation, and the improvement was greatest (-3.8 points) in subjects who felt ‘much better’ compared to those who only felt a ‘little better’ (-1.3 points) or no different (-2.3 points) [119]. If it is assumed that the MCID for the CAT score can be interpolated from the SGRQ, then the MCID for CAT is 1.6 (corresponding to 4 units on the SGRQ) [123] which is therefore of a similar magnitude to the change in the group who felt a little better following pulmonary rehabilitation [119]. Although ‘feeling a little better’ could be considered a ‘just-noticeable’ difference [121], it should be noted that in the study of Dodd et al [119], 96% of subjects felt a little or much better, while only 4% felt the same or worse which would question the validity of this conclusion. Further work is therefore required to demonstrate whether or not CAT is an effective and sensitive measure in longitudinal studies.



### **1.3.5 Measures of Exercise Capacity**

#### *1.3.5.1 6 Minute Walk Test (6MWT)*

The 6MWT measures the distance that a patient can walk in a period of 6 minutes (i.e. the 6 minute walk distance (6MWD)), and guidelines (United States) recommend the use of standard instructions to improve repeatability [124], although this may require some modification to account for different populations:

‘The object of this test is to walk as far as possible for 6 minutes. You will walk back and forth in this hallway. Six minutes is a long time to walk, so you will be exerting yourself. You will probably get out of breath or become exhausted. You are permitted to slow down, to stop, and to rest as necessary. You may lean against the wall while resting, but resume walking as soon as you are able.

You will be walking back and forth around the cones. You should pivot briskly around the cones and continue back the other way without hesitation. Now I'm going to show you. Please watch the way I turn without hesitation.’ [124]

A multicentre study conducted in 7 countries [125] demonstrated the mean 6MWD in healthy controls was 571 metres (Standard Error (SE) 90), with a range of 380 to 782 metres. Importantly, there were significant differences between countries which may be explained by cultural factors (usual speed of walking, lifestyle etc), and this should be considered when conducting the 6MWT in subjects from a variety of ethnic backgrounds. Additionally, there was a significant increase in the 6MWD on repeat testing, demonstrating there is a learning effect within this test [125], which must be accounted for, particularly in longitudinal studies.

The 6MWT test offers advantages over other forms of exercise testing. Firstly, there are ventilatory and metabolic differences between assessments of walking (such as the 6MWT) and cycle ergometry [126], and since the 6MWT measures an activity central to daily living this is *probably* the more useful assessment. Secondly, the test has been evaluated in patients with COPD and relates well to self-ranking of symptom severity (compared to their peers with COPD), with differences of more than 54 metres considered to be the MCID [116]. Finally, it is a simple, safe assessment that requires little equipment and although there is a learning effect, this is only modest.

#### *1.3.5.2 Incremental Shuttle Walk Test (ISWT)*

Although the 6MWT is partly an objective assessment, the test may be influenced by motivation, cultural norms and repeated testing [125]. The ISWT protocol was an attempt to overcome these issues and requires the subject to walk up and down a 10 metre course, at a speed dictated by a pre-recorded audio signal (a beep) [127]. The time between the ‘beeps’ decreases as the test progresses and the test ends when the subject can no longer keep up with the beeps or they are too breathless to continue. The test relates well to maximal heart rate, and since subjects do not have to pace themselves the test will theoretically provide a better measure of their true disability [127]. However, a study comparing 6MWT, with ISWT and cycle ergometry showed that subjects performing the 6MWT were able to match exertion and dyspnoea to reach a more sustainable (and natural) maximum intensity, although there was no difference between peak heart rate or dyspnoea between the 3 tests [128] which indicates that effort was also similar. The

theoretical advantages of the ISWT have therefore not been proven and hence the more straightforward 6MWT is probably the more appropriate form of assessment.

### **1.3.6 Composite Scores: BODE**

The BODE score is a multidimensional score of COPD severity comprising the Body mass index (BMI), airflow Obstruction ( $FEV_1$  % predicted), Dyspnoea (modified MRC score) and Exercise capacity (6 minute walking test) [129]. When compared to  $FEV_1$ , this assessment tool has a stronger relationship with the need for hospital admission [130], exacerbations [131] and mortality [130, 132]. The BODE index is expressed as the sum of the individual component scores (Table 3) and therefore ranges from 0 to 10. BODE is a good measure of cross-sectional disease severity, however, there is stronger evidence for the use of the index expressed as quartiles (usually scores of 0 – 2, 3 – 4, 5 – 6 and 7 – 10) due to small numbers included in subsequent follow-up studies and this allows for less discrimination between individuals. This is particularly important in longitudinal studies where sensitive measures of disease progression or treatment efficacy are required, and may partly explain why there is a *statistically* significant increase in BODE in only 14% of patients with moderate to severe COPD over a median study period of 64 months [88], although this may also relate to heterogeneous disease progression. Also even within those whose physiology declined, the average increase in BODE score was only 0.5 points per year, which is of doubtful *clinical* significance, and there was no relationship between decline in BODE and  $FEV_1$  progression. However and importantly,  $FEV_1$  progression within this study was also non-linear and variable [88], which indicates both the heterogeneity of

disease progression in subjects with COPD and the need for markers of disease activity rather than severity (discussed later in this thesis). Finally, all the components of BODE are simple to perform but in combination are labour intensive and therefore this score may not be suitable for widespread use in the assessment of this prevalent disease.

Parameter	BODE Index Score			
	0	1	2	3
Body mass index	>21	<21		
Airflow obstruction (FEV <sub>1</sub> % predicted)	≥65	50 – 64	36 – 49	≤35
Modified MRC dyspnoea scale	0 – 1	2	3	4
Exercise Tolerance (Distance walked in 6 minutes (metres))	≥350	250 – 349	150 – 249	≤149

*Table 3 The BODE index. This multidimensional assessment tool of COPD disease severity also relates to mortality [129].*

### 1.3.7 Biomarkers

A biomarker is ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention’ [133]. While this term may include any clinical endpoint, it is generally considered that a biomarker is something (usually a protein) that can be measured [134] whether in sputum (induced or spontaneous), exhaled breath, bronchoalveolar lavage (BAL), bronchial biopsies, blood (serum or plasma), or urine. In contrast to markers of disease severity, the biomarkers have the potential to be markers of disease activity (which may therefore predict future disease progression) and may also allow a more sensitive assessment of treatment effects.

#### *1.3.7.1 Sputum Markers*

Up to 60% of current smokers with COPD have chronic bronchitis and therefore regularly produce sputum spontaneously [135], however sputum production may also be induced by the inhalation of an induction agent (often hypertonic saline). In those who are ‘producers’, spontaneous sputum is generally easy to obtain at minimal cost and without discomfort. However, sputum induction may not be acceptable to all patients and furthermore in subjects with COPD it may be associated with a temporary but significant worsening of FEV<sub>1</sub>, FVC and oxygen saturations [136], which could be temporarily detrimental in those with the most severe lung disease. Also biomarker concentrations in spontaneous sputum are not influenced by the dilutional or inflammatory effects of sputum induction agents, although they may both be diluted by physiological secretions from the oropharynx. In addition, processing methods [137, 138] and the dilutional effect of the inhalation and eventual expectoration of the induction agent (usually saline) may also affect the results [139]. Furthermore, inflammation is only detectable in induced sputum samples obtained from those who also spontaneously expectorate, however, the induction process adds a further dilutional error [140]. Although analysis (in the stable state) of spontaneous sputum is limited to those with chronic bronchitis, importantly (if patients expectorate) the cellular results and validity are the same in expectorated and induced sputum [141], however this is not a universal finding [136]. Additionally, the accuracy of spontaneous sputum analysis may be increased by repeated testing [141], however sputum induction may itself trigger inflammation with raised neutrophils that persist for 24 hours which precludes repeated testing, at least within a short timeframe [142].

A number of inflammatory mediators are raised in the sputum of patients with COPD, particularly during exacerbations, although few have been fully validated in longitudinal studies. Of these markers, sputum IL-8 has been more extensively studied and is present in higher concentrations in subjects with COPD, and relates to FEV<sub>1</sub>/FVC in cross-sectional studies [143] and to disease progression measured by CT densitometry, but not lung function, in longitudinal studies [21]. Also sputum myeloperoxidase (MPO) and LTB<sub>4</sub> correlate with FEV<sub>1</sub> and TLCO decline respectively [21]. However, at present, limited data exists regarding the long-term variability of markers in induced sputum [144], which currently restricts their use as biomarkers.

#### *1.3.7.2 Exhaled Breath Condensates (EBC)*

Exhaled air contains aerosolised particles from airways' lining fluid as well as water vapour and, when cooled, the water vapour condenses trapping non-volatile substances in the EBC [145]. Guidelines for the use of EBC were produced by the ERS in 2005 [146], however there are still outstanding concerns regarding the variability in both sample collection and analysis [145]. For example, the dilution of exhaled biomarkers (and therefore their concentration) may be influenced by either the water content of the airways' lining fluid or exhaled breath, however there is no accepted endogenous factor to compensate for this [145]. Intra-patient variability has also been shown to be greater in patients with COPD than healthy controls, largely due to changes in airway pH of uncertain aetiology [147].

LTB<sub>4</sub> is raised in the EBC from patients with COPD [148], increases during exacerbations [149] and correlates with sputum neutrophils [148]. However, studies reveal a wide range of absolute values (despite the use of the same ELISA and similar methodology) and this variability currently precludes the routine use of EBC in COPD [145]. Airway cytokines are also raised in EBC from patients in COPD relative to controls, although at concentrations that are below or close to the lower limit of detection and quantification for many assays, which again may lead to unacceptable variability or interpretation [145, 150]. Nevertheless, the EBC IL-6 concentration correlates with FEV<sub>1</sub> in current smokers in cross-sectional studies [151], although no longitudinal data are available.

The nitrogen oxides nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) may also be measured in EBC and while nitrite is raised in COPD relative to healthy controls [152], nitrate is not [153], and it has been suggested that exhaled nitric oxide relates more to concomitant asthma than COPD [154]. Although certain EBC markers may relate to physiological markers of COPD, and proteomic analysis of EBC has established that several proteins are elevated in subjects with A1AT deficiency compared to controls [155], further studies are required to prove the validity of the methodology, reproducibility, predictive value and response to treatment.

#### *1.3.7.3 Bronchial Biopsies and BAL*

Bronchial biopsies demonstrate an inverse relationship between FEV<sub>1</sub> and the number of neutrophils, macrophages and NK-lymphocytes in the subepithelium in smokers, both with and

without airflow limitation [156]. Also, *in situ* NF- $\kappa$ B activation is greater in subjects with COPD, and increases with disease severity [157]. However, bronchial biopsies are performed via bronchoscopy and therefore they are not without risk, particularly in those with more severe disease or co-morbidities [158]. Furthermore, they provide only a limited anatomical and pathological ‘snapshot’ and the reproducibility of measurements of inflammatory cells is poor requiring multiple biopsies [159].

Betsuyaku et al demonstrated that subjects with a high level of A1AT/NE (A1AT/NE) complex in BAL have a greater rate of decline in FEV<sub>1</sub> than those with a low level, although the high group included only six subjects and there was no correlation between FEV<sub>1</sub> decline and A1AT/NE complex when expressed as continuous variables [160]. Also, BAL from current smokers with COPD contains the greatest proportion of neutrophils, while ex-smokers with COPD still have a greater proportion of these inflammatory cells than ‘healthy’ smokers. Moreover, the absolute number of neutrophils correlates with pack years of smoking, and there is greater macrophage expression of MMP-12 [161] and MMP-9 but less TIMP-1 [162] in those with COPD compared to non-smokers (the role of these inflammatory mediators was discussed in 1.2.4 and Figure 3). However, BAL is invasive and may lead to a transient fever and also carries the associated risks of bronchoscopy (albeit small), such as haemorrhage and respiratory failure, which increase with disease severity [158]. Also BAL recovery decreases with greater emphysema severity [163], and again there is no accepted measure of the dilutional effect of the saline lavage, hence interpretation of biomarker concentrations in the recovered fluid presents a major challenge [164].

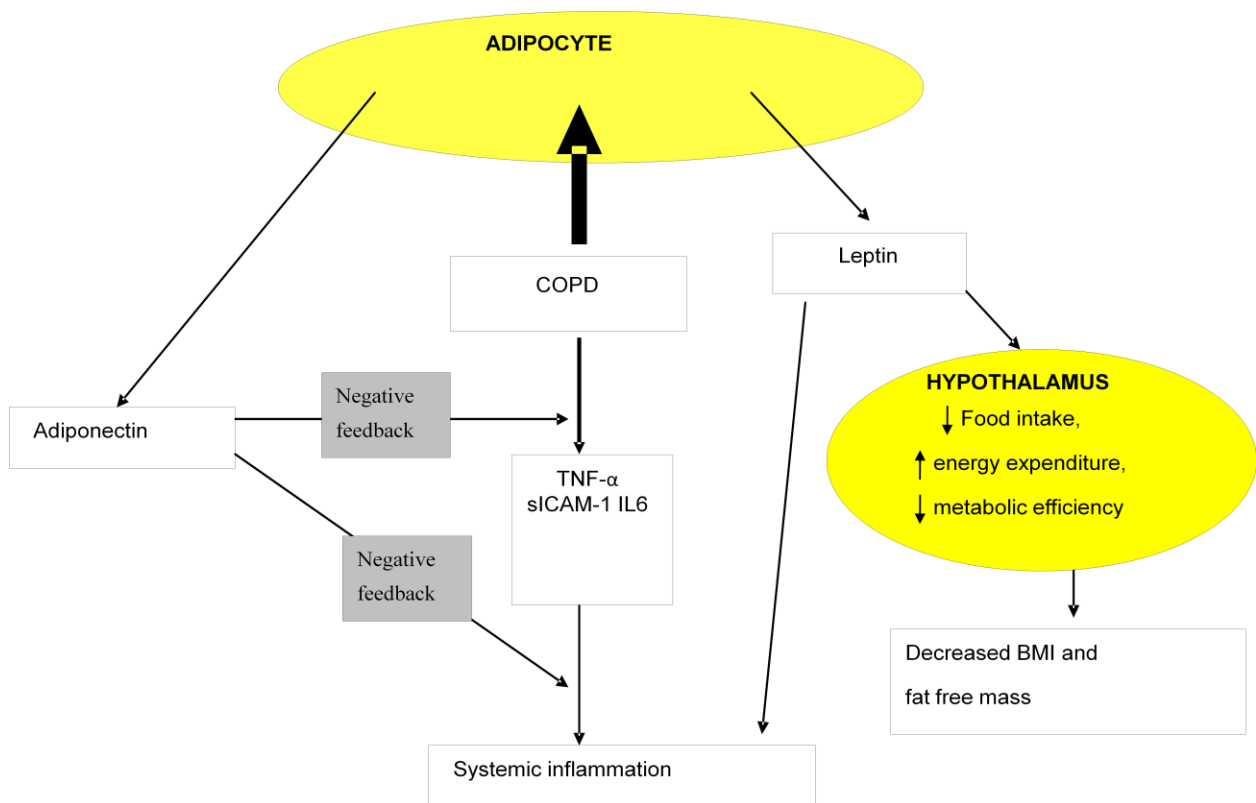


#### *1.3.7.4 Circulating Markers*

There has been increasing interest in peripheral blood biomarkers for use in patients with COPD, since they have many advantages in terms of collection (reproducible methodology of collection; minimal staff training; limited equipment required at the point of collection) and patient acceptability, even for repeated measures. A number of plasma or serum markers have been evaluated in many studies of patients with COPD:

##### *(a) Circulating Cytokines*

Raised levels of TNF- $\alpha$  and its receptor have been observed in patients with COPD associated with hypoxaemia [165], cachexia, skeletal muscle wasting and weakness [166], however, this may reflect association rather than causation (see 7.3.4). Also, elevated levels of the pro-inflammatory cytokine IL-8 (and other similar chemokines) have also been associated with muscle weakness in patients with COPD [167]. IL-8 is produced by leucocytes, as well as airway epithelial cells, in response to cigarette smoke [168], TNF- $\alpha$ , IL-1 $\beta$  [169] or bacteria [170] and these potential stimuli are either present or increased in subjects with COPD or have an associated gene polymorphism [171]. Additionally, the adipokine leptin, which is important in the regulation of energy balance, is found in lower concentrations in plasma from subjects with COPD [165], and its potential role in inflammation is summarised in Figure 6.



*Figure 6: The inflammatory role of the adipocyte [172]. Leptin and adiponectin appear to be important in systemic inflammatory processes such as COPD, particularly during acute exacerbations. The primary role of **LEPTIN** is to influence energy balance via the hypothalamus by decreasing food intake and metabolic efficiency while increasing energy expenditure, however leptin may also be important in systemic inflammation since it can stimulate neutrophil and macrophage chemotaxis and activation, and influence T lymphocytes. Leptin therefore stimulates both inflammation and weight loss, while **ADIPONECTIN** inhibits production of TNF- $\alpha$ , IL6 and ICAM-1, and may also inhibit the pro-inflammatory activities of these cytokines, and is therefore anti-inflammatory. However, disorders associated with increased NE activity (including COPD) may be associated with reduced adiponectin anti-inflammatory activity since NE can cleave adiponectin. This dysregulated leptin/adiponectin cycle may therefore be self-perpetuating in subjects with COPD [172].*

Levels of circulating cytokines may be increased through a ‘systemic overspill’ of mediators originally released within the lung [173]. However, it appears more complex since the levels of circulating cytokines do not necessarily relate to their concentration within the airways, a relationship which would be expected if overspill occurs. However, there may be a number of

explanations for this disparity including the variability of the biomarkers within sputum [141], facilitated protein transport from (or to) the lung, local autocrine and paracrine effects and systemic uptake [173]. Also, it is questionable whether these cytokines are central to the pathology of COPD or are secondary phenomena which relate to other features of COPD (such as bacterial colonisation) and therefore do not truly reflect concomitant COPD disease activity. Further longitudinal studies are therefore required to explore the relationship (if any) of these systemic cytokines to the disease process in subjects with COPD.

*(b) Circulating Acute Phase Proteins*

In comparison to healthy controls, subjects with COPD have higher average plasma IL-6, an inflammatory mediator which increases the expression of acute phase proteins (such as C-Reactive Protein (CRP)) and is associated with both reduced muscle strength and exercise capacity [174]. Also, the concentration of CRP relates to mortality in people with mild-to-moderate (but not severe) COPD [132]; health status; exercise capacity [175]; and risk of cardiovascular disease [176]. Elevated levels of CRP occur in some patients during acute exacerbations of COPD, and it has been shown to be a sensitive marker of exacerbations in the presence of one or more symptoms [177], although the diagnostic sensitivity was found to be less in other studies [178]. Additionally, CRP relates cross-sectionally to FEV<sub>1</sub>, however does not predict a more rapid subsequent FEV<sub>1</sub> decline [179].

Fibrinogen is also an acute phase protein which is elevated during exacerbations and is associated with an increased risk of hospitalisation, frequent exacerbations and inversely with FEV<sub>1</sub> [180]. However, studies have not shown a relationship between fibrinogen and disease progression [181] nor is it a sensitive marker of therapeutic intervention [182].

Surfactant protein D (SPD) is a large hydrophilic protein found within the endoplasmic reticulum of type II pneumocytes and secretory granules of Clara cells and has been suggested as a lung specific biomarker. Lomas et al [183] demonstrated that this marker is slightly, though significantly higher in subjects with COPD relative to controls (121.1 vs. 114.3 ng/ml) and higher levels were associated with increased risk of exacerbations. However, the greatest difference was seen between non-smokers and current smokers and the marker did not correlate with FEV<sub>1</sub>, visual emphysema or objective emphysema scores. Although values decreased in subjects receiving steroid therapy, it was not certain whether this reflects reduced leakage from the lung, reduced production or molecular and hence immunological change [184]. Additionally, while reasonable short term reproducibility has been demonstrated, there are no long term data.

More recently, the ECLIPSE study demonstrated an association between disease progression measured by FEV<sub>1</sub> decline over a 3 year period and Clara Cell Secretory Protein 16 (CC-16), but not fibrinogen, IL-6, IL-8, TNF- $\alpha$ , CRP or SPD [181]. However, the association was weak, the statistical testing did not account for multiple analyses, and furthermore, there was no relationship between CC-16 and emphysema severity measured by CT densitometry [185]. CC-16 is produced by a number of tissues, although serum levels are primarily influenced by secretion from cells of the lower respiratory tract [186], and it has immunomodulatory effects

including protection against oxidative stress. Although CC-16 may be relatively specific to the lung, any pathological process within the lung has the potential to cause depleted concentrations [185], and its relationship to disease activity in subjects with COPD is questionable since it may simply be a measure of general inflammation or reflect past events causing airway damage.

*(c) Circulating Cells*

A number of abnormalities may occur within circulating leucocytes, which relate to overall mortality in subjects with COPD [187]. Also, an inverse relationship exists between FEV<sub>1</sub> and circulating neutrophil numbers [188]. In addition, neutrophils from subjects with COPD exhibit both abnormal chemotactic responses [58] and enhanced production of reactive oxygen species [189] compared to those from 'healthy' smokers and non-smokers. Monocytes also accumulate in the lungs of patients with COPD owing to an enhanced chemotactic response to CXCL-1 and CXCL-7, which may be explained by an abnormally high turnover of the monocyte receptor CXCR-2 [190]. Circulating [191] and alveolar macrophages [192] from patients with COPD also show greater spontaneous production of MMP-9 compared to healthy controls indicating a degree of activation and/or the production of potentially damaging proteases [162]. However, while these cells are important in the pathogenesis of COPD (see 1.2.4), their absolute numbers do not relate well to COPD severity or progression and therefore cannot be considered as biomarkers.

#### *1.3.7.5 Biomarkers of NE Activity*

##### **(a) Connective Tissue Destruction**

Elastin monomers are cross-linked by 2 amino acids (isodesmosine and desmosine) to form insoluble elastic fibres which are a key component of the skin, blood vessels and lung [193]. Elastin is degraded even in healthy individuals by NE and other proteases (although this is increased in pathological processes such as COPD) releasing isodesmosine and desmosine which may be detected in plasma, urine and sputum [194]. Although these amino acids are elastin specific, and have therefore been considered potential biomarkers of COPD, they are not specific to a pathological process and therefore urinary desmosine may be influenced by diet [195], renal function [196] or reflect tissue breakdown in other organs [197]. Urinary desmosine differs between healthy non-smokers, smokers with normal lung function and subjects with COPD [198], however it demonstrates poor stability in individuals over a 2 week period [199] (although this accuracy has been improved more recently using mass spectrometry (MS) technology [200]). Also, while urinary desmosine is higher in PiZ individuals than ‘healthy’ smokers [138], it is not influenced by A1AT augmentation therapy [140, 150]. Early studies did not show a correlation between desmosine and FEV<sub>1</sub> [35, 155, 198], although more recently a relationship was shown in subjects with COPD, but not healthy controls [201]. However, the relationships in this more recent study, while statistically significant, were relatively weak and also desmosine related to age, sex and weight (even in the healthy controls), demonstrating that this marker is not specific to disease states but may also reflect physiological processes.

Elastin peptides may also be useful biomarkers since some are found in subjects with COPD but not in healthy controls [202]. However, the studies are relatively small and further work would be required to validate these markers prospectively.

(b) Alpha-1-antitrypsin/neutrophil elastase complex (A1AT/NE complex)

When A1AT and NE interact, they bind (largely) irreversibly on an equimolar basis to form the A1AT/NE complex which can be measured in plasma or other bodily fluids. Although the complex concentration will be a surrogate measure of the total amount of NE released, it will not provide information about the duration of exposure of proteins to NE (and hence potential tissue damage) prior to its inhibition by A1AT. A1AT/NE complexes have previously been shown to be higher in subjects with an infective exacerbation of COPD compared to those in the stable state, however, there was no difference in A1AT/NE complex between subjects with COPD and healthy controls [203] which suggests that a measure of total NE release is an inadequate measure of the destructive potential of NE in the stable state. The A1AT/NE complex assay and the distinction between a measure of total NE release and pre-inhibition NE activity is considered later in the current thesis.

## **1.4 VALIDATION OF BIOMARKERS**

### **1.4.1 Markers of Disease Activity, Severity and Impact**

The markers used in COPD and emphysema (many of which are outlined above), could be categorised into 3 separate groups (disease activity, severity and impact) which provide differing information about the patient with COPD [204]. Disease *activity* describes the underlying pathophysiological process leading to end-organ damage (measured by a marker of disease *severity*) leading to an impact producing an adverse influence on the quality of life of a patient.

Disease activity is recognised to be distinct from disease severity since subjects with only limited impairment of a marker of disease severity (e.g. FEV1) may have very active disease and may or may not progress at a greater rate than those with ‘severe’ disease [89, 204], although true disease activity remains unclear in the absence of an effective marker. An ideal marker of disease activity would be a measure (biomarker) of the underlying pathophysiological process which leads to end-organ damage, with the associated development of symptoms and changes in a marker of disease severity such as FEV1, gas transfer or CT densitometry. The rate of change of markers of disease severity (e.g. change in FEV1) have also been considered to be markers of disease activity [204], with the caveat that they measure a single aspect of disease activity in COPD (namely disease activity leading to increased airflow obstruction for FEV1) rather than measuring overall disease activity. Furthermore, the rate of change of FEV1 could not be considered a gold standard marker of the activity of the disease leading to emphysema development, nor could it be



considered a direct marker of underlying pathophysiological disease activity. An assessment of overall disease activity would therefore require a composite score, which as a minimum should include measures of emphysema progression and airflow obstruction. The current thesis therefore considers the rate of change of FEV1 as well as gas transfer in an attempt to overcome some of these issues.

There is also controversy regarding the most appropriate methods to measure the rates of change of markers of disease severity such as CT densitometry (see 1.3.3) and FEV1 since the change of an absolute value of FEV1 is dependent on the baseline value [95]. For example, a 60 ml change in FEV1 is a relatively lesser change in someone with mild airflow obstruction compared to an individual with severe airflow obstruction. The use of change in % predicted or standardised residual values partly (but not completely [205]) compensates for this differential and is therefore used in the current thesis, however, this anomaly requires careful consideration when discussing disease activity. With this in mind, and in the absence of sensitive markers, the description of different rates of disease activity at different disease severity stages should be considered with caution. Furthermore, the rate of change of FEV1 is essentially a surrogate, rather than direct, marker of a single aspect of the underlying pathophysiological disease activity in patients with COPD since a change in FEV1 will only occur in situations where the underlying disease process specifically alters airflow obstruction rather than being a direct measure of other underlying processes. Additionally, FEV1 relates to overall mortality and is therefore at least partly a surrogate marker of overall health, rather than a specific marker of disease severity (or activity in the case of rate of change of FEV1) [206]. All these factors further support the need for new

biomarkers of disease activity for use in highly characterised patients who have or are at risk of developing COPD and emphysema.

Measures of disease severity would include spirometry, gas transfer and HRCT thorax scans, while it has been suggested that measures of disease impact would include the mMRC, daily activity score and COPD assessment test [204]. However, the 6 minute walk test (and equivalent tests) could arguably be described as either a measure of disease severity (since those with more severe disease may be expected to walk a shorter distance in 6 minutes) or a measure of impact since it is a measure of the influence of the severity of the disease (muscular dysfunction, breathlessness, fatigability) on an everyday activity. Additionally, the impact of a disease relates to the complex interaction between the individual's disease severity, their perception of symptoms and the influence of the disease on their everyday life (which may also vary depending on lifestyle choices). This may differ considerably between people who otherwise have similar disease severity or even similar symptoms, and could also be considered in terms of disability (symptoms) and handicap (impact). Therefore the 'impact' of a disease may only be truly measured by a marker of quality of life, while a 4<sup>th</sup> group of tests may be required to measure the symptoms of the disease, since moderate symptoms may have little impact on the quality of life experienced by some subjects yet warrant inclusion in any composite measure of COPD.

### **1.4.2 Validation of the Ideal Biomarker of Disease Activity**

A large number of studies have now reported data demonstrating a relationship between a range of proteins and inflammatory mediators and disease activity or severity in patients with COPD, and also allude to their potential as biomarkers, however few have been effectively validated. This validation step is critical, and although less validation may be required depending on the purpose of the biomarker, an ideal biomarker of COPD disease activity that will serve all purposes must [207]:

1. be central to the pathophysiological disease process
2. be either a direct measure of that disease process (or a close surrogate)
3. be stable and reproducible, but vary with events associated with disease progression
4. relate to disease severity
5. be responsive to therapies known to modify disease progression
6. predict individuals who will progress more quickly

It should be recognised that the relationship between a marker of disease severity and disease activity will be complex, since there is likely to be a relationship between a marker of current disease activity and future disease severity (assuming relative stability of disease activity between the point of measurement of the marker of activity and marker of future severity). Additionally, there will be at least a partial relationship between disease activity and contemporary disease severity in a cross-sectional study if disease activity is stable over a more prolonged period of time. These concepts are discussed further in subsequent sections of the current thesis.

Alternate criteria have also been suggested for the validation of biomarkers. Bucher et al [208] proposed that the biomarker concentration must firstly relate to the clinical endpoint and secondly would demonstrate a treatment effect allied with evidence of clinical improvement in an RCT. If applied to biomarkers of disease activity in COPD, the use of these criteria must therefore also assume stability of the disease activity and the marker over time (since a change in a clinical outcome marker such as FEV1 or mortality may only be measured after observation of the patients for several years). Also, since no therapies have been conclusively shown to influence disease activity and survival in patients with COPD these criteria are of limited use to describe valid biomarkers for COPD.

Sin et al [209] also proposed criteria for an ideal biomarker specifically for use in COPD which include 5 criteria similar to those proposed by Stockley [207]. Rather than a relationship to disease severity, however, they suggest an equivalent requirement that there should be 'a strong, consistent and independent association between the biomarker and chronic obstructive pulmonary disease'. Again, although this criterion is not fully defined, it would seem to assume a stable marker and stable disease activity. This will be discussed further in section 7.3.4.

To date no biomarker has fulfilled the validation criteria proposed by Stockley [207] or by Sin et al [209], and therefore the implications of the results of many biomarker studies remain unclear, and it is uncertain whether many inflammatory mediators are integral to the pathophysiological process or are simply secondary to either the disease process or smoking itself. This is critical since a non-specific biomarker, such as CRP, may be elevated in patients with COPD through a

variety of mechanisms, not all of which may be directly associated with COPD disease activity (i.e. ongoing damage to the airways or lung parenchyma). CRP cannot therefore be considered central to the pathophysiology of COPD and hence does not meet the first or second criteria. There is therefore an urgent need for new biomarkers of disease activity for use in patients with COPD which meet these validation criteria, particularly for phase II and III clinical trials where an early appraisal of treatment efficacy is crucial. It is also important to base further development of biomarkers on the current state of knowledge of the pathophysiology of COPD.

### 1.4.3 Biomarker Validation: Summary

Table 4 and Table 5 compare a selection of biomarkers and markers of disease severity against the criteria for an ideal biomarker [207], however the 3<sup>rd</sup> criterion is modified slightly, to separate it into stability and exacerbations. Exacerbations are included as one example of an event which is known to relate to disease progression in subjects with COPD.

Biomarker Criteria	1	2	3 – stability	3 – Exacerbations	4	5	6
Physiological	X	X	X (stability poor relative to progression)	√	√	√ (prolonged follow-up)	X
Radiological	X	X	√ (using same scanner)	X	√	√	X
Patient reported outcomes	X	X	X	√ (relatively insensitive)	√	√ Relatively insensitive	X
Measures of exercise capacity	X	X	X	√	√	X	X
BODE	X	X	X	√	√	X	Partly

*Table 4: This table assesses the physiological, radiological and patient reported measures of COPD severity against the criteria for an ideal biomarker (in top row) comprising: 1. Central to the pathophysiology 2. A direct measure of the disease process 3a. Stability. 3b. Vary with (one) event known to relate to disease progression (i.e. exacerbations). 4. Relate to disease severity. 5. Responsive to therapies known to modify disease progression. 6. Predict individuals who will progress more quickly.*

Plasma Biomarker	1	2	3 – Stability	3 – Exacerbations	4	5	6
<b>TNF-<math>\alpha</math></b>	X	X	√ [210]	X [211]	Relates to some severe COPD phenotypes	X [212]	X [181]
<b>CRP</b>	X	X	X [211]	√ [211]	√ [181]	X [184, 213]	X [181]
<b>Surfactant protein D</b>	X	X	√ [211]	√ [211]	X [181]	Possible [183, 184]	X [181]
<b>CC-16</b>	X	X	√ [185]	X [211]	√ [181]	Insufficient data	Weak association [181]
<b>IL-6</b>	X	X	X [211]	√ [211]	X [181]	X [184, 213]	X [181]
<b>IL-8</b>	X	X	X [211]	X [211]	√ [181]	Insufficient data	X [181]
<b>Fibrinogen</b>	X	X	√ [211]	√ [211]	√ [181]	X [182]	X [181]

*Table 5: This table assesses a selection of plasma biomarkers against the criteria for an ideal biomarker (in top row) comprising: 1. Central to the pathophysiology 2. A direct measure of the disease process 3a. Stability (>50% of subjects have a repeat value within 25% of baseline). 3b. Vary with (one) event known to relate to disease progression (i.e. exacerbations). 4. Relate to disease severity. 5. Responsive to therapies known to modify disease progression. 6. Predict individuals who will progress more quickly. X= does not meet criteria, √ = meets criteria.*

## **1.5 DEVELOPMENT OF A NOVEL ASSAY FOR NE ACTIVITY**

### **1.5.1 Protease-antiprotease Imbalance is Central to the Pathophysiology of COPD**

The pathophysiology of COPD is complex, however it is increasingly accepted that protease-antiprotease imbalance, in particular relative local excesses of NE and Pr3, is central to the pathophysiology of COPD (see 1.2.4). However, the absence of specific assays for the *in vivo* measurement of NE and Pr3 activity prior to their inhibition has restricted the evaluation of their role, particularly in pathological states such as COPD, and therefore an alternate approach utilising surrogate markers of activity is required. Our research group, together with Merck (USA), therefore developed and validated a novel assay to a specific peptide formed when fibrinogen is degraded by NE, which provides a specific measure of NE activity prior to its inhibition. It is this validation which is described in the current thesis.

### **1.5.2 Development of A $\alpha$ -Val<sup>360</sup>: a specific marker of pre-inhibition NE activity**

**Data pertaining to this section has previously been published [214].**

Fibrinogen is a glycoprotein consisting of 3 chains, which may be cleaved by NE at multiple sites, however, the A $\alpha$ -Val<sup>360</sup> site was chosen for the assay since it is relatively large with a disulfide network and therefore more likely to be stable than other smaller cleavage products.

Rabbit antisera were generated to the free carboxyl group of A $\alpha$ -Val<sup>360</sup>, following conjugation of C(Nle)T<sup>355</sup>SESSV<sup>360</sup> to thyroglobulin using Sulfo-MBS, which ensures conjugation occurs at the amine group of the peptide therefore exposing its carboxyl group. A Europium based classical sandwich Elisa was then generated using the antisera. The specificity of the antisera, in particular to the terminal valine of the peptide sequence, is shown in Figure 7 [214], and further discussed in section 3.3.

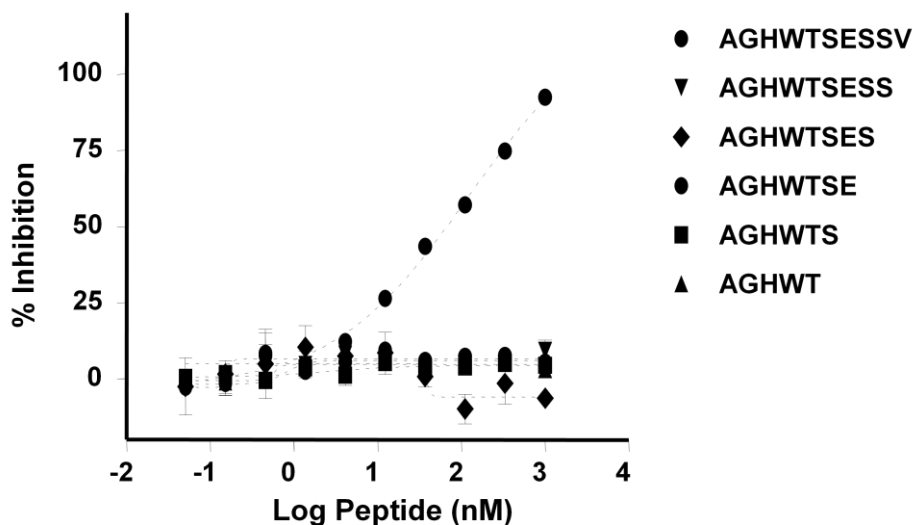


Figure 7: The specificity of the A $\alpha$ -Val<sup>360</sup> antibody. Removal of the terminal valine from the peptide sequence completely abrogates antibody binding.



### 1.5.3 Previous Studies Exploring Fibrinogen Cleavage Products as Potential Markers of NE Activity

NE cleaves the A $\alpha$  chain of fibrinogen at multiple sites, including A $\alpha$  -21 with the subsequent generation of a 21 amino acid fragment. This fragment includes fibrinopeptide A (FPA) which is the first 16 amino acids of the A $\alpha$  chain of fibrinogen (excluding the signal peptide) and a normal thrombin cleavage product of fibrinogen. Weitz et al [215] considered measurement of the A $\alpha$ -21 fragment concentrations may be an effective surrogate marker of NE activity *in vivo*, however they initially developed an assay (thrombin-increasable FPA immunoreactivity (TIFPA)) that measured baseline FPA concentrations indirectly. This assay required a 2 step analysis to determine the concentration of A $\alpha$ -21, necessitating precipitation of the remaining whole fibrinogen followed by incubation with thrombin to generate FPA, and derivation of the A $\alpha$ -21 concentration from the measured concentration of FPA by interpolation. TIFPA could therefore not be considered a specific measure of an NE cleavage product but rather a measure of the non-specific fragment FPA (the concentration of which would also reflect *in vivo* thrombin activity).

Weitz et al subsequently developed 2 further assays, one based on a specific antibody for A $\alpha$ -21 and a second assay (termed 'elastase-specific fibrinopeptide' (ESF)) which utilised an antibody with cross-reactivity to A $\alpha$ -21 in addition to smaller FPA containing fragments [216]. Of the 3 assays developed by Weitz et al, only A $\alpha$ -21 could therefore *be considered NE specific* since this is the only assay to include an antibody which binds to an NE specific cleavage point. Interestingly, *in vivo* experiments demonstrated that although TIFPA and ESF were highly correlated neither related well to the NE specific assay A $\alpha$ -21. The poor performance of the A $\alpha$ -

21 assay was explained by the rapid *in vivo* degradation of A $\alpha$ -21 to smaller peptides, a lack of stability which makes this a poor target for development as a biomarker. Also, the strong relationship between a measure of FPA (TIFPA) and ESF indicated that ESF was not entirely specific to NE cleavage. Furthermore, while they demonstrated a relationship between FEV<sub>1</sub> and ESF (in a study including only 12 PiZ subjects), no relationship was described between either TIFPA or A $\alpha$ -21 and FEV<sub>1</sub>. The work was never pursued on the basis of these uncertainties and there is therefore insufficient clinical evidence to support the use of the A $\alpha$ -21 peptide (or its derivatives) as a biomarker and there are also more fundamental concerns about the specificity of the assay.

Both animal and human studies have demonstrated the stability of the larger A $\alpha$ -Val<sup>360</sup> fragment of fibrinogen [214], which is an important characteristic of a biomarker. Also, the A $\alpha$ -Val<sup>360</sup> antibody is specific to the NE cleavage site on the A $\alpha$  chain of fibrinogen, and does not cross-react with other fragments of fibrinogen [214], and therefore is predominantly NE specific.

Although these data support the specificity of the A $\alpha$ -Val<sup>360</sup> ELISA to NE degradation products, more work was required to demonstrate the validity of A $\alpha$ -Val<sup>360</sup> as a biomarker in COPD and emphysema and this formed the basis for my thesis.

## **1.6 IDENTIFICATION OF PR3 SPECIFIC FIBRINOGEN CLEAVAGE PRODUCTS**

Pr3 is also likely to be central to the pathophysiology of COPD and emphysema and since it is less readily inhibited, particularly in the airways, a marker of Pr3 activity may relate better to the airways disease activity of COPD and emphysema than a marker of NE activity. The current thesis therefore also describes the identification of specific Pr3 fibrinogen cleavage products which may be used in future studies to develop a specific marker of Pr3 activity.

## **1.7 PURPOSES OF THE CURRENT THESIS**

### **1.7.1 Aims and Objectives: Studies Evaluating A $\alpha$ -Val<sup>360</sup>**

#### *1.7.1.1 Hypothesis*

A $\alpha$ -Val<sup>360</sup> is an ideal *in vivo* biomarker of pre-inhibition NE activity and hence COPD and emphysema disease activity in subjects with and without A1AT deficiency.

#### *1.7.1.2 Aims*

To evaluate:

- A $\alpha$ -Val<sup>360</sup> assay variability
  - *In vitro*
  - In blood (plasma and serum)
- Factors that influence the concentration of A $\alpha$ -Val<sup>360</sup>
  - Within an individual
    - Stability over time
    - Exacerbations
    - The influence of a therapeutic intervention
  - Between individuals (cross-sectional and longitudinal)
    - A1AT level and phenotype

- Demographic factors
- Smoking
- Physiological and radiological disease severity

## **1.7.2 Aims and Objectives: Identification of a Pr3 Specific Fibrinogen Cleavage Product**

### *1.7.2.1 Hypothesis*

In a similar manner to NE and A $\alpha$ -Val<sup>360</sup> (subsequent chapters and also [214, 217]), proteinase 3 (Pr3) will also produce specific fibrinogen cleavage products that can be identified using mass spectrometry (MS), and developed into a specific assay to determine pre-inhibition Pr3 activity *in vivo*.

