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The