### *1.7.2.2 Aims*

1. To generate specific Pr3 cleavage products of fibrinogen *in vitro*
2. To identify the *in vitro* Pr3 cleavage products using MS
3. To identify the terminal amino acid sequence of a large Pr3 fibrinogen cleavage product (which is more likely to be stable *in vivo*) by fragmentation and MS analysis

## **1.8 DEFINING THE STUDY POPULATIONS**

As discussed in section 1.1, most guidelines suggest that COPD is only diagnosed in the presence of airflow obstruction, where the  $FEV_1/FVC$  is less than 0.7 or the lower limit of normal. However, this definition will exclude subjects who nevertheless have significant pathology particularly those with emphysema without airflow obstruction [10] and it is therefore important to include patients in studies who do not necessarily meet these arbitrary airflow criteria. This may also allow the identification of patients with early (rather than mild) disease which may be more responsive to treatment [25].

The inclusion criteria for the individual studies reported in the current thesis are described in the methods section, however to ensure the studies were generalisable, the following only included subjects with a post-bronchodilator  $FEV_1/FVC < 0.7$  or healthy controls:

1. PiM subjects with COPD (section 3.1.3)
2. Variability Study (section 4.1)
3. Exacerbation Study (section 4.3)
4.  $A\alpha$ -Val<sup>360</sup> as a marker of A1AT augmentation therapy (Chapter 6) [inclusion criterion was a post bronchodilator  $FEV_1$ :slow vital capacity below 0.7]

The study investigating the use of  $A\alpha$ -Val<sup>360</sup> in subjects with symptoms of COPD unrelated to A1AT deficiency (Chapter 5) included patients who were current or ex-smokers (>10 pack year smoking history) with chronic bronchitis and associated dyspnoea. Spirometric criteria were not

used for this study to allow the inclusion of a broad range of phenotypes, however alternate and concomitant diseases were excluded by HRCT thorax scans and subgroup analyses were also performed for patients with a post-bronchodilator FEV1/FVC below the LLN.

The cross-sectional and longitudinal analyses in the study investigating subjects with A1AT deficiency (Chapter 7) included only PiZ A1AT deficient subjects (with and without airflow obstruction or emphysema) to investigate the differences in A $\alpha$ -Val<sup>360</sup> formation across a broad range of phenotypes.

## **CHAPTER 2.METHODS**

### **2.1 GENERAL METHODS**

#### **2.1.1 The Alpha-1-antitrypsin Deficiency Assessment and Programme for Treatment (ADAPT) Project**

The ADAPT project is a UK based registry established in 1996 which now includes over 900 A1AT deficient subjects (predominantly PiZ), who were either diagnosed following presentation with suggestive symptoms (index patients) or through family screening (non-index). Following the provision of informed consent, subjects attending the programme undergo annual full lung function testing, health status assessment and physical examination and also provide serum, plasma and sputum (where possible) at each visit. All subjects also have HRCT scans of the thorax, with most performed for quantitative assessment at baseline and/or subsequent visits. Subjects continue to attend for annual follow-up at the ADAPT centre until they die or withdraw from the study.

Data for the ADAPT subjects is included in 2 separate databases. The first clinical database was commenced in 1996 but was superseded by a more comprehensive database in 2000 (which includes all subsequent clinical data). For the current studies, a further (anonymised) database was established in Microsoft Access 2007 to unify both data sources and also include all laboratory analyses.



### **2.1.2 Ethical Approval and Informed Consent**

Ethical approval for studies relating to patients included in the ADAPT Registry was provided by the University Hospitals Birmingham NHS Trust Research Ethics Committee (local research ethics committee reference 3359). Ethical approval for all other studies was obtained from the South Birmingham Research Ethics Committee. Informed consent was provided by all participants.

### **2.1.3 Pulmonary Function Tests**

Lung function testing was performed by the physiologists in the Lung Function and Sleep Department, Queen Elizabeth Hospital Birmingham, United Kingdom in accordance with Association of Respiratory Technicians' and Physiologists' Guidelines and, to permit accurate longitudinal analysis, on the same equipment at each time point (where possible). Spirometry was performed post-bronchodilator (salbutamol 2.5 mg and ipratropium 500 mcg via a nebuliser unless otherwise stated) on a wedge bellows spirometer (Vitalograph, Buckinghamshire, UK). For many studies spirometry was also performed pre-bronchodilator. Lung volumes were performed by helium dilution and/or whole body plethysmography (Morgan Medical, Kent, UK). Gas transfer was measured by the single breath carbon monoxide method. The European Community for Steel and Coal reference equations [16, 218] were used to derive predicted values for spirometry, lung volumes and TLCO, while Cotes' reference equation [219] was used to calculate predicted values for KCO.

#### **2.1.4 HRCT Thorax Scans**

Scans at the ADAPT centre were performed in one millimetre slices at 10 mm intervals whilst the subject was breath-holding at full inspiration. Prior to December 2001, scans were performed on a 3<sup>rd</sup> generation scanner (General Electric Prospeed Scanner, General Electric Medical Systems, Milwaukee, USA) with a single row of Xenon detectors. Subsequent scans were performed on a General Electric Lightspeed scanner which is a 3<sup>rd</sup> generation helical scanner with 4 rows of detectors. CT scans were reported by an experienced thoracic radiologist for the presence or absence of visible emphysema using established criteria [220].

Although emphysema distribution within the lung may be heterogeneous, it characteristically involves the apical (usual COPD) or lower zones (A1AT deficient) [221] and therefore scanning of selected areas within these regions has been demonstrated to be highly sensitive while avoiding the need to assess the whole lung including less ‘active’ areas of emphysema progression and the accompanying increase in radiation exposure [222]. In the current thesis, the radiological lung density was therefore measured quantitatively in both the upper zone (level of aortic arch) and the lower zone (level of the inferior pulmonary vein) by assessing the proportion of the low density areas below a threshold of -910 Hounsfield Units and/or -950 Hounsfield Units using Density Mask Analysis. The 15<sup>th</sup> percentile point was also measured on many of the later scans using semi-automated computer software (Pulmo-CMS, MEDIS Medical Imaging Systems BV, Leiden, the Netherlands).

Volume correction is generally only required in longitudinal studies and was not performed for the majority of HRCT thorax scans described in the current thesis as most were performed on one occasion and correction requires 2 CT scans with differing volumes[223]. In the current thesis, this may reduce the sensitivity of the CT densitometry for detecting progression and increase variability [223] however uncorrected scans remain a valid form of assessment for patients with COPD and have been widely used in other studies.

## **2.1.5 Objective Quality of Life Assessment**

### *2.1.5.1 The St George's Respiratory Questionnaire (SGRQ)*

The SGRQ has been validated for use in subjects with asthma, COPD and bronchiectasis [224]. Subjects were provided with the questionnaire which they were asked to self-complete, following the guidelines provided with the SGRQ [225].

### *2.1.5.2 COPD Assessment Test (CAT)*

The CAT is a validated assessment tool also designed for self-completion which was introduced more recently to assess patients with COPD [123]. Since it includes only 8 items (Figure 8), it can be conducted in a timelier manner and is therefore suitable for either research or clinical management. Each item is accompanied by 2 statements which describe the best and worst scenario and patients decide where they lie on the scale (0 to 5) between these 2 scenarios. The overall score therefore ranges from 0 (best health status) to 40 (worst health status).

Your name:

Today's date:


  
COPD Assessment Test

## How is your COPD? Take the COPD Assessment Test™ (CAT)

This questionnaire will help you and your healthcare professional measure the impact COPD (Chronic Obstructive Pulmonary Disease) is having on your wellbeing and daily life. Your answers, and test score, can be used by you and your healthcare professional to help improve the management of your COPD and get the greatest benefit from treatment.

For each item below, place a mark (X) in the box that best describes you currently. Be sure to only select one response for each question.

Example:

I am very happy

0

X

2

3

4

5

I am very sad

I never cough	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	I cough all the time	SCORE
I have no phlegm (mucus) in my chest at all	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	My chest is completely full of phlegm (mucus)	
My chest does not feel tight at all	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	My chest feels very tight	
When I walk up a hill or one flight of stairs I am not breathless	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	When I walk up a hill or one flight of stairs I am very breathless	
I am not limited doing any activities at home	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	I am very limited doing activities at home	
I am confident leaving my home despite my lung condition	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	I am not at all confident leaving my home because of my lung condition	
I sleep soundly	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	I don't sleep soundly because of my lung condition	
I have lots of energy	<div>0</div> <div>1</div> <div>2</div> <div>3</div> <div>4</div> <div>5</div>	I have no energy at all	
			TOTAL SCORE

COPD Assessment Test and CAT logo is a trademark of the GlaxoSmithKline group of companies.  
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 RES/QST/09/43163/1 Date of preparation: September 2009.

Figure 8 The CAT is a validated tool designed to assess the quality of life experienced by patients with COPD. The questionnaire is intended for rapid self-completion, and is therefore appropriate for both research and clinical settings [123].

### **2.1.6 Sample Collection and Processing**

Spontaneous sputum samples were collected over a 4 hour period (from rising in the morning), and the sputum colour was assessed by experienced research staff using a standardised sputum colour chart (Bronkotest, Heredilab, Utah, USA). One aliquot was then ultracentrifuged at 50,000 g for 90 minutes in a Beckman-Coulter Avanti JE centrifuge at 4°C to prepare a sol phase sample. The remaining sputum was used for quantitative culture which was performed by an experienced microbiology technician as described previously [226]. Plasma samples were either collected in lithium heparin or EDTA coated tubes and cooled on ice prior to ultracentrifugation (within 2 hours of sample collection). All samples were stored at -70°C prior to measurement of mediator concentrations. Unless otherwise specified, the A $\alpha$ -Val<sup>360</sup> concentration was measured in plasma samples.

### **2.1.7 Statistical Analysis**

Statistical analysis was performed using PASW Statistics 18.0.0 for Microsoft Windows (Chicago, IL, USA). Normally distributed data were presented as mean ( $\pm$ SE), while non-parametric data were presented as median (interquartile range). Normality was confirmed using the Kolmogorov-Smirnov test and statistical significance was taken as  $p < 0.05$ . Statistical differences between means were assessed using independent t-tests (Mann-Whitney for non-parametric data), while paired t-tests (Wilcoxon Rank Tests for non-parametric data) were used for dependent variables. The significance of correlations was assessed using a Pearson correlation

for normally distributed data or Spearman's Rho for non-parametric data. Multivariate analysis was performed using linear regression (backward stepwise) analysis unless otherwise stated.

## **2.2 AA-VAL<sup>360</sup> ASSAY METHODS**

The assay was performed using a modified version of a protocol (outlined below) originally developed by Professor Rob Stockley (Queen Elizabeth Hospital Birmingham, UK) in conjunction with Merck (Rahway, NJ, USA).

Human fibrinogen (Merck, UK) in phosphate buffered saline (PBS) was incubated with human NE (Elastin Products Company, Missouri, USA) in PBS at a 200:1 molar ratio for 30 minutes at 37°C to produce cleaved fibrinogen. Aliquots of cleaved fibrinogen were frozen at -70°C until use.

Voller's buffer was prepared by adding 15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, and 3 mM NaN<sub>3</sub> to deionised water at a pH of 9.6. Fifty microlitres of the solution of cleaved human fibrinogen (diluted 1:1000 in Voller's buffer) were added to each well of an Opti-96 plate (labelled 'Plate A'), with 2 empty wells forming the negative control wells. Plate A was spun at 5000 g for 5 minutes to ensure uniform distribution of the cleaved fibrinogen solution and incubated overnight at 4°C with shaking.

Block buffer was prepared by adding 10 g of bovine serum albumin (Sigma-Aldrich, UK), 2 ml 10% NaN<sub>3</sub> and 0.5 ml of Tween 20 to one litre of 1x TBS. The CJTSESSV standard peptide (AltaBioscience, University of Birmingham, UK) was diluted in block buffer to form a 1:3 dilution series, with the highest concentration of peptide at 333.33 nM and the lowest 0.15 nM. Seventy five microlitres of each dilution of the standard peptide or 50 µl of plasma sample (diluted with 25 µl of block buffer) or 75 µl of block buffer alone (the positive control) were added to a low binding polypropylene plate ('Plate B') in triplicate. Seventy five microlitres of Aα-Val<sup>360</sup> antibody, diluted 1:2500 in block buffer, was added to each well of Plate B and this plate was incubated overnight at 4°C.

Following overnight incubation, Plate A (coated with cleaved fibrinogen) was washed 3 times with 300 µl/well of wash buffer (0.5 ml Tween 20 in 1000 ml 1x TBS), and after the final wash the plate was inverted and blotted against clean paper towels. All the wells of plate A were blocked with 200 µl/well of block buffer for one hour at 37°C and then washed (x3). One hundred microlitres of standard peptide/sample/control and antibody were then transferred from plate B to the corresponding wells on plate A. Plate A was then incubated at room temperature for 2 hours with shaking, before further washing (x3). One hundred microlitres of anti-Rabbit IgG Europium Antibody (Perkin-Elmer, UK) diluted 1:1500 in block buffer was then added to each well of plate A, prior to incubation at room temperature for one hour with shaking. Plate A was again washed (x3), 100 µl of enhancement solution (Perkin-Elmer, UK) added to each well and the plate was incubated at room temperature in the dark for 20 minutes with shaking. Plate A was read on a Biotek Synergy 2 Multimode Microplate Reader (Biotek, Winooski, VT, USA) at 25°C with the excitation wavelength set to 340 nm and the emission filter set to 620/40. The Aα-



Val<sup>360</sup> concentration was calculated by interpolation from the standard curve of the CJTSESSV peptide (which was generated for each plate).

#### *2.2.1.1 Aα-Val<sup>360</sup> Assay Variability*

See chapter 3.

## **2.3 OTHER BIOCHEMICAL ASSAYS**

### **2.3.1 Samples under or over standard range**

Repeat tests were performed for any samples falling outside of the standard working range of the assay, using a higher or lower dilution as applicable.

### **2.3.2 A1AT**

To ensure accuracy and repeatability, the A1AT levels and phenotype were performed by a single reference laboratory (Heredilab, Salt Lake City, USA). Where specified, an in house assay was also performed to quantify the A1AT concentration according to the following methods:

Coating antibody solution was prepared by adding 2 µL/ml of sheep antihuman A1AT to coating buffer (0.05 M Na<sub>2</sub>CO<sub>3</sub>; 0.05 M NaHCO<sub>3</sub>), and 200 µL was added to the inner wells of a Maxisorp microtitre plate (Nunc, UK) which was then incubated overnight at 4°C.

Wash buffer was prepared by adding 0.5 ml of Tween 20 (Sigma, UK), and one gram of bovine serum albumin (Sigma, UK) to one litre of PBS. Samples were diluted with wash buffer at a ratio of 1:20000. Each well was washed 3 times with 300 µL of wash buffer, and then 200 µL of sample or standard was added to the inner wells only. The plate was incubated for 2 hours at room temperature with shaking, and then washed (x3). Two hundred microlitres of horseradish-peroxidase conjugate (Sigma, UK) was added to each well, and the plate incubated for 2 hours at room temperature with shaking. After washing, 100 µL of tetramethylbenzidine solution (Sigma, UK) was added to each well and the plate incubated until a colour change occurred (approximately 2 minutes). The reaction was stopped with 50 µL/well of 0.1 M H<sub>2</sub>SO<sub>4</sub>, the absorbance measured on a plate reader at 450 nm (with 570 nm wavelength correction) and the A1AT concentrations interpolated from the standard curve.

Working range	2 ng/ml to 14 ng/ml (post-dilution)
Inter-assay Coefficient of Variation (CV)	10.20% (at upper end of working range)
Inter-assay CV	4.35% (at lower end of working range)

### 2.3.3 Alpha-1-antitrypsin-neutrophil Elastase Complex (A1AT/NE complex)

A1AT/NE complex was measured by ELISA using a commercially available kit (Merck4biosciences, UK). One hundred microlitres of standard or sample (diluted by sample diluent JA6404) were added to duplicate wells of a plate pre-coated with an antibody specific to human NE. The plate was incubated at room temperature for one hour with shaking.

Wash buffer was prepared by diluting wash buffer concentrate with deionised water, and each well was washed 4 times. One hundred and fifty microlitres of horseradish-peroxidase conjugated with rabbit anti-A1AT antibody was added to each well and the plate was incubated at room temperature for one hour with shaking. The plate was then washed (x4) and 200  $\mu$ L of tetramethylbenzidine (TMB) added to each well. The plate was incubated at room temperature until a colour change was observed within the lowest standard (approximately 20 minutes), and the reaction was then stopped by the addition of 50  $\mu$ L of 2 Molar (M) hydrochloric acid. The absorbance of each well was measured using a microplate reader set at 450 nanometres (with 570 nanometre (nm) wavelength correction). The concentration of A1AT/NE complex was then determined for each duplicate sample by interpolation from the standard curve.

Working range	0.00187 to 0.120 nM (post-dilution)
Inter-assay CV	14.68% (at lower end of working range)
Inter-assay CV	9.34% (at upper end of working range)

#### **2.3.4 MPO**

MPO was measured by ELISA using a commercially available kit (R&D Systems, UK). Samples were diluted in calibrator diluent RD6-57 and 50 µL of Assay Diluent RD1-62 was added to each well. Fifty microlitres of standard, control or diluted sample were added, and the plate incubated for 2 hours at room temperature with shaking. The wells were washed 4 times with 400 µL of wash buffer and then 200 µL of MPO conjugate added to each well. The plate was then incubated at room temperature for 2 hours with shaking and then washed (x4). Two hundred microlitres of substrate solution was added to each well, and the plate was then incubated for 20 minutes at room temperature in the dark, after which 50 µL of stop solution was added to each well. The absorbance was measured on a microplate reader set to 450 nm with wavelength correction set to 540 nm, and the MPO concentration determined from the standard curve.

Working range	10.42 nM – 666.67 nM (post-dilution)
Inter-assay CV	5.68% (at lower end of working range)
Inter-assay CV	4.59% (at upper end of working range)

#### **2.3.5 High Sensitivity C-Reactive Protein (hsCRP)**

This assay was performed using a commercially available kit (R&D Systems Europe, Abingdon, UK).

All samples were diluted with calibrator diluent (100 fold dilution). One hundred microlitres of assay diluent was added to each well, followed by 50  $\mu$ L of standard, control or diluted sample. The plate was covered and incubated for 2 hours at room temperature. The plate was then washed 4 times using an autoplate washer, and following the final wash the plate was inverted and blotted against clean paper towels. Two hundred microlitres of CRP conjugate was then added to each well, and the plate was incubated for 2 hours at room temperature. The wash step was repeated (x4) and then 200  $\mu$ L of substrate solution was added to each well, prior to incubation for 30 minutes at room temperature in the dark. Fifty microlitres of stop solution was then added to each well in a rapid action to ensure thorough mixing. A microplate reader set to 450 nm (with wavelength correction at 570 nm) was used to determine the optical density of each well. The CRP concentrations were determined by interpolation from the standard curve.

Working range	1.56 to 50 ng/ml (post-dilution)
Inter-assay CV	6.00% (at lower end of working range)
Inter-assay CV	6.60% (at upper end of working range)

### **2.3.6 LTB<sub>4</sub>**

LTB<sub>4</sub> was measured using a commercially available kit (R&D Systems Europe, Abingdon, UK).

One hundred microlitres of calibrator diluent was added to the non-specific binding (NSB) wells, while 50  $\mu$ L of calibrator diluent was added to the zero standard blank control ('B0') wells and

50  $\mu$ L of standard ('B'), control or sample was added to the remaining wells. Fifty microlitres of the primary antibody solution was added to all wells (except the NSB wells) and the plates were covered and incubated for one hour at room temperature on a plate shaker. Fifty microlitres of LTB4 conjugate was added to each well, the plate was re-covered and then incubated at room temperature for 3 hours on a shaker. The plate was washed (x4) on an autoplate washer, and after the final wash the plate was inverted and blotted on a clean paper towel. Two hundred microlitres of substrate solution was then added to each well and the plate was incubated for 30 minutes at room temperature in the dark. One hundred microlitres of stop solution was then added to each well and the optical density (OD) determined using a microplate reader set to 450 nm (with wavelength correction at 570 nm).

The duplicate readings for each standard ('B'), control and sample were averaged and then the NSB optical density was subtracted to produce the 'corrected OD'. The %B/B0 was calculated by dividing the corrected 'B' OD by the corrected 'B0' OD multiplied by 100. A standard curve was generated of %B/B0 against the log transformed known concentration of standards, from which the LTB4 concentration of the samples was obtained by interpolation.

Working range	10.3 to 2500 pg/ml (post-dilution)
Inter-assay CV	8.60% (at lower end of working range)
Inter-assay CV	8.40% (at upper end of working range)

### 2.3.7 Calprotectin

Calprotectin was measured using a commercially available ELISA (the PhiCalTest ELISA, Calpro AS, Norway).

Fifty microlitres of each standard, control or diluted sample were added in duplicate to the plate, prior to incubation at room temperature on a plate shaker for 45 minutes. The plate was washed 5 times on an autoplater washer and after the final wash, the plate was inverted and blotted on a dry paper towel. Fifty microlitres of the enzyme conjugate was then added to each well and the plate was then covered and incubated for 45 minutes on a plate shaker at room temperature. The plate was washed 5 times and then 100  $\mu$ L of enzyme substrate solution was added to each well. The plate was incubated for 30 minutes at room temperature in the dark and then 100  $\mu$ L of one molar NaOH stop solution was added to each well and the OD values were read at 400 nm (with wavelength correction at 470 nm). The calprotectin concentration in the samples was then determined by interpolation from the standard curve.

Working range	216 – 13,889 nM (post-dilution)
Inter-assay variability	14.8%

### 2.3.8 NE Activity Assay

The activity of NE was measured spectrophotometrically using an in-house method based on the original assay described by McGillivray et al [227].

NE buffer was prepared by combining 0.01 M Tris-HCl, 0.5 M NaCl and 0.1% Triton x100 at pH8.6. Doubling dilutions of NE (one  $\mu$ M to 15.62 nM) were made in NE buffer as standards. Thirty microlitres of standard or sample were added in triplicate to the plate followed by 150  $\mu$ L of either the synthetic substrate N-succinyl-ala<sub>3</sub>-p-nitroanilide (in PBS at pH 8.6) in 2 wells or buffer alone in one well (to provide the optical density control for each sample). The plates were incubated for 45 minutes at 37°C. The absorbance of each sample or standard was then measured at 410 nm, the absorbance of the relevant control well subtracted and the NE activity determined by interpolation from the standard curve.

Working range	15 nM to 1000 nM (post-dilution)
Inter-assay CV	4.56% (at lower end of working range)
Inter-assay CV	4.76% (at upper end of working range)



### 2.3.9 Interleukin-8 (IL-8) Assay

IL-8 was measured using a commercially available kit (R&D Systems Europe, Abingdon, UK). One hundred microlitres of assay diluent was added to each well, followed by 50  $\mu$ L of standard, control or sample. The plate was covered and incubated for 2 hours at room temperature. The plate was then washed 4 times using an autoplater washer and after the final wash, the plate was inverted and blotted against clean paper towels. One hundred microlitres of IL-8 conjugate was then added to all wells, the plate was re-covered and incubated for one hour at room temperature. The plate was again washed (x4) and 200  $\mu$ L of substrate solution added to each well, followed by a further 30 minute incubation at room temperature (in the dark). Fifty microlitres of stop solution was rapidly added to each well (to ensure thorough mixing) and the OD of each well was determined using a microplate reader at 450 nm (with wavelength correction at 570 nm). The optical densities of the (duplicate) samples were averaged and the concentrations determined by interpolation from the standard curve.

Working range	31.30 to 2000 pg/ml (post-dilution)
Inter-assay CV	6.24% (at lower end of working range)
Inter-assay CV	7.22% (at upper end of working range)

## **2.4 AA-VAL<sup>360</sup> ASSAY REPEATABILITY**

### **2.4.1 Interplate and Intraplate Repeatability (Peptide Standard)**

Four serial dilutions of the CJTSESSV peptide standard (AltaBioscience, University of Birmingham, UK) were generated, and the concentrations of A $\alpha$ -Val<sup>360</sup> were measured in each sample following the standard protocol. One serial dilution was designated to be the standard curve and the concentrations of A $\alpha$ -Val<sup>360</sup> in the remaining 3 serial dilutions were derived by extrapolation from this standard curve. These steps were repeated in 2 further assays and the intra-plate and inter-plate CV were calculated. Further assays were performed following minor adjustments to the assay protocol and plate reader settings to obtain the best CV. These settings were then used in all future experiments and are described earlier in this chapter (section 2.2).

### **2.4.2 Intraplate Repeatability (Plasma)**

If repeated testing is carried out on the same sample, all biochemical assays will exhibit a degree of unavoidable variation whether tested within the same plate (intra-assay variability) or in different assay runs (inter-assay variability). However this variability may be reduced by using the average measurements of 2 or more aliquots per plate to determine the concentration of the target peptide or protein within a sample. In order to establish the optimal number of sample aliquots per plate for the A $\alpha$ -Val<sup>360</sup> assay, plasma was obtained from one healthy volunteer into 4

ml Vacuette tubes (Greiner Bio-One, UK) coated with 7.2 mg of EDTA on a single occasion. Twelve 50 µl aliquots of this plasma sample were placed in separate wells and analysed on a single plate following the standard Aα-Val<sup>360</sup> protocol. The Aα-Val<sup>360</sup> value was then determined by interpolation from the standard curve using the averaged fluorescence output of 2, 3 or 4 wells (chosen at random from the 12 aliquots). These steps were repeated on 2 further plates to ensure that representative values were obtained, and the intraplate CVs were calculated for each potential combination.

In a further analysis, the Aα-Val<sup>360</sup> concentration was obtained by interpolation from the standard curve using the mean concentration of all 12 aliquots of the single plasma sample on a single plate. Variation from this derived concentration was then calculated using progressively fewer aliquots (selected from the same plate using random number generation). These data were used to establish the number of aliquots required to provide an acceptable balance between minimising the intra-assay CV for the use of least plasma.

### **2.4.3 Interplate Repeatability (Healthy Control Plasma)**

A plasma sample obtained from a healthy PiM individual was analysed in 9 separate assays to determine the *interplate* variability. Three aliquots of the plasma sample were analysed per plate since earlier experiments determined this minimised *intraplate* variability whilst using the least plasma. The interplate CV of any assay may be reduced by determining the average mediator

concentrations across 2 or more plates. The average A $\alpha$ -Val<sup>360</sup> was therefore derived from one, 2 or 3 plates (selected at random) and the interplate CV was then calculated across these groupings.

#### **2.4.4 Intraplate and Interplate Variability (PiM Subjects with COPD)**

It is possible that intraplate and interplate variability for the A $\alpha$ -Val<sup>360</sup> assay may be different in plasma obtained from subjects with COPD compared to PiM healthy controls. To determine whether this is the case, samples were obtained from 8 consecutive PiM subjects attending a COPD outpatient clinic. All provided written informed consent, were current or ex-smokers, and had a clinical history consistent with COPD with a post-bronchodilator FEV<sub>1</sub>/FVC below 0.7.

The A $\alpha$ -Val<sup>360</sup> concentration was measured in plasma samples obtained from all 8 subjects. The measurements were performed in 9 plates for each sample (with 3 aliquots of sample per plate), with the average measurement from 3 plates used to calculate the A $\alpha$ -Val<sup>360</sup> concentration and the interplate CV.

The A $\alpha$ -Val<sup>360</sup> concentration was also compared with the FEV<sub>1</sub>.

#### **2.4.5 Serum versus Plasma**

Analysis of mediator concentrations in blood may be performed using either serum or plasma samples and the choice of media may influence the assay performance. To demonstrate the

influence of serum or plasma on the A $\alpha$ -Val<sup>360</sup> assay, plasma (4 ml EDTA tube) and serum (10 ml tube containing serum clot activator) were obtained from 4 PiZ A1AT deficient individuals and 2 PiM healthy controls. Samples were obtained by venepuncture at a single time point for each individual and processed within 2 hours of collection. The concentration of A $\alpha$ -Val<sup>360</sup> was determined using the mean fluorescence output of 9 aliquots on a single plate. The serum and plasma were both analysed on the same assay to minimise the influence of assay variability, since the intraplate CV is less than the interplate CV.

## **2.5 PILOT STUDIES: AA-VAL<sup>360</sup> IN HEALTHY AND A1AT DEFICIENT SUBJECTS**

In this initial pilot study, plasma was obtained from 12 healthy controls and 15 PiZ A1AT deficient subjects. The A $\alpha$ -Val<sup>360</sup> (using the optimal methodology (section 2.2)), MPO and Calprotectin were measured in plasma both before and after the addition of a neutrophil activator (calcium ionophore A23187). Blood samples were drawn from all subjects into 2 lithium heparin tubes and 150 micromoles ( $\mu$ M) of calcium ionophore was added to one (within 90 minutes of sample collection). Both tubes were incubated at 37°C for 60 minutes after the addition of ionophore to one, and then centrifuged (500 g for 10 minutes) to obtain plasma which was then stored at -70°C until analysis. The FEV<sub>1</sub>, A1AT concentration, A $\alpha$ -Val<sup>360</sup>, MPO, A1AT/NE complex and Calprotectin were also measured in a larger group of 40 PiZ and 24 healthy individuals.

## **2.6 IN VIVO VARIABILITY**

### **2.6.1 The Variability Study**

It is important to assess for changes in marker concentrations over time, particularly in the presence or absence of factors known to influence progression. The Variability Study was designed to assess the variability of inflammatory indices in both plasma and spontaneous sputum samples from subjects with A1AT deficiency over an 84 day time period.

Subjects were assessed and both blood and sputum samples were obtained on 11 occasions (daily for 5 days, then on days 7, 14, 21, 28, 56 and 84). Clinical stability was assured by the use of a daily diary card [228] in which symptoms (including dyspnoea, sputum colour and volume) were recorded by each patient.

#### *Inclusion and Exclusion Criteria*

PiZ A1AT deficient patients with an  $FEV_1/FVC < 0.7$  who had a history of chronic bronchitis (as defined by MRC criteria [229]) and were daily sputum producers were recruited. Both clinical assessment and HRCT scanning of the thorax were used to exclude concomitant lung disease and to characterise the patients. All subjects attended the ADAPT assessment programme and provided further consent to participate in this study.

### **2.6.2 Variability over 3 consecutive annual measurements**

This study was designed to assess the variability of A $\alpha$ -Val<sup>360</sup> over a 2 year period. Plasma was obtained from 40 individuals who were selected at random from the ADAPT database of samples.

#### *Inclusion and Exclusion Criteria*

All PiZ A1AT deficient patients were eligible if plasma samples were available for a minimum of 3 consecutive annual visits.

## **2.7 THE EXACERBATION STUDY (SUBJECTS WITH PIZ A1AT DEFICIENCY)**

Individuals who suffer more frequent exacerbations of COPD have a greater rate of disease progression [118]. Furthermore, these episodes are often associated with an inflammatory response [230] and hence it would be predicted that such episodes would be associated with an increase in A $\alpha$ -Val<sup>360</sup> as a marker of the NE activity during these episodes. To investigate this possibility, A $\alpha$ -Val<sup>360</sup> was measured in plasma obtained from 8 PiZ A1AT deficient subjects with chronic bronchitis during an acute exacerbation. Sputum and plasma samples were collected within 48 hours of the start of an exacerbation and the patient was then commenced on oral

antibiotics (amoxicillin or cefuroxime for 14 days) but not steroids. Sputum and plasma samples were also collected on 6 further occasions over a 28 day period. Sputum NE activity was measured spectrophotometrically as described earlier (section 0).

### *Inclusion and Exclusion Criteria*

Subjects with PiZ A1AT deficiency (and a post-bronchodilator  $FEV_1/FVC < 0.7$ ) were identified from the ADAPT registry and included in the study following further informed consent. An acute exacerbation of COPD was defined in the current study as a sustained increase in all three of the symptoms of sputum purulence, sputum volume and breathlessness compared to the usual clinical state [3].

## **2.8 AA-VAL<sup>360</sup> IN SUBJECTS WITH SYMPTOMS OF COPD UNRELATED TO A1AT DEFICIENCY**

Patients aged 40 to 80 years who were smokers or ex-smokers with a diagnosis of COPD (based on symptoms with and without supportive spirometry) and who presented in primary care with an acute exacerbation were recruited to the study. All had a history of chronic bronchitis [229] and exertional breathlessness and had the normal PiM A1AT phenotype (Heredilab, Salt Lake City, USA). Exacerbations were defined by the presence of increased dyspnoea, cough and sputum production (although the volume of the latter had not always increased) and new or increased sputum purulence that persisted for at least 2 days was a feature of a proportion [231].



Spirometric confirmation of COPD was not used as an entry requirement to allow the inclusion of participants with a broad range of phenotypes, and therefore subgroup analyses were also performed for patients with chronic bronchitis and dyspnoea but with an FEV<sub>1</sub> and FEV<sub>1</sub>/FVC within the normal range, and additionally for subjects with COPD defined spirometrically. Physiological normality was defined as within  $\pm 1.64$  standardised residuals as recommended by ATS/ERS guidelines to overcome sex and age differences in lung function, a threshold which is often termed the 'lower limit of normal' (LLN).[17]

Subjects were assessed at presentation with the exacerbation and all patients provided a spontaneous sample of sputum over a 4 hour period after waking. Sputum samples were analysed macroscopically using a standardised colour chart used to classify sputum colour (Bronkotest, Heredilab, Utah, USA) and those with mucopurulent or purulent sputum (grade 3 to 8) were treated with an antibiotic (oral cefuroxime), while those with mucoid sputum (grade 0 to 2) were not. The patients were assessed in detail 8 weeks after the episode (when in the stable clinical state) with full lung function tests and an HRCT scan of the thorax.

A $\alpha$ -Val<sup>360</sup> was measured in plasma samples obtained both at presentation with the exacerbation and when clinically stable using the highly specific assay as described earlier in section 2.2. In addition, comparisons were made with physiological parameters and visual assessment of the HRCT scan. All scans were assessed for the presence or absence of visible emphysema by an experienced thoracic radiologist using established criteria [220]. A $\alpha$ -Val<sup>360</sup> was also measured in plasma samples obtained from 39 healthy controls.

Finally, the patients were reviewed 4 years later (where possible) and repeat lung function testing and an HRCT scan of the thorax was performed at full inspiration using the same General Electric Prospeed Scanner. Densitometric analysis was undertaken to assess the severity of the emphysema and its progression accurately using the voxel index (-950 Hounsfield Units (HU)) and the 15<sup>th</sup> percentile point in both the upper zone (level of aortic arch) and lower zone (level of inferior pulmonary vein) (see 2.1.4). Post-bronchodilator (salbutamol 400 mcg and ipratropium 60 mcg via a large volume spacer) spirometry was performed using a wedge bellows spirometer (Vitalograph, Buckinghamshire, UK) and gas transfer by the single breath carbon monoxide method.

## **2.9 AA-VAL<sup>360</sup> AS A MEASURE OF THE EFFICACY OF AUGMENTATION THERAPY**

A $\alpha$ -Val<sup>360</sup> was measured in plasma samples obtained from participants in the EXACTLE trial [111], which was a randomised, double-blind, placebo controlled parallel group study conducted in Copenhagen (Denmark), Birmingham (UK) and Malmo (Sweden). Study participants were randomised to receive weekly intravenous infusions of either A1AT (60 mg/kg body weight of Prolastin; Talecris Biotherapeutics, NC, USA) or placebo (2% albumin) over a 24 to 36 month period. CT scans were performed at baseline, 12 and 24 months, while post-bronchodilator lung function was assessed at baseline, and at months 6, 12, 18, 24 and 36 (where participants continued to month 36).

In the EXACTLE trial, plasma samples were obtained at baseline (one month prior to the commencement of the interventional study product), month 6, month 12 and at the study termination visit (one week after the last infusion of the interventional study product). A 24 month sample was therefore not available from all 36 individuals in whom the trial was extended to 36 months. It was therefore decided to measure A $\alpha$ -Val<sup>360</sup> and A1AT/NE complexes at baseline and at 6 months to maximise the number of patients still participating at this time point and available samples.

#### *Inclusion and Exclusion Criteria*

The EXACTLE trial [111] required PiZ A1AT deficient subjects to be at least 18 years old, with a history of at least one exacerbation in the 2 years prior to screening for the study. Subjects were only included if they demonstrated a KCO below 80% predicted and/or a post-bronchodilator FEV<sub>1</sub> between 25% and 80% predicted with an FEV<sub>1</sub>:slow vital capacity below 0.7. Patients were excluded if their body mass was below 42 kg or greater than 92 kg; if they had undergone (or were on the waiting list for) any thoracic surgery; if they had smoked in the 6 months prior to screening; or had detectable cotinine in plasma.

Subjects were only included in the current study ('A $\alpha$ -Val<sup>360</sup> as a Measure of the Efficacy of Augmentation Therapy') if plasma samples were available at both baseline (pre-study intervention) and at month 6.

## **2.10 AA-VAL<sup>360</sup> IN A1AT DEFICIENT SUBJECTS: CROSS-SECTIONAL AND LONGITUDINAL RELATIONSHIPS**

Patients with PiZ A1AT deficiency who had been assessed at 3 or more annual visits were identified from the UK A1AT registry (ADAPT) which has been recruiting patients since 1997. All patients had a baseline HRCT thorax performed and at each visit had undergone full lung function testing and clinical assessment. Although the registry primarily includes PiZ subjects, a number of patients with alternate A1AT phenotypes had also undergone assessment and were included in the cross-sectional component of the current study, along with 24 never smoking PiM healthy controls (asymptomatic with lung function tests within the normal range). A1AT phenotypes were confirmed or determined in all subjects by a single reference laboratory (Heredilab, Salt Lake City, USA).

All subjects were assessed when in the clinically stable state, at least 6 weeks after any exacerbation, and patients who had received A1AT augmentation therapy were not included in the current study.

A $\alpha$ -Val<sup>360</sup> was measured in plasma samples using the highly specific assay as described earlier (section 2.2). Other inflammatory markers relevant to neutrophilic inflammation including MPO, IL-8 and LTB<sub>4</sub> in sol phase sputum samples and plasma hsCRP and A1AT/NE complexes were also measured as described in section 2.3.

Detailed cross-sectional and longitudinal analyses were only performed in the PiZ A1AT deficient subjects to reduce confounding. All patients completed the SGRQ [224], and a subset (those who had attended more recently after 2009) was also assessed using the CAT, which is a validated objective measures of the quality of life of patients with COPD [123].

### *Inclusion and Exclusion Criteria*

Patients with A1AT deficiency were identified from the UK A1AT registry (ADAPT). The aim of the current study was to evaluate A $\alpha$ -Val<sup>360</sup> in subjects with A1AT deficiency and therefore the inclusion criteria did not require participants to have either a diagnosis of COPD or abnormal lung function, to enable inclusion of patients with a broad range of clinical phenotypes. The detailed cross-sectional and longitudinal analyses only included PiZ subjects who had attended for assessment at 3 or more annual visits.

## **2.11 GENERATION AND IDENTIFICATION OF PR3 SPECIFIC FIBRINOGEN CLEAVAGE PRODUCTS**

### **2.11.1 Generation of a Pr3 Fibrinogen Cleavage Product**

Three test tubes containing solutions of 3.37  $\mu$ M human fibrinogen in PBS were warmed to 37°C in a water bath and NE (16.83 nM), Pr3 (16.83 nM) or PBS were added prior to incubation for 15 minutes. A 100  $\mu$ L aliquot was then extracted from each tube and mixed with an excess of 0.1% trifluoroacetic acid (TFA) to inhibit enzyme activity, and each sample was then frozen at -70°C until further analysis. Further 100  $\mu$ L aliquots were removed from the 3 test tubes at 30, 60, 120 and 180 minutes, inhibited as above and stored until analysed. This process was repeated on several occasions to produce samples for duplicate analysis to enable the assessment of the repeatability of fragment formation.

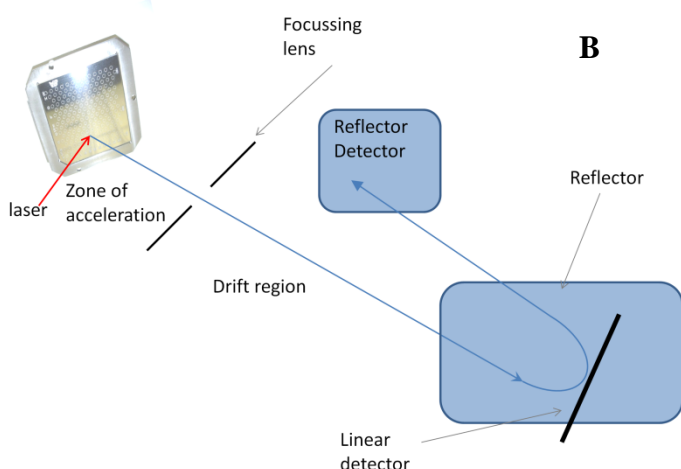
### **2.11.2 Mass Spectrometry**

An initial matrix assisted laser desorption/ionisation (MALDI)-MS analysis was performed to detect low molecular weight products in unmodified solutions of the fibrinogen cleavage products. Since the sensitivity and resolution of MALDI-MS decreases as the target peptide mass increases and also MS-MS does not easily identify peptides much larger than 30 to 40 residues

[232], analysis of larger molecular fragments was performed following gel electrophoresis and in-gel digestion (which is described in section 2.11.4).

A solution of  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) was prepared by adding 500  $\mu$ L of 100% acetonitrile to 5 mg of CHCA and 500  $\mu$ L of deionised water, followed by sonication for 5 minutes and then centrifugation (one minute at 13000 RPM). A solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) was prepared by adding 5 mg of sinapinic acid to 200  $\mu$ L of 50% acetonitrile and 0.5% TFA. This was sonicated for 5 minutes, and then centrifuged for one minute at 13000 RPM. One microlitre of each sample was then added to a MALDI plate (Figure 9) and overlaid with either one microlitre of sinapinic acid solution or 1.5  $\mu$ L of CHCA solution and 1.5  $\mu$ L of 10 mM ammonium bicarbonate. The CHCA and sinapinic acid are both aromatic ‘matrix’ compounds which absorb laser energy, and help prevent fragmentation of the peptides prior to analysis.

The plate (or the ‘target’) was then placed in the vacuum chamber of a MALDI-MS (UltrafleXtreme, Bruker). The analyte/matrix mixture was targeted with a laser which converted the analyte into gas phase ions (Figure 9). A delayed electrical field then accelerated the ions, which were focussed through a lens system before leaving the source, and which then travelled through a field-free region to the detector. The time of flight (TOF) of the singly charged ions produced in this process is inversely proportional to the square root of their molecular mass [233]. In addition to the samples, calibrants of known molecular weights were added to each plate to externally calibrate the spectra.



*Figure 9A: The plates used for MALDI TOF mass spectrometry. Each sample is added to a single 'spot' on each plate and then overlaid with an aromatic laser absorbing 'matrix' consisting of either sinapinic acid or CHCA with ammonium bicarbonate. 2B. The initial burst of ions generated by the laser pulse are accelerated into the flight tube towards the linear detector. For more detailed analysis, a constant electrostatic field is generated by the reflector which further separates parent ions and their fragments by their mass:charge ratio.*

MS was performed with the instrument in linear mode for fragments >5 kilodaltons (kDa) giving average masses at moderate resolution. For smaller peptides MS was performed in reflectron mode where an ion mirror focuses the ions to give much higher resolution (>15000 full width at half-maximum) allowing charge state and monoisotopic masses to be determined.



### *2.11.2.1 MALDI TOF-TOF and High Performance Liquid Chromatography (HPLC) MS-MS*

Tandem MS (MALDI TOF-TOF or MALDI MS-MS) was also used to identify peptides using the instrument in 'LIFT' mode. Following laser induced fragmentation of the peptides, the fragments corresponding to a particular parent ion are isolated by an initial time of flight 'gating', focussed and re-accelerated via the ion reflector to the detector, and this generates an MS peak list of the fragment ions.

Peptide identification was also assisted by HPLC MS-MS which fractionates the peptides, prior to MS analysis. This process enabled MS-MS spectra to be produced from a greater proportion of the peptides in complex mixtures, being particularly useful for separating peptides of a similar mass. The samples were centrifuged to remove large particles and were then mixed with 100  $\mu$ L of 0.5% TFA, prior to injection into an Acclaim PepMap100 C18 reverse-phase HPLC (75  $\mu$ M x 25 cm) column. The HPLC matrix was prepared by adding 10  $\mu$ L of 100 mM ammonium phosphate, 45  $\mu$ L of CHCA solution, 10  $\mu$ L of 10% TFA and 47  $\mu$ L of deionised water to 888  $\mu$ L of 100% acetonitrile. Two solvents were used in the column, namely 0.1% TFA in water and 0.1% TFA in 100% acetonitrile, at a flow rate of 350 nL/min. The HPLC eluent and matrix were added directly to a MALDI plate in up to 384 spots using an automated procedure (Proteineer fc II), followed by MS-MS analysis.

#### *2.11.2.2 Data Analysis*

Analysis was performed using peptide mass fingerprinting: a peak list was generated from the mass spectra and searched against the SWISS-PROT human sequence database using MASCOT to identify the intact peptides by their unique mass signature. The computer software has the capability to virtually ‘digest’ the intact protein or peptide using designated enzymes (e.g. trypsin), to produce lists of expected peptide fragments with specific and accurate molecular weights. This allows the identification of peptide fragments in an unknown solution using their precise molecular weight. Peptides were also identified by searching MS-MS data against the SWISS-PROT human database using MASCOT, using a 5% significance level for a peptide match.

#### **2.11.3 Gel Electrophoresis of Fibrinogen Cleavage Products**

Non-reducing and reducing polyacrylamide gel electrophoresis (PAGE) were used to fractionate NE and Pr3 cleavage products. A running buffer was prepared by adding 760 ml of deionised water to 40 ml of NuPage SDS running buffer. A 10% Bis-Tris Gel (1.0 mm x 10 wells; Nupage Novex, Invitrogen) was bathed in deionised water containing running buffer (with the addition of an antioxidant to the inner well of the electrophoresis apparatus for the reducing gel). Five microlitres of loading buffer was added to 25 µL of each sample, prior to loading into the gel. For the reducing gels, 3 µL of a reducing agent (500 mM DTT) was also added to the samples prior

to heating at 70°C for 10 minutes, which were subsequently loaded into the gel together with 10 µL of a molecular weight marker.

The gels were run at 200 volts until an acceptable separation of the bands of the molecular weight marker had occurred (around 2 hours).

#### **2.11.4 Mass Spectrometry of High Molecular Weight Products**

Although the resolution of MS decreases with increasing molecular weight, the larger molecular weight fibrinogen cleavage products are likely to be of greater importance as potential biomarkers since they would be expected to represent the first Pr3 related cleavage products of an intact fibrinogen molecule (and hence have the greatest specificity for Pr3) and are more likely to be stable with a greater circulating half-life [214]. Since direct MS analysis of large molecular weight proteins is difficult, an alternate approach was to use in-gel digestion followed by MS.

##### *2.11.4.1 In-Gel Digestion of Proteins Separated on PAGE*

The gel was placed on a clean, glass plate and the bands of interest were excised from the gel, prior to washing twice with 50% acetonitrile/50 mM ammonium bicarbonate for 45 minutes at 37°C. The bands were dried and then 50 mM dithiothreitol (DTT) was added followed by an incubation for one hour at 56°C. Iodoacetamide (100 mM) was then added followed by a further incubation for 30 minutes at room temperature. The bands were washed three times with 10%

acetonitrile/40 mM ammonium bicarbonate. Acetonitrile (100%) was added until the bands became opaque and shrunken, and they were then dried at 60°C. A known concentration of an enzyme (Asp-N, trypsin or Arg-C) was then added to the bands followed by incubation for one hour at room temperature. A solution of 10% acetonitrile and 40 mM ammonium bicarbonate was then added to the solution and the bands were incubated for 18 hours at 37°C. The supernatant was then extracted and retained, and the band mixed with 3% formic acid for one hour at 37°C. The supernatant was again extracted and retained before the final 30 minute incubation with 3% formic acid. The collected supernatant was then frozen at -70°C prior to further analysis.

#### **2.11.5 MS Analysis of Proteins Isolated by PAGE**

The samples were added to an MS plate and overlaid with either sinapinic acid or 10 mM ammonium phosphate and 5 mg/ml CHCA in 50% acetonitrile, prior to analysis using MALDI MS, MS-MS and HPLC MS-MS (as described earlier).

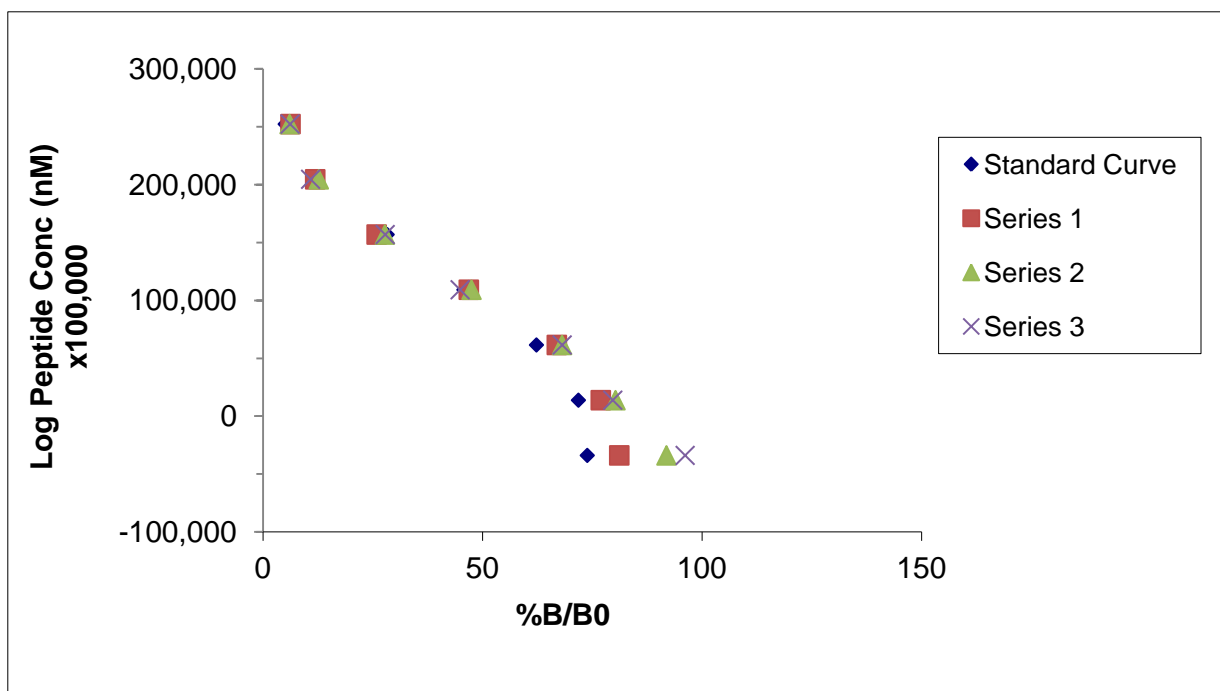
# CHAPTER 3. ASSAY VALIDATION AND THE PILOT

## STUDY: RESULTS AND DISCUSSION

### 3.1 ASSAY REPEATABILITY RESULTS

#### 3.1.1 Intra- and Interplate Repeatability (Peptide Standard)

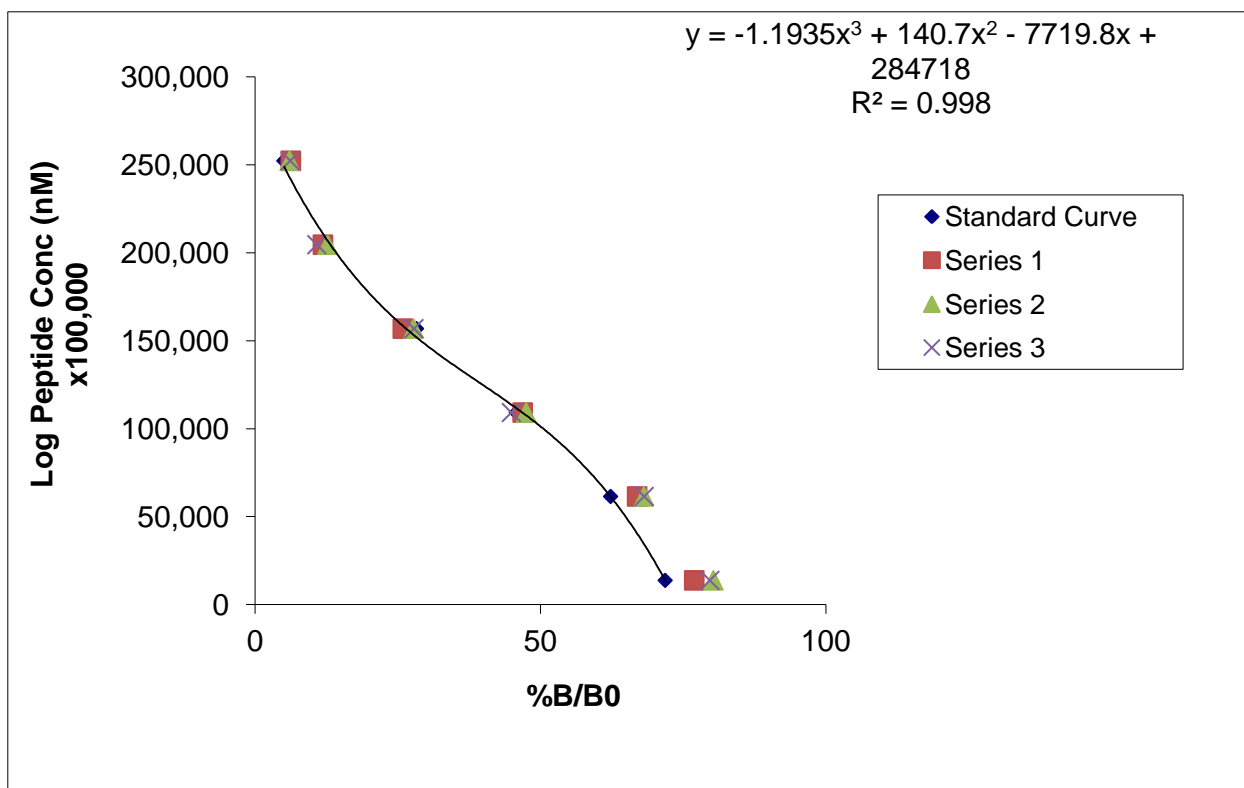
Following optimisation of the standard operating procedure, the A $\alpha$ -Val<sup>360</sup> assay demonstrated good *intra*-plate repeatability for the peptide standard, with CVs of 19.16% and 12.12% when measuring the fluorescence of 2 and 3 aliquots respectively, and repeated analysis demonstrated these CVs were consistent. However, even using 3 aliquots, the within plate CV increased as the peptide concentration decreased: 13.61%, 26.09% and 89.84% at 4.12, 1.37 and 0.46 nM respectively (**Figure 10**).



**Figure 10:** Variability of the A $\alpha$ -Val<sup>360</sup> peptide standard (range 0.46 nM to 333.33 nM). The chart shows the result of 3 series of experiments using different concentrations of the peptide. Each point represents the OD (expressed as %B/B0) against the log of the known concentration of the peptide. The variability increases as the known concentration decreases.

The *inter*-plate CV across the 3 assay plates (using the average fluorescence of 3 aliquots on each plate) also increased as the peptide concentration decreased and ranged from 9.87% for 333.33 nM to 44.07% for 1.37 nM and 95.57% for the 0.46 nM A $\alpha$ -Val<sup>360</sup> standard.

It was therefore determined that working range of the assay should include peptide concentrations between 1.37 nM and 333.33 nM (**Figure 11**). Within this range, the repeatability of the assay was deemed acceptable and consistent with the repeatability demonstrated by other assays or physiological tests [141, 211].



**Figure 11:** An example standard curve for the Aα-Val<sup>360</sup> assay, including the equation of the curve and its correlation coefficient ( $R^2$ ). Each point represents the OD (expressed as %B/B0) and known peptide concentration (expressed on a logarithmic scale). For comparison, the fluorescence measures for the 3 further serial dilutions of the peptide standard are included (range 1.37 nM to 333.33 nM).

### 3.1.2 Assay Repeatability (Plasma from a Single Healthy Control)

#### 3.1.2.1 Intraplate CV (Plasma)

The concentration of Aα-Val<sup>360</sup> was determined using the fluorescent readings from 2, 3 or 4 aliquots of the same sample on the same plate. The *intraplate* CV for the plasma sample varied depending on the number of aliquots analysed, with CVs of 13.39%, 9.44% and 6.43% using the average fluorescence of 2, 3 or 4 aliquots of the plasma standard respectively. However, there was little difference in the Aα-Val<sup>360</sup> concentration determined using 3 aliquots of a sample on a

single plate when compared to the A $\alpha$ -Val<sup>360</sup> concentration determined using the full 12 aliquots of the same sample (**Table 6**). Future A $\alpha$ -Val<sup>360</sup> assays were therefore performed by measuring the average fluorescence output of 3 aliquots of the sample on the same plate, since this offered both acceptable repeatability and economic sample use.

Number of aliquots Averaged	Concentration of A $\alpha$ -Val <sup>360</sup> (nM)	Variation from A $\alpha$ -Val <sup>360</sup> calculated using 12 aliquots (%)
2	5.92	13.15
3	7.26	6.63
4	7.14	4.90
5	6.38	6.37
6	7.27	6.75
8	7.27	6.78
9	7.23	6.17
10	6.91	1.43
11	6.66	2.26
12	6.81	0

**Table 6** The A $\alpha$ -Val<sup>360</sup> concentration was interpolated from the standard curve using the mean fluorescence output of a progressively greater number of aliquots of the plasma sample (selected at random) and the percentage variation from the measurement derived using the mean of 12 aliquots was calculated.

### 3.1.2.2 Interplate Repeatability (Plasma from a Single Healthy Control)

The A $\alpha$ -Val<sup>360</sup> concentration in the plasma from the single healthy control was measured using a total of 9 plates. Based on the data of the *intraplate* CV of the A $\alpha$ -Val<sup>360</sup> assay (section 3.1.2.1), 3 aliquots of sample were used per plate. The mean concentration of A $\alpha$ -Val<sup>360</sup> in the standard plasma sample measured in the 9 individual plates was 10.11 nM, with an interplate CV of 35.07%. The interplate CV was reduced to 23.59% by averaging the measurements of A $\alpha$ -Val<sup>360</sup> from 3 plates, however using the average of 2 plates did not influence the CV (35.02%).



### 3.1.3 Interplate CVs (Plasma from PiM Subjects with COPD)

The 8 patients with COPD (7 males and one female) included in this section of the study were recruited consecutively at a single specialist COPD clinic. Spirometry was available from 7 subjects (all male) as one subject was unable to produce consistent results (**Table 7**). The A $\alpha$ -Val<sup>360</sup> concentration was measured following the standard operating procedure in a single sample obtained from each individual and the concentration was derived from the average result of 3 plates. This was repeated on 2 further occasions (i.e. 9 plates in total) and the mean, standard deviation and interplate CV was calculated between these 3 results for each individual. When the average result from 3 plates was used to calculate the A $\alpha$ -Val<sup>360</sup> concentration, the mean interplate CV was 22.37% which is consistent with results from the healthy controls.

There was a negative correlation between the A $\alpha$ -Val<sup>360</sup> and the absolute FEV<sub>1</sub> values although this did not reach statistical significance in this relatively small group (r=-0.414; p=0.178; Figure 12) and there was no relationship with FEV<sub>1</sub> expressed as % predicted.

Subject number	Mean A $\alpha$ -Val <sup>360</sup> concentration nM (SD)	FEV <sub>1</sub> (L)	FEV <sub>1</sub> (% predicted)	Age (yrs)
1	5.12 (1.44)	1.42	82	84
2	2.85 (0.83)	0.61	23	68
3	5.00 (0.73)	NA	NA	71
4	3.00 (0.23)	1.72	50	50
5	4.60 (0.87)	1.04	56	86
6	2.46 (0.61)	1.12	26	47
7	4.25 (1.41)	1.11	45	79
8	8.87 (2.00)	0.59	23	69

**Table 7:** The mean A $\alpha$ -Val<sup>360</sup> concentration, FEV<sub>1</sub> and age of the 8 PiM subjects with COPD.

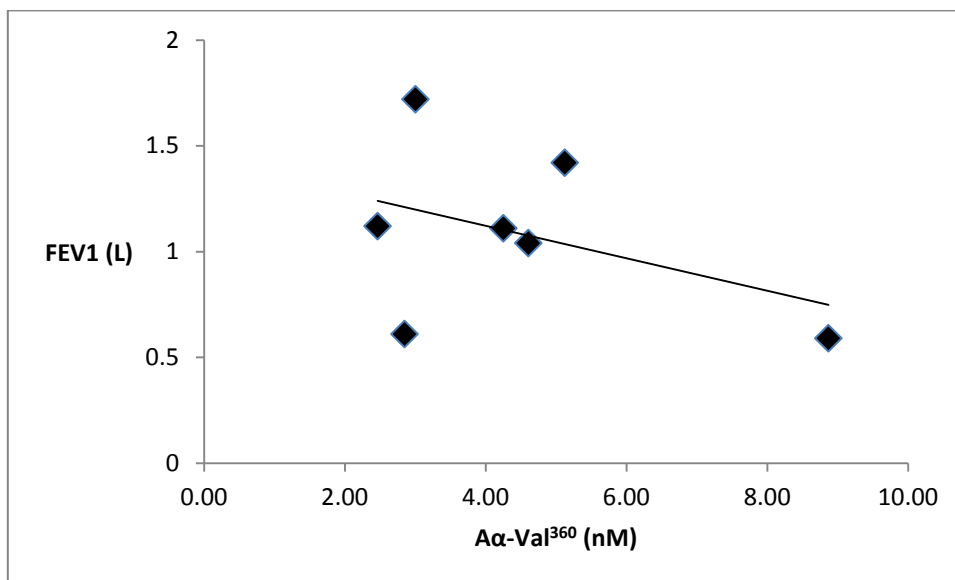


Figure 12: The relationship between the plasma Aα-Val<sup>360</sup> concentration and the FEV<sub>1</sub> in the 7 subjects with COPD for whom data were available ( $r=-0.414$ ,  $p=0.178$ ).

### 3.1.4 Serum versus Plasma

The repeatability and accuracy of the assay in serum compared to plasma was assessed using samples obtained from 6 subjects (4 PiZ A1AT deficient and 2 healthy PiM control subjects). The plasma and serum from a subject was analysed on the same plate to minimise variability. The intraplate CV for the plasma samples was similar to that obtained in earlier experiments (8.59%). However, the intraplate CV for Aα-Val<sup>360</sup> within serum was consistently higher than plasma while the detected concentration was lower. Similar differences were observed between plasma and serum samples from both PiZ and PiM patients (**Table 8**).

Subject number	Mean Plasma concentration (nM)	Mean Serum concentration (nM)	Serum standard deviation	Serum Intraplate CV	A1AT phenotype
1	12.11	3.46	0.57	16.33	PiZ
2	10.21	2.77	0.72	26.13	PiZ
3	12.41	4.25	0.77	18.22	PiZ
4	7.88	5.09	1.20	23.59	PiZ
5	1.96	0.36	0.13	36.29	PiM
6	2.17	0.11	0.04	31.61	PiM

**Table 8** The A $\alpha$ -Val<sup>360</sup> concentration was measured in both plasma and serum obtained from 6 subjects (4 PiZ and 2 PiM healthy control subjects). Serum and plasma samples from each individual were analysed in 9 aliquots in the same assay run and the mean A $\alpha$ -Val<sup>360</sup> concentration, standard deviation and CV were calculated. There was a significant reduction in the A $\alpha$ -Val<sup>360</sup> concentration detected in serum compared to plasma ( $p=0.013$ ). The results obtained for each individual in a single assay run are shown in the table, however, similar results were obtained when the samples were reanalysed in 2 further runs.

### **3.2 PILOT STUDIES: AA-VAL<sup>360</sup> IN HEALTHY AND A1AT DEFICIENT SUBJECTS RESULTS.**

**These data have been published in a high impact factor peer reviewed journal (Carter RI, et al. The fibrinogen cleavage product A $\alpha$ -Val<sup>360</sup>, a specific marker of neutrophil elastase activity in vivo. Thorax 2011; 66: 686 – 91).**

Following neutrophil stimulation with calcium ionophore, the production of both A $\alpha$ -Val<sup>360</sup> and A1AT/NE complex was both time and ionophore concentration dependent, with the maximal formation of A $\alpha$ -Val<sup>360</sup> occurring within 15 minutes [214]. The LDH concentration within the sample did not increase, which suggests NE was released by degranulation of the azurophil granule rather than neutrophil death. The data also demonstrate that A $\alpha$ -Val<sup>360</sup> is generated in the presence of high concentrations of the plasma NE inhibitors A1AT and  $\alpha$ 2-macroglobulin, which supports the premise that A $\alpha$ -Val<sup>360</sup> could also be generated within the target tissue.

A $\alpha$ -Val<sup>360</sup> was detected at a range of 3.5 to 18.9 nM in (non-stimulated) plasma samples from both healthy controls and PiZ subjects, however there was no relationship to the numbers of circulating neutrophils in either population. This suggests that A $\alpha$ -Val<sup>360</sup> is not generated within the blood sample following collection and also that the generation of A $\alpha$ -Val<sup>360</sup> reflects the amount of neutrophil degranulation rather than neutrophil numbers (at least in subjects who are in the stable state). The hypothesised relationship to neutrophil degranulation was further supported by the increase in A $\alpha$ -Val<sup>360</sup> following the addition of calcium ionophore. This rise was

accompanied by increases in both MPO and calprotectin, which are both markers of neutrophil degranulation (Table 9).

	A $\alpha$ -Val <sup>360</sup> (nM)			MPO (nM)			Calprotectin (nM)		
	Basal	Stimulated	<i>p</i> (basal vs. stimulated)	Basal	Stimulated	<i>p</i> (basal vs. stimulated)	Basal	Stimulated	<i>p</i> (basal vs. stimulated)
<b>Normal</b> ( <i>n</i> =12)	3.64 (0.38)	76.51 (6.03)	1.02x10 <sup>-7</sup>	15.57 (2.41)	321.05 (34.95)	2.67x10 <sup>-6</sup>	69.92 (13.94)	261.63 (29.42)	3.32x10 <sup>-4</sup>
<b>PiZ</b> ( <i>n</i> =15)	9.46 (1.06)	157.43 (20.60)	1.98x10 <sup>-5</sup>	20.16 (2.77)	291.40 (17.32)	1.19x10 <sup>-7</sup>	70.04 (18.28)	287.05 (80.33)	0.016

Table 9: The table shows the mean (SE) concentrations of A $\alpha$ -Val<sup>360</sup>, myeloperoxidase (MPO) and calprotectin measured in samples (stimulated and unstimulated) obtained from normal healthy individuals and PiZ A1AT deficient subjects.

In the 2<sup>nd</sup> part of the pilot study, which included 40 PiZ individuals and 24 healthy controls, the A $\alpha$ -Val<sup>360</sup> related to the concentrations of calprotectin, MPO and A1AT/NE complex. The A $\alpha$ -Val<sup>360</sup> concentration was also significantly greater ( $p < 0.001$ ) in plasma from subjects with A1AT deficiency with a mean of 9.28 nM (SE 0.61) compared to healthy controls with a mean of 3.16 (SE 0.25). Importantly, the A1AT/NE complex concentration did not vary between PiZ subjects

with a mean of 11.57  $\mu\text{M}$  (SE 1.40) compared to healthy controls with a mean of 13.05 (SE 1.96), although there was a slightly greater ( $p=0.042$ ) concentration of MPO with a mean of 21.12 (SE 1.94) and 15.62 (SE 2.05) respectively. The absence of a significant difference in A1AT/NE complex between PiZ patients and healthy controls, despite the significant difference in  $\text{A}\alpha\text{-Val}^{360}$ , suggests that there is greater pre-inhibition NE activity in PiZ subjects without an increase in total NE release. The differing relationships between  $\text{A}\alpha\text{-Val}^{360}$  and A1AT/NE complex concentrations are demonstrated in Figure 13 and would be consistent with the process of quantum proteolysis described by Campbell et al [52-54].

	$\text{A}\alpha\text{-Val}^{360}$ vs. A1AT/NE Complex	$\text{A}\alpha\text{-Val}^{360}$ vs. MPO	$\text{A}\alpha\text{-Val}^{360}$ vs. Calprotectin
<b>Normal</b> ( $n=24$ )	$r=0.414$ $p=0.045$	$r=0.694$ $p=1.66 \times 10^{-4}$	$r=0.535$ $p=0.007$
<b>PiZ</b> ( $n=40$ )	$r=0.644$ $p=7.25 \times 10^{-6}$	$r=0.762$ $p=1.11 \times 10^{-8}$	$r=0.492$ $p=0.001$

*Table 10: The table shows the Spearman correlation coefficients ( $r$ ) and significance ( $p$ ) for  $\text{A}\alpha\text{-Val}^{360}$  with A1AT/NE complex, myeloperoxidase (MPO), and Calprotectin (CP) for subjects with and without A1AT deficiency.*



### **3.3 DISCUSSION: ASSAY VALIDATION AND THE PILOT STUDY**

Prior to the evaluation of any assay in patient populations it is essential that adequate validation is performed which should include assessment of the lower limit of quantification, reproducibility and performance in relevant biological fluids [6]. There are a number of commercially available assays which are widely used to measure inflammatory indices, owing to their simple methodologies and reported reproducibility. Although many assays may be described by manufacturers to have been ‘validated’, this is usually performed *in vitro* and few quote the assay reproducibility in biological fluids [234]. Analysis of assay variability in biological fluids is important since mediator recovery may be influenced by protein binding or the presence of proteolytic enzymes. As a new assay, it was therefore important to subject A $\alpha$ -Val<sup>360</sup> to the vigorous validation outlined in this thesis.

A $\alpha$ -Val<sup>360</sup> is a unique assay which is based on a specific fibrinogen cleavage product, and demonstrates good intraplate and interplate variability. Although the CV of the peptide standard increased as the peptide concentration decreased, the CV was deemed acceptable within the working range of the assay (1.37 nM to 333.33 nM) and consistent with other assays [211]. The intraplate CV was improved by measuring 3 aliquots of the same sample on each plate, however measuring greater numbers did not influence the repeatability. Similarly the measurement of A $\alpha$ -Val<sup>360</sup> across 3 plates reduced the interplate variability still further (to around 22%) and this was consistent in samples obtained from healthy controls and PiM subjects with COPD. Further increases in the number of replicates of sample which were analysed did not influence the



variability and therefore subsequent assays were conducted using 3 aliquots of sample on each plate, with an average value taken from 3 plates. The total sample required for this analysis was 450  $\mu$ L.

### **3.3.1 Plasma versus Serum**

Lower concentrations of A $\alpha$ -Val<sup>360</sup> were detected in serum samples compared with plasma samples obtained from the same individual at the same time point. This difference is likely to be explained by clotting since serum is obtained from clotted blood using a clot activator prior to centrifugation. During clotting, the A $\alpha$  chain of fibrinogen is cleaved at A $\alpha$ -16R-17G by thrombin which produces fibrinopeptide A and initiates fibrin assembly [235] and furthermore, a number of other proteins (for example factor Xa) interact with fibrinogen, fibrin and/or their degradation products [199]. In these circumstances, binding of the A $\alpha$ -Val<sup>360</sup> peptide to the clot is likely to occur, and this may 'hide' or remove the A $\alpha$ -Val<sup>360</sup> neoepitope and hence reduce interaction with the A $\alpha$ -Val<sup>360</sup> antibody in the remaining serum.

### **3.3.2 Other Published Data**

Published data on the A $\alpha$ -Val<sup>360</sup> neoepitope also provide further evidence of the validity of this assay [214]. The A $\alpha$ -Val<sup>360</sup> antibody is highly specific and does not recognise peptides lacking the C-terminal valine. Furthermore, other proteinases (including plasmin and cathepsins B, H and L) do not generate A $\alpha$ -Val<sup>360</sup>, although Pr3 produces this neoepitope at 15% of the rate of NE.

Also the addition of L233, a specific intracellular NE inhibitor, completely blocked the formation of A1AT/NE complex and the generation of A $\alpha$ -Val<sup>360</sup> (even in the presence of the neutrophil stimulant calcium ionophore) which further confirms the NE specificity of this assay [214].

### **3.3.3 Relationship with FEV<sub>1</sub>**

Interestingly, even within the small group of subjects (n=8) with COPD in the initial part of the study, there was a negative correlation between the A $\alpha$ -Val<sup>360</sup> and the absolute FEV<sub>1</sub> (a widely used measure of disease severity in patients with COPD). Although this relationship did not reach statistical significance and there was no relationship with FEV<sub>1</sub> (% predicted), the sample size was small and therefore follow-up in a larger population was warranted and is described in this thesis.

### **3.3.4 Relationship with Other Markers of Neutrophil Degranulation**

Data from the pilot study demonstrate a good correlation between A $\alpha$ -Val<sup>360</sup> and MPO and calprotectin which are independent markers of neutrophil activation. MPO is stored within the azurophil granule and is released at degranulation along with NE, while Calprotectin accounts for 60% of the protein within the cytosol of neutrophils (although it is also found in monocytes and macrophages) and has both bacteriostatic and fungistatic properties [236]. The relationship between A $\alpha$ -Val<sup>360</sup>, MPO and calprotectin therefore indicates that A $\alpha$ -Val<sup>360</sup> is (at least partly) a marker of neutrophil degranulation.

### 3.3.5 Quantum Proteolysis

A marker of neutrophil degranulation provides indirect quantification of the total amount of NE likely to have been released, however this is an inadequate indicator of NE activity and hence tissue damage prior to NE inactivation. This was shown in elegant mathematical and *in vitro* studies by Campbell et al [52-54] which demonstrated an area of quantum proteolytic damage in the immediate vicinity of the neutrophil at degranulation, because NE is released at an exceptionally high concentration which exceeds the surrounding inhibitor concentration by 100 fold. Although the area of proteolytic damage is exponentially greater in subjects who are A1AT deficient, this phenomenon also occurs in individuals with a normal A1AT concentration. However, in either population when in the stable clinical state, all NE is eventually inhibited within the neutrophil microenvironment, (Figure 14) [52-54].

The mathematical and *in vitro* data of Campbell et al [52-54] are supported by the *in vivo* data from the current study which demonstrated that the A $\alpha$ -Val<sup>360</sup> concentration (and therefore pre-inhibition NE activity) was higher in subjects who were A1AT deficient than healthy controls, despite similar levels of NE release (A1AT/NE complex). The absence of a difference in A1AT/NE complex concentrations suggests that subjects with A1AT deficiency who are in the stable state do not have an overall increase in either neutrophil activity or degranulation. The development of the pathological changes of COPD in subjects with A1AT deficiency therefore relates to the greater volume of proteolytic damage around each point of NE release.

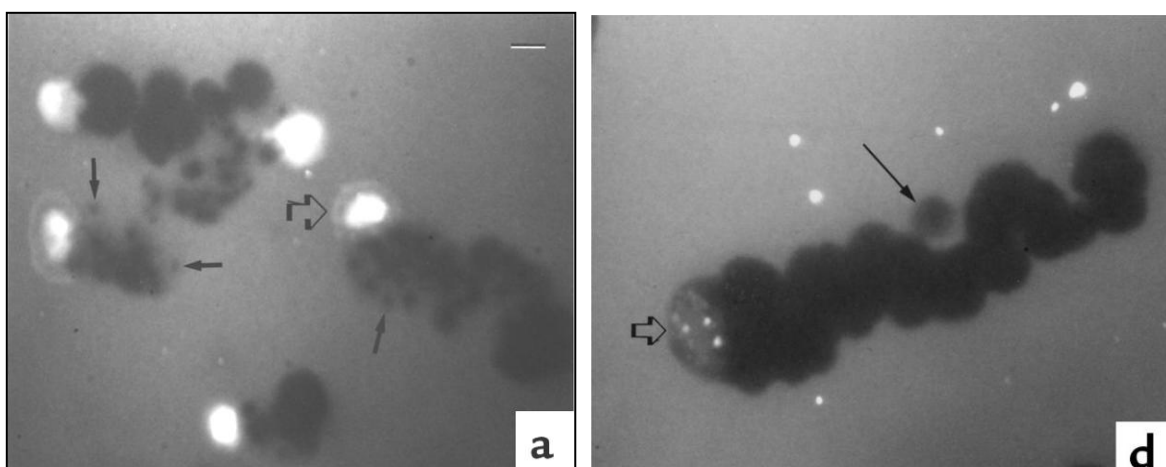


Figure 14: The figures illustrate time lapse photographs from the *in vitro* studies by Campbell et al [54]. Neutrophils were placed in sera from PiM individuals (a) and PiZ individuals (d) and incubated at 37°C for 30 minutes on opsonised fluoresceinated fibronectin. Neutrophils remaining on the surface are white because of ingested fibronectin fragments (broad arrows). The areas of quantum proteolysis (thin arrows) are exponentially larger in the PiZ serum than the PiM serum. Published with permission of JCI 1999; 104 (3):337-44.

### 3.4 SUMMARY: ASSAY VALIDATION AND THE PILOT STUDY

These initial data support the robustness of the A $\alpha$ -Val<sup>360</sup> assay which demonstrates an acceptable variability both *in vitro* and *in vivo* (in plasma from subjects with and without COPD). Although there is potentially some minor cross-reactivity with Pr3 mediated cleavage of fibrinogen, the assay is otherwise highly NE specific which was supported by the absence of A $\alpha$ -Val<sup>360</sup> generation by other enzymes or by neutrophils in the presence of a specific NE inhibitor. The initial data also suggest a relationship between the A $\alpha$ -Val<sup>360</sup> concentration and markers of disease severity (FEV<sub>1</sub>) which may reflect the disease process but required confirmation in the larger studies reported in subsequent chapters of the current thesis.

## CHAPTER 4. *IN VIVO* VARIABILITY

### 4.1 THE VARIABILITY STUDY

This study included 8 male and 2 female PiZ A1AT deficient subjects with chronic bronchitis and a post-bronchodilator  $FEV_1:FVC < 0.7$ . There were 2 current smokers, 5 ex-smokers and one never smoker, while 8 of the subjects were on inhaled steroids (one of whom was also being treated with a long-acting antimuscarinic). Other baseline demographic data are listed in Table 11. No subject experienced an exacerbation or medication change during the study or in the 8 weeks prior to commencement of the study, and there were no significant changes in symptoms on the patients' diary cards. All subjects were also seen on 11 occasions over an 84 day period when stability was confirmed by clinical examination and medication review.

Parameter	Mean (SE)
Age	52.03 (3.86)
BMI	23.29 (1.69)
Pack year smoking history	26.91 (5.27)
$FEV_1$ (litres)	1.24 (0.21)
$FEV_1$ (% predicted)	36.30 (4.22)
$FEV_1/FVC$	0.30 (0.04)
KCO (mmol/min/kPa/L)	0.94 (0.14)
KCO (% predicted)	56.82 (8.76)

*Table 11: Baseline demographic data for the 10 PiZ subjects included in the 'Variability Study'*

### 4.1.1 Cross-sectional Relationships

The mean biomarker concentrations (and within patient CVs) for each subject over the 11 visits are listed in Table 12. The mean A $\alpha$ -Val<sup>360</sup> concentration correlated well with both the baseline FEV<sub>1</sub> expressed as % predicted ( $r = -0.662$ ;  $p = 0.019$ ) and the baseline KCO expressed as % predicted ( $r = -0.514$ ,  $p = 0.064$ ). However, there were no cross-sectional relationships between the sputum markers and lung function, nor sputum markers and A $\alpha$ -Val<sup>360</sup>. There were however significant relationships between sputum MPO and both sputum IL-8 ( $r = 0.818$ ,  $p = 0.002$ ) and sputum LTB4 ( $r = 0.794$ ,  $p = 0.003$ ). Sputum IL-8 also correlated with sputum LTB4 ( $r = 0.731$ ,  $p = 0.008$ ).

Subject	Plasma A $\alpha$ -Val <sup>360</sup> (nM) Mean (SE/CV)	Sol MPO (nM) Mean (SE/CV)	Sol IL-8 (nM) Mean (SE/CV)	Sol LTB4 (pg/ml) Mean (SE/CV)
1	11.50 (0.20/5.77)	1.51 (0.14/31.39)	13.58 (1.39/33.93)	29.82 (6.70/74.49)
2	10.24 (0.53/17.18)	4.18 (0.23/18.06)	28.71 (3.78/43.72)	42.37 (7.40/57.91)
3	10.10 (0.85/27.77)	1.65 (0.10/20.83)	15.70 (2.10/44.40)	48.27 (13.81/94.87)
4	14.93 (0.70/15.51)	3.96 (0.89/74.76)	13.60 (1.86/45.23)	76.23 (24.69/107.41)
5	9.09 (0.21/7.53)	0.28 (0.03/36.84)	0.50 (0.15/97.27)	3.66 (1.21/110.41)
6	12.76 (0.91/23.56)	0.27 (0.03/39.52)	1.69 (0.31/61.24)	1.94 (0.31/53.48)
7	19.12 (1.26/21.88)	0.71 (0.03/12.31)	10.21 (1.55/50.32)	34.13 (6.11/59.40)
8	9.48 (0.16/5.72)	0.53 (0.02/15.74)	10.39 (1.85/59.14)	33.47 (23.09/228.90)
9	23.15 (0.91/13.01)	0.76 (0.03/11.77)	35.79 (3.47/32.14)	67.92 (16.01/78.15)
10	17.46 (1.06/20.18)	0.61 (0.05/27/25)	2.59 (0.81/104.23)	22.65 (7.35/107.60)
Mean	13.78 (1.51/15.81)	1.44 (0.46/28.85)	13.28 (3.62/57.16)	36.05 (7.65/97.26)

Table 12: The table displays the mean (SE/CV) concentration of the markers **by patient** over the 11 visits of the Variability study. The final row lists the mean marker concentration and SE for all 10 subjects as a group.

### 4.1.2 Longitudinal Analysis

The mean CV of 15.81% for the A $\alpha$ -Val<sup>360</sup> assay is consistent with the previously established inter- and intraplate variability, and was less than any other marker (Table 13). There were no longitudinal relationships between any of the biomarkers studied.

Day	Plasma A $\alpha$ -Val <sup>360</sup> (nM)	Sol MPO (nM)	Sol IL-8 (nM)	Sol LTB4 (pg/ml)
	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)
1	16.00 (2.27)	1.44 (0.44)	9.31 (3.56)	31.21 (12.37)
2	14.13 (1.99)	1.32 (0.43)	9.65 (3.03)	20.48 (6.77)
3	11.43 (1.94)	1.36 (0.45)	14.50 (4.76)	38.34 (13.70)
4	17.76 (3.81)	1.43 (0.49)	16.03 (5.99)	33.38 (8.24)
5	13.79 (1.52)	1.59 (0.63)	13.39 (3.49)	47.50 (25.17)
7	12.44 (1.34)	2.43 (1.19)	15.36 (5.00)	53.04 (29.96)
14	10.91 (1.54)	1.06 (0.32)	14.89 (4.72)	45.47 (19.02)
21	12.74 (1.28)	1.40 (0.43)	11.28 (3.19)	34.70 (11.19)
28	17.08 (1.71)	1.43 (0.45)	12.20 (2.62)	36.08 (10.77)
56	16.82 (3.09)	1.30 (0.34)	17.35 (4.79)	31.89 (8.68)
84	16.50 (3.45)	1.20 (0.31)	12.06 (3.04)	24.44 (7.30)
CV	15.81% (2.44)	28.85% (5.97)	57.16% (7.85)	97.26% (16.15)

*Table 13: The mean biomarker concentrations are listed **by day** of the study and include average data for all 10 subjects. The final row lists the mean (SE) CV for each marker (the CV was calculated for each patient over the 11 visits and then these results were averaged).*

The study also explored the use of a ‘rolling mean’ which has previously been used as a method of reducing the influence of inter-visit variability [141]. The 3 day rolling mean was calculated

for each subject by averaging the biomarker concentration for 3 visits, starting with visits one, 2 and 3 (termed visit 3.1) then visits 2, 3 and 4 (termed visit 3.2) and this process was continued until all visits were included. A similar process was conducted using a 5 day rolling mean (termed visit 5.1 onwards). Analysis was also performed of a 3 day mean in which there was no overlap of data (i.e. the mean biomarker concentration was calculated for visits one, 2 and 3, then 4, 5 and 6 etc until all visits were included). Analysis of the rolling mean data demonstrated a reduction in the mean CV for A $\alpha$ -Val<sup>360</sup> (Table 14 and Figure 15), however greater reductions were seen in the CVs for the sputum markers using this technique where greater variability of a single sample may be expected [141].

Method	A $\alpha$ -Val <sup>360</sup> mean CV (SE)	MPO mean CV (SE)	IL-8 mean CV (SE)	LTB4 mean CV (SE)
<b>Raw</b>	15.81 (2.45)	28.85 (5.97)	57.16 (7.85)	97.26 (16.16)
<b>3 day rolling</b>	14.44 (2.18)	15.80 (3.30)	28.12 (3.06)	48.16 (7.58)
<b>5 day rolling</b>	8.62 (1.48)	9.20 (1.92)	17.01 (1.65)	32.32 (4.12)
<b>3 day non-overlapping</b>	12.04 (1.91)	18.99 (4.49)	29.56 (4.21)	55.61 (8.46)

*Table 14. The table includes the mean (SE) CV data for each marker as raw data, as a 3 day rolling mean, 5 day rolling mean and 3 day non-overlapping mean. The CV for each marker was reduced by the average biomarker concentration from samples obtained over 3 or 5 days, rather than on one occasion.*



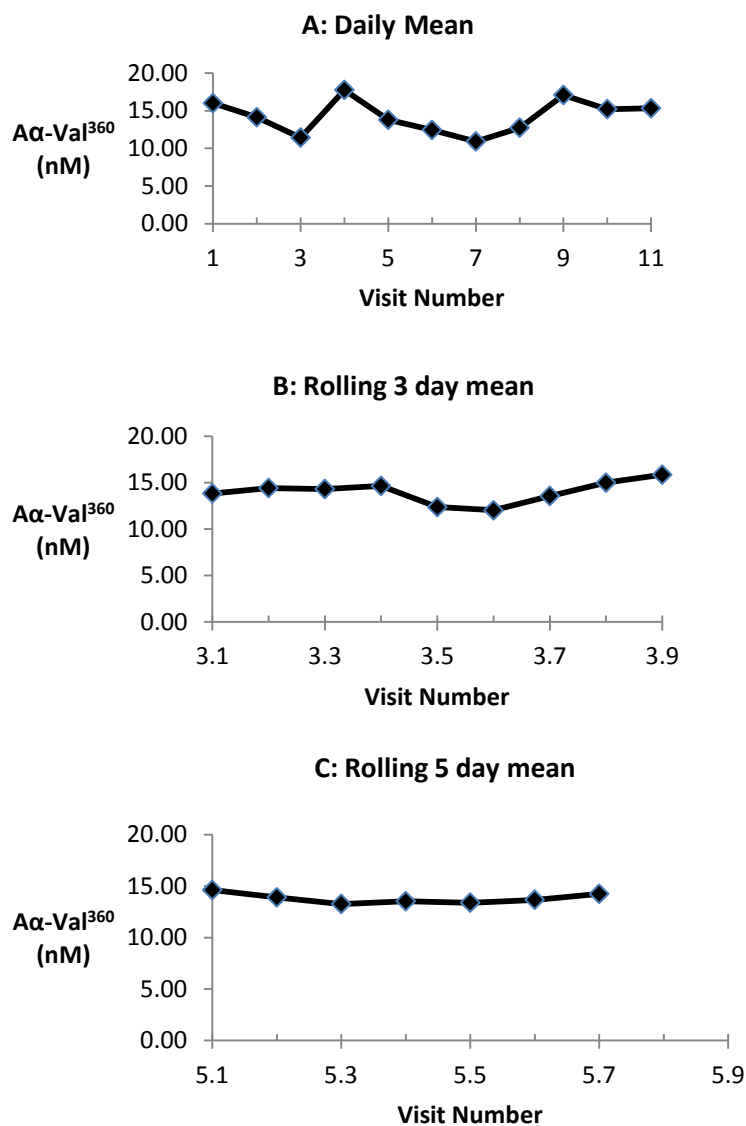


Figure 15A, each point represents the mean Aα-Val<sup>360</sup> for the 10 PiZ A1AT deficient patients at each visit. In B, each point represents the rolling mean of 3 visits (3.1 represents the mean of visits 1,2 and 3; 3.2 represents the mean of visits 2,3 and 4 etc). The mean CV was reduced to 14.44 (SE 2.18). In C, each point represents the rolling mean of 5 visits which reduced the mean CV to 8.62 (SE 1.48)

## 4.2 VARIABILITY OF AA-VAL360 OVER 3 CONSECUTIVE ANNUAL MEASUREMENTS

The study included 40 PiZ A1AT deficient subjects who were selected at random from the ADAPT UK registry. Their demographic data is described in Table 15. There was no significant difference in the A $\alpha$ -Val<sup>360</sup> concentrations over the 2 year period ( $p=0.554$ , Friedman's test) demonstrating the stability of this marker (Figure 16). The data for each individual is also shown (Figure 17).

	Mean (SE)
Age	52.48 (1.55)
FEV <sub>1</sub> (litres)	2.23 (0.25)
FEV <sub>1</sub> (% predicted)	70.13 (7.09)
FEV <sub>1</sub> /FVC	0.50 (0.04)
KCO (mmol/min/kPa/L)	1.13 (0.05)
KCO (% predicted)	71.00 (2.77)
Mean A $\alpha$ -Val <sup>360</sup> (nM) at 1 <sup>st</sup> visit	15.01 (1.53)

Table 15 Demographic data for subjects included in the 2 year variability study

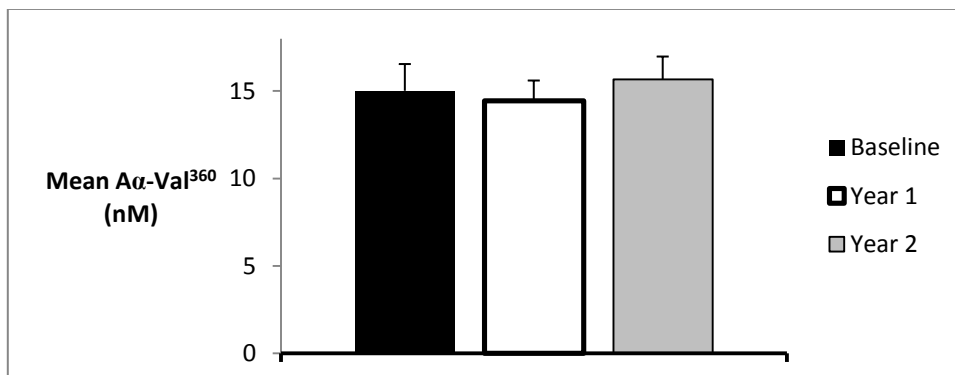


Figure 16: The mean (SE) A $\alpha$ -Val<sup>360</sup> concentrations for plasma obtained from 40 PiZ individuals at 3 consecutive annual visits demonstrating the stability of this marker of disease activity ( $p=0.554$ , Friedman's test).

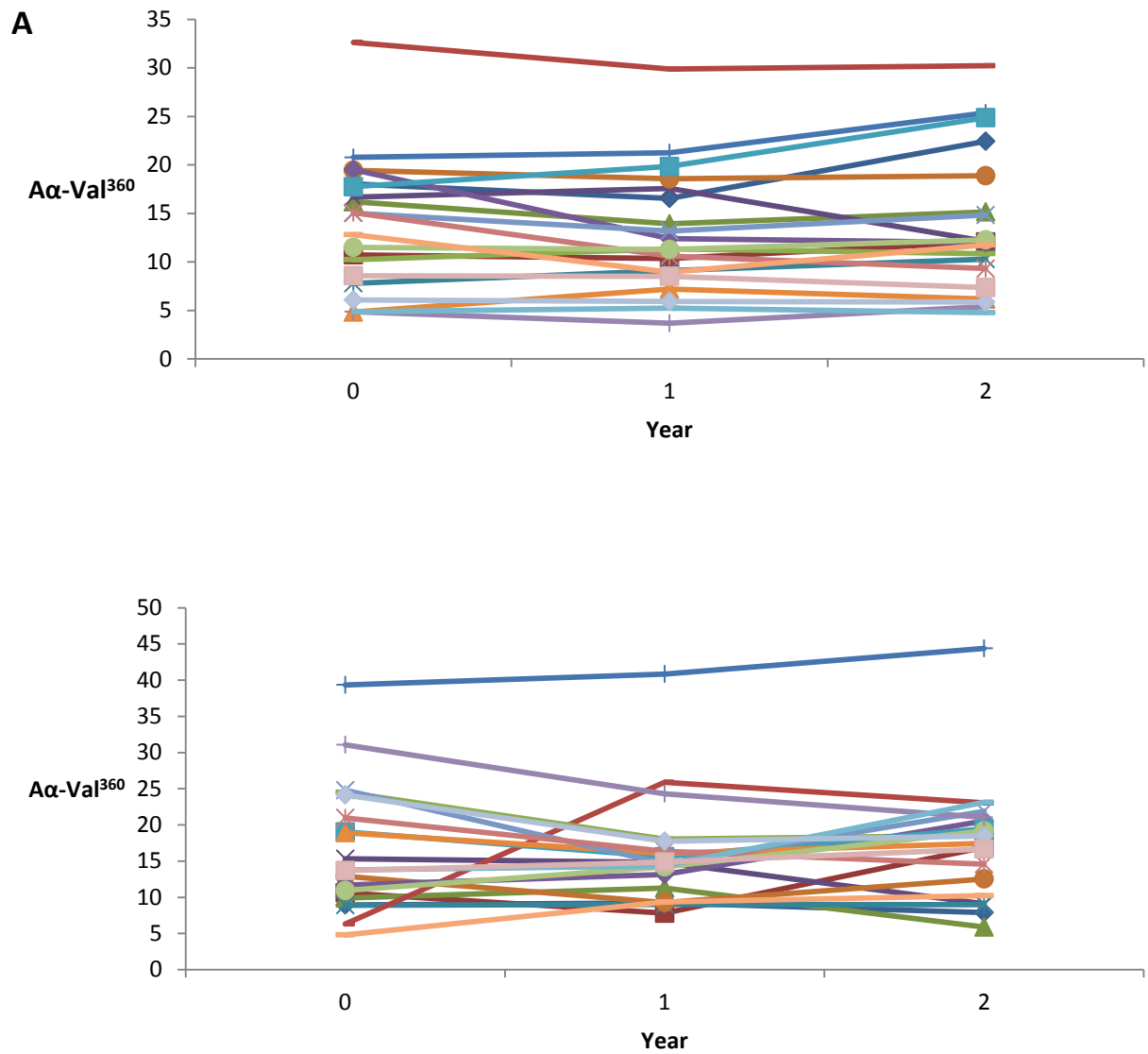


Figure 17A and B show the 1<sup>st</sup> 20 subjects and 2<sup>nd</sup> 20 subjects respectively who were included in the 2 year variability study, with each line representing a single subject. The CV of 17.13% (mean of all subjects) is within the range expected for the intra-plate variability of the assay which is approximately 12 to 15%.

### 4.3 THE EXACERBATION STUDY

The study include 6 male and 2 female PiZ A1AT deficient subjects, all of whom had chronic bronchitis and an FEV<sub>1</sub>/FVC<0.7. Their demographic details are listed in Table 16.

	Mean (SE)
<b>Age</b>	49.77 (3.18)
<b>FEV<sub>1</sub> (litres)</b>	1.41 (0.35)
<b>FEV<sub>1</sub> (% predicted)</b>	41.01 (8.35)
<b>FEV<sub>1</sub>/FVC</b>	0.41 (0.07)
<b>KCO (mmol/min/kPa/L)</b>	1.07 (0.20)
<b>KCO (% predicted)</b>	66.02 (11.68)

*Table 16: Demographic data for the 8 PiZ A1AT deficient subjects included in the 'exacerbation study'*

Following their initial presentation with an exacerbation, there was an improvement in the self-reported symptoms of the subjects over the course of the study (as assessed by diary card) which was associated with a decrease in the A $\alpha$ -Val<sup>360</sup> concentration (Figure 18A; Table 17). Although there was no statistically significant difference in the A $\alpha$ -Val<sup>360</sup> concentrations on the 1<sup>st</sup> day of presentation with the exacerbation ('day 1') when compared with day 28 (p=0.164), there was variation between individuals, with some demonstrating a large increase in A $\alpha$ -Val<sup>360</sup> compared to the stable state (n=5), while others did not demonstrate any rise (n=3).

In this group of individuals with chronic bronchitis who were experiencing an exacerbation, it was also possible to measure sputum sol NE and this related to their plasma A $\alpha$ -Val<sup>360</sup> concentrations (Figure 18B). Again, there was no significant difference in the sputum NE

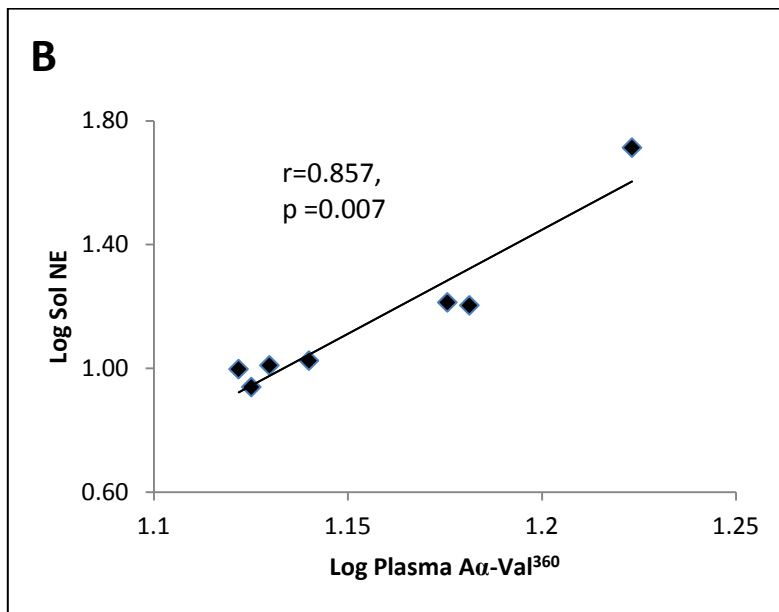
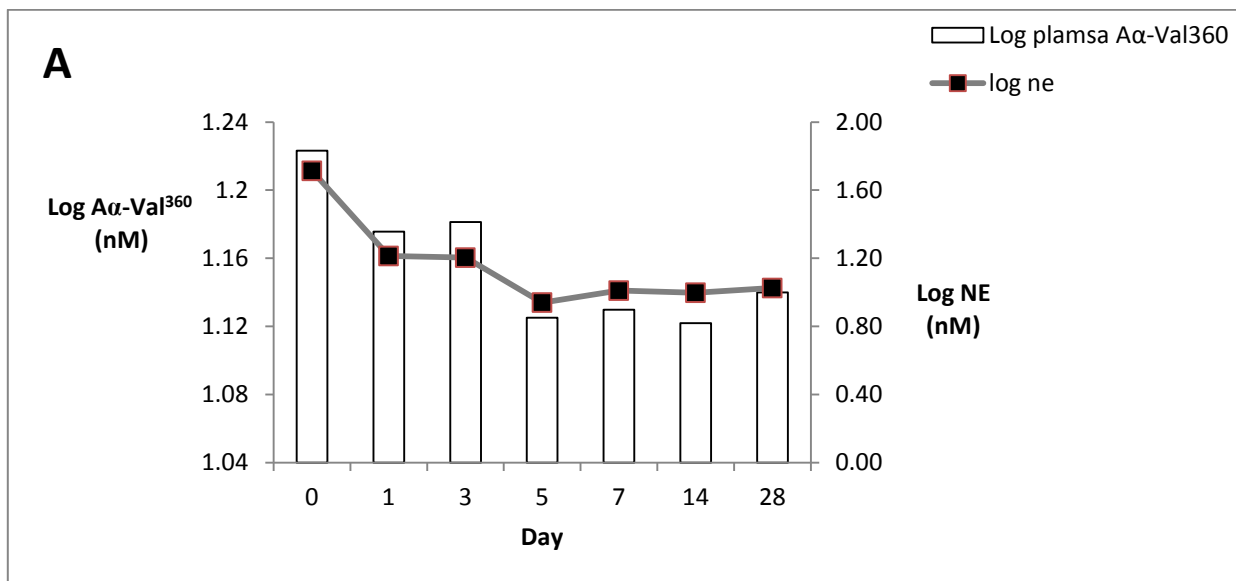
concentration on day one versus day 28 ( $p=0.173$ ) (Figure 18). However, the sputum sol NE concentrations were low in the stable state (day 28), and only above the lower limit of quantification in 3 of the 8 subjects and therefore the stable state NE data should be interpreted with caution.

Day	1	2	3	5	7	14	28
<b>Mean A<math>\alpha</math>-Val<sup>360</sup> nM (SE)</b>	20.61 (6.16)	17.56 (3.95)	18.40 (5.67)	14.58 (2.36)	14.38 (2.12)	13.83 (1.75)	14.78 (2.09)
<b>Mean CRP (mg/L) (SE)</b>	22.47 (14.46)	33.63 (25.97)	13.20 (7.69)	5.26 (2.58)	6.20 (2.87)	5.45 (3.11)	3.79 (1.82)
<b>Mean NE (nM) (SE)</b>	51.67 (28.00)	16.33 (5.53)	15.97 (3.66)	8.70 (2.81)	10.22 (3.48)	9.94 (3.25)	10.59 (3.43)

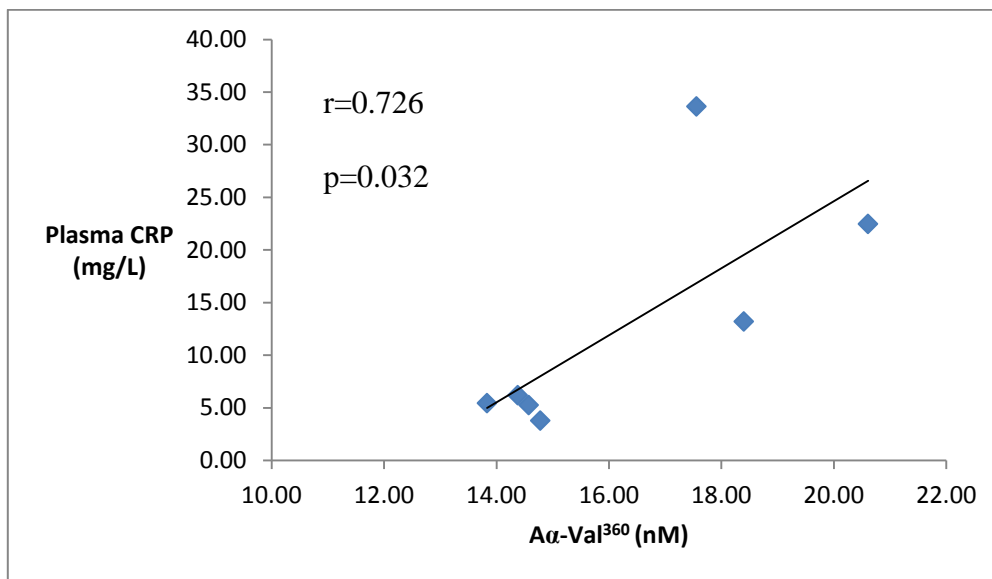
*Table 17: The mean (SE) biomarker concentrations for the 8 subjects who took part in the exacerbation study. All subjects completed a 14 day course of antibiotics.*

The mean A $\alpha$ -Val<sup>360</sup> concentrations correlated highly with the plasma CRP concentrations (Figure 19), and a significant correlation also existed between the plasma CRP concentration and the sputum sol phase NE concentration ( $r=0.714$ ,  $p=0.036$ ). The plasma CRP concentration was significantly greater on day one of the exacerbation compared to day 28 ( $p=0.043$ ), however 3 individuals did not demonstrate any change (of whom 2 also did not have an increase in A $\alpha$ -Val<sup>360</sup>). Two individuals demonstrated persistently low CRP values throughout the study period and also had little variance in their A $\alpha$ -Val<sup>360</sup> concentrations.

For each individual, the CVs were calculated over the 7 days of the study period. There was a significant correlation ( $r=0.683$ ,  $p=0.031$ ) between the CVs for CRP and A $\alpha$ -Val<sup>360</sup>, demonstrating the longitudinal variation of these biomarker is similar within each subject.



**Figure 18: A:** The figure depicts the mean Aα-Val<sup>360</sup> and sputum sol phase NE concentrations for each day of the exacerbation study. Although the Aα-Val<sup>360</sup> was higher on day one of an exacerbation, this did not reach statistical significance compared to day 28 when patients had returned to the clinically stable state. **B** Each point represents the mean plasma Aα-Val<sup>360</sup> and sputum NE concentrations at for a single day of the study. There was a significant correlation between the plasma Aα-Val<sup>360</sup> and sputum sol phase NE concentrations.



*Figure 19. Each point on this figure represents the mean plasma Aα-Val<sup>360</sup> and CRP concentrations for a single day of the exacerbation study (n=8). The correlation coefficient (r) and the p-value are shown.*

## **4.4 IN VIVO VARIABILITY: DISCUSSION**

### **4.4.1 A $\alpha$ -Val<sup>360</sup> in the Stable State**

#### *4.4.1.1 Selection of Sputum Markers*

A number of inflammatory mediators are raised in the sputum of patients with COPD, particularly during exacerbations, although few have been fully validated or monitored in longitudinal studies. Sputum IL-8 was measured in the current study, as it is increased in subjects with COPD and relates to FEV<sub>1</sub>/FVC in cross-sectional studies [143] and progression of emphysema measured by lung densitometry (but not lung function) in longitudinal studies [21]. Also other markers of neutrophilic inflammation including sputum MPO and LTB<sub>4</sub> have been shown to correlate with FEV<sub>1</sub> and TLCO decline respectively [21].

#### *4.4.1.2 Variability of A $\alpha$ -Val<sup>360</sup> and Sputum Biomarkers*

In this study which only included clinically stable patients, there were strong cross-sectional relationships between the mean A $\alpha$ -Val<sup>360</sup> concentration and baseline markers of physiological disease *severity* of COPD, which is an important feature of any potential biomarker of disease *activity*. Additionally, stability is an important characteristic of an ideal biomarker of disease activity since this allows clinically useful measurements to be taken at any time, and the Variability Study showed that within patient variation was low, at least when in the clinically



stable state. Further evidence of the stability and repeatability of the A $\alpha$ -Val<sup>360</sup> marker was provided by data over a 2 year period in 40 PiZ A1AT deficient individuals.

The assay variability is likely to be the main explanation for the observed variation in values of the A $\alpha$ -Val<sup>360</sup> assay. However, the variability of A $\alpha$ -Val<sup>360</sup> remained within acceptable limits, was much lower than any of the sputum markers and is consistent with the previously reported variability of a number of other plasma biomarkers [211]. These data therefore add further support for the use of a plasma marker (such as A $\alpha$ -Val<sup>360</sup>) rather than a sputum marker, since plasma is less influenced by changes in water content, which can result in major changes in marker concentrations within the airways, or the sample collection process.

Although changes in the water content of airway secretions may partly account for the variability of the spontaneous sputum markers, this cannot be the only explanation since it would be expected that dilutional changes would affect all markers equally at the same time point. This would be demonstrated by correlations in the longitudinal variability between the sputum biomarkers, however this was not observed in the current study. It is thus possible that the variability in the sputum markers related to changes in the underlying disease activity or processes within individuals, although again, the absence of correlations in the longitudinal variability would not support the hypothesis that this reflects the central pathophysiological process. Importantly, all subjects were carefully reviewed at each visit to ensure that they had not developed new symptoms or started taking new medications and this was reinforced by the use of daily diary cards, suggesting that clinical features of changes in disease activity did not account for the variability of the sputum markers. Finally, oropharyngeal contamination may contribute to

the intra-patient variability of the sputum markers, despite the use of mouth rinsing in the current study to minimise this influence, although again this would be expected to affect all mediators equally and hence result in significant longitudinal correlations of these biomarkers.

#### *4.4.1.3 Cross-sectional Relationships*

Interestingly, in the current study there were cross-sectional relationships *between* the sputum biomarkers but not to either the A $\alpha$ -Val<sup>360</sup> concentration or the physiological markers such as FEV<sub>1</sub>. Although this may be partly explained by the variability of the sputum biomarkers, the mean biomarker concentrations for all 11 visits were used in these calculations (rather than the values for individual visits) which would have minimised the influence of collection variability. However, since the study involved relatively small numbers of individuals, and demonstrated marked variability of sputum measures, the study is likely to be underpowered to completely *exclude* any associations between sputum markers and other parameters such as physiology and A $\alpha$ -Val<sup>360</sup>.

#### *4.4.1.4 Rolling Mean Data*

This study demonstrated that the use of 3 or 5 day average data reduced the intra-patient variability of all the biomarkers studied, including A $\alpha$ -Val<sup>360</sup>, although it had the greatest influence on the sputum markers. This difference is almost certainly related to the greater underlying variability of the absolute values of the sputum biomarkers demonstrated in the

current study. Although the improvement may be partly accounted by the use of *overlapping* data (which will always be associated with a reduction in variability), there was a similar reduction in variability observed with the non-overlapping data suggesting that this is a true effect rather than an artefact of the methodology.

The relatively small improvement in A $\alpha$ -Val<sup>360</sup> repeatability when the average is calculated from 3 samples over 3 days, as opposed to using the absolute values, further demonstrates the stability of this marker within the variability of the assay measurement. The greater repeatability offered by the 5 day rolling mean data may be of particular use in interventional clinical trials where a high sensitivity for the detection of change is critical. However, this would usually be unnecessary under most circumstances as the advantages associated with less variability would usually be offset by the logistical difficulties of obtaining samples over a prolonged period and the expected change with time during an interventional study.

#### **4.4.2 A $\alpha$ -Val<sup>360</sup> in Subjects Experiencing an Acute Exacerbation**

The A $\alpha$ -Val<sup>360</sup> concentration was generally higher on the 1<sup>st</sup> day of an acute exacerbation and decreased overall as symptomatic improvement occurred (as recorded in the patient diary cards). It is recognised that at least some exacerbations are associated with an excess of neutrophilic activity [230] and patients with a greater number of exacerbations decline at a greater rate [118]. Thus the A $\alpha$ -Val<sup>360</sup> data reported here demonstrate that exacerbations are also associated with an increase in NE associated *disease activity*.

The A $\alpha$ -Val<sup>360</sup> did not increase in all study subjects which may be partly explained by the diverse nature of acute exacerbations of COPD and the absence of a universally accepted definition. The inclusion criteria for the current study required subjects to have experienced an increase in all three of the symptoms of sputum purulence, sputum volume and breathlessness, which is consistent with the definition of the widely referenced ‘type I exacerbation’ devised by Anthonisen et al [231]. Anthonisen also suggested the terms type II exacerbation (to include 2 of the type I symptoms) and type III exacerbation (to include one of the type I symptoms plus one or more of: an upper respiratory tract infection in the last 5 days, fever without other cause, increased wheezing or cough, or an increase in heart rate or respiratory rate by 20% compared to baseline). The use of ‘type I’ criteria in the current study aimed to ensure the inclusion of patients with a similar clinical phenotype. However, and despite the use of diary cards, these terms (particularly increase in breathlessness) are still open to subjective interpretation, and furthermore will encompass a wide range of severities. An exacerbation may therefore be associated with an impact which is slightly greater than the nature of symptoms experienced during normal day-to-day variation, and it would be interesting to determine whether ‘mild’ clinical exacerbations are similarly associated with ‘mild’ inflammatory and neutrophilic response.

#### *4.4.2.1 Relationships with other Biomarkers*

The current study still demonstrated a highly significant relationship between the CRP and A $\alpha$ -Val<sup>360</sup> concentrations. Furthermore, the longitudinal variation in both inflammatory mediators was similar over the course of the Variability Study. The CRP (although non-specific) is the most

sensitive marker of acute exacerbations of COPD (when ranked against 35 other biomarkers) [177], and in the current study a significant difference was demonstrated between CRP on day one of the onset of the exacerbation and day 28.

The data demonstrated that 3 individuals did not have an increased CRP on the 1<sup>st</sup> day of the study, of whom 2 also did not generate an increase in A $\alpha$ -Val<sup>360</sup>. It is difficult to comment on the relative sensitivity of the CRP compared to the A $\alpha$ -Val<sup>360</sup> for detecting acute exacerbations, since relatively few subjects were included in the current study. It is however interesting that some subjects experience an exacerbation without a significant rise in any marker despite clinically equivalent symptoms and this indicates key issues in the use of biomarkers to define exacerbations in subjects with COPD or potentially as markers of severity. Importantly, it raises the question of whether diseases (and their exacerbations) should be defined by their clinical features or whether this will be surmounted by the use of blood tests alone.

There was a relationship between sputum NE concentrations and both the plasma A $\alpha$ -Val<sup>360</sup> and CRP. While this is important, since it shows the plasma biomarkers relate to active inflammation within the airways, sputum NE would be an impractical marker of disease activity in clinical practice since it is present at the limit of quantification in the sputum of subjects in the stable state (even in subjects who produce sputum) [71].

## 4.5 SUMMARY

The data from these studies demonstrate that  $A\alpha\text{-Val}^{360}$  is a stable footprint of pre-inhibition NE activity in subjects who are not experiencing an acute exacerbation. The low level variability which has been demonstrated in the current studies is almost certainly accounted for by the variability of the assay itself, rather than reflecting true changes in the concentration of this biomarker or in the underlying disease activity. In these same subjects,  $A\alpha\text{-Val}^{360}$  also generally related to physiological markers of disease severity but not sputum inflammatory markers, which may be accounted for by the high variability of the sputum markers or the anatomical relationship to the samples collected and the site of disease activity (e.g. airway or lung parenchyma).

$A\alpha\text{-Val}^{360}$  was higher at the onset of an acute exacerbation, and related to both sputum NE and plasma CRP concentrations. This demonstrates that exacerbations characterised by all 3 of the Anthonisen criteria are associated with an increase in disease activity which is consistent with the observation that subjects with these exacerbations decline more rapidly [118]. The data also suggest a potential role for biomarkers to both diagnose exacerbations and classify their severity and hence guide treatment.

The relationship of  $A\alpha\text{-Val}^{360}$  with acute exacerbations will be discussed further in Chapter 7.

# **CHAPTER 5. AA-VAL<sup>360</sup> IN SUBJECTS WITH SYMPTOMS OF COPD UNRELATED TO A1AT DEFICIENCY: RESULTS AND DISCUSSION**

These data have been accepted for publication by a high impact factor peer reviewed journal (Carter RI, et al. *A $\alpha$ -Val<sup>360</sup>: A Marker Of Neutrophil Elastase And COPD Disease Activity*. Manuscript published online ahead of print by the ERJ, April 2012).

## **5.1 RESULTS**

### **5.1.1 Initial Stable State Assessment**

Eighty one subjects (36 female and 45 male) with chronic bronchitis and exertional dyspnoea but a broad range of spirometric disease severity (Table 18) were included in the study. Of these patients, 58 had spirometric criteria consistent with COPD as defined by current guidelines (with an FEV<sub>1</sub>/FVC<LLN), while 61 subjects had an FEV<sub>1</sub>/FVC<0.7 and therefore met the alternate spirometric threshold for COPD.

<i>Parameter</i>	<i>Mean (SE)</i>
<i>Age (years)</i>	<i>65.75 (0.92)</i>
<i>Height (metres)</i>	<i>1.64 (0.01)</i>
<i>FEV<sub>1</sub> (L)</i>	<i>1.79 (0.08)</i>
<i>FEV<sub>1</sub> (% predicted)</i>	<i>73.28 (2.86)</i>
<i>FEV<sub>1</sub> (SR)</i>	<i>-1.44 (0.16)</i>
<i>FEV<sub>1</sub>/FVC</i>	<i>0.55 (0.02)</i>
<i>FEV<sub>1</sub>/FVC (% predicted)</i>	<i>72.27 (2.35)</i>
<i>FEV<sub>1</sub>/FVC (SR)</i>	<i>-3.01 (0.25)</i>
<i>Residual Volume (L)</i>	<i>2.20 (0.04)</i>
<i>RV (% predicted)</i>	<i>126.20 (3.78)</i>
<i>RV (SR)</i>	<i>1.52 (0.22)</i>
<i>TLC (L)</i>	<i>6.03 (0.15)</i>
<i>TLC (% predicted)</i>	<i>107.24 (1.66)</i>
<i>TLC (SR)</i>	<i>0.60 (0.14)</i>
<i>KCO (mmol/min/kPa/L)</i>	<i>1.41 (0.48)</i>
<i>KCO (% predicted)</i>	<i>96.69 (3.21)</i>
<i>KCO (SR)</i>	<i>-0.28 (0.20)</i>
<i>Plasma Aα-Val<sup>360</sup> (nM)</i>	<i>24.39 (2.20)</i>
<i>Plasma AIAT/NE Complex (nM)</i>	<i>2.63 (0.18)</i>
<i>Plasma hsCRP (mg/L)</i>	<i>730.60 (146.67)</i>
<i>Sputum MPO (nM)</i>	<i>0.82 (0.17)</i>
<i>Sputum LTB<sub>4</sub> (pg/ml)</i>	<i>9.41 (2.22)</i>
<i>Sputum IL-8 (nM)</i>	<i>6.79 (1.04)</i>

*Table 18: Aα-Val<sup>360</sup> was measured in 81 stable state subjects with symptoms of COPD and their baseline demographic and lung function data (performed on the same day as the plasma sample was obtained) are displayed. Plasma samples refer to stable state results at baseline. Sputum results refer to the 55 subjects able to produce spontaneous sputum in the stable state at baseline. Data represent the group mean (SE).*

The stable state plasma Aα-Val<sup>360</sup> concentration related to baseline FEV<sub>1</sub> (% predicted) (r=-0.340; p=0.001) and KCO (% predicted) (-0.246; p=0.013). Multivariate analysis accounting for age, sex, smoking history, height and sputum colour demonstrated that the stable state Aα-Val<sup>360</sup>



was an independent predictor of KCO (standardised B co-efficient=-0.243,  $R^2$  change=0.048,  $p=0.037$ ) however the independent relationship with FEV<sub>1</sub> fell short of conventional levels of significance (standardised B co-efficient=-0.231,  $R^2$  change=0.037,  $p=0.070$ ). Importantly, in subjects with an FEV<sub>1</sub>/FVC below the normal range, similar relationships were also observed between A $\alpha$ -Val<sup>360</sup> and KCO (% predicted) ( $r=-0.214$ ;  $p=0.054$ ) and FEV<sub>1</sub> (% predicted) ( $r=-0.297$ ;  $p=0.013$ ).

In the full cohort, plasma A $\alpha$ -Val<sup>360</sup> showed a reasonable correlation with plasma A1AT/NE complex in the stable state ( $r=0.459$ ,  $p<0.001$ ; Figure 20). Also, the A1AT/NE complex did not relate to either FEV<sub>1</sub> % predicted ( $r=-0.087$ ,  $p=0.451$ ) or KCO % predicted ( $r=-0.172$ ,  $p=0.126$ ). Furthermore, the A $\alpha$ -Val<sup>360</sup> was significantly higher ( $p<0.001$ ) in subjects with chronic bronchitis and dyspnoea ( $n=80$ ) with a median A $\alpha$ -Val<sup>360</sup> value of 20.76 nM (IQR 13.99 – 25.44) than healthy controls ( $n=39$ ) with a median value of 3.50 nM (IQR 2.35 – 5.14).

There was no relationship of A $\alpha$ -Val<sup>360</sup> with high sensitivity CRP ( $r=0.004$ ,  $p=0.361$ ), a non-specific measure of inflammation. The hsCRP did not relate to FEV<sub>1</sub> % predicted ( $r=0.111$ ,  $p=0.165$ ) but there was a *positive* correlation with KCO % predicted ( $r=0.235$ ,  $p=0.036$ ).

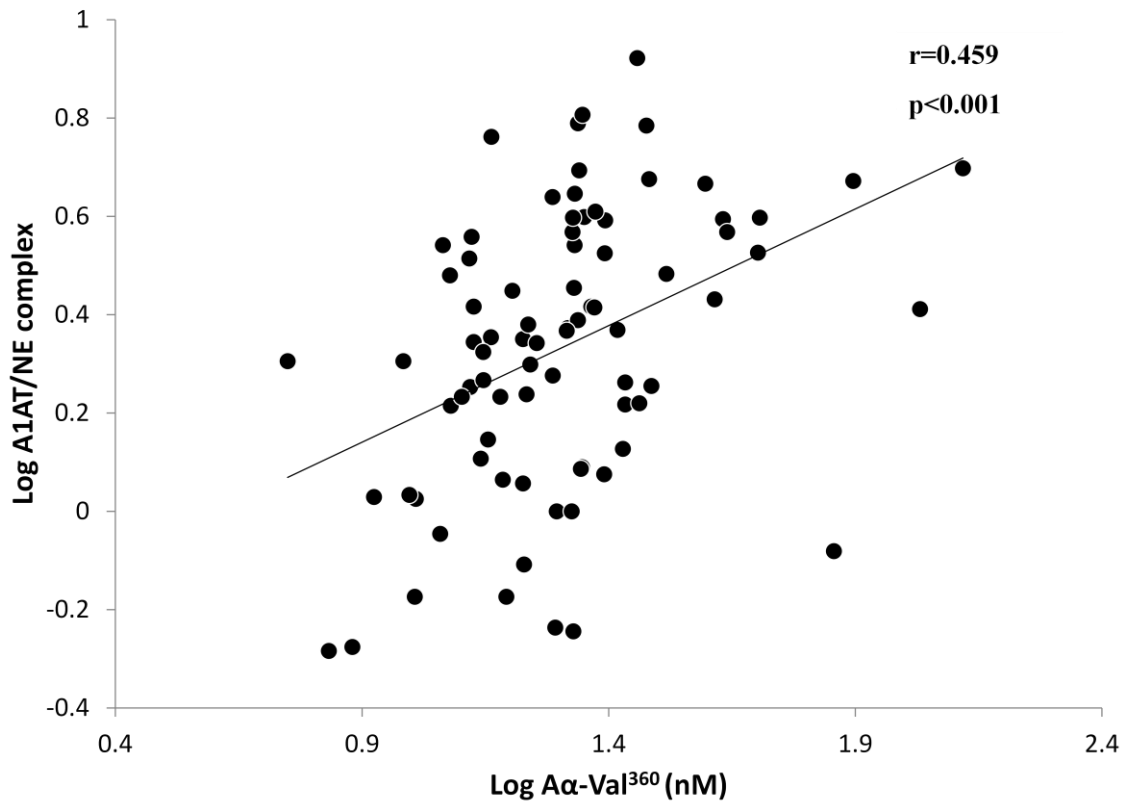


Figure 20: The Aα-Val<sup>360</sup> concentration (a marker of NE activity) related to the A1AT/NE complex concentration (a marker of total NE release). The correlation co-efficient ( $r$ ) and  $p$  value are shown. All samples were obtained from subjects when in the clinically stable state.

### 5.1.2 Aα-Val<sup>360</sup> in Subjects With and Without Visible Emphysema on HRCT

In the overall group, the plasma Aα-Val<sup>360</sup> concentration was greater ( $p=0.013$ ) in those with visible emphysema on HRCT ( $n=43$ ) compared to those without ( $n=38$ ). Also, subjects with visible emphysema had a significantly lower FEV<sub>1</sub> (% predicted;  $p=0.014$ ), FEV<sub>1</sub>/FVC ( $p<0.001$ ) and KCO (% predicted;  $p<0.001$ ) than those without (Table 19). However, there was no difference in the plasma CRP or A1AT/NE complex or sputum MPO, LTB<sub>4</sub> and IL-8 (in the

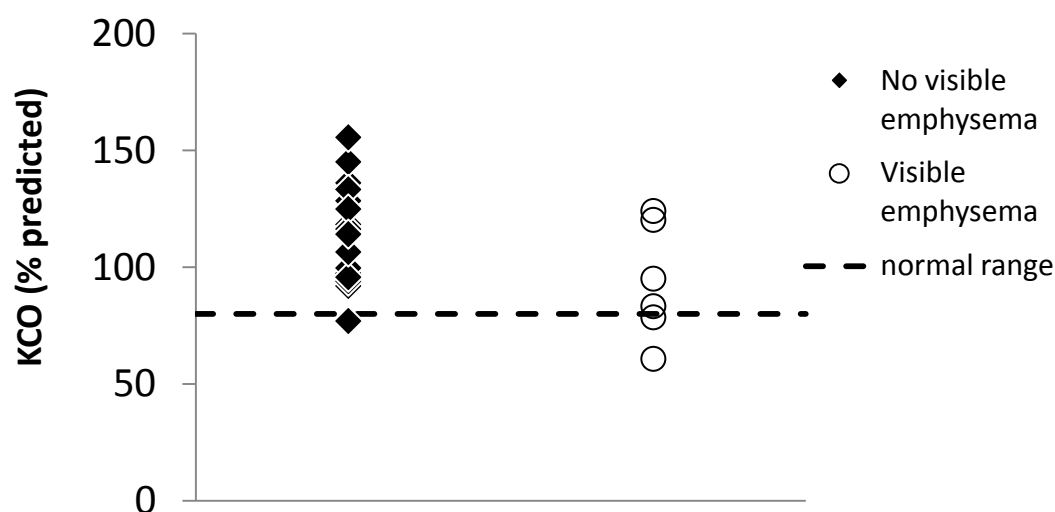
55 subjects able to produce a spontaneous sputum sample for the stable state assessment) between those with and without emphysema.

<i>Subset of Patients</i>	<i>Parameter</i>	<i>No visible emphysema on</i>	<i>Visible emphysema on</i>	<i>P</i>
		<i>HRCT</i>	<i>HRCT</i>	
<i>All</i>	<i>Aα-Val<sup>360</sup> (nM)</i>	<i>16.98 (IQR 13.15 to 22.29)</i>	<i>21.77 (IQR 15.58 – 27.13)</i>	<i>0.013</i>
	<i>FEV<sub>1</sub> (% predicted)</i>	<i>79.75 (±4.12)</i>	<i>67.27 (±3.80)</i>	<i>0.014</i>
	<i>FEV<sub>1</sub>/FVC</i>	<i>0.62 (±0.03)</i>	<i>0.48 (±0.14)</i>	<i>&lt;0.001</i>
	<i>KCO (% predicted)</i>	<i>114.76 (± 3.55)</i>	<i>80.71 (±3.76)</i>	<i>&lt;0.001</i>
<i>Subjects with an</i>	<i>Aα-Val<sup>360</sup> (nM)</i>	<i>13.98 (IQR 12.31 to 21.00)</i>	<i>22.88 (IQR 14.09 to 42.17)</i>	<i>0.071</i>
<i>FEV<sub>1</sub> and</i>	<i>FEV<sub>1</sub> (% predicted)</i>	<i>100.77 (2.98)</i>	<i>100.61 (2.38)</i>	<i>0.488</i>
<i>FEV<sub>1</sub>/FVC within</i>	<i>FEV<sub>1</sub>/FVC</i>	<i>0.77 (0.01)</i>	<i>0.70 (0.02)</i>	<i>0.003</i>
<i>normal range</i>	<i>KCO (% predicted)</i>	<i>114.88 (5.09)</i>	<i>93.83 (10.43)</i>	<i>0.030</i>
<i>(&gt;LLN)</i>				
<i>Subjects with an</i>	<i>Aα-Val<sup>360</sup> (nM)</i>	<i>17.96 (15.26 – 24.13)</i>	<i>21.77 (16.12 – 27.13)</i>	<i>0.141</i>
<i>FEV<sub>1</sub>/FVC below</i>	<i>FEV<sub>1</sub> (% predicted)</i>	<i>62.73 (4.31)</i>	<i>61.56 (3.63)</i>	<i>0.420</i>
<i>the normal range</i>	<i>FEV<sub>1</sub>/FVC</i>	<i>0.50 (0.02)</i>	<i>0.45 (0.02)</i>	<i>0.06</i>
	<i>KCO (% predicted)</i>	<i>114.67 (5.04)</i>	<i>78.59 (3.98)</i>	<i>&lt;0.001</i>

*Table 19: Data are shown for all subjects, for subjects with an FEV<sub>1</sub> and FEV<sub>1</sub>/FVC within the normal range (>LLN) and for subjects with an FEV<sub>1</sub>/FVC below the normal range (<LLN). Mean and standard error or median and interquartile range are shown for normally distributed and non-parametric data respectively. In the whole cohort, subjects with visible emphysema on HRCT had significantly worse lung function tests and a greater Aα-Val<sup>360</sup>. Similar differences were seen in subjects with an FEV<sub>1</sub> and FEV<sub>1</sub>/FVC within the normal range and those with an FEV<sub>1</sub>/FVC below the normal range.*

Subgroup analysis was performed for subjects with chronic bronchitis and dyspnoea but an FEV<sub>1</sub> and FEV<sub>1</sub>/FVC in the normal range as confirmed by SRs (Table 19). This demonstrated that the Aα-Val<sup>360</sup> was also greater in those with visible emphysema (median 22.88 nM; IQR 14.09 to

42.17; n=6) on HRCT compared to those without (13.98; IQR 12.31 to 21.00; n=17), which is similar to that seen in the larger cohort but the difference just failed to achieve statistical significance in this subgroup ( $p=0.09$ ). There was no significant difference in the FEV<sub>1</sub> (% predicted) in those with visible emphysema compared to those without. However, the FEV<sub>1</sub>/FVC was lower in those with emphysema (mean  $0.70 \pm 0.01$ ) than those without ( $0.77 \pm 0.02$ ;  $p=0.003$ ). In addition, the KCO (% predicted) was also lower (Figure 21) in those with visible emphysema ( $93.83 \pm 10.43$ ) than those without ( $114.88 \pm 5.09$ ;  $p=0.030$ ).



*Figure 21: In subjects with both an FEV<sub>1</sub> and FEV<sub>1</sub>/FVC within the normal range, the KCO (% predicted) is significantly lower ( $p=0.030$ ) in subjects with visible emphysema on CT thorax (open circles) compared to those without (closed diamonds), although the KCO (% predicted) largely remains above 80% (dotted line).*

Analysis of patients with an FEV<sub>1</sub>/FVC below the normal range demonstrated that the average Aα-Val<sup>360</sup> was also greater in those with visible emphysema (n=37) compared to those without (n=21), but this difference was not significant ( $p=0.141$ ). There was no difference in the FEV<sub>1</sub> (% predicted), or the sputum markers between the 2 groups of patients although the FEV<sub>1</sub>/FVC

( $p=0.06$ ) and KCO (% predicted) ( $p<0.001$ ) were lower in subjects with visible emphysema on HRCT (Table 19).

### **5.1.3 A $\alpha$ -Val<sup>360</sup> During an Acute Exacerbation**

Both the A $\alpha$ -Val<sup>360</sup> and hsCRP were higher at presentation with the exacerbation than in the stable state even when stratified by sputum colour into visibly purulent or non-purulent episodes (Table 20). Furthermore, both the A $\alpha$ -Val<sup>360</sup> and CRP at presentation with the exacerbation were significantly greater ( $p=0.030$  and  $p=0.019$  respectively) in subjects who presented with purulent sputum compared to those with mucoid sputum. Interestingly, although A $\alpha$ -Val<sup>360</sup> fell in both groups following resolution of the exacerbation, the difference between these 2 groups persisted ( $p=0.024$ ; Table 20). In addition, the sputum IL-8 ( $p<0.001$ ) and plasma A1AT/NE complex ( $p=0.036$ ) were also higher at resolution in those who had experienced an exacerbation associated with purulent sputum. However, importantly there was no longer a difference in the plasma hsCRP ( $p=0.573$ ), sputum colour or sputum MPO and LTB<sub>4</sub> concentrations in the stable state. In the stable state, no correlation was seen between A $\alpha$ -Val<sup>360</sup> and sputum MPO ( $r=0.059$ ;  $p=0.337$ ), however there remained positive correlations with the key neutrophil chemoattractants: LTB<sub>4</sub> ( $r=0.227$ ;  $p=0.048$ ) and especially IL-8 ( $r=0.486$ ;  $p<0.001$ ).

There was a weak correlation between the hsCRP and plasma A $\alpha$ -Val<sup>360</sup> at presentation with the exacerbation ( $r=0.225$ ,  $p=0.054$ ). Interestingly, there was also a correlation between the hsCRP at the presentation with the exacerbation and both the stable state A $\alpha$ -Val<sup>360</sup> ( $r=0.234$ ,  $p=0.047$ ) and

hsCRP (r=0.211, p=0.038). However, the hsCRP at exacerbation onset did not correlate with either the stable state FEV<sub>1</sub> or KCO.

	<i>Aα-Val</i> <sup>360</sup> (nM)	<i>Aα-Val</i> <sup>360</sup> (nM)	<i>P</i>	<i>hsCRP</i> (mg/L)	<i>hsCRP</i> (mg/L)	<i>P</i>
	<i>Exacerbation presentation</i>	<i>Stable State</i>	<i>(Aα-Val</i> <sup>360</sup> <i>Exacerbation vs stable state)</i>	<i>exacerbation presentation</i>	<i>stable state</i>	<i>(hsCRP presentation vs stable state)</i>
<i>All</i>	23.72 (18.1 – 35.41)	21.28 (13.99 – 24.65)	0.005	2459.8 (809.72-7029.20)	212.24 (90.34 - 809.05)	<0.001
<i>Non-purulent sputum group</i>	21.22 (17.42 – 27.44)	20.00 (12.83 – 21.69)	0.022	1246.79 (530.70 - 5292.33)	234.94 (120.12 - 902.03)	<i>p</i> =0.003
<i>Purulent sputum group</i>	26.29 (19.43 – 39.07)	21.83 (14.91 – 28.45)	0.043	3539.97 (1313.65 - 8324.34)	198.49 (78.77 - 752.32)	<i>P</i> <0.001

Table 20: The table demonstrates the significantly higher median *hsCRP* and *Aα-Val*<sup>360</sup> (IQR) in subjects with COPD at presentation with the exacerbation compared to the stable state (in all subjects and in those with non-purulent sputum or purulent sputum at the onset of the exacerbation).

#### 5.1.4 Longitudinal Analysis

Forty individuals were alive and consented to assessment with full lung function tests and densitometric analysis of HRCT scans at both baseline (stable state) and at follow-up 4 years later. *Aα-Val*<sup>360</sup> obtained from subjects at baseline related cross sectionally to both baseline and

follow-up physiological and radiological measures (Table 21). However, there was no relationship between hsCRP or A1AT/NE complex concentration and any physiological or radiological parameter at either baseline or follow-up. Both physiological and radiological markers demonstrated disease progression in the 40 subjects (Table 22), however statistical significance was not observed for all parameters. The absence of a significant change may at least partly be explained by the lack of sensitivity of these physiological and radiological tests for detecting disease progression in subjects with COPD and emphysema, and again supports the need for new markers of disease severity and activity.

	<i>Correlation with Baseline (Stable State) Plasma A<math>\alpha</math>-Val<sup>360</sup></i>			
	<i>Baseline</i>		<i>Follow-up</i>	
	<i>R</i>	<i>p</i>	<i>r</i>	<i>p</i>
<i>FEV<sub>1</sub> (% predicted)</i>	-0.319	0.022	-0.357	0.012
<i>KCO (% predicted)</i>	-0.401	0.005	-0.403	0.008
<i>UZVI (-950 HU)</i>	0.416	0.004	0.434	0.003
<i>LZVI (-950 HU)</i>	0.279	0.041	0.382	0.008
<i>UZ 15th percentile point (HU)</i>	-0.338	0.017	-0.350	0.013
<i>LZ 15th percentile point (HU)</i>	-0.224	0.083	-0.299	0.031

*Table 21: UZ = upper zone; LZ=lower zone; VI=voxel index; HU=Hounsfield Units. In 40 individuals, plasma A $\alpha$ -Val<sup>360</sup> was measured at baseline (stable state) and related to physiology and CT densitometry performed both at baseline (stable state) and at follow-up 4 years later. The correlations (r) and p values are shown for these relationships. Physiological parameters were expressed as % predicted (corrected for age, height and sex).*

Parameter	Baseline	4 Year Follow Up	P
	Mean (SE)	Mean (SE)	
<b>Age</b>	64.07 (1.20)	68.36 (1.20)	
<b>FEV<sub>1</sub> (L)</b>	1.93 (0.11)	1.79 (0.11)	<0.001
<b>FEV<sub>1</sub> (% predicted)</b>	78.23 (4.16)	75.79 (4.49)	0.071
<b>FEV<sub>1</sub> (SR)</b>	-1.24 (0.22)	-1.25 (0.23)	0.453
<b>FEV<sub>1</sub>/FVC</b>	0.57 (0.03)	0.55 (0.03)	0.005
<b>FEV<sub>1</sub>/FVC (SR)</b>	-2.84 (3.78)	-3.11 (0.36)	0.024
<b>KCO (mmol/min/kPa/L)</b>	1.42 (0.07)	1.36 (0.08)	0.006
<b>KCO (% predicted)</b>	95.35 (4.73)	93.14 (5.48)	0.273
<b>KCO (SR)</b>	-0.35 (0.30)	-0.55 (0.40)	0.273
<b>UZ 15 PCP</b>	-920.34 (5.70)	929.07 (5.03)	0.002
<b>UZ VI (-950)</b>	9.49 (1.92)	11.59 (2.08)	<0.001
<b>UZ VI (-910)</b>	24.70 (2.85)	27.44 (2.69)	0.004
<b>LZ 15 PCP</b>	-907.51 (5.44)	-913.05 (4.75)	0.021
<b>LZ VI (-950)</b>	7.96 (1.21)	9.53 (1.36)	<0.001
<b>LZ VI (-910)</b>	22.96 (2.63)	25.73 (2.58)	0.007
<b>A<math>\alpha</math>-Val<sup>360</sup> (nM)</b>	25.73 (4.32)	N/A	N/A
<b>A1AT/NE Complex (nM)</b>	2.37 (0.24)	N/A	N/A

Table 22: The baseline and follow up data for 40 subjects (19 female and 21 male).

There was a significant decrease in the FEV<sub>1</sub> ( $p<0.001$ ) and KCO ( $p<0.001$ ) over the 4 year period. There was also significant emphysema progression as measured by absolute change in the voxel index (-950 HU) and 15<sup>th</sup> percentile point in both the upper ( $p<0.001$  and  $p=0.002$  respectively) and lower ( $p<0.001$  and  $p=0.021$ ) zones. The baseline A $\alpha$ -Val<sup>360</sup> related to both the subsequent decline in KCO (% predicted) ( $r=-0.406$ ,  $p=0.008$ ) and progression in lower zone emphysema expressed as change in the voxel index at -950 HU ( $r=0.306$ ,  $p=0.027$ ), however the relationship of the baseline A $\alpha$ -Val<sup>360</sup> with change in the lower zone 15<sup>th</sup> percentile point did not achieve statistical significance ( $r=-0.212$ ,  $p=0.095$ ). There was no significant association between A $\alpha$ -Val<sup>360</sup> and decline in FEV<sub>1</sub> (% predicted) ( $r=-0.196$ ,  $p=0.113$ ), upper zone voxel index ( $r=0.103$ ,  $p=0.264$ ) or 15<sup>th</sup> percentile point ( $r=-0.049$ ,  $p=0.381$ ). There was no relationship between the A1AT/NE complex and any measure of decline. Receiver operating characteristic



analysis of A $\alpha$ -Val<sup>360</sup> in subjects who demonstrated a decline in KCO (% predicted) over the 4 year period compared to non-decliners gave an area under the curve of 0.711 (p=0.037). An A $\alpha$ -Val<sup>360</sup> threshold of 11 would have a sensitivity and specificity of 91% and 43% respectively for the identification of subjects who will demonstrate a decline in KCO (% predicted) over a 4 year period, while a threshold of 22 would have a sensitivity of 33% and specificity of 93%.

In the subgroup analyses of patients with chronic bronchitis and dyspnoea but a baseline FEV<sub>1</sub>/FVC SR and FEV<sub>1</sub> SR within the normal range (n=14), the baseline A $\alpha$ -Val<sup>360</sup> also correlated with subsequent decline in KCO (% predicted) (r=-0.534, p=0.025) but not to spirometric or radiological progression. In those with a baseline FEV<sub>1</sub>/FVC SR below the LLN (n=26), the A $\alpha$ -Val<sup>360</sup> related to radiological progression (change in lower zone voxel index r=0.348, p=0.041) but there was no significant relationships with other measurements.

## 5.2 DISCUSSION

This is a unique assay based on an NE specific fibrinogen degradation product ( $A\alpha\text{-Val}^{360}$ ) which measures the damaging potential of NE at the point of release from the neutrophil prior to its inhibition by the surrounding protease inhibitors [214]. Previous authors have investigated the use of an alternate fibrinogen degradation product (formed by the cleavage of the fibrinogen alpha chain at  $A\alpha\text{-21}$ ), however this was not pursued further because of low NE specificity and the very short half-life of this smaller fibrinogen fragment [216]. In contrast, the  $A\alpha\text{-Val}^{360}$  neoepitope is highly specific and shows stability over time, which are both important features of a biomarker [214].

It is possible that a synergistic relationship may exist between the fibrinogen cleavage product  $A\alpha\text{-Val}^{360}$  and the fibrinogen concentration, since fibrinogen may also relate to COPD disease severity [237]. However, although NE is released from the azurophil granule at an initial concentration which exceeds most substrates, the total amount of NE released is very low and therefore the abundant fibrinogen protein is present in excess. Thus fibrinogen is only likely to influence  $A\alpha\text{-Val}^{360}$  formation if the clotting factor concentration varies between individuals by several orders of magnitude. However, published data show this not to be the case since the median plasma concentration of fibrinogen in stable state subjects with COPD who had suffered a severe exacerbation in the last 12 months was reported as 3.75 compared to 3.45 g/L in those who had not [237]. Also, while it is recognised that fibrinogen is an acute phase protein, the fibrinogen concentration only increases from 4.55 to 5.45 g/L in subjects experiencing an acute

exacerbation of COPD [238], which again suggests the fibrinogen concentration has little influence on A $\alpha$ -Val<sup>360</sup> formation. Furthermore, stimulation of neutrophils in plasma with ionophore increases the A $\alpha$ -Val<sup>360</sup> concentration (Table 9) to levels greater than stable state physiological concentrations, in the presence of constant levels of fibrinogen, which indicates the substrate concentration is not rate limiting. Also, the A $\alpha$ -Val<sup>360</sup> is detected in plasma at nanomolar concentrations, which again suggests that the formation of this product will not be dependent on the micromolar concentrations of fibrinogen.

In common with many other inflammatory mediators, fibrinogen is a non-specific marker of inflammation which may be elevated in the absence of ongoing tissue damage. In contrast A $\alpha$ -Val<sup>360</sup> is a specific marker of NE activity with a more certain association with tissue damage.

### **5.2.1 Relationship to Markers of Disease Severity**

In the absence of more suitable gold standard markers, the A $\alpha$ -Val<sup>360</sup> was related to physiological and radiological measures of COPD disease severity since (despite their flaws) they are widely used and reasonably well validated. The A $\alpha$ -Val<sup>360</sup> was not compared with other potential biomarkers since (to date) none have been effectively validated and therefore interpretation of relationships (if any) would be difficult. For example, while urinary desmosine may differ between healthy individuals, smokers with normal lung function and subjects with COPD, it is not influenced by augmentation therapy [197] (perhaps because it is neither organ nor disease specific).

In the current study, it has been demonstrated that  $A\alpha\text{-Val}^{360}$  relates to several specific features of COPD in subjects without A1AT deficiency. Firstly it relates cross-sectionally to physiological and radiological markers of current COPD severity. This relationship is likely to be complex (and therefore consistent with the strength of the observed associations) since a marker of activity may also relate to the process leading to the current disease state (or severity) or to future disease progression. The relationship between a marker of disease activity and disease severity will also depend on both the stability of the marker and the underlying pathophysiological disease activity with respect to time. If (in the stable clinical state) both marker and true disease activity are stable over time then there will be a relationship between a marker of disease activity, current disease severity and future disease severity. This is not necessarily the case and will therefore be discussed further in section 7.3.4. Nevertheless, the strongest relationship was observed between  $A\alpha\text{-Val}^{360}$  and markers of the severity of the emphysematous process (gas transfer and the voxel index), and in particular  $A\alpha\text{-Val}^{360}$  was the best independent predictor of gas transfer. These data suggest  $A\alpha\text{-Val}^{360}$  is likely to be an important surrogate marker of the pathogenesis of COPD however it is of greatest relevance to the development of emphysema.

### **5.2.2 $A\alpha\text{-Val}^{360}$ as a Marker of Emphysema**

Further confirmation of the relationship between NE activity and the emphysematous process was provided by the observation that  $A\alpha\text{-Val}^{360}$  is greater in subjects with visible emphysema on HRCT compared to those without. Although the difference was less marked in subjects who also had obstructive spirometry, this may be explained by the smaller number and discordance

between the severity of the airway obstruction and alveolar destruction within individuals since the average observed differences were similar to that seen for the whole cohort.

It is increasingly recognised that COPD is a heterogeneous disease, and the current study demonstrated that visible emphysema was present even in 6 of the 23 subjects with spirometry within the normal range, and those with visible emphysema also had a greater A $\alpha$ -Val<sup>360</sup> and significantly lower gas transfer and lower FEV<sub>1</sub>/FVC than those without visible emphysema, even though these physiological tests remained either largely or entirely within the normal range. This observation supports the use of a symptomatic diagnosis of COPD (chronic bronchitis and exertional dyspnoea) for inclusion of patients in the current study since spirometric criteria would have excluded a number of subjects with either 'early' disease or an emphysema predominant phenotype. The difference in A $\alpha$ -Val<sup>360</sup> between these 2 subgroups with normal spirometry fell just short of conventional levels of statistical significance, however the absolute difference in A $\alpha$ -Val<sup>360</sup> concentrations mirrored that observed in the entire cohort suggesting that this is not due to chance alone but rather reflects the smaller number of subjects identified. There is therefore likely to be a subset of patients with an active NE related disease process, yet relatively mild physiological changes who do not meet current spirometric criteria for the diagnosis of COPD, but may be identified by A $\alpha$ -Val<sup>360</sup> and benefit from targeted therapeutic intervention to prevent deterioration to the more classical stages of COPD. Clearly further studies are required to investigate this possibility in depth.

### 5.2.3 A $\alpha$ -Val<sup>360</sup> and the A1AT/NE Complex

A $\alpha$ -Val<sup>360</sup> is a specific marker of pre-inhibition NE *activity*, while the A1AT/NE complex is a marker of total NE release as a result of neutrophil degranulation. In general, the A $\alpha$ -Val<sup>360</sup> was related to the plasma A1AT/NE complex and subjects with symptoms suggestive of COPD had higher levels of both A $\alpha$ -Val<sup>360</sup> and A1AT/NE complex than healthy controls indicating both greater NE activity and neutrophil enzyme release, as may be expected. However, the differences between A $\alpha$ -Val<sup>360</sup> and other markers of neutrophil activation were emphasised by the absence of any correlation between either MPO (a marker of neutrophil degranulation) or A1AT/NE complex and the physiological and radiological markers of COPD disease severity (either cross-sectionally or longitudinally), demonstrating that a measure of NE release alone is a poor surrogate of the enzyme's proteolytic potential.

### 5.2.4 A $\alpha$ -Val<sup>360</sup> and Acute Exacerbations

The A $\alpha$ -Val<sup>360</sup> also related to exacerbations which are episodes known to relate to FEV<sub>1</sub> progression [118]. A $\alpha$ -Val<sup>360</sup> was higher at the presentation with an exacerbation than in the stable state 8 weeks later, reflecting greater NE activity which may at least partly impact on subsequent evidence of disease progression. The hsCRP was also greater at the onset of an exacerbation, and correlated (albeit weakly) with the A $\alpha$ -Val<sup>360</sup> (both at presentation with the exacerbation and in the stable state) which is of interest, since one study has suggested that CRP may relate to exacerbation severity [177] although the relatively non-specific nature of CRP may

limit its use as a surrogate of disease activity in clinical practice. Importantly, the A $\alpha$ -Val<sup>360</sup> was higher not only at the presentation with an exacerbation in subjects who experienced an exacerbation associated with purulent (neutrophilic) sputum compared to those with mucoid sputum but remained higher even in the stable state supporting the concept that these episodes may mark subjects with a greater likelihood of progression [118]. Furthermore, the higher A $\alpha$ -Val<sup>360</sup> in this group of patients was associated with a higher stable state sputum IL-8 and plasma A1AT/NE complex concentration (but not hsCRP), demonstrating a greater ongoing process leading to neutrophil recruitment, enzyme release and hence potential tissue damage.

Although these data could represent a slower recovery, it is unlikely since all subjects were seen 2 months after the exacerbation onset when they were confirmed to be clinically stable, supported by the observation that there was no difference in the hsCRP (which has previously been demonstrated to be higher in acute exacerbations of COPD [177]) between these 2 groups. The A $\alpha$ -Val<sup>360</sup> related both to the stable state and 4 year follow-up physiological and radiological measures, and these relationships would be less likely if patients had not been in the stable state at the time of assessment (with further resolution after the study). In particular, there was no evidence of an ongoing bacterial trigger since there was no difference in the subjective assessment of the stable state sputum colour or objective measurement of MPO between the 2 groups of patients. Although further studies are required, it is probable that patients who experience an exacerbation associated with purulent sputum have greater NE activity in general (leading to tissue damage which enhances the subsequent risk of a bacterial infection) and therefore experience exacerbations associated with purulent rather than mucoid sputum. It is also possible that subjects who experience a more severe exacerbation have a greater A $\alpha$ -Val<sup>360</sup> signal

even following recovery and hence decline at a greater rate. However, although CRP may partly relate to the severity of exacerbations [177], this has yet to be proven definitively and therefore there is currently no accepted inflammatory marker of exacerbation onset or severity in subjects with COPD to confirm this concept, although it is possible that A $\alpha$ -Val<sup>360</sup> itself may fill this role in specific targeted studies.

### **5.2.5 A $\alpha$ -Val<sup>360</sup> as a Marker of Disease Progression**

There appears to be a relationship between NE activity (measured by A $\alpha$ -Val<sup>360</sup>) and disease progression. Current pathophysiological activity is likely to reflect not only preceding but also future disease progression and in the current study it was demonstrated that baseline A $\alpha$ -Val<sup>360</sup> related to deterioration and subsequent severity measured by gas transfer. The data also suggest a relationship with A $\alpha$ -Val<sup>360</sup> and disease progression measured by CT densitometry, although this is slightly inconsistent and would warrant further exploration in a larger prospective study. A $\alpha$ -Val<sup>360</sup> did not relate to spirometric decline, suggesting tissue damage reflected by A $\alpha$ -Val<sup>360</sup> is more indicative of the emphysematous process, not only in the presence of established COPD but importantly even in those with emphysema but without airflow obstruction. Clearly larger studies, especially in this latter group, are now indicated including data on longitudinal progression.



### 5.2.6 Summary

In summary, the current study reports the first *in vivo* data in human subjects which support the role of NE in the pathophysiology of chronic bronchitis, COPD and emphysema in human subjects without A1AT deficiency. Furthermore, when considered in combination with previous circumstantial data (see 1.5.1), the current study suggests NE may represent a final common pathway leading to tissue destruction in this disease process.  $A\alpha\text{-Val}^{360}$  is the first specific biomarker of pre-inhibition NE activity which relates to cross-sectional measures of disease severity and exacerbations and appears to relate to disease progression in the current study. Although further work in a larger cohort of patients is required, particularly to explore the relationship with longitudinal markers of disease progression in subjects at risk,  $A\alpha\text{-Val}^{360}$  therefore represents a new concept of specific biomarkers that may be central to the pathophysiology of emphysema and potentially COPD.

## **CHAPTER 6. A $\alpha$ -VAL<sup>360</sup> AS A MEASURE OF THE EFFICACY OF A1AT AUGMENTATION**

**Data pertaining to the A $\alpha$ -Val<sup>360</sup> in the EXACTLE study have been published (Carter RI, et al. The fibrinogen cleavage product A $\alpha$ -Val<sup>360</sup>, a specific marker of neutrophil elastase activity in vivo. Thorax 2011; 66: 686 – 91.)**

The EXACTLE study included 77 PiZ A1AT deficient subjects from the UK, Sweden and Denmark who were randomised to receive weekly infusions of either placebo or 60 mg/kg human A1AT augmentation therapy (Prolastin) for 2 to 2.5 years [111]. The current study included 67 of these subjects, excluding 10 of the original patients for whom suitable plasma samples were not available for analysis at either baseline (prior to commencing the interventional study product) or 6 months later. For 6 of the 10 subjects who were excluded, samples were deemed unsuitable for analysis due to visible, heavy bloodstaining (which leads to a rise in A $\alpha$ -Val<sup>360</sup>), while paired baseline and month 6 plasma samples were not available for 4 subjects.

## 6.1 RESULTS

The following data only relate to the 67 subjects included in the current A $\alpha$ -Val<sup>360</sup> study in whom paired viable samples were available.

As reported in the original EXACTLE study [111], there was a significant difference in the distribution of males and females between the 2 arms of the current A $\alpha$ -Val<sup>360</sup> study, however there were no differences in other baseline characteristics (Table 23).

	Placebo	Prolastin	P (placebo vs. Prolastin)
<b>N</b>	34	33	
<b>Males n (%)</b>	14 (41.18)	22 (66.67%)	0.036
<b>Females n (%)</b>	20 (58.82)	11 (33.33%)	
<b>Mean age (SE) in yrs</b>	54.44 (1.67)	55.03 (1.32)	0.783
<b>Smoking history</b>			0.969
<b>Current</b>	0	0	
<b>Ex</b>	31	30	
<b>Never</b>	3	3	
<b>Median baseline FEV<sub>1</sub> % predicted (IQR)</b>	38.32 (30.88 - 55.45)	38.89 (30.33 - 61.28)	0.964
<b>Median FEV<sub>1</sub>/FVC (IQR)</b>	0.35 (0.27 - 0.54)	0.37 (0.26 - 0.50)	0.821
<b>Median baseline KCO % predicted (IQR)</b>	56.67 (43.99 - 66.94)	53.67 (43.55 - 62.92)	0.854

*Table 23: the baseline characteristics of the 67 PiZ A1AT subjects who took part in the A $\alpha$ -Val<sup>360</sup> study (where paired, viable samples were available). There was a significant difference in the sex distribution between the 2 groups, but all the other baseline parameters were similar.*

### 6.1.1 Change in Lung Function and CT Densitometry

The data were expressed and analysed in 3 different ways. Firstly, the data were compared cross-sectionally between the placebo and treatment groups at each time point. Secondly the data were compared longitudinally between baseline and 6 months within each arm of the trial. However, since HRCT scans were performed at baseline and month 12 (but not month 6), data comparisons were performed with the CT densitometry at these time points in those subjects with both paired plasma and HRCT scans (n=31 placebo, n=31 Prolastin). Finally the baseline A $\alpha$ -Val<sup>360</sup> was related to the rate of change of each parameter from baseline over the full 2 year period of the original trial.

There was no significant difference in the physiological measures between the 2 treatment groups at either baseline or month 6 (Table 24). Also, there was no significant change in FEV<sub>1</sub> (% predicted) or KCO (% predicted) in either treatment group between baseline and month 6. At month 6 and 12, the rates of change in FEV<sub>1</sub> and KCO were the same in both treatment groups (data not shown).

Visit	FEV <sub>1</sub> (% predicted)			KCO (% predicted)		
	Placebo	Prolastin	P (placebo vs. Prolastin)	Placebo	Prolastin	P (placebo vs. Prolastin)
Baseline	46.73 (3.83)	45.22 (3.42)	0.769	56.13 (2.75)	55.20 (3.83)	0.844
Month 6	47.16 (3.73)	44.11 (3.31)	0.544	57.52 (2.73)	56.45 (3.67)	0.816
P (Baseline vs. month 6)	0.621	0.117		0.288	0.434	

Table 24: The table shows the mean (SE) FEV<sub>1</sub> and KCO (% predicted) for the subjects who were included in the EXACTLE study [111] in whom paired, viable plasma samples were available at baseline and at month 6. There was no significant difference in the FEV<sub>1</sub> or KCO between the 2 treatment groups or from baseline to month 6.

Visit	Upper Zone 15 <sup>th</sup> Percentile Point			Lower Zone 15 <sup>th</sup> Percentile Point		
	Placebo	Prolastin	P (placebo vs. Prolastin)	Placebo	Prolastin	P (placebo vs. Prolastin)
<b>Baseline</b>	65.39 (3.60)	61.14 (4.93)	0.489	35.72 (2.74)	36.24 (3.09)	0.900
<b>Month 12</b>	65.13 (4.01)	59.98 (5.18)	0.435	35.12 (2.82)	36.00 (3.19)	0.837
<b>P (Baseline vs. month 12)</b>	0.873	0.380		0.521	0.783	

*Table 25: The table shows the mean (SE) 15<sup>th</sup> percentile point in the upper and lower zones for subjects who took part in the EXACTLE trial [111] in whom paired baseline and month 6 plasma samples were available AND paired baseline and month 12 HRCT scans were performed (n=31 placebo, n=31 Prolastin). There was no difference in the 15<sup>th</sup> percentile point in either the upper or lower zone between the 2 groups at any time during study. There was a decrease in the 15<sup>th</sup> percentile point in both the upper and lower zone by month 24 but not month 12.*

In the subjects included in the current study (with paired plasma samples), there was no significant difference in the 15<sup>th</sup> percentile point (a measure of lung density) between the placebo and treatment group at either baseline or month 12 (Table 25). Also there was no significant change in the 15<sup>th</sup> percentile point between baseline and month 12 in either treatment group. The rate of change of the lower zone and the whole lung 15<sup>th</sup> percentile point was greater in the placebo group than the Prolastin group over the 2 year study period, (p=0.034 and p=0.09 respectively), however there was no difference in the rate of change in the upper zone (p=0.599).

### **6.1.2 A $\alpha$ -Val<sup>360</sup> as a Measure of the Efficacy of Augmentation Therapy**

At baseline, there was no difference in the median A $\alpha$ -Val<sup>360</sup> between the 2 study groups. Between baseline and month 6, the A $\alpha$ -Val<sup>360</sup> decreased significantly in the treatment group (p=0.022) but not the placebo group (p=0.313; Figure 22). Also, the A $\alpha$ -Val<sup>360</sup> was lower in the

Prolastin group than the placebo group at month 6, although this just fell short of conventional statistical significance ( $p=0.084$ ).

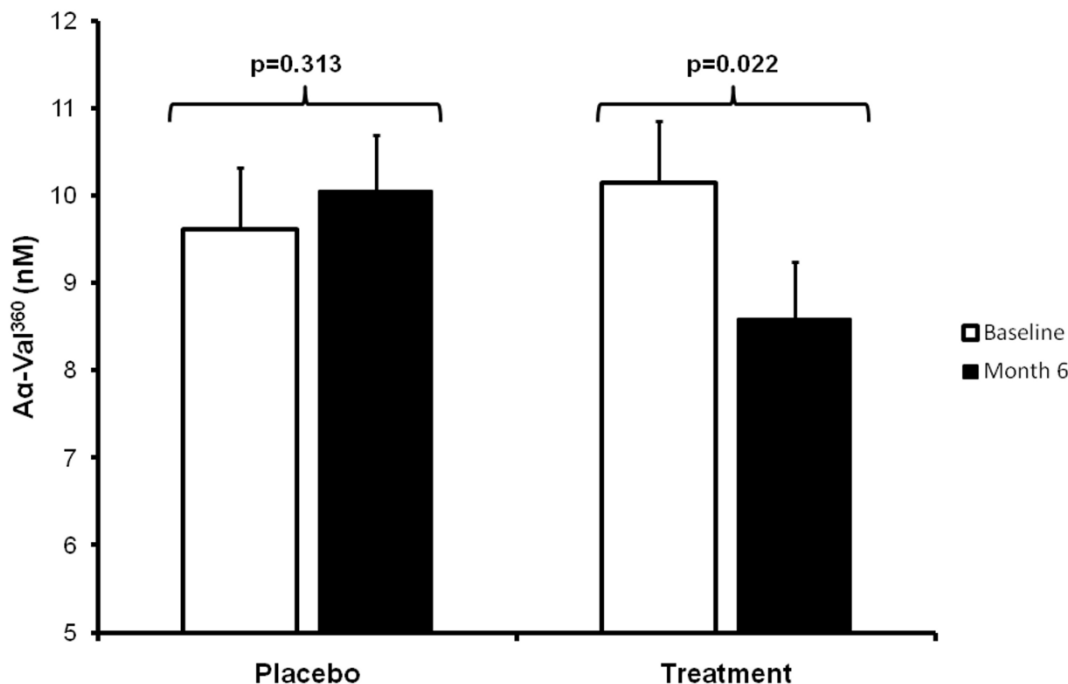


Figure 22 The mean (SE) Aα-Val<sup>360</sup> concentrations are shown for the 2 treatment arms at baseline and at month 6. There was a significant reduction in the Aα-Val<sup>360</sup> between baseline and month 6 in the treatment but not the placebo arm in the 67 subjects from the EXACTLE trial.

There was no significant difference in the median Aα-Val<sup>360</sup> between men and women at either baseline or month 6. Within these relatively small groups, there were no relationships between the baseline physiological or radiological measures and Aα-Val<sup>360</sup>.

Within the placebo arm, the data suggested a potential relationship between Aα-Val<sup>360</sup> and the rate of decline of FEV<sub>1</sub> (% predicted) and possibly the upper zone 15<sup>th</sup> percentile point, although

these relationships were far from clear (Table 26). The A $\alpha$ -Val<sup>360</sup> did not relate to the rate of decline of any other parameter.

Relationship of baseline A $\alpha$ -Val <sup>360</sup> with rate of decline between:	FEV <sub>1</sub> (% Predicted)		Upper zone 15 <sup>th</sup> percentile point	
	r	p	r	p
Baseline and month 6	-0.314	0.035	-	-
Baseline and month 12	-0.164	0.190	0.240	0.097
Baseline and month 18	-0.331	0.037	-	-
Baseline and month 24	-0.151	0.218	0.199	0.151

Table 26: the table depicts the relationship (r) between the baseline A $\alpha$ -Val<sup>360</sup> and the rate of decline of either the FEV<sub>1</sub> (% predicted) or upper zone 15<sup>th</sup> percentile point at various time points. Although the relationships at each time point were not all statistically significant, the trends were nevertheless similar.

## **6.2 AA-VAL<sup>360</sup> AS A MEASURE OF THE EFFICACY OF A1AT AUGMENTATION: DISCUSSION**

### **6.2.1 Current Evidence Supporting the Use of Augmentation Therapy**

The association between a deficiency of A1AT (the main inhibitor of NE) and early onset emphysema was first noted in the 1960s [68], and this was closely followed by the demonstration in animal studies that NE may directly induce the development of emphysema [69]. Also, both mathematical and *in vitro* modelling demonstrate that the area of quantum proteolytic damage (around the point of NE release) is exponentially greater in subjects with A1AT deficiency compared to healthy controls [52-54]. On the basis of this evidence alone, it would therefore seem logical to suggest that A1AT augmentation therapy would either effectively prevent the onset of COPD or slow disease progression in subjects with PiZ A1AT deficiency. However, to date there is little evidence from randomised controlled trials to support this hypothesis.

Weekly augmentation therapy with pooled A1AT in PiZ subjects has been shown to raise trough plasma levels to around 0.7 grams/litre (13.46  $\mu$ mol) [239], which therefore exceeds the putative protective threshold of 11  $\mu$ mol. Also, further evidence supporting the use of augmentation therapy has been provided by national registry data which showed that German PiZ A1AT deficient subjects (who received augmentation therapy) demonstrated a lesser FEV<sub>1</sub> decline than matched Danish control subjects [240]. However, these data were comparative, the study was not randomised, and furthermore, the German subjects included more women, had a significantly greater FEV<sub>1</sub> at baseline and were followed up for a shorter period than the Danish subjects,



which may all influence determination of the rates of decline. In 1999, the Dutch-Danish randomised controlled trial of monthly infusions of placebo versus A1AT augmentation therapy (250 mg/kg) demonstrated a reduction in the rate of emphysema progression on CT scan. However, this did not quite achieve conventional statistical significance ( $p=0.07$ ) and there was no difference in the rate of disease progression measured by physiological parameters [241]. More recently, a randomised controlled trial of placebo versus A1AT augmentation therapy (the EXACTLE study [111]) was conducted using CT densitometry as the primary outcome measure since this is a more sensitive marker of emphysema progression than physiological tests [106]. However, there remains controversy regarding the most appropriate method to measure longitudinal densitometric change and therefore this study explored 4 different methodologies. All 4 methods demonstrated a trend towards decreased progression in the treated population, however only one methodology (which used only the baseline and final 15<sup>th</sup> percentile point measurements) showed statistically significant differences between the 2 groups. This method was also the most sensitive measure of progression within the placebo group [111]. Finally, a study using pooled raw data from the Dutch-Danish and the EXACTLE study demonstrated a statistically significant decrease in emphysema progression measured by the 15<sup>th</sup> percentile point (whole lung) [242].

### **6.2.2 Physiological and Radiological Disease Progression**

The current study investigated the A $\alpha$ -Val<sup>360</sup> concentrations in plasma obtained from 67 subjects during the EXACTLE study. As there were fewer patients included in the A $\alpha$ -Val<sup>360</sup> study

(compared to the clinical study), the influence of treatment on the physiological and radiological measures of disease severity was also re-examined.

There was no difference in the FEV<sub>1</sub> or KCO (% predicted) in the placebo group compared to the treatment group at baseline or month 6, nor was there a significant change in these physiological measures *between* baseline and month 6 in either treatment group. Additionally, there was no significant change in FEV<sub>1</sub> or KCO over the 2 year period of the clinical study [111], FEV<sub>1</sub> and these data therefore demonstrate the insensitivity of physiological parameters for monitoring disease progression in patients with COPD especially if they deteriorate at a slow rate.

In the current (A $\alpha$ -Val<sup>360</sup>) study, the rate of change of the 15<sup>th</sup> percentile point was greater in the placebo group than the treatment group (although this only achieved statistical significance in the lower zone). The data are therefore consistent with the original EXACTLE study which showed a slowing of disease progression measured by CT, and this is important since it suggests the population studied here is comparable to that of the original EXACTLE study despite the exclusion of some subjects.

### **6.2.3 A $\alpha$ -Val<sup>360</sup> as a Measure of Treatment efficacy**

In these small subgroups, the A $\alpha$ -Val<sup>360</sup> did not relate to the physiological and radiological markers of disease severity. It is plausible that the study was insufficiently powered to examine cross-sectional relationships, particularly in this multi-centre trial where variation in the

physiological and radiological measures is likely to be much greater and potentially compounded by a centre effect. Therefore further study in larger groups was required (discussed in this thesis).

The A $\alpha$ -Val<sup>360</sup> did not change significantly over the 6 month period in the placebo arm of the trial which confirms the stability of this marker of disease *activity*. In contrast to markers of disease *severity* (such as KCO) which would be anticipated to decline as the disease progresses, an effective marker of disease *activity* would be stable over time, yet higher in subjects who deteriorate at a greater rate. There was a significant decrease in the A $\alpha$ -Val<sup>360</sup> in the treatment arm of the trial by month 6. This is in contrast to the physiological measures of disease severity at month 6 which neither demonstrated a significant decline in the placebo arm nor a treatment response in the Prolastin arm. Also, in the clinical study there was no demonstrable difference in the CT lung densitometry between the 2 arms of the trial until 2 years after commencement and no difference in the physiological measures at any time [111]. These data support the greater sensitivity of A $\alpha$ -Val<sup>360</sup> as a potential measure of treatment efficacy compared to either physiological or radiological measures of disease severity, although further longitudinal studies are required.

The A $\alpha$ -Val<sup>360</sup> concentration at baseline (at least partly) related to the subsequent rate of decline of both the FEV<sub>1</sub> and the upper zone 15<sup>th</sup> percentile point in patients within the placebo arm. Since it would be anticipated that a marker of disease activity would predict subjects who would decline at a greater rate, and there was trend towards a relationship at all time points this is unlikely to be explained by chance alone. The absence of more significant relationships may be explained by sample size and the relatively short duration of the study, particularly when there

was no significant difference in the FEV<sub>1</sub> between baseline and month 24. Furthermore, decline in FEV<sub>1</sub> and CT densitometry may not be linear [181] and may also account for the variation in these relationships.

Of interest, even in the treated population, the A $\alpha$ -Val<sup>360</sup> did not fall to the concentrations observed in healthy controls (even after 6 months of treatment). A number of factors may account for this: firstly variability of the assay (or its insensitivity), secondly that a greater duration of treatment is required to allow disease activity (and its markers) to decrease to ‘normal’ levels, thirdly there was inadequate dosing with A1AT or finally that once the COPD disease process is initiated, additional factors (other than the A1AT concentration) are also important stimuli of neutrophilic COPD disease activity.

#### **6.2.4 There is Ongoing Disease Activity Despite Augmentation Therapy**

Inadequate dosing or an ongoing pathophysiological process are plausible reasons for the failure of A $\alpha$ -Val<sup>360</sup> to return to the concentration of healthy controls, since the CT densitometry continues to decline despite augmentation therapy [111]. Current A1AT augmentation doses are based on the quantity required to achieve a plasma level over the likely protective threshold [243]. This threshold is partly based on the observation that PiZ subjects with a mean plasma concentration of 5  $\mu$ mol are at risk of developing emphysema while PiSZ subjects (who largely have an A1AT concentration greater than 11  $\mu$ mol) are not particularly susceptible, however this remains controversial [56]. Furthermore, it has been suggested that although current dosing

regimens may provide adequate basal levels of A1AT, weekly or monthly augmentation therapy would not mirror the physiological acute phase rise in A1AT concentrations that occur in healthy individuals during periods of increased (and potentially damaging) inflammatory activity [243]. Exacerbations in deficient individuals would therefore be associated with an increase in the release of NE which is not mirrored by a rise in its predominant inhibitor (A1AT), and may explain why exacerbations are associated with a more rapid decline [118].

More recent studies have suggested that A1AT polymers are chemoattractants [244] and have indicated that polymers and neutrophils tend to aggregate in the interstitium of the lung in A1AT deficient subjects [245], which may be a further potential cause for ongoing pathological activity despite augmentation therapy. However, it is also possible that these findings simply reflect the increased neutrophil traffic within the lung in subjects with A1AT deficiency that continues or only slowly declines to normal.

An inflammatory process is likely to be central to the pathophysiology of usual COPD in subjects who smoke (but have normal A1AT concentrations), however many inflammatory markers remain elevated in ex-smokers [246], which is thought to explain the ongoing progressive disease in some subjects even after smoking cessation. It is therefore possible that subjects with A1AT deficiency who develop COPD have a sustained general inflammatory response even after initiation of augmentation therapy. Although analysis of sputum samples in A1AT deficient subjects receiving augmentation therapy has demonstrated a decrease in LTB<sub>4</sub> (a key neutrophil chemoattractant in A1AT deficiency), there is no decrease in MPO (which is a marker of neutrophil activity) [247] and this suggests (at least in the short term) that there is a continued

inflammatory response. Additionally, studies have not shown a decrease in the number of exacerbations in subjects receiving augmentation therapy [111], which may be important because exacerbations are periods of intense inflammatory activity [230]. A marker of disease activity (such as A $\alpha$ -Val<sup>360</sup>) would be a potential method of further investigating this ongoing inflammatory process.

### **6.2.5 The Efficacy of ‘Early’ Augmentation Therapy**

It is possible that the initiation of augmentation therapy in subjects with A1AT deficiency prior to the development of COPD will be more effective than treating people with established lung disease. This would seem a logical step, since the initial pathophysiological process leading to emphysema within these individuals almost certainly relates directly to the deficiency of A1AT with associated exponentially larger areas of tissue destruction at points of NE release. If this is also the initial trigger for an ongoing inflammatory process (if present), then early augmentation therapy may prevent the onset of this process and further lung destruction. Early treatment may also prevent aggregation of A1AT polymers within the lung interstitium since A1AT polymer formation is concentration dependent (at least *in vitro*) [49], however this remains controversial [243, 245] and is difficult to fully assess in the absence of an effective *in vivo* marker of polymerised A1AT. Additionally, the *in vitro* marker of A1AT polymers cross-reacts with A1AT/NE complex and possibly with other antigens [245] which further complicates assessment of this potential mechanism. Assessment of early treatment would also be complicated by smoking history since, at standard doses, A1AT augmentation will not prevent smoking induced

lung damage, which is a significant risk factor for more rapid disease progression in A1AT deficient subjects [248].

Subgroup analysis of observational studies which have been conducted in subjects with PiZ A1AT deficiency have in part considered the implications of treating people with early disease. For example, the National Heart Lung and Blood Institute A1AT deficiency registry study group reported that subjects with an  $FEV_1 > 80\%$  deteriorate at a *greater* rate following the initiation of augmentation therapy. However, there were only 11 subjects with an  $FEV_1 > 80\%$  included in the study registry who received augmentation therapy, and although decline was greater it did not achieve conventional statistical significance ( $p=0.09$ ), suggesting this was a chance finding. In support of the concept of early treatment, however, Wencker et al [25] demonstrated that some subjects with ‘early’ disease (defined by the study team as  $FEV_1 > 65\%$ ) decline at a greater rate (irrespective of smoking status), and these rapid decliners respond more effectively than others to augmentation therapy. Nevertheless, the study design (which explored the rates of decline within individuals pre- and post augmentation therapy) does not allow definitive conclusions on treatment effect to be reached since  $FEV_1$  decline is not linear and therefore may have slowed with or without augmentation.

Recent registry data have also suggested that subjects with an  $FEV_1 > 65\%$  who receive augmentation therapy decline at a *greater* rate than those who do not [249]. However, subjects in the registry are initiated on augmentation therapy on a clinical (and not random) basis and therefore treatment may be more likely to be commenced in subjects who are already declining at a more rapid rate. Also, it would be difficult to consider a biologically plausible hypothesis to

support the proposition that augmentation therapy truly causes more rapid decline in subjects with a higher FEV<sub>1</sub> at baseline, although this requires a more pragmatic approach to studying patients before and after augmentation therapy.

#### **6.2.6 Defining ‘Early Disease’**

Data from observational studies conducted in subjects with PiZ A1AT deficiency therefore neither support nor refute the hypothesis that early treatment with augmentation therapy is beneficial. Furthermore, these studies have classified disease severity using FEV<sub>1</sub> alone, which is a poor measure of the degree of emphysema [10] and may therefore have misclassified some subjects as having early disease when pathologically it is advanced. This also raises the question of the definition of early disease since there may be a difference between subjects with mild disease (who currently have few symptoms and do not progress) and those with early disease (who initially have minimal physiological and symptomatic disease but progress rapidly) in whom early treatment may be beneficial [25].

There is also the issue of identifying subjects at an early stage, prior to the development of significant pathological lung disease, since under-diagnosis of A1AT deficiency and COPD in general has long been recognised. Although this could be overcome by screening, the distinction between early and mild disease would then become even more important since people with A1AT deficiency who have never smoked and are identified through screening have a markedly different prognosis and achieve a normal average lifespan [250]. Irrespective of screening, it is



interesting to speculate that a marker of disease activity (such as A $\alpha$ -Val<sup>360</sup>) would effectively identify patients with early disease who are progressing rapidly and would potentially benefit from early treatment [25]. The definitive answers to these questions would be provided by a randomised controlled trial of augmentation therapy incorporating a marker of disease activity, although this is clearly beyond the scope of the current thesis. However, given the financial burden and implications of lifelong augmentation therapy, these are questions that need to be addressed.

#### **6.2.7 Future Clinical Trials**

To date no study has demonstrated statistically significant improvements in clinically relevant outcomes in PiZ A1AT deficient subjects receiving augmentation therapy. It has been shown that such a study would require 147 subjects per treatment arm over a 4 year period to demonstrate a 23 ml/year difference in FEV<sub>1</sub> or 684 subjects in a 5 year study to demonstrate a 40% reduction in mortality [251]. Such a study is probably feasible, although it would require significant international co-operation (in countries where augmentation is currently unavailable) and funding. However, this is unlikely to be forthcoming since A1AT augmentation therapy is available in many countries throughout the world, which is a disincentive to both study participants and potential funders from the pharmaceutical industry. Again surrogate endpoints (including biomarkers) may help to address these questions, but are currently not validated sufficiently to be acceptable to regulatory authorities and clinicians alike.

### 6.2.8 Summary: A $\alpha$ -Val<sup>360</sup> as a Measure of the Efficacy of A1AT Augmentation

Evidence from biochemical, observational and randomised controlled studies suggest that augmentation therapy is *probably* effective, and the current study provides further evidence of treatment efficacy since there was a statistically significant decrease in A $\alpha$ -Val<sup>360</sup> (a marker of the potential destructive process in these patients) after just 6 months of treatment. The study also confirmed the insensitivity of FEV<sub>1</sub> (which is widely used in clinical trials) since it did not significantly change over the 2 year study period in either the placebo or treatment groups [111] or alternatively demonstrated the relative stability of the disease in these patients.

Further work is required to ensure that where augmentation therapy is initiated, subjects are treated with optimal A1AT doses (potentially supplemented during periods of acute infection or exacerbations) and to establish definitively if augmentation can return disease activity to normal levels, with an associated improvement in clinical outcomes. Finally, there are likely to be some patients with early or no lung disease who may decline more rapidly and potentially benefit from earlier treatment and a marker of disease activity (such as A $\alpha$ -Val<sup>360</sup>) may effectively identify this subgroup. However, a significantly larger and longer trial would be required to investigate the concept of early augmentation therapy, particularly using conventional markers of disease progression.

# CHAPTER 7. AA-VAL<sup>360</sup> IN SUBJECTS WITH A1AT DEFICIENCY: RESULTS AND DISCUSSION

## 7.1 PIZ AND NON-PIZ A1AT DEFICIENT SUBJECTS RESULTS

The Aα-Val<sup>360</sup> concentration was measured in a plasma sample obtained at the baseline visit from the first 463 subjects recruited to ADAPT with a variety of A1AT phenotypes and concentrations (Table 27). . There was an inverse exponential relationship between A1AT and Aα-Val<sup>360</sup> (Figure 23) in the form  $A\alpha\text{-Val}^{360} = e^{k/A1AT}$  (where k is a constant) as demonstrated by the *linear* relationship between A1AT and the function  $1/\ln(A\alpha\text{-Val}^{360})$  (Figure 24) which was predicted by previous mathematical and *in vitro* modelling [52-54]. There were highly significant ( $p < 0.001$ ) differences between the Aα-Val<sup>360</sup> concentrations in subjects with PiZ A1AT deficiency compared to the other phenotypes (Table 27 and Figure 25). However, differences were also present between subjects with PiSZ and PiS deficiency and other A1AT phenotypes.

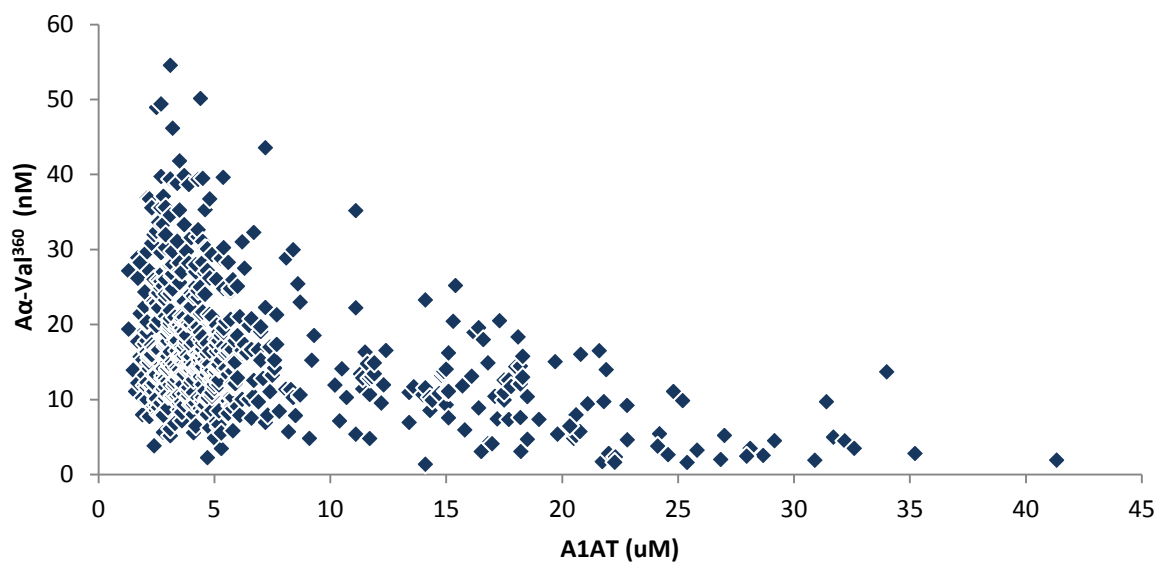


Figure 23: The exponential relationship between the A1AT concentration and  $A\alpha\text{-Val}^{360}$  concentration in subjects with differing A1AT phenotypes.

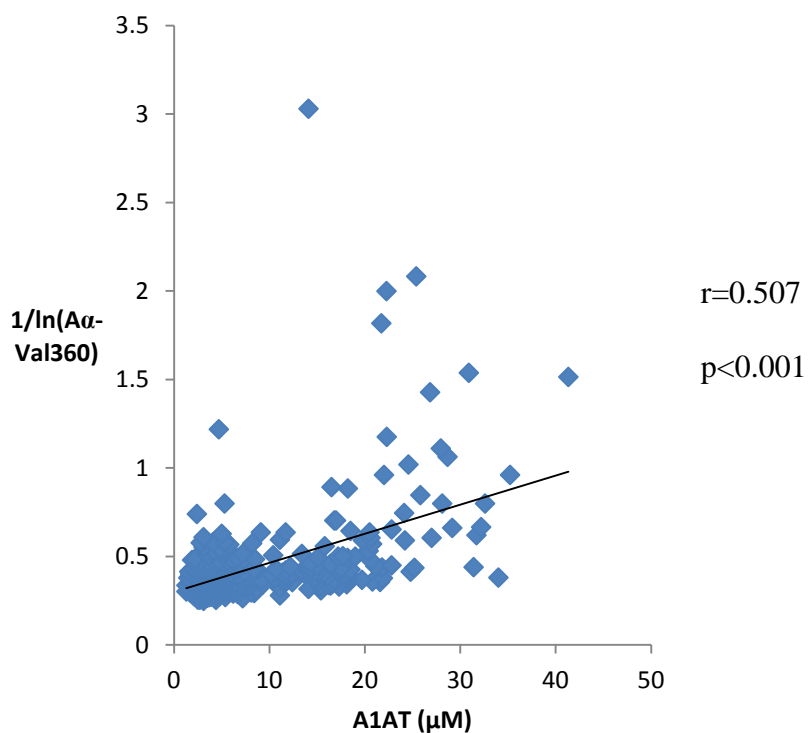


Figure 24. An exponential relationship exists between A1AT and the  $A\alpha\text{-Val}^{360}$  concentration since there is a linear relationship between A1AT and the function  $1/\ln(A\alpha\text{-Val}^{360})$  where  $\ln$  is the natural logarithm.

	PiZ	PiSZ	PiS	PiMZ	PiM healthy controls
<b>n</b>	350	51	8	30	24
<b>n with FEV<sub>1</sub>/FVC &lt;0.7 (%)</b>	284 (81.14)	26 (50.98)	7 (87.50)	14 (46.67)	0 (0)
<b>Mean FEV<sub>1</sub> [L] (SE)</b>	2.01 <sup>SZ, M</sup> (0.06)	2.76 <sup>Z</sup> (0.17)	2.04 <sup>M</sup> (0.33)	2.57 (0.26)	3.38 <sup>Z, S</sup> (0.24)
<b>Mean FEV<sub>1</sub> % predicted (SE)</b>	63.13 <sup>SZ, MZ, M</sup> (1.71)	86.82 <sup>Z, S, M</sup> (4.22)	64.54 <sup>SZ, M</sup> (9.37)	86.90 <sup>Z, M</sup> (7.05)	112.27 <sup>Z, SZ, S, MZ</sup> (3.34)
<b>Median A1AT [<math>\mu</math>M] (IQR)</b>	3.85 <sup>SZ, S, MZ, M</sup> (2.90 – 5.00)	14.20 <sup>Z, S, MZ, M</sup> (11.58 – 15.80)	17.80 <sup>Z, SZ, M</sup> (14.40 – 18.20)	18.20 <sup>Z, SZ, M</sup> (15.80 – 20.77)	24.98 <sup>Z, SZ, S, MZ</sup> (21.80 – 29.04)
<b>Median Aa-Val<sup>360</sup> [nM] (IQR)</b>	16.60 <sup>SZ, S, MZ, M</sup> (12.72 – 23.09)	11.95 <sup>Z, MZ, M</sup> (10.62 – 14.49)	11.92 <sup>Z, M</sup> (10.22 – 12.70)	9.58 <sup>Z, SZ</sup> (5.90 – 19.79)	2.96 <sup>Z, SZ, S, MZ</sup> (2.10 – 4.04)
<b>Smoking Status:</b>					
Current (%)	88 (25.1)	13 (25.5)	2 (25)	11 (36.7)	0 (0)
Ex (%)	240 (68.6)	28 (54.9)	6 (75)	14 (46.7)	0 (0)
Never (%)	22 (6.3)	10 (19.6)	0 (0)	5 (16.7)	24 (100)

Table 27: The Aa-Val<sup>360</sup> concentration was measured in a variety of subjects with varying A1AT phenotypes. Letters in superscript indicate significant differences (significance was taken as  $p < 0.05$ ). The median age was similar between the groups although was slightly (not significantly) higher in PiS subjects

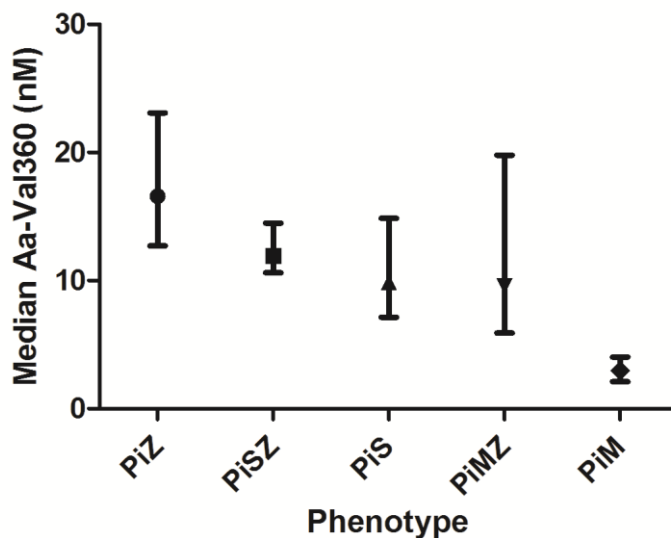


Figure 25: The median (IQR) Aa-Val<sup>360</sup> by A1AT phenotype

The A $\alpha$ -Val<sup>360</sup> was significantly higher ( $p<0.001$ ) in subjects with an A1AT concentration below 11  $\mu$ mol/litre (median 15.91; IQR 11.97 – 21.62) compared to those with a level greater than this putative protective threshold who had a median of 10.39 nM (IQR 5.12 – 13.57). Also, PiZ A1AT deficient subjects ( $n=53$ ) who had never smoked and had an FEV<sub>1</sub> (% predicted) above 80% had a median A $\alpha$ -Val<sup>360</sup> concentration of 15.25 nM (IQR 10.69 – 19.36), which was significantly higher ( $p<0.001$ ) than the normal healthy controls.

The numbers of index cases (who were identified following presentation with respiratory symptoms) and non-index cases (who were identified through family screening) varied by A1AT phenotype ( $p<0.001$ ; Table 28).

A1AT phenotype	Non-index cases <i>n</i> (%)	Index cases <i>n</i> (%)	Total <i>n</i>
<b>MZ</b>	11 (36.67)	19 (63.33)	30
<b>S</b>	4 (50)	4 (50)	8
<b>SZ</b>	41 (80.39)	10 (19.61)	51
<b>Z</b>	100 (28.57)	250 (71.43)	350

Table 28: There were differences ( $p<0.001$ ) in the numbers of non-index and index cases between the A1AT phenotypes which reflects the referral patterns to the ADAPT project.

There were also differences in the numbers of index and non-index cases within each A1AT phenotype when stratified into those with and without an  $FEV_1/FVC<0.7$  (commonly used physiological criteria for COPD). However, the majority of subjects with an  $FEV_1/FVC<0.7$  were both index cases and current or ex-smokers regardless of A1AT phenotype (Table 29).

FEV <sub>1</sub> /FVC<0.7	A1AT phenotype	Non-index cases <i>n</i> (%)	Index cases <i>n</i> (%)	Current or ex Smokers <i>n</i> (%)	Total <i>n</i>
<b>No</b>	MZ	11 (68.75)	5 (31.25)	7 (43.75)	16
	S	1 (100)	0 (0)	0 (0)	1
	SZ	20 (80)	5 (20)	15 (60.00)	25
	Z	49 (74.24)	17 (25.75)	26 (39.39)	66
<b>Yes</b>	MZ	0 (0)	14 (100)	12 (85.71)	14
	S	3 (42.86)	4 (57.14)	6 (85.71)	7
	SZ	21 (80.77)	5 (19.23)	23 (88.46)	26
	Z	51 (17.96)	233 (82.04)	236 (83.10)	284

Table 29: The proportion of subjects with COPD (defined using  $FEV_1/FVC<0.7$ ) varied by index and non-index status as well as by A1AT phenotype. The majority of subjects with an  $FEV_1/FVC<0.7$  were current or ex-smokers irrespective of A1AT phenotype.

## **7.2 SUBJECTS WITH PIZ ALPHA-1-ANTITRYPSIN DEFICIENCY**

Detailed cross-sectional and longitudinal analyses were performed on the 350 PiZ A1AT deficient subjects who were assessed with full lung function tests at 3 or more annual visits (median follow-up 6 years). There was no significant difference ( $p=0.246$ ) in the plasma A $\alpha$ -Val<sup>360</sup> concentration between the 141 females with a median of 16.51 nM (IQR 12.09 – 21.33) and the 209 males with a median of 16.87 (IQR 13.15 – 24.13). The A $\alpha$ -Val<sup>360</sup> was significantly higher ( $p=0.007$ ) in current smokers with a median of 21.6 nM (IQR 16.32 to 32.8) compared to ex-smokers with a median of 16.65 (12.85 – 23.76) and never smokers (median=15.3; IQR 10.80 – 19.88). There was also a weak, though statistically significant, relationship between A $\alpha$ -Val<sup>360</sup> and pack year history ( $r=0.131$ ,  $p=0.007$ ), however this relationship was not observed if never smokers were excluded from the analysis ( $p=0.123$ ).

At baseline, there were 137 patients (39.14%) on inhaled steroids, 91 (26%) on a long-acting beta agonist (LABA), 96 (27.43%) on a combined steroid and LABA, and 76 (21.71%) on a long acting antimuscarinic. The proportion of subjects on inhaled combination therapy (steroid and LABA) rose to 52.6% by the end of the study period. For the purposes of the current study, subjects were therefore considered to be on a particular therapy if on treatment for at least 50% of the study period. There was no difference in A $\alpha$ -Val<sup>360</sup> at baseline in subjects receiving inhaled corticosteroids (compared to those who were not). However, the baseline A $\alpha$ -Val<sup>360</sup> was greater ( $p=0.018$ ) in those receiving combination therapy with a median of 18.02 nM (IQR 13.98 -26.98) compared to those not on combination therapy with a median of 16.22 (12.23 - 22.23). The A $\alpha$ -



Val<sup>360</sup> was also greater ( $p=0.013$ ) in those receiving a long-acting antimuscarinic with a median of 18.81 (14.28 to 27.92) compared to those who were not (median 16.2; 12.44 to 21.59).

The median A $\alpha$ -Val<sup>360</sup> was higher ( $p<0.001$ ) in index cases ( $n=250$ ) who presented with symptoms of chronic lung disease (17.72 nM; IQR 19.93 – 26.00) compared to non-index cases ( $n=100$ ) who were identified through family screening (13.87; IQR 9.99 – 17.84). This difference ( $p<0.001$ ) persisted in never smokers with a median A $\alpha$ -Val<sup>360</sup> of 17.32 nM (IQR 14.30 – 22.25) in the 46 PiZ index cases compared to 13.32 (9.93 – 17.82) in the 42 non-index cases.

### **7.2.1 Cross-Sectional Relationships in PiZ Subjects**

The A $\alpha$ -Val<sup>360</sup> related to both physiological and radiological measures of COPD disease severity as well the SGRQ, which is a quality of life score (Table 30) [224]. The A $\alpha$ -Val<sup>360</sup> concentration also related to symptom severity measured by the COPD assessment tool (CAT) [123], in the subset ( $n=156$ ) who underwent this evaluation ( $r=0.289$ ,  $p<0.001$ ). Although the A $\alpha$ -Val<sup>360</sup> concentration did not relate to age ( $p=0.213$ ), height ( $p=0.389$ ) or weight ( $p=0.085$ ), a weak relationship was observed between A $\alpha$ -Val<sup>360</sup> and BMI ( $r=-0.091$ ,  $p=0.047$ ).

Multivariate analysis accounting for age, sex, height, smoking history, and A1AT concentration demonstrated significant relationships between A $\alpha$ -Val<sup>360</sup> and both the KCO (adjusted  $R^2$  -0.166;  $p<0.001$ ) and FEV<sub>1</sub> (adjusted  $R^2$  -0.129;  $p=0.005$ ). However, the patient's age was the strongest predictor of both physiological measures.

The A $\alpha$ -Val<sup>360</sup> related to the A1AT/NE complex concentration ( $r=0.339$ ,  $p=0.004$ ), a measure of total NE release, however there was no relationship with the plasma CRP ( $r=-0.023$ ,  $p=0.378$ ) which is a non-specific measure of inflammation. Furthermore, and of interest, the A $\alpha$ -Val<sup>360</sup> also related to the plasma A1AT concentration ( $r=-0.133$ ,  $p=0.007$ ) even within this group of subjects with severe (PiZ) A1AT deficiency. However, the absolute A1AT concentration did not relate to physiological or radiological measures of disease severity. The A $\alpha$ -Val<sup>360</sup> related better to sputum LTB<sub>4</sub> ( $r=0.328$ ,  $p=0.008$ ) than to sputum IL-8 ( $r=0.222$ ,  $p=0.053$ ) or sputum MPO ( $r=0.243$ ,  $p=0.061$ ) in the 50 subjects in whom spontaneous sputum was available and analysed.

Parameter	r	p
FEV <sub>1</sub> (litres)	-0.210	<0.001
FEV <sub>1</sub> (% predicted)	-0.229	<0.001
KCO (mmol/min/kPa/litre)	-0.243	<0.001
KCO (% predicted)	-0.229	<0.001
Upper zone voxel index (-910 Hounsfield units)	0.197	0.002
Lower zone voxel index (-910 Hounsfield units)	0.197	0.002
St George's respiratory questionnaire (symptoms)	0.257	<0.001
St George's respiratory questionnaire (activity)	0.235	<0.001
St George's respiratory questionnaire (impact)	0.251	<0.001
St George's respiratory questionnaire (total)	0.267	<0.001

*Table 30: The relationship ( $r$ ) between A $\alpha$ -Val<sup>360</sup> and physiological, radiological and symptomatic markers of disease severity in PiZ subjects with A1AT deficiency.*

## 7.2.2 Longitudinal Relationships

There was significant progression of all physiological parameters in the 350 PiZ subjects who were reviewed annually over a median period of 6.00 years (IQR 3.97 – 8.61) (Table 31). The median annual decline in FEV<sub>1</sub> (% predicted) was 0.52% (IQR 0.11 – 1.63), while the annual decline in absolute FEV<sub>1</sub> was 33.5 ml/yr (IQR 10.0 – 68) and the annual decline in KCO (% predicted) was 1.30% (IQR 0.22 – 2.29).

Parameter	Baseline Median (IQR)	Follow-up Median (IQR)	p (Baseline vs Follow-up)
<b>Age (years)</b>	51.26 (43.92 – 57.33)	58.05 (49.14 to 63.85)	
<b>FEV<sub>1</sub> (litres)</b>	1.69 (1.17 to 2.61)	1.40 (1.05 to 2.22)	<0.001
<b>FEV<sub>1</sub> (% predicted)</b>	55.62 (36.84 to 85.51)	47.82 (33.23 to 81.45)	<0.001
<b>KCO (mmol/min/kPa/litre)</b>	1.08 (0.86 to 1.36)	0.91 (0.72 to 1.18)	<0.001
<b>KCO (% predicted)</b>	67.06 (54.47 to 82.41)	59.50 (47.80 to 73.73)	<0.001
<b>FEV<sub>1</sub>:FVC</b>	41.25 (30.59 to 61.29)	35.42 (26.72 to 55.17)	<0.001

*Table 31: Aα-Val<sup>360</sup> was measured in 350 subjects with PiZ alpha-1-antitrypsin deficiency with a wide range of disease severity. The age and full lung function tests at baseline and at follow-up (median of 6 years later) are quoted. The statistical significance refers to the difference between follow-up and baseline values.*

The Aα-Val<sup>360</sup> concentration related to physiological parameters at both baseline and follow-up, however it did not relate to progression of either FEV<sub>1</sub> or KCO.

Linear regression analysis was performed using the following parameters: Aα-Val<sup>360</sup>, BMI, index status, smoking status (current, ex, never), age, sex, inhaled therapy (combination therapy, long

acting antimuscarinic or steroid) and baseline lung function (either FEV<sub>1</sub> or KCO). This identified inhaled steroid use, increasing age, index case status and higher baseline KCO as independent predictors of a more rapid decline in KCO (standardised b values of -0.114, -0.142, -0.132 and -0.225; p values 0.04, 0.022, 0.029, 0.001 respectively). Linear regression identified being male, a current smoker, and having a higher baseline FEV<sub>1</sub> as independent predictors of a more rapid decline in FEV<sub>1</sub> (standardised b values -0.158, -0.139, -0.144; p values 0.004, 0.020, 0.017 respectively).

### **7.2.3 Cross-Sectional and Longitudinal Relationships in Subjects with a ‘normal’ FEV<sub>1</sub>**

There was a subgroup of 96 PiZ subjects with an FEV<sub>1</sub> (% predicted) within the normal range (>80%), of whom 53 (55.2%) were never smokers, 39 (40.6%) were ex-smokers and 4 (4.2%) were current smokers. There were 62 non-index and 34 index patients. Within this subgroup, there was no relationship between Aα-Val<sup>360</sup> and age, weight, BMI or pack year smoking history, however, there were significant relationships between Aα-Val<sup>360</sup> and measures of disease severity (Table 32).

Parameter	r	p
FEV <sub>1</sub> (% predicted)	-0.181	0.039
KCO (% predicted)	-0.187	0.034
Upper zone voxel index (-910 Hounsfield units)	0.262	0.028
Lower zone voxel index (-910 Hounsfield units)	0.197	0.032
St George's respiratory questionnaire (symptoms)	0.268	0.004
St George's respiratory questionnaire (activity)	0.216	0.017
St George's respiratory questionnaire (impact)	0.241	0.010
St George's respiratory questionnaire (total)	0.265	0.005

*Table 32: Relationships between Aα-Val<sup>360</sup> and markers of disease severity in subjects with an FEV<sub>1</sub> (% predicted) within the normal range*

These 96 subjects were followed up on an annual basis for a median of 6.64 years (IQR 4.49 – 9.50). The median annual change within this subgroup was -0.25 (IQR -1.68 – 0.60) for FEV<sub>1</sub> (% predicted) and -0.96 (-1.86 – -0.04) for KCO (% predicted). The annual change in FEV<sub>1</sub> (%predicted) was greater (p=0.011) in index patients with a median of -1.04 (IQR -2.71 to 0.11) compared to non-index patients with a median of 0.03 (-1.20 to 0.84). There were no significant relationships between Aα-Val<sup>360</sup> or other parameters, however, and of importance, the baseline Aα-Val<sup>360</sup> concentration related to the annual change in KCO (% predicted) (r=-0.172, p=0.047) in this subgroup, although no factor was independently associated in linear regression analysis.

### **7.3 AA-VAL<sup>360</sup> IN SUBJECTS WITH A1AT DEFICIENCY: DISCUSSION**

The role of NE in the pathogenesis of COPD, particularly related to A1AT deficiency, has long been recognised and elegant mathematical and *in vitro* modelling predicted an exponential relationship between the A1AT concentration and extent of neutrophil mediated proteolytic damage [52-54]. Although NE is released from the neutrophil at a high concentration, its concentration rapidly decreases with diffusion and complete inhibition occurs within the neutrophil micro-environment when an equimolar concentration is reached with its main inhibitor (A1AT), even in A1AT deficient subjects. Therefore free NE activity cannot be detected regularly even in sputum obtained from subjects in the stable clinical state, and is never detectable in plasma samples [71]. The current study describes the use of a unique surrogate marker of NE activity (A $\alpha$ -Val<sup>360</sup>) which demonstrates exponentially greater NE activity in subjects with severe PiZ A1AT deficiency compared to those with less severe A1AT deficiencies or healthy controls, and is therefore consistent with the earlier mathematical model [52-54].

#### **7.3.1 A $\alpha$ -Val<sup>360</sup> in PiZ and non-PiZ A1AT Deficiencies**

There were a number of subjects with non-PiZ phenotypes who met the spirometric criteria for the diagnosis of COPD, however this relates to the number of smokers within this study population. Interestingly, the proportion of current or ex-smokers with an FEV<sub>1</sub>/FVC<0.7 was similar irrespective of A1AT phenotype (although, as expected, was slightly less in the PiZ subjects) which indicates the importance of tobacco smoke in disease pathogenesis, even in the

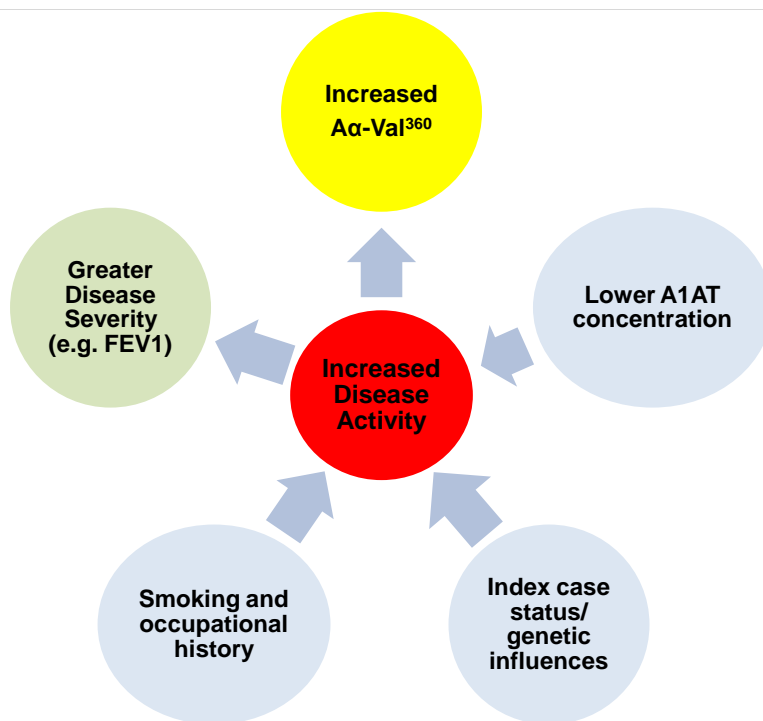
A1AT deficient population (as reported previously [248, 252]). The non-random referral pattern to the ADAPT project may also influence the distribution of emphysema seen amongst the different phenotypes, since subjects in the UK are not identified through population screening. Instead, patients are included in the registry if they are referred with an abnormal A1AT phenotype after presenting to their clinician with symptoms (index cases) or through family screening (non-index cases). Although this may not reflect the true distribution of the disease by phenotype within the general population, the data are nevertheless highly relevant since this large group of patients will be representative of those who present in clinical practice (i.e. with symptoms or as family members of those identified as having A1AT deficiency).

There remains controversy regarding the risk conferred by the PiSZ phenotype for the development of COPD [56], and it is therefore of interest that the current study shows that subjects with PiSZ A1AT deficiency have a greater A $\alpha$ -Val<sup>360</sup> concentration than either PiMZ or PiM subjects. The difference between PiSZ and healthy (never smoking) PiM subjects may partly be accounted for by differences in their smoking history or the presence of COPD (since these subjects were not matched). However, there were significant differences in A $\alpha$ -Val<sup>360</sup> concentrations between PiSZ and PiMZ subjects despite similar smoking patterns, spirometry, and distribution of subjects with an FEV<sub>1</sub>/FVC below 0.7 between the 2 groups. Although, a greater proportion of the PiMZ subjects were index cases (63.33% vs. 19.61%; p<0.001), it would be anticipated that this would be associated with a higher (and not lower A $\alpha$ -Val<sup>360</sup> concentration) since in the current study, index status in *PiZ* subjects was associated with a greater A $\alpha$ -Val<sup>360</sup> concentration. The greater A $\alpha$ -Val<sup>360</sup> in PiSZ subjects may indicate an increased risk of developing progressive lung disease compared to similar patients with less

severe deficiencies or normal A1AT concentrations. However, the  $A\alpha\text{-Val}^{360}$  is greatest in PiZ subjects, and furthermore the data demonstrated a relationship between the A1AT concentration and this marker of NE activity even within PiZ subjects suggesting the greatest risk remains for those with the most severe A1AT deficiencies. This is also consistent with the differences in clinical phenotype observed between PiZ, PiSZ patients and other A1AT phenotypes [56]. It should also be noted that physiological impairment is reported to be even greater in patients with the null versions of A1AT deficiency [253] and it would be of interest to study  $A\alpha\text{-Val}^{360}$  in these individuals where it would be predicted to be higher still.

Subjects with PiSZ A1AT deficiency have a significantly lower A1AT concentration than those with PiS A1AT deficiency yet have a similar  $A\alpha\text{-Val}^{360}$  concentration. However, in this relatively small group of patients, confounding factors have the potential to have a greater influence. For example, the higher than expected  $A\alpha\text{-Val}^{360}$  concentration in the PiS subjects may be explained by the chance inclusion in the registry of PiS patients with more severe disease since they have a significantly lower FEV<sub>1</sub> (% predicted) and number of subjects with an FEV<sub>1</sub>/FVC<0.7 than the PiSZ population and in this respect they behave more like patients with usual COPD (Chapter 5). Additionally, there were greater numbers of index cases with a PiS rather than PiSZ phenotype which may reflect pathophysiological processes and would also explain differences in neutrophilic disease activity between these populations (Figure 26 and Figure 27).





*Figure 26: The complex relationship between disease severity, activity and risk factors. In subjects with A1AT deficiency, there are a number of risk factors (blue circles) that are associated with greater disease progression and disease activity (red circle). In turn greater disease activity will be reflected by increases in Aα-Val<sup>360</sup> and eventually by changes in markers of disease severity (such as FEV<sub>1</sub> or CT densitometry). In the current study, PiS subjects had a higher Aα-Val<sup>360</sup> concentration and worse disease severity than PiSZ subject. It is likely that other factors (perhaps genetic or as yet unrecognised) have led to greater disease/neutrophil activity (and hence more severe disease and greater Aα-Val<sup>360</sup>) in the PiS population or acquisition to the registry. In the latter instance, there is likely to be a relationship between current disease activity and disease severity irrespective of phenotype (Figure 27) although this is discussed further in section 7.3.4.*

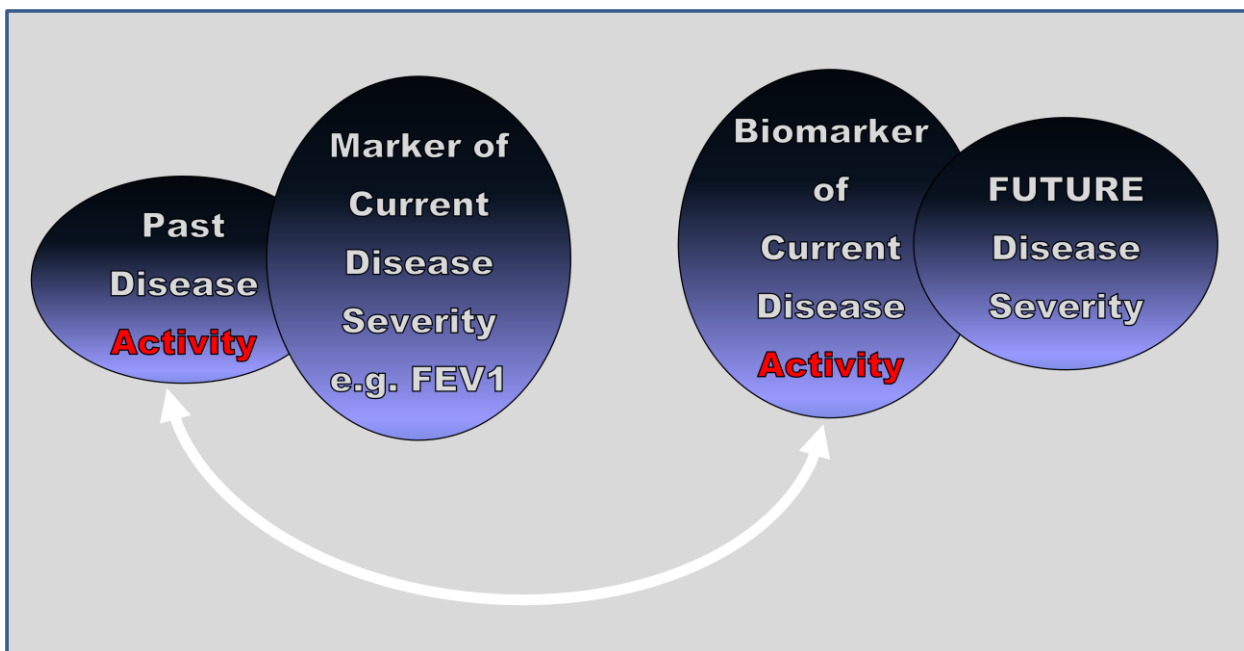


Figure 27: A marker of current disease activity would relate to future disease severity (since an active disease process would eventually lead to progressive tissue damage detectable by a marker of disease severity in the future). If the marker of disease activity is stable then it would also relate to past disease activity and hence there would be at least a partial relationship with current disease severity. This is discussed further in section 7.3.4.

### 7.3.2 A $\alpha$ -Val<sup>360</sup> in PiZ A1AT Deficient Subjects

There was a relationship between A $\alpha$ -Val<sup>360</sup> and pack year smoking history, however this was not observed if never smokers were excluded and suggests a binary population effect rather than a continuum. This is also consistent with previous studies which demonstrate a threshold (irrespective of pack year history) beyond which the risk of COPD increases exponentially [254]. It is therefore likely that significant previous cigarette exposure is sufficient to create ongoing evidence of NE activity, although the evidence of NE activity is greater in those who continue to smoke (i.e. greater A $\alpha$ -Val<sup>360</sup> concentrations in current versus ex-smokers).

A1AT deficiency is the only recognised genetic risk factor for the development of COPD, however the current study demonstrates that non-index subjects with PiZ deficiency have a lower A $\alpha$ -Val<sup>360</sup> concentration than other PiZ subjects. Of significant importance, it was also shown that never-smoking index cases have significantly greater A $\alpha$ -Val<sup>360</sup> concentrations than never-smoking non-index cases. This suggests the presence of additional neutrophilic risk factors (genetic or environmental) in index patients that have yet to be identified, and is consistent with previous work suggesting non-index, never smokers with PiZ deficiency have a normal life expectancy [250].

### **7.3.3 Stability of A $\alpha$ -Val<sup>360</sup>**

The stability of A $\alpha$ -Val<sup>360</sup> over time has previously been demonstrated (Chapter 4 and [214]) and in the current study the concentration of A $\alpha$ -Val<sup>360</sup> does not relate to age. Since in a cross-sectional study, age is partly a reflection of disease duration, this again indicates the stability of A $\alpha$ -Val<sup>360</sup> which is important for a biomarker.

### **7.3.4 The Cross-sectional Relationship of A $\alpha$ -Val<sup>360</sup> with Markers of COPD Disease Severity**

It is likely that an important characteristic of an ideal biomarker of disease activity is to relate to cross-sectional markers of disease severity (such as FEV<sub>1</sub>) [207, 209]. A marker of severity at a

single time point will broadly reflect disease activity but will be influenced by age (length of disease progression) and predisposing factors such as tobacco exposure, but will not predict progression (Figure 27).

However, this is also complicated by the absence of a gold standard marker of COPD disease severity or activity. For example, FEV<sub>1</sub> may exhibit greater day-to-day variability than its decline over several years [255], and pharmacological interventions may significantly increase FEV<sub>1</sub> yet fail to influence mortality or subsequent progression [101]. However, FEV<sub>1</sub> is widely used and relates to overall and lung specific mortality [206], and therefore importantly there was a relationship between FEV<sub>1</sub> and Aα-Val<sup>360</sup>. Additionally, Aα-Val<sup>360</sup> also related to other widely accepted markers of disease severity in this large cohort of patients including gas transfer and CT densitometry demonstrating that this marker is (at least partly) a measure of current emphysema severity. Furthermore, there were highly significant relationships with 2 independent measures of quality of life in subjects with COPD: the SGRQ [224] and the CAT score [123]. This is important since it is well recognised that patients may have relatively severe symptoms despite relatively mild physiological changes [91], and therefore an ideal biomarker of disease severity or activity should relate to measures of symptoms, as well as markers of disease severity (radiological, physiological or otherwise).

Some caveats should be applied to the hypothesised relationship between disease activity and disease severity since it assumes disease activity is stable throughout or alternatively that the biomarker measures the *predominant* level of disease activity over the course of the disease process. If activity is not stable, when biomarkers are measured in large cross-sectional studies it

is more likely that they will be obtained at a point which reflects the *predominant* (and presumably longstanding) level of disease activity with a resulting relationship between disease severity and activity across the population, even if this relationship is not observed for each individual. Currently, there is no gold standard marker of disease activity and therefore it is difficult to conclusively address these important issues, however the current thesis provides some data to suggest that the marker A $\alpha$ -Val<sup>360</sup> (and hence disease activity) is stable at least over a period of 2 years (section 4.2) and is stable in repeated measures over a period of 84 days (section 4.1). Importantly, there is no relationship to age in the large population of PiZ A1AT deficient subjects which suggests that there is no systematic variation in disease activity over time in this cross-sectional study. Also, while patients experience exacerbations and an associated increase in A $\alpha$ -Val<sup>360</sup>, it returns relatively rapidly to baseline which is consistent with both the stability of underlying disease activity and the small contribution of exacerbations to decline in FEV1 [181].

Data from studies investigating other potential biomarkers of disease activity also suggest a relationship between disease activity and disease severity, since urinary desmosine concentration relates to disease severity (albeit relatively weakly) [201] which again indicates that activity may be relatively stable throughout the course of the disease (or alternatively that urinary desmosine is increased in more severe disease). There is also the potential that underlying *pathophysiological* disease activity may be stable, but changes in markers of severity such as FEV1 may not be linear with respect to time, owing to the complex relationship between airflow obstruction and pathological changes within the airway and parenchyma. With this in mind, more recent data suggest that the natural history of the decline in physiological parameters is more heterogeneous than previously thought [88]. However, further data are required to establish the true longitudinal

variability of progression in patients with COPD using a range of measures including change in FEV1, gas transfer and CT densitometry in combination with serial measurement of biomarkers ideally over a period of 15 to 20 years. This clearly is beyond the scope of the current thesis.

### **7.3.5 A $\alpha$ -Val<sup>360</sup> and BMI**

The current study also demonstrates that A $\alpha$ -Val<sup>360</sup> relates to BMI, which is of interest since a low BMI is associated with a higher mortality in subjects with COPD [129] and is therefore indicative of the systemic nature of this disease process. This concept is increasingly recognised since weight loss is a relatively common phenomenon in subjects with COPD, and relates to both excessive energy expenditure and insufficient calorie intake [256]. Also, a link between antinuclear autoantibodies and low BMI in subjects with COPD has been reported, which is not present in subjects without COPD [257], although this was not replicated in a second study [258]. If true, this may partly explain the systemic aspect of the disease process since subjects with COPD and a lower BMI are more likely to be positive for an autoantibody, have an emphysema predominant phenotype (indicating lung parenchymal destruction which may be associated with an autoimmune process) [259], and have a higher A $\alpha$ -Val<sup>360</sup> (as a measure of the final common pathway leading to connective tissue destruction). However, the underlying pathological mechanism explaining the relationship between BMI, emphysema and autoantibodies has yet to be identified, and therefore this can only be considered an association at this stage rather than causation.

An association between TNF- $\alpha$  and a lower BMI in subjects with COPD has also been described, since TNF- $\alpha$  can trigger a decreased energy intake, and adversely influence protein and lipid metabolism [260]. Interestingly, it was also shown that subjects with a low BMI also had a lower KCO (indicating a more severe emphysematous process) compared to those with a normal BMI, although the association with emphysema was not confirmed since the study pre-dated quantitative CT scanning. Also, causation has not been definitively proven, since starvation itself may lead to an elevated level of TNF- $\alpha$  [261]. Additionally, trials of anti-TNF- $\alpha$  therapy have had disappointing (and possibly even detrimental) results [212], however, the absence of a demonstrable therapeutic effect may partly reflect generally low levels of TNF- $\alpha$ . On the other hand, the presence of TNF polymorphisms (such as rs361525) is associated with chronic bronchitis, a lower BMI and faster disease progression, in addition to increased airways secretions of TNF- $\alpha$  [262]. The detection of such polymorphisms for TNF- $\alpha$  may therefore enable the use of more targeted therapy for COPD, with the efficacy measured using a marker of disease activity.

### **7.3.6 Relationship of A $\alpha$ -Val<sup>360</sup> to other biochemical markers**

Although sputum biomarkers are impractical since (for example) not all patients produce sputum, they nevertheless relate to disease progression in COPD [21] and in the current study they related to A $\alpha$ -Val<sup>360</sup>. Interestingly, there was a stronger relationship between A $\alpha$ -Val<sup>360</sup> and LTB<sub>4</sub> than between A $\alpha$ -Val<sup>360</sup> and IL-8 which is the converse of findings in subjects with usual COPD (Chapter 5 and [217]). Both IL-8 and LTB<sub>4</sub> promote transendothelial migration of neutrophils,

and are increased in subjects with COPD related to A1AT deficiency compared to those with usual COPD (with an associated increase in sputum chemotactic activity) [263]. However, *in vitro* studies demonstrate that the relative contributions of IL-8 and LTB4 towards chemotaxis in the *airways* are similar in both usual COPD and A1AT deficiency, with the greatest contribution provided by LTB4 in both groups [263]. The disparity suggested by the *in vitro* data and A $\alpha$ -Val<sup>360</sup> data regarding the relative roles of LTB4 and IL-8 may be explained by differences in chemotaxis *in vitro* compared to the true *in vivo* contributions, particularly in the lung parenchyma rather than the airway. The plasma marker A $\alpha$ -Val<sup>360</sup> may be more influenced by neutrophilic activity within the lung parenchyma, while clearly sputum markers will be more influenced by changes within the airway. Alternatively, the better relationship between A $\alpha$ -Val<sup>360</sup> and LTB4 in A1AT deficiency may reflect greater activation of neutrophils rather than stronger chemotaxis, since LTB4 can also stimulate both neutrophil degranulation and oxidative metabolism [264]. This neutrophil ‘activation’ would be associated with greater tissue damage (and higher A $\alpha$ -Val<sup>360</sup> concentrations) in the A1AT deficient subject compared to the non-deficient individual because of the differences in quantum proteolytic events between these groups as explained previously.

The A $\alpha$ -Val<sup>360</sup> also partly relates to the A1AT/NE complex which is a marker of total NE release. However, the complex does not relate to any measure of disease severity which suggests that it is important to measure the activity of NE prior to its inhibition rather than total release.



### 7.3.7 Longitudinal Relationships

Over the 6 years of the study there was significant disease progression as measured by all physiological tests. Linear regression demonstrated index case status, increasing age, higher baseline KCO and use of inhaled steroids to be significant factors for progressive disease measured by gas transfer which again highlights the importance of index case status in determining prognosis. The correlation with age is perhaps unsurprising since COPD is more likely to develop in A1AT deficient subjects with time, and reflects duration of the patient's exposure to risk factors. The relationship with a higher baseline KCO is important and may be explained by the presence of increased disease activity in subjects with earlier disease. The relationship of decline with inhaled steroids is of interest, although it should be interpreted with significant caution, since this was a weak association in a non-randomised trial. This may therefore represent confounding rather than a true association since, for example, subjects who were deteriorating more quickly may be more likely to be commenced on inhaled corticosteroid treatment. Additionally, inhaled steroids were more likely to be prescribed to subjects with an 'asthmatic' component which makes further analysis more difficult, since there is controversy regarding the diagnosis of asthma in subjects with COPD, emphysema and/or A1AT deficiency. Although NICE guidelines [2] suggest asthma may be distinguished from COPD by symptoms (Table 33), many of these symptoms are shared and they are less helpful in the 35 years or older, ex or current smoker who is commonly referred to the ADAPT registry. Also, it is increasingly recognised that patients with COPD have diurnal variability [265] and quantification of 'significant variability' may be arbitrary. Furthermore, while significant bronchodilator reversibility of over 400 ml may help to distinguish asthma and COPD [2], lesser reversibility

commonly occurs in patients with asthma and may also be observed in patients with COPD [95]. Finally asthma and COPD probably co-exist in a number of patients [266].

Clinical Feature	COPD	Asthma
Smoker or ex-smoker	Common	Possibly
Symptoms<35 yrs old	Rare	Often
Chronic productive cough	Common	Uncommon
Breathlessness	Persistent and progressive	Variable
Night-time waking with breathlessness or wheeze	Uncommon	Common
Significant diurnal or day-to-day variability	Uncommon	Common

*Table 33. Clinical features which have been suggested to distinguish between asthma and COPD [2]*

Linear regression analysis identified being male and a current smoker as independent risk factors for disease progression measured by spirometry. The  $A\alpha\text{-Val}^{360}$  was greater in subjects who continued to smoke, and these subjects therefore have both ongoing NE activity and greater spirometric decline. The association between a higher baseline  $FEV_1$  (% predicted) and greater rate of decline is again of importance since these are likely to be subjects in whom targeted intervention may be more effective.

Although the  $A\alpha\text{-Val}^{360}$  related to disease progression measured by physiological markers of disease severity in subjects with an  $FEV_1 > 80\%$ , it did not appear to relate to disease progression in the overall cohort, however, the relationships between markers of disease activity and disease severity are likely to be complex. Firstly, the absence of a relationship may be at least partly explained by confounding factors within the heterogeneous nature of the cohort which included subjects with a wide range of disease severity and a mix of index and non-index cases and current

and never smokers. Secondly, it may also be explained by the inter-patient variability of A $\alpha$ -Val<sup>360</sup> or the variability and relative insensitivity of physiological tests for detecting changes in disease severity, since the annual loss of FEV<sub>1</sub> was only 0.52 (IQR 0.11 – 1.63) % predicted/year (which is close to normal) while the annual change in KCO (% predicted) was -1.30 (IQR 0.22 – 2.29) % predicted. Therefore these physiological tests may not allow discrimination between rapid and slow decliners, even within the scope of this large study. Additionally, physiological decline may not be linear, which would further complicate the relationships. Thirdly, the discrepancy between index and non-index cases both in terms of A $\alpha$ -Val<sup>360</sup> concentrations and disease severity also suggests that there are further confounding factors (presumably genetic) that are not adequately accounted for by the multivariate model used within the current study. Fourthly, it is important to consider that a marker of disease activity may predict overall disease progression, which may not be adequately measured by a single physiological marker such as either FEV<sub>1</sub> or KCO, since some patients may exhibit significant changes in FEV<sub>1</sub> and yet have little change in KCO. Fifthly, it is possible that a marker of neutrophilic disease activity (such as A $\alpha$ -Val<sup>360</sup>) is a more important predictor of disease progression only in subjects with mild spirometric changes who may have an early and more distinct disease process.

Sixthly, disease activity in patients may be highly variable and in these circumstances there would be no relationship between a measure of disease activity at a single time point and subsequent disease progression. However, the data reported in the current thesis suggest that, at least over the short term, A $\alpha$ -Val<sup>360</sup> is stable and therefore a relationship between A $\alpha$ -Val<sup>360</sup> and disease progression over a 2 or 3 year period may be expected if it reflects the key underlying

disease activity. Further data is required however to confirm stability over a longer period of time and to clarify this relationship.

Finally,  $A\alpha\text{-Val}^{360}$  may be a good marker of NE related disease activity in the stable state, however, this may provide insufficient data to allow predictions to be made about disease progression. For example, a model of disease progression based purely on stable state  $A\alpha\text{-Val}^{360}$  data will not necessarily account for the number of exacerbations (since both disease activity and  $A\alpha\text{-Val}^{360}$  rise significantly during these periods). With this in mind it should be noted that subjects with symptoms of COPD who experienced a purulent exacerbation had a higher stable state  $A\alpha\text{-Val}^{360}$  (Chapter 5), indicating this biomarker (at least partly) reflects the nature of exacerbations even when measured in clinically stable subjects. Also, enzymes other than NE may also be important in the pathogenesis of COPD (including Pr3 and MMPs), which may not be adequately accounted for by the  $A\alpha\text{-Val}^{360}$  model. However, there were clear (and highly statistically significant) relationships between  $A\alpha\text{-Val}^{360}$  and current markers of disease severity in COPD, and furthermore there is a trend towards a relationship with disease progression in the population with symptoms of COPD (Chapter 5) and the A1AT population with an  $FEV_1 > 80\%$  indicating that  $A\alpha\text{-Val}^{360}$  may be a marker of early disease activity (and may particularly relate to emphysema progression), although greater prognostic accuracy may be achieved by combining the results with a number of other relevant activity markers.

### 7.3.8 A $\alpha$ -Val<sup>360</sup> as a Marker of Early Disease

Support for the concept of A $\alpha$ -Val<sup>360</sup> as a marker of activity in *early* disease was provided by the highly significant relationships with physiological, radiological and symptomatic markers of COPD disease *severity* in subjects with an FEV<sub>1</sub> within the normal range (>80%). Furthermore within this subgroup, there was a significant association between A $\alpha$ -Val<sup>360</sup> and disease progression measured by decline in gas transfer. These data suggest that there are patients with little physiological evidence of disease who have an ongoing disease process which can be detected by a sensitive marker of disease activity such as A $\alpha$ -Val<sup>360</sup>. Also, it demonstrates the potential heterogeneous nature of the pathophysiological processes in this group of patients which includes those with both *early* and *mild* disease. This suggests that intervention in this group of patients with earlier disease and a higher A $\alpha$ -Val<sup>360</sup> would be of greatest benefit, however clearly large prospective studies are required to investigate this further.

### 7.3.9 Summary

In the current study, the A $\alpha$ -Val<sup>360</sup> relates to the prevailing A1AT concentration *in vivo* confirming previous *in vitro* and observational data. Also, data from this large cohort of subjects with PiZ A1AT deficiency demonstrate that A $\alpha$ -Val<sup>360</sup> relates cross-sectionally to physiological, radiological and symptomatic markers of disease severity. Importantly, these relationships are also present in subjects with mild or no physiological change, in whom the A $\alpha$ -Val<sup>360</sup> concentration also relates to disease progression. A $\alpha$ -Val<sup>360</sup> may be of particular use in

identifying subjects with progressive early stage disease especially as a measure of the emphysematous component of COPD. This marker maybe of greatest relevance in clinical trials, to develop and identify treatment interventions which may be effective prior to significant physiological or symptomatic decline. However, further work is required to understand the complex relationships between this marker and disease activity, severity and progression in subjects with A1AT deficiency in general, potentially by exploring the activity of other important enzymes as well as the impact of exacerbations. However, this is beyond the scope of the current thesis.

# **CHAPTER 8.IDENTIFYING SPECIFIC PR3**

## **FIBRINOGEN CLEAVAGE PRODUCTS: RESULTS**

### **AND DISCUSSION**

#### **8.1 RESULTS**

##### **8.1.1 Low Molecular Weight Peptides**

MS analysis detected a number of peptides in the solutions of fibrinogen following incubation with NE or Pr3 at 37°C, and many of these peptides were successfully identified (Figure 28) using peptide mass fingerprinting (MASCOT database). The identified peptides were derived from fibrinogen (the beta, gamma and alpha chains), Pr3 (termed myeloblastin by MASCOT) as well as minor contaminants of the human fibrinogen preparation (transthyretin, complement, immunoglobulin and keratin). The peak list for all the peptides or fragments identified by MASCOT in the solutions with a mass:charge below 5000 is shown in Figure 29, although there were also a number of peptides that were detected by MS but not identified. Although the peak lists for the NE/fibrinogen and the Pr3/fibrinogen solutions were similar, there were also peaks which were unique to either NE or Pr3 (Figure 30) indicating that certain products formed by the cleavage of fibrinogen are enzyme specific.

**Taxonomy** : Homo sapiens (human) (20266 sequences)  
**Timestamp** : 7 Jan 2011 at 15:36:27 GMT  
**Protein hits** : [FIBA HUMAN](#) Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2  
[CO4B HUMAN](#) Complement C4-B OS=Homo sapiens GN=C4B PE=1 SV=1  
[TTHY HUMAN](#) Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1  
[FIBG HUMAN](#) Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3  
[K2C6A HUMAN](#) Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3  
[PRTN3 HUMAN](#) Myeloblastin OS=Homo sapiens GN=PRTN3 PE=1 SV=3  
[ANT3 HUMAN](#) Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 SV=1  
[FIBB HUMAN](#) Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2  
[IGHG1 HUMAN](#) Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1

[FIBA HUMAN](#) Mass: 94914 Score: 195 Matches: 28(5) Sequences: 28(5) emPAI: 0.57

Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2

Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1067.5225	1066.5152	1066.5117	3.28	0	30	2.3	1	U	I.MEILRGDFS.S
1159.5593	1158.5521	1158.5710	-16.34	0	30	2.9	1	U	T.ASTGKTFPGFF.S
1341.6891	1340.6818	1340.6837	-1.42	0	40	0.31	1	U	V.SFRGADYSLRAV.R
1350.6152	1349.6079	1349.6212	-9.83	0	57	0.004	1	U	D.SGEGDFLAEGGGVR.G
1385.6126	1384.6053	1384.6106	-3.83	0	36	0.38	1	U	V.SETESRGSESGIF.T
1465.6632	1464.6559	1464.6481	5.30	0	55	0.006	1	U	A.DSGEGDFLAEGGGVR.G
1474.6894	1473.6822	1473.6963	-9.57	0	26	5.8	1	U	T.ASTGKTFPGFFSPM.L
1478.7182	1477.7109	1477.7062	3.17	0	44	0.11	1	U	S.SSHHPGIAEFPSRG.K
1536.6952	1535.6879	1535.6852	1.77	0	52	0.014	1	U	T.ADSGEGDFLAEGGGVR.G
1573.7984	1572.7911	1572.8082	-10.91	0	34	1.3	1	U	T.SLGGWLLIQQRMDG.S
1587.7549	1586.7476	1586.7803	-20.62	0	26	6.6	1	U	T.ASTGKTFPGFFSPML.G
1640.7170	1639.7097	1639.7114	-1.02	0	32	0.89	1	U	T.SYNRGDSTFESKSY.K
1644.7587	1643.7514	1643.8018	-30.65	0	29	3.1	2	U	T.ASTGKTFPGFFSPMLG.E
1773.7824	1772.7751	1772.7968	-12.20	0	32	0.91	1	U	F.PGFFSPMLGEFVSETE.S
1822.8862	1821.8789	1821.8758	1.73	0	42	0.19	1	U	T.KESSSHHPGIAEFPSRG.K
1846.8431	1845.8359	1845.8606	-13.39	0	61	0.0017	1	U	T.ADSGEGDFLAEGGGVVRGPR.V
1899.8703	1898.8630	1898.8468	8.53	0	46	0.05	1	U	T.SYNRGDSTFESKSYKM.A
1915.8280	1914.8207	1914.8232	-1.27	0	26	2.8	1	U	T.SSTSYNRGDSTFESKSY.K
1920.9035	1919.8962	1919.9128	-8.66	0	33	1.4	1	U	T.ASTGKTFPGFFSPMLGEF.V
1958.8871	1957.8798	1957.8865	-3.42	0	46	0.046	1	U	V.SETESRGSESGIFTNTKE.S
1970.9065	1969.8992	1969.8840	7.73	0	34	0.89	1	U	T.SYNRGDSTFESKSYKMA.D
2037.9753	2036.9680	2036.9664	0.80	0	36	0.85	1	U	T.NTKESSSHHPGIAEFPSRG.K
2044.9705	2043.9632	2043.9974	-16.74	0	28	4.3	1	U	T.ADSGEGDFLAEGGGVVRGPRV.V
2045.9252	2044.9179	2044.9185	-0.31	0	43	0.098	1	U	V.SETESRGSESGIFTNTKES.S
2174.9913	2173.9841	2173.9586	11.7	0	26	4.9	1	U	T.SSTSYNRGDSTFESKSYKM.A
2553.2097	2552.2024	2552.1781	9.50	0	99	3.6e-007	1	U	T.ASTGKTFPGFFSPMLGEFVSETES.R
2648.1877	2647.1804	2647.1746	2.18	0	85	5.9e-006	1	U	V.SETESRGSESGIFTNTKESSHHPG.I
2766.3100	2765.3028	2765.3007	0.74	0	43	0.14	1	U	T.ASTGKTFPGFFSPMLGEFVSETESRG.S

2. [CO4B HUMAN](#) Mass: 192673 Score: 91 Matches: 4(2) Sequences: 4(2) emPAI: 0.05

Complement C4-B OS=Homo sapiens GN=C4B PE=1 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1739.9273	1738.9200	1738.9227	-1.54	0	26	8.7	1	U	R.NGFKSHALQLNNRQI.R
1890.9627	1889.9554	1890.0098	-28.78	0	80	3.5e-005	1	U	R.GLEELQFSLGSKINVK.V
1896.0054	1894.9981	1895.0238	-13.52	0	29	4.2	1	U	R.NGFKSHALQLNNRQIR.G
2751.2254	2750.2181	2750.2858	-24.61	0	57	0.0031	1	U	R.TLEIPGNSDPNMPDGFNSYVRVT.A

Proteins matching the same set of peptides:

[CO4A HUMAN](#) Mass: 192650 Score: 91 Matches: 4(2) Sequences: 4(2)

Complement C4-A OS=Homo sapiens GN=C4A PE=1 SV=1

3. [TTHY HUMAN](#) Mass: 15877 Score: 73 Matches: 5(2) Sequences: 5(2) emPAI: 0.69

Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1

Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
958.5001	957.4928	957.5131	-21.18	0	41	0.24	1	U	T.TAVVTNPKE.-
1063.5149	1062.5076	1062.5094	-1.73	0	68	0.0005	1	U	V.VFTANDSGPR.R
1250.5439	1249.5366	1249.5615	-19.94	0	33	0.81	1	U	R.KAADTWEFFA.S
1969.9546	1968.9474	1969.0044	-28.97	0	43	0.17	1	U	A.LLSPYSYSTTAVVTNPKE.-
2225.0911	2224.0838	2224.1627	-35.47	0	55	0.011	1	U	T.IAALLSPYSYSTTAVVTNPKE.-

4.



<b>FIBG HUMAN</b> <b>Mass:</b> 51479 <b>Score:</b> 66 <b>Matches:</b> 2(1) <b>Sequences:</b> 2(1) <b>emPAI:</b> 0.09 Fibrinogen gamma chain OS=Homo sapiens GN=FGG PE=1 SV=3									
Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1117.6130	1116.6057	1116.6291	-20.96	0	38	0.51	1	U	T.QSAIPYALRV.E
2690.2878	2689.2805	2689.2886	-3.03	0	66	0.00078	1	U	T.ADYAMFKVGPEADKYRLTYAYFA.G
<hr/>									
<b>5. K2C6A HUMAN</b> <b>Mass:</b> 60008 <b>Score:</b> 52 <b>Matches:</b> 2(0) <b>Sequences:</b> 2(0) <b>emPAI:</b> 0.07 Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3									
Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1407.6915	1406.6842	1406.7041	-14.18	0	52	0.021	1	U	K.ADTLTDEINFLR.A
1701.7731	1700.7658	1700.8006	-20.46	0	32	1.4	1	U	G.LGLGGSSSYSGSLGVGGG.F
<hr/>									
Proteins matching the same set of peptides: <b>K2C6B HUMAN</b> <b>Mass:</b> 60030 <b>Score:</b> 52 <b>Matches:</b> 2(0) <b>Sequences:</b> 2(0) Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5									
<hr/>									
<b>6. PRTN3 HUMAN</b> <b>Mass:</b> 27789 <b>Score:</b> 52 <b>Matches:</b> 1(0) <b>Sequences:</b> 1(0) <b>emPAI:</b> 0.16 Myeloblastin OS=Homo sapiens GN=PRTN3 PE=1 SV=3									
Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1042.5196	1041.5123	1041.5284	-15.39	0	52	0.018	1	U	T.RLFPDFFT.R
<hr/>									
<b>7. ANT3 HUMAN</b> <b>Mass:</b> 52569 <b>Score:</b> 50 <b>Matches:</b> 1(0) <b>Sequences:</b> 1(0) <b>emPAI:</b> 0.08 Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 SV=1									
Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2291.1072	2290.0999	2290.1441	-19.28	0	50	0.032	1	U	K.AFLEVNEEGSEAAASTAVVIAGR.S
<hr/>									
<b>8. FIBB HUMAN</b> <b>Mass:</b> 55892 <b>Score:</b> 45 <b>Matches:</b> 2(0) <b>Sequences:</b> 2(0) <b>emPAI:</b> 0.08 Fibrinogen beta chain OS=Homo sapiens GN=FGB PE=1 SV=2									
Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1160.6520	1159.6448	1159.6502	-4.69	0	39	0.32	1	U	M.KIRFFFPQQ.-
2551.1382	2550.1309	2550.2238	-36.40	0	45	0.057	1	U	V.KDNENVNEYSSLEKHQLYI.D
<hr/>									
<b>10. IGHG1 HUMAN</b> <b>Mass:</b> 36083 <b>Score:</b> 43 <b>Matches:</b> 2(0) <b>Sequences:</b> 1(0) <b>emPAI:</b> 0.12 Ig gamma-1 chain C region OS=Homo sapiens GN=IGHG1 PE=1 SV=1									
Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2130.9800	2129.9727	2130.0534	-37.90	0	44	0.1	1	U	D.PEVKFNWYVDGVEVHNAK.T
2130.9846	2129.9773	2130.0534	-35.73	0	(37)	0.54	1	U	D.PEVKFNWYVDGVEVHNAK.T

Figure 28: Peptide mass fingerprinting: the figure includes the results of a MASCOT search using data generated by MS of the Pr3/fibrinogen solution (15 minute incubation at 37°C). The displayed data only include the results where peptide mass fingerprinting successfully identified the peptide and parent protein. Peptides derived from the fibrinogen beta, gamma and alpha chain as well as from Pr3 (myeloblastin) were detected and identified. The detection of minor contaminants of the human fibrinogen (transthyretin, complement, immunoglobulin and keratin) demonstrates the sensitivity of MS. The data include the experimental mass:charge ratio ('observed'); the experimental mass:charge transformed to a relative molecular mass ('Mr (expt)'); the calculated relative molecular mass for the peptide sequence ('Mr (calc)'); number of missed cleavage site ('miss'); probability score expressed as  $-10 \times \log(\text{probability})$  ('Score'); the number of times the expected 'score' would be obtained purely by chance, where a lower score is more significant ('expect'); rank of the ions match, where 1 is the best match ('rank'); U=peptide is unique to the protein ('unique'); the sequence of the peptide ('peptide').

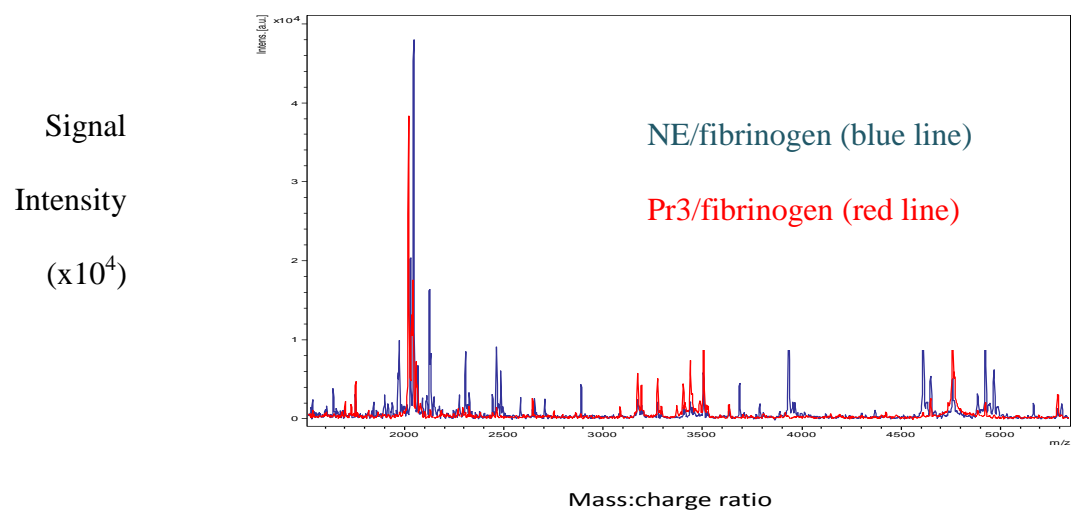


Figure 29: Mass spectrometry of solutions of NE/fibrinogen or Pr3/fibrinogen (15 minute incubation at 37°C, followed by inhibition with 0.1% TFA). Each peak represents the detection of a fragment or peptide at the specific mass:charge ratio, while the signal intensity gives an indication of the number of ions detected. Peaks with a higher intensity can be confidently interpreted as representing a true finding (rather than background interference). Since the intensity largely reflects the ease of ionisation of the peptide, this measure only partly relates to the concentration of the peptide. Peaks at the same masses were detected in both solutions, however there were also peaks specifically formed through cleavage by Pr3 (red line) or NE (blue line) (Figure 30).

A peak with a mass of 2021.3 daltons was detected in the Pr3/fibrinogen but not the NE/fibrinogen solution (following incubation at 37°C for 15 minutes) [Figure 30]. MS analysis of plasma from subjects with A1AT deficiency detected a similar peak, indicating a peptide of the same mass is also present *in vivo*, suggesting a specific Pr3 degradation product may be detectable *in vivo*. However, it was not possible to identify this peak using peptide mass fingerprinting and the MS-MS analysis was also unsuccessful as the fragmentation of the target was poor and therefore this fragment cannot conclusively be related to either Pr3 or fibrinogen. There was also a specific peak in the NE/fibrinogen solution and while this could not be detected *in vivo*, this may be accounted for by further *in vivo* degradation.

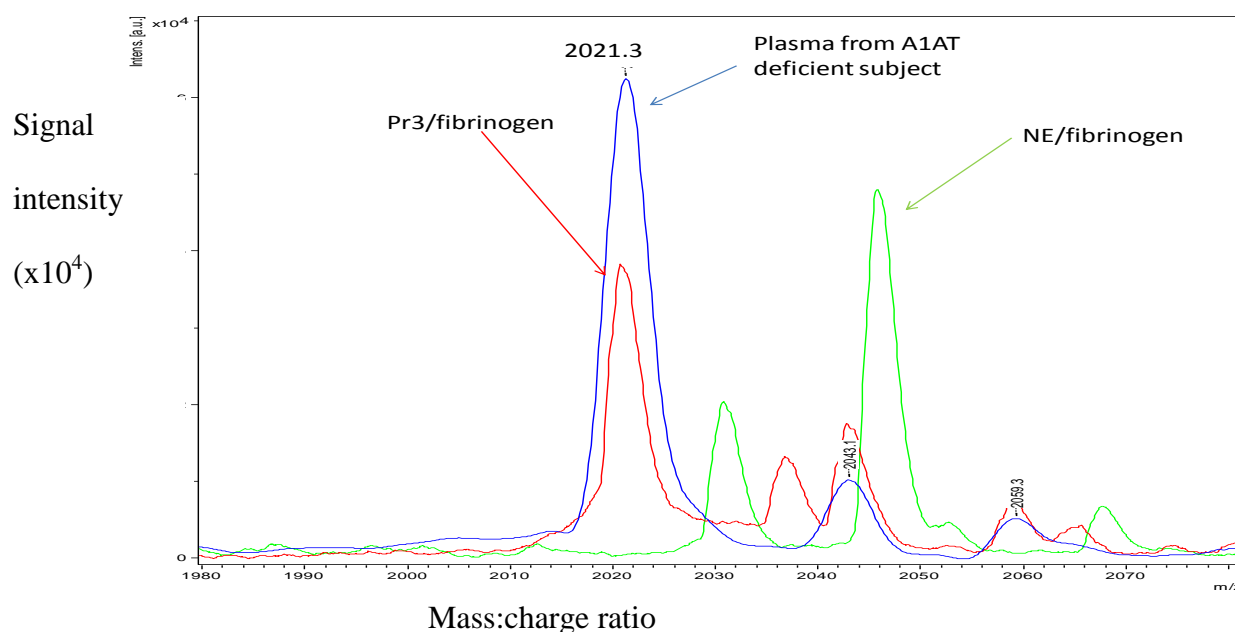


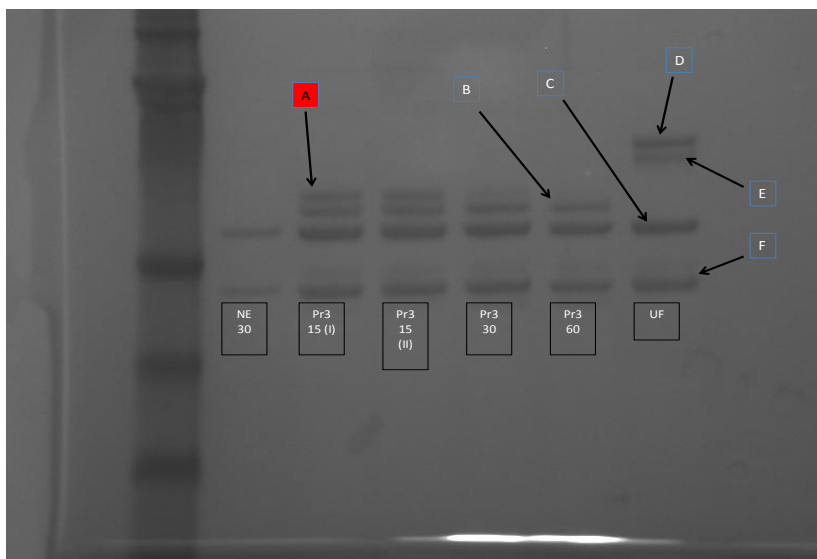
Figure 30: The MS analysis of the solution of Pr3/fibrinogen detected a peak at a molecular weight of 2021.3 Daltons. This peak was also present in plasma from subjects with alpha-1-antitrypsin deficiency but was not identified in the NE/fibrinogen solution. Similarly, an NE specific fibrinogen degradation product was also formed with a molecular weight of 2046 daltons, and while this fragment was not identified in plasma this may be explained by further *in vivo* degradation of the fragment. These data suggest that there may be a fragment (with a molecular weight of 2021.3 daltons) which is formed by the actions of Pr3 on fibrinogen (but not NE) which can be detected *in vivo* in patients with A1AT deficiency. However, in the absence of specific MS identification, this cannot be concluded categorically since it is also possible that an alternate substance (unrelated to fibrinogen or Pr3) with the same mass:charge ratio is present in the plasma of patients with A1AT deficiency.

### 8.1.2 Isolation of Cleavage Products using Gel Electrophoresis

In the current thesis, further analysis focussed on the identification of larger molecular weight cleavage products since there are more likely to have a greater circulating half-life *in vivo* and to be more enzyme specific (since they will be generated by the initial Pr3 cleavage of fibrinogen) [214].

The non-reducing gel electrophoresis did not demonstrate any bands below 160 kDa. However, the reducing gel electrophoresis demonstrated several bands in both the enzymatically cleaved and uncleaved fibrinogen which were labelled *A* to *F* (Figure 31).

Importantly, bands *A* and *B* were present in the Pr3/fibrinogen solutions but were not in the NE/fibrinogen solution or the uncleaved fibrinogen. Band *B* persisted in the 120 and 180 minute samples of Pr3/fibrinogen, however band *A* became progressively fainter over the same time period and was barely visible even at 60 minutes. Band *A* was also present after incubation of Pr3 with fibrinogen for only one minute.



*Figure 31: Reducing Polyacrylamide Gel Electrophoresis of enzymatically cleaved and uncleaved fibrinogen. From left to right, the columns are labelled NE 30 (NE/fibrinogen incubated for 30 minutes); Pr3 15 (I) (Pr3/fibrinogen incubated for 15 minutes (1<sup>st</sup> sample)); Pr3 15 (II) (Pr3/fibrinogen incubated for 15 minutes (2<sup>nd</sup> sample)); Pr3 30 (Pr3 and fibrinogen incubated for 30 minutes); Pr3 60 (Pr3 and fibrinogen incubated for 60 minutes); UF (uncleaved fibrinogen). The Pr3 and fibrinogen were incubated on a number of occasions to ensure repeatability and in this figure, samples from 2 different incubations (both taken at 15 minutes) are shown on the same electrophoresis gel (labelled as I and II). The bands of interest are labelled A to F. Similar bands were generated for NE/fibrinogen at 15 minutes and 30 minutes. Electrophoresis demonstrated identical bands with repeat testing.*

### 8.1.3 Peptide Mass Fingerprinting

Peptide mass fingerprinting of the in-gel digests successfully identified the bands labelled *A* to *F* (Figure 30). Bands *D* and *E* both represent isoform 2 of the complete fibrinogen alpha chain, however sequences within *E* could only be definitively identified up to the lysine residue at 598 which may be explained by an alternate or truncated terminal sequence of the alpha chain which is only expressed by some individuals (and identified by gel electrophoresis of this pooled human fibrinogen solution). Band *C* represents the complete fibrinogen beta chain while *F* represents the complete gamma chain. Gel electrophoresis demonstrated that bands *A* and *B* both have masses between that of *C* (the complete fibrinogen beta chain) and *D* (the complete fibrinogen alpha chain). Since the masses of the alpha and beta chain are 69,757 and 55,892 daltons respectively, the molecular weight of the bands *A* and *B* is likely to be between these 2 values.

Following in-gel digestion with trypsin (repeated on several occasions), peptide mass fingerprinting of band *A* consistently identified peptides derived from G72 to K527 of the fibrinogen alpha chain (Figure 32), which are recognised trypsin cleavage sites of fibrinogen. In-gel digestion was also performed using Asp-N which demonstrated peptides between D26 and F520, which again are both known Asp-N cleavage points of fibrinogen. These peptide mass fingerprinting data were confirmed using HPLC MS-MS. Band *A* must therefore be generated by cleavage of the fibrinogen alpha chain on the carboxyl side of K527 by Pr3. However, these data also show that the amino terminus of *A* is relatively intact (with the exception of the initial 19 amino acids that form part of the signal peptide of fibrinogen).

```

1 MFSMRIVCLV LSVVGTAWTA DSGEGDFLAE GGGVRGPRVV ERHQSACKDS
51 DWPFCSDEDW NYKCPSGCRM KGLIDEVNQD FTNRINKLKN SLFEYQKNNK
101 DSHSLTTNIM EILRGDFSSA NNRDNTYNRV SEDLRSRIEV LKRKVIEKVQ
151 HIQLLQKNVR AQLVDMKRLE VDIDIKIRSC RGSCSRALAR EVDLKDYEDQ
201 QKQLEQVIAK DLLPSRDRQH LPLIKMKPVP DLVPGNFKSQ LQKVPPEWKA
251 LTDMPQMRME LERPGGNEIT RGGSTSYGTG SETESPRNPS SAGSWNSGSS
301 GPGSTGNRNP GSSGTGGTAT WKPSSSGPGS TGSWNSGSSG TGSTGNQNP
351 SPRPGSTGTW NPGSSERGA GHWTSESSVS GSTGQWHSES GSFRPDSPGS
401 GNARPNPDW GTFEEVSGNV SPGTRREYHT EKLVTSGDK ELRTGKEKVT
451 SGSTTTTTRS CSKTVTKVI GPDGHKEVTK EVTSEDGSD CPEAMDGLTL
501 SGIGTLDGFR HRHPDEAAFF DTASTGKTFP GFFSPMLGEF VSETESRGSE
551 SGIFTNTKES SSHHPGIAEF PSRGKSSSYS KQFTSSTSYN RGDSTFESKS
601 YKMADEAGSE ADHEGTHSTK RGHAKSRPVR DCDDVLQTHP SGTQS

```

Figure 32: Peptide mass fingerprinting of a trypsin digest of Band A demonstrated this band is generated from the fibrinogen alpha chain. The peptide sequences which were both detected and identified are highlighted in red and there is good sequence coverage between G72 and K527 (both known cleavage sites for trypsin), suggesting the Pr3 cleavage point is on the carboxyl side of K527. Asp-N cleavage demonstrated peptide sequences from D26 to F520.

Peptide mass fingerprinting identified band *B* as a fragment of the fibrinogen alpha chain, although on repeated testing, the results of the MS analysis were less consistent than for band *A*. The apparent carboxyl terminus of band *B* was between K480 and K527 following digestion with trypsin, however, the peptide fragments detected beyond K480 varied with repeated testing.

#### 8.1.4 Data Analysis

Since the specific cleavage sites of trypsin and Asp-N and the full amino acid sequence of human fibrinogen are known, potential sites of Pr3 cleavage of the carboxyl terminus of the fibrinogen alpha chain were then considered using the accrued data, including the peptide mass fingerprinting data and the molecular weight range of band *A* (demonstrated by gel electrophoresis and MS) (Figure 33).

<u>10</u>	<u>20</u>	<u>30</u>	<u>40</u>	<u>50</u>	<u>60</u>
MFSMRIVCLV	LSVVGTAWT	A	DSGEGDFLAE	GGVVRGPRVV	ERHQSACKDS
70	80	90	100	110	120
NYKCPSGCRM	KGLIDEVNQD	FTNRINKLKN	SLFEYQKNNK	DSHSLTTNIM	EILRGDFSSA
130	140	150	160	170	180
NNRDNTYNRV	SEDLRSRIEV	LKRKVIEKVQ	HIQLLQKNVR	AQLVDMKRLE	VDIDIKIRSC
190	200	210	220	230	240
RGSCSRALAR	EVDLKDYEDQ	QKQLEQVIAK	DLLPSRDRQH	LPLIKMKPVP	DLVPGNFKSQ
250	260	270	280	290	300
LQKVPPEWKA	LTDMPQMRME	LERPGGNEIT	RGGSTSYGTG	SETESPRNPS	SAGSWNSGSS
310	320	330	340	350	360
GPGSTGNRNP	GSSGTGGTAT	WKPGSSGPGS	TGSWNSGSSG	TGSTGNQNPG	SPRPGSTGTW
370	380	390	400	410	420
NPGSSERGSA	GHWTSESSVS	GSTGQWHSES	GSFRPDSPGS	GNARPNNPDW	GTFEVSGNV
430	440	450	460	470	480
SPGTRREYHT	EKLVTSGDK	ELRTGKEKVT	SGSTTTTRS	CSKTVTKTVI	GPDGHKEVTK
490	500	510	520	530	540
EVVTSEDGSD	CPEAMDGLTL	SGIGTLDGFR	HRHPDEAAAF	<b>F</b>	<b>D</b> TASTG <b>KT</b> FP
550	560	570	580	590	600
VSETES <b>RG</b> SE	SGIFTNT <b>KES</b>	SSHHPGIAEF	PS <b>RGKS</b> SSYS	<b>KQ</b> FTSSTS <b>YN</b>	<b>RGD</b> STFES <b>KS</b>
610	620	630	640		
YK <b>MA</b> DEAGSE	<b>AD</b> HEGTHST <b>K</b>	<b>RG</b> HAKSRP <b>VR</b>	<b>DC</b> DDVLQTHP	SGTQS	

Figure 33: The diagram shows the sequence of the fibrinogen alpha chain with the signal peptide (which is removed following translocation across the endoplasmic reticulum [267]) in green text. The potential sites of trypsin and Asp-N cleavage around the likely carboxyl terminus of band A, are highlighted in yellow and blue respectively. The sites of trypsin and Asp-N cleavage identified by peptide mass fingerprinting which were closest to the likely carboxyl terminus are highlighted and in bold. Gel electrophoresis and MS analysis demonstrated that the molecular weight of band A is between 55,928 and 69,757 daltons which suggests the carboxyl terminus of band A lies between M536 and G548 (underlined) since the amino terminus of the fibrinogen alpha chain remains relatively intact when A is generated. The carboxyl terminus is also unlikely to be beyond R547 since this represents a further potential trypsin cleavage site, and therefore in-gel digestion would generate a further fragment (T528 to R547).

### 8.1.5 Identification of the Carboxyl Terminus Peptide Fragment

The data suggest that there is a high probability that the true carboxyl terminus of A (and therefore the point of Pr3 cleavage) lies in the amino acid sequence between the 2 trypsin specific cleavage sites K527 and G548. Therefore following in-gel digestion, a cleavage fragment must be generated which would not be identified by peptide mass fingerprinting (since the carboxyl terminus of the peptide fragment will be Pr3 specific and Pr3 cleavage data are not included in the online sequencing database). As demonstrated by peptide mass fingerprinting, the amino terminus of this fragment would either be D521 (when Asp-N is used for in-gel digestion) or T528 (trypsin) while the carboxyl terminus must lie between M536 and G548 (Pr3)

The predicted cleavage points indicated the Asp-N digest was the most likely to produce the carboxyl fragment at a detectable molecular weight. The potential terminal fragments in the Asp N digest are likely to lie between D521 (after the final detectable amino acid in the Asp N digestion) and the subsequent *trypsin* cleavage site (R547) (Figure 33), since if the Pr3 cleavage site were on the carboxyl side of R547, a further complete trypsin-trypsin fragment would have been detected by peptide mass fingerprinting. The potential molecular weights of the fragment were calculated, assuming that following Asp-N digestion, its amino terminus lies at F520 (Figure 34).

No identifiable fragment was detected in the trypsin digest.



Peptide Fragment	Molecular Weight (daltons)
D	134.04
DT	235.09
DTA	306.13
DTAS	393.16
DTAST	494.21
DTASTG	551.23
DTASTGK	679.33
DTASTGKT	780.37
DTASTGKTF	927.44
DTASTGKTFP	1024.49
DTASTGKTFP G	1081.52
DTASTGKTFP GF	1228.58
DTASTGKTFP GFF	1375.65
DTASTGKTFP GFFS	1462.68
DTASTGKTFP GFFSP	1559.74
DTASTGKTFP GFFSPM	1690.78
DTASTGKTFP GFFSPML	1803.86
DTASTGKTFP GFFSPMLG	1860.88
DTASTGKTFP GFFSPMLGE	1989.93
DTASTGKTFP GFFSPMLGEF	2136.99
DTASTGKTFP GFFSPMLGEFV	2236.06
DTASTGKTFP GFFSPMLGEFVS	2323.09
DTASTGKTFP GFFSPMLGEFVSE	2452.14
DTASTGKTFP GFFSPMLGEFVSET	2553.19
DTASTGKTFP GFFSPMLGEFVSETE	2682.23
DTASTGKTFP GFFSPMLGEFVSETV*	2652.25

*Figure 34: The masses and amino acid sequences of peptide fragments that could potentially represent the carboxyl terminal fragment of band A (following cleavage with Asp-N). The molecular weight will be specific to the fragment generated by Asp-N cleavage at the amino terminus (at D521) and Pr3 cleavage at the carboxyl terminus. \*is the molecular weight of a polymorphism with a valine substitution. These data were generated using <http://expasy.org/cgi-bin/peptide-mass.pl>*

MS analysis of the Asp-N digest of band A detected a number of peaks of varying molecular weights [Figure 35], and many of these peaks were identified by peptide mass fingerprinting. However, a peak with a molecular weight 2236.08 (which would be consistent with a peptide fragment between D521 and V541 of the fibrinogen alpha chain) was detected by MS [Figure 35], but not identified by peptide mass fingerprinting as would be anticipated if it were formed following cleavage by Pr3 because the peptide database does not include data on peptide fragments formed by Pr3. Furthermore, this peak at 2236.08 was the only peak of significant intensity that was detected, but not matched to a predicted Asp-N peptide fragment (using peptide

mass fingerprinting), and additionally was of a mass consistent with a potential carboxyl terminal fragment of band A (DTASTGKTFP GFFSPMLGEFV).

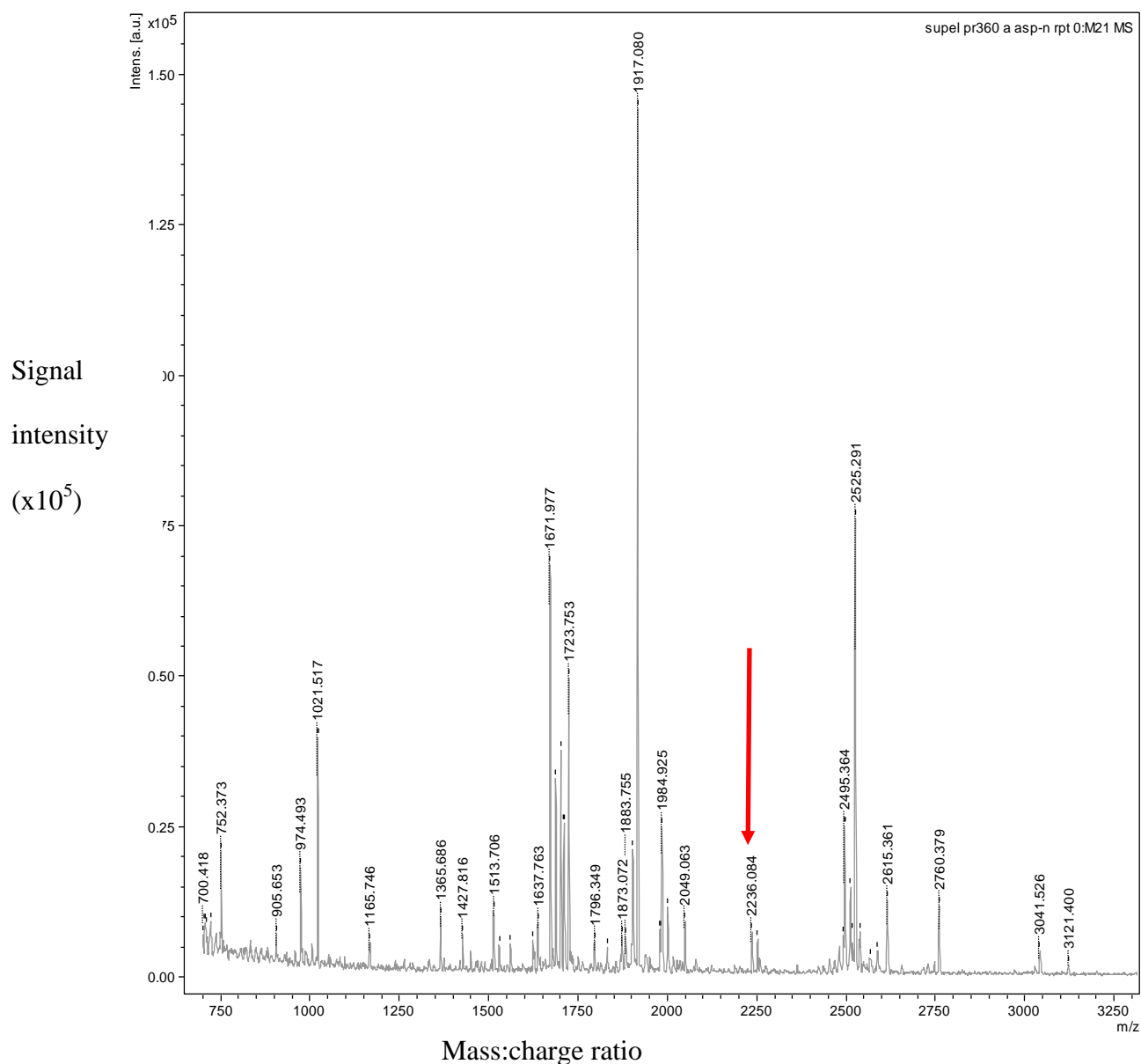


Figure 35: MS analysis of the Asp-N digest of band A detected a number of peaks representing distinct peptides. The peak at 2236.08 (arrowed) would be consistent with the predicted molecular weight of a peptide fragment between D521 and V541, and also was not identified using peptide mass fingerprinting. Peptide mass fingerprinting identifies peptides by searching databases that include the likely fragments that would be formed following digestion using a known enzyme. However, a fragment produced by Pr3 cannot be identified since the database does not include data relating to digestion with Pr3.

### 8.1.6 MALDI MS-MS Analysis

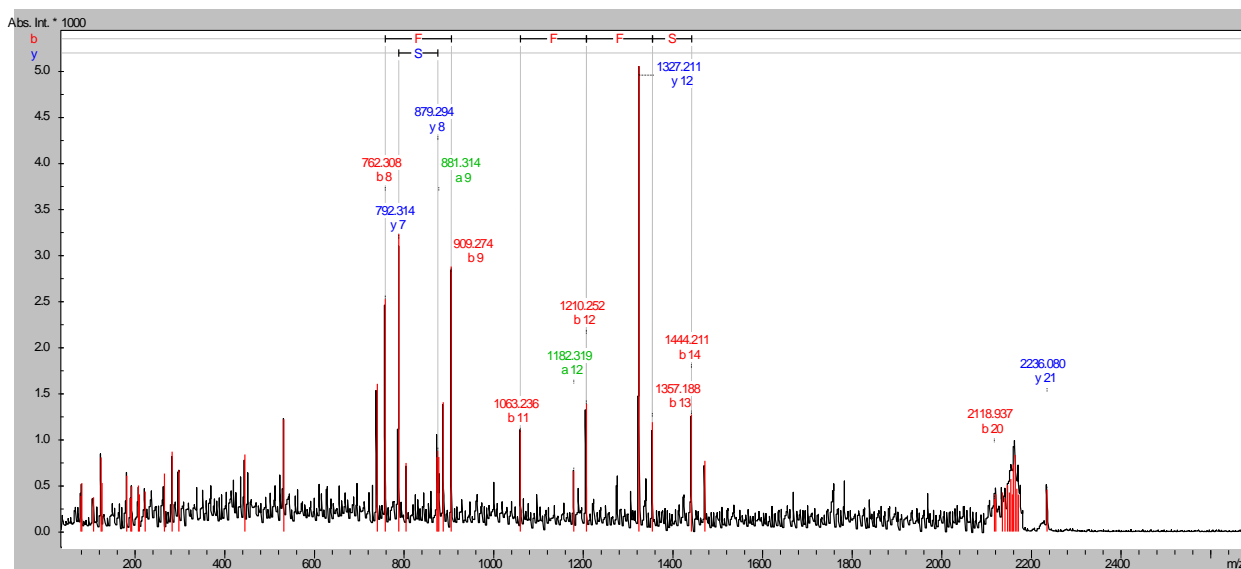


Figure 36: MALDI MS-MS analysis of the 2236.08 Molecular Weight Peptide

MS-MS analysis was also performed and, although this did not produce a comprehensive fragmentation (Figure 36), fragment ion analysis identified 10 of the expected 21 amino acids (DTASTGKTFP GFFSPMLGEFV) within the peptide with a mass of 2236.08.

## 8.2 DISCUSSION

The central role of protease-antiprotease imbalance in the development of COPD has been increasingly recognized and although there may be a number of underlying mechanisms or pathways it is acknowledged that the proteases probably represent a final common pathophysiological pathway. Although NE has long been considered important in this process [68], the more recently discovered and often overlooked enzyme Pr3 may be of greater relevance since it is also capable of cleaving a number of extracellular matrix proteins *in vitro* [81]; producing emphysematous change [70] and mucus hypersecretion [268] in animal models; and apoptosis [269] and cytolysis [270] of human cells *in vitro*. Also, Pr3 is released at a greater concentration than NE [83], yet is less readily inhibited [81, 85], and therefore Pr3 may be more important than NE in the tissue destructive process. Nevertheless, both free NE and Pr3 are inhibited within the neutrophil microenvironment especially in plasma [54, 85] and this currently precludes the direct measure of their activity, which hinders the evaluation of their potential roles *in vivo*.

The current thesis reports the validation of A $\alpha$ -Val<sup>360</sup>, which is a fibrinogen cleavage product and a specific marker of pre-inhibition NE activity *in vivo*. A $\alpha$ -Val<sup>360</sup> is influenced by exacerbations and treatment, and also relates to current markers of COPD disease severity, with a possible relationship to disease progression (as described in earlier chapters and [214, 217]). In contrast to other human proteases which fail to generate A $\alpha$ -Val<sup>360</sup> Pr3 will produce some A $\alpha$ -Val<sup>360</sup>, although only at 15% of the rate of NE [214], demonstrating that this enzyme is also capable of

cleaving fibrinogen. It was therefore hypothesised that there would be a Pr3 specific fibrinogen cleavage product that could be a marker of Pr3 activity *in vivo*.

### **8.2.1 Identification of Low Molecular Weight Peptides**

MS analysis of the solutions of NE/fibrinogen and Pr3/fibrinogen (formed *in vitro*) detected a number of peaks demonstrating that cleavage of fibrinogen by these enzymes produces several similar peptide products. This would be expected since human Pr3 and NE share a similar structure and functionality, and until recently there was no specific substrate available that was preferentially cleaved by Pr3 but not NE [271]. These same characteristics also partly account for the lack of progress in exploring the role of Pr3 in many disease processes (including COPD).

Importantly, there were peaks detected by MS which were enzyme specific, including a peak at a mass of 2021.3 daltons which was detected in the Pr3/fibrinogen but not NE/fibrinogen solutions. A peptide with the same mass was also detected in the plasma of subjects with A1AT deficiency demonstrating that this cleavage product may also be present *in vivo*. Although the peptide with a mass of 2021.3 daltons could not be specifically identified using peptide mass fingerprinting, this would be expected for a peptide with a carboxyl or amino terminus which is generated by cleavage of fibrinogen by Pr3, since the SWISS PROT database include data on the peptides formed through cleavage of known peptides by specific enzymes (but not Pr3). The data provide further support for the hypothesis that this fragment was a Pr3 specific peptide. Unfortunately,

MS-MS analysis of the 2021.3 peptide also did not identify this fragment, which was largely due to poor fragmentation of the target.

Since a larger peptide fragment would represent a more enzyme specific biomarker (it is more likely to be formed in the initial Pr3 cleavage of fibrinogen) and will also have a longer circulating half life [214], further study in the current thesis, focussed on the larger peptide cleavage products.

## **8.2.2 Identification of Larger Molecular Weight Peptides**

### *8.2.2.1 Gel Electrophoresis*

In the absence of a reducing agent, no fragments below 160 kDa were detected using gel electrophoresis. However, the reducing gel demonstrated a number of bands in the NE/fibrinogen, Pr3/fibrinogen and uncleaved fibrinogen solutions, and furthermore, 2 of these bands (termed band *A* and band *B*) were only demonstrated in the Pr3/fibrinogen solution. The contrasting results of the non-reducing and reducing gels suggest that the peptide chains (including the cleavage products termed bands *A* and *B*) remain bound to other chains of fibrinogen by disulphide bonds in a stable large molecular weight structure, which is consistent with the known structure of fibrinogen [272]. The stability which these bonds afford to bands *A* and *B* are advantageous for a plasma biomarker since animal studies demonstrated the long

circulating half life of A $\alpha$ -Val<sup>360</sup> (which remains similarly bound to the beta chain by disulphide bonds), a characteristic associated with reduced longitudinal variability [214].

The fibrinogen and NE or Pr3 were incubated for 15 minutes at physiological temperatures as this length of time produces the optimal yield of the NE/fibrinogen products [214], and the aim of the current study was to generate a Pr3 cleavage product under similar conditions. Band A was present from one minute and although it was still present at 30 minutes (demonstrating the relative stability of this product even in the presence of active enzyme), the appearance of the band was consistently more faint suggesting ongoing proteolytic destruction of band A, and again supported a 15 minute incubation as the optimum period for the generation and detection of Pr3 specific fragments.

It should be recognised that the *in vitro* digestion of fibrinogen by Pr3 cannot precisely reflect the *in vivo* conditions in which Pr3 is released at a very high concentration from the neutrophil, but may also be membrane bound. *In vivo*, as diffusion occurs, free Pr3 is rapidly inhibited by A1AT within the neutrophil microenvironment in a similar manner to NE [54], however membrane bound Pr3 is resistant to inhibition by A1AT [271] leading to ongoing digestion of surrounding peptides. Although in the absence of an effective marker of Pr3 activity, it is difficult to assess the exact contribution to tissue destruction of membrane-bound Pr3 compared to the free enzyme, a comparable methodology resulted in the development of a good marker of pre-inhibition NE activity (despite similar pitfalls). Also, the persistence of a large peptide fragment (band A) at

both one minute and 15 minutes suggest that this is a relatively stable cleavage product of fibrinogen, and therefore a potential biomarker of Pr3 activity.

#### 8.2.2.2 *Peptide Identification: Fibrinogen Chains*

Edman degradation has been the traditional method of choice for protein sequencing, however over the last decade this has been surpassed by rapid advances in MS technology [233]. Although a number of factors, including the sensitivity and resolution of the MS machines, generally preclude the sequencing of whole proteins using MS, this is relatively easily overcome by the use of enzymes that generate a number of small peptides at known and specific cleavage points. These smaller peptides may then be identified by peptide mass fingerprinting which couples the highly precise MS mass measurements with data from sequence databases that allow ‘virtual’ cleavage of almost the whole proteome by certain specific enzymes [232, 233].

In the current study, MS-MS and peptide mass fingerprinting following in-gel digestion identified the protein bands in the uncleaved fibrinogen solution to be the alpha, beta and gamma chains of fibrinogen. Although the complete beta and gamma chains were identified in all the solutions, the complete alpha chain was only present in the uncleaved fibrinogen. Also, following in-gel digestion of band *E* (only detected in the uncleaved fibrinogen solution), peptide mass fingerprinting identified the cleavage products to be derived from the fibrinogen alpha chain, however minimal coverage of the sequence of the carboxyl terminus of the alpha chain was detected in this band. It is possible that this band represents a polymorphism of the fibrinogen



alpha chain (since it was detected in pooled human fibrinogen) which is either a truncated form of the more common alpha chain variant or has an alternate carboxyl sequence. Although sequence databases include data on a number of alternate sequences, not all polymorphisms are included in the online databases (in this instance the SWISS PROT database), which is one drawback to peptide mass fingerprinting.

#### *8.2.2.3 Peptide Identification of Bands A and B*

Fragments of the fibrinogen alpha chain from D26 to K527 were identified by peptide mass fingerprinting (with good sequence coverage) from the in-gel digest of band A. Since the signal peptide of fibrinogen (from M1 to T19) is removed following translocation across the endoplasmic reticulum [267], these data suggest the amino end of band A lies at or within a few residues of the amino end of the intact parent chain. Peptide mass fingerprinting also consistently identified F520 and K527 as the apparent carboxyl termini of band A, following Asp-N and trypsin cleavage respectively. The true carboxyl terminus (i.e. the site of Pr3 cleavage) would not be demonstrated by peptide mass fingerprinting since this method will only identify peptides cleaved at both the amino and carboxyl ends by specific enzymes which (unlike Pr3) are included in the sequencing database SWISS PROT. Nevertheless, the consistency of these data indicate that the true carboxyl terminus of band A lies between K527 and the subsequent trypsin cleavage point (R546), since if the Pr3 cleavage site lay on the carboxyl side of R546, a further trypsin-trypsin cleavage fragment would be identified by peptide mass fingerprinting.

Gel electrophoresis separated bands *A* and *B* by similar distances, indicating that they have similar mass:charge ratios. Peptide mass fingerprinting also identified band *B* as a fragment of the fibrinogen alpha chain, although the data were less consistent compared to *A* and indicated an apparent carboxyl terminus between K480 and K527 following trypsin digest (which varied with repeated testing of different Pr3/fibrinogen digests even at the same time points). The variable results and incomplete identification suggest that band *B* is either a fragment of the alpha chain cleaved at a different site to *A* or a cleavage product of band *E* (the fibrinogen alpha chain polymorphism). It is also possible that *B* is a further Pr3 breakdown product of *A*, which may also explain the decreasing intensity of band *A* with increasing incubation time (Figure 31) however this is less likely since there was no corresponding increase in the intensity of band *B*. If band *A* results from the initial Pr3 cleavage of fibrinogen, this peptide would be more relevant as a target biomarker since initial degradation products are more likely to represent an *in vivo* degradation product formed prior to the rapid inhibition of Pr3. If bands *A* and *B* represent cleavage products of different polymorphisms of fibrinogen, the more consistent and complete peptide mass fingerprinting data for Band *A* and its derivation from the standard polymorphism suggest that *A* is the preferential target.

#### 8.2.2.4 Data Analysis

Gel electrophoresis indicated bands *A* and *B* lie between the beta chain (molecular weight of 55,892) and the alpha chain (molecular weight 69,757). The mass of a peptide fragment between A20 (the first amino acid of fibrinogen alpha chain following the signal peptide) and M536 is

56,050 which is just greater than the mass of the beta chain. Therefore, the true carboxyl terminus of band A post Pr3 cleavage is likely to lie on the carboxyl side of M536 (given the otherwise intact amino terminus demonstrated by trypsin and Asp-N cleavage). The peptide mass fingerprinting data detected peptides up to K527, but repeated testing failed to demonstrate any complete trypsin-trypsin or Asp-N–Asp-N cleaved peptides beyond this amino acid, suggesting the true carboxyl terminus lies on the amino side of R547 (the subsequent trypsin cleavage site). These data suggest the true (Pr3) carboxyl terminus of band A lies between M536 and R547.

MS detected a peptide with a mass of 2236.08 which represents the exact mass of the peptide D521 to V541 (DTASTGKTFP GFFSPMLGEFV) which would be formed following Asp N cleavage of band A, if Pr3 cleaved at V541. MS-MS analysis demonstrated that this peptide is highly likely to represent the sequence with 10 positive hits. The MS analysis also provided further support for this hypothesis since no further peptide fragments consistent with other Pr3 cleavage sites were detected. These data demonstrate that V541 represents the carboxyl terminus of band A and therefore the site of Pr3 cleavage data. This would also be consistent with previous data demonstrating that Pr3 preferentially cleaves peptide bonds where alanine, valine, leucine or serine are the amino acids are at the P<sub>1</sub> and P<sub>1</sub>' sites [81].

#### 8.2.2.5 *Disadvantages of MS*

Peptide mass fingerprinting is a highly accurate technique but does have imperfections. Following, in-gel digestion, the concentration of fragments may be very low and at the limits of

detection for MS [233]. Also, not all polymorphic variants of protein sequences are included in the commonly used databases, which will prevent complete match identification, although this was only an issue in the current study for Band *B* and not Band *A*. Further problems with matching can occur because of non-specific modifications that are generated during protein extraction and processing, although these can partly be overcome by the use of compensatory computer algorithms or deliberate chemical effects that prevent this process [232, 233]. For example, in the current study, iodoacetamide was used during the in-gel digestion to modify cysteine, which minimised other (non-specific) modifications and allowed the detection of the specific fibrinogen degradation products despite the difficulties presented by the use of MS.

#### 8.2.2.6 *Summary*

These data (if correct) will allow the generation of a monoclonal antibody, necessary to develop a specific assay for Pr3 which would represent a new potential marker of protease related disease activity for use in patients with COPD and other diseases in which the neutrophil and its proteinases are implicated (including vasculitis). However, this assay would subsequently require extensive formal validation (as described for A $\alpha$ -Val<sup>360</sup> in earlier chapters) and this is therefore beyond the scope of the current thesis.

## CHAPTER 9. CONCLUSION AND FUTURE WORK

### 9.1.1 A $\alpha$ -Val<sup>360</sup>: Summary

It is increasingly recognised that there is an urgent need for new markers of disease activity and severity in patients with COPD and emphysema [89] and the current thesis therefore describes the validation of A $\alpha$ -Val<sup>360</sup> which is a specific *in vivo* biomarker of pre-inhibition NE activity. Although other criteria for biomarkers have been described [209], an ideal biomarker of COPD disease activity that will serve all purposes should [207]:

1. be central to the pathophysiological disease process
2. be either a direct measure of that disease process (or a close surrogate)
3. be stable and reproducible, but vary with events associated with disease progression
4. relate to disease severity (assuming disease activity is relatively stable, or in a large cross-sectional study that a biomarker would measure the predominant level of disease activity over the time course of the disease process)
5. be responsive to therapies known to modify disease progression
6. predict individuals who will progress more quickly

There is increasing evidence that proteases are central to the pathophysiology of COPD and may represent a final common pathway leading to tissue destruction (see 1.2.4), and therefore as a marker of pre-inhibition NE activity, A $\alpha$ -Val<sup>360</sup> is a close surrogate of the disease process of

COPD fulfilling the second criterion. Importantly, the data reported in the current thesis demonstrate that the repeatability of the A $\alpha$ -Val<sup>360</sup> assay is consistent with other plasma biomarkers [211], and the A $\alpha$ -Val<sup>360</sup> concentration is constant within individuals over time, when in the clinically stable state. However, A $\alpha$ -Val<sup>360</sup> also rises significantly during an exacerbation (which are events known to relate to disease progression [118]) yet returns rapidly to baseline (or close to baseline) as recovery occurs, fulfilling the 3<sup>rd</sup> criterion. Also, A $\alpha$ -Val<sup>360</sup> relates to physiological and radiological measures of disease severity and patient reported outcomes both in those with symptoms of COPD (Chapter 5) and in those with A1AT deficiency demonstrating its relationship with markers of COPD/emphysema disease severity (the 4<sup>th</sup> criterion). There was a significant decrease in the A $\alpha$ -Val<sup>360</sup> in PiZ A1AT deficient subjects who received A1AT augmentation therapy which did not occur in controls, indicating that it may be a biomarker of an effective intervention (the fifth criterion). Finally, there was a possible relationship between A $\alpha$ -Val<sup>360</sup> and disease progression measured by gas transfer in patients with symptoms of COPD and in subjects with A1AT deficiency with *early* disease (with mild spirometric changes) indicating that A $\alpha$ -Val<sup>360</sup> may be a marker of the disease activity driving the emphysematous process in early stage disease. Although there was no relationship with progression in the overall cohort of patients with A1AT deficiency, this may be explained by a number of factors (see 7.3.7) and prognostication may be enhanced by assessing activity with a number of different biomarkers (perhaps including Pr3) to ensure a comprehensive evaluation of the overall pathophysiology.

### 9.1.2 A $\alpha$ -Val<sup>360</sup>: Further Work

Although this thesis and other published work [214, 217] provide evidence to support the use of A $\alpha$ -Val<sup>360</sup> as a biomarker of *neutrophilic* disease activity in chronic bronchitis, COPD and emphysema. Although there appears to be a relationship with disease progression measured by gas transfer in patients with symptoms of usual COPD and patients with early stage COPD and A1AT deficiency, the absence of more clear relationships indicates that further work is required to enable a better understanding of the underlying pathophysiology of COPD and to establish the populations in which this marker of disease activity would be most useful.

Firstly, the relationships between disease activity and severity are complex, and further investigation is hindered by the current absence of gold standard markers. Nevertheless, it is increasingly clear that disease progression (i.e. increasing disease severity measured by physiological or radiological tests) is not linear, and displays marked heterogeneity between individuals with COPD [181]. Disease *activity* is almost certainly as heterogeneous as disease progression between individuals (as demonstrated by the range of A $\alpha$ -Val<sup>360</sup> values reported in the current thesis), however there are insufficient data at present to establish whether disease activity remains stable over the medium to long term, although at least in the short term (over a few years) neutrophilic disease activity (measured by A $\alpha$ -Val<sup>360</sup> and reported in the current thesis) appears to be stable unless patients are experiencing exacerbations. This is important since the linearity of the 2 measures will influence their relationship, and this may be determined in future studies by assessing the cross-sectional and longitudinal relationships of repeated measures of both disease severity, rate of decline of disease severity (gas transfer, CT densitometry and

spirometry) and biomarkers of disease activity such as A $\alpha$ -Val<sup>360</sup> at several time-points, in a number of patients over a prolonged period of time.

Secondly, while the within and between batch variability of the current A $\alpha$ -Val<sup>360</sup> assay is acceptable (and within the range of variability of other plasma and serum biomarkers [211]), patients with COPD progress at a relatively slow rate and therefore even less assay variability and more stable markers of disease activity may be required. This may be at least partly achieved by the development of a simpler and more robust assay using a monoclonal A $\alpha$ -Val<sup>360</sup> antibody and an ELISA technique (usual inter and intra-assay variability of about 10%) rather than the current polyclonal assay (variability of about 24%), and this work is currently underway.

Thirdly, although the current data (Chapter 7) suggest that there may be a subgroup of PiZ patients with early disease in whom A $\alpha$ -Val<sup>360</sup> is associated with disease progression, there is no relationship within the overall PiZ population. However, the progression in the overall group was low (close to that of normal healthy individuals) and therefore a larger study may be required to effectively investigate these relationships, which is now achievable since the ADAPT database has around 800 PiZ individuals. Also, a larger population would allow the identification of other specific subsets or phenotypes (if any) in whom neutrophilic disease activity measured by A $\alpha$ -Val<sup>360</sup> is more relevant.

Fourthly, further studies are required to investigate A $\alpha$ -Val<sup>360</sup> in patients with usual 'COPD' with little or no spirometric change which would include patients with symptoms of COPD (cough, dyspnoea and chronic bronchitis) with a significant risk factor (e.g. smoking). The natural history



of the disease process in this group of patients is particularly poorly understood, and therefore it is important to accurately establish disease progression within this population more accurately and to determine whether a marker of disease activity (such as A $\alpha$ -Val<sup>360</sup>) will help to distinguish between the potential phenotypes and prognosis of early or mild disease. Such patients could be identified through population screening, and assessed using both biomarkers and physiological markers of disease severity.

Fifthly, it will be important to explore the value of A $\alpha$ -Val<sup>360</sup> as a marker of therapeutic efficacy in other clinical trials. Although there are few effective therapies for COPD, it is interesting to speculate that novel NE inhibitors may be successfully used to modify this disease process. Although a recent trial of one NE inhibitor over a 3 month period demonstrated a good safety profile, predictably there was no change in the physiological markers of disease severity within this timeframe [273], which again indicates the need for early readout biomarkers for use in phase I to III clinical trials.

Finally, further studies are required to demonstrate whether A $\alpha$ -Val<sup>360</sup> is also an effective marker of activity in other disease processes within which the neutrophil has been implicated including cystic fibrosis, vasculitis, bronchiectasis and acute lung injury. Again these studies are underway, however are beyond the scope of the current thesis.

### **9.1.3 Pr3: Summary**

Pr3 may be a key mediator of tissue damage, and is released at a higher concentration than NE, is less readily inhibited by A1AT (particularly when cell membrane bound) but (in contrast to NE) is not inhibited by the major airway serine protease inhibitor SLPI which indicates Pr3 may be of particular importance in the larger airways (see 1.2.4.4 and 8.2). In addition to measuring pre-inhibition NE activity, it is therefore also likely to be important to measure Pr3 activity in subjects with COPD since this will provide further information about potential tissue damage, particularly within the airway. The current thesis therefore describes the identification of a specific pre-inhibition Pr3 fibrinogen cleavage product using MS technology which will allow the generation of a specific assay of Pr3 activity.

#### **9.1.4 Pr3: Future Work**

Ideally, prior to the development of a specific antibody, MS analysis would be used to confirm the presence of the neoepitope in human plasma, however, with current technology this would be difficult owing to the range of other peptides and proteins which are present in plasma and the difficulties of firmly identifying large molecular weight products using MS based techniques. Also, although the peptide is not formed by when fibrinogen is cleaved NE, it will also be important to demonstrate whether any peptide is also a product of other proteases including the cathepsins, plasmin and trypsin.

Further work would then be focussed on generating a specific monoclonal antibody and following this, western blot analysis will be required to establish the presence of the neoepitope in human plasma. Also, it would be important to demonstrate the generation of the product in human plasma following the addition of a neutrophil activator, and to show whether this process is abrogated in the presence of a specific inhibitor of Pr3.

Following the successful generation of a specific antibody, an ELISA could be created along conventional lines. A plate would be coated in Pr3 cleaved fibrinogen and then incubated with the sample premixed with the specific Pr3 antibody. Following washing the plate would be incubated with a secondary (labelled) antibody to determine the amount of the Pr3 specific antibody that had bound to the plate not the sample and the Pr3 fibrinogen cleavage product concentration of the sample determined by interpolation from results using a standard peptide.

Further work would then be required to validate the assay in the same manner described in the current thesis for A $\alpha$ -Val<sup>360</sup>.

#### **9.1.5 Other Enzymes**

Similar techniques could be used to develop assays of the activity of other enzymes which are believed to play a role in the tissue damage in COPD, including the MMPs and cathepsins (1.2.4). A composite measure of the activity of a number of different enzymes may then provide more detailed information about the overall disease activity or their interactions in patients with COPD.

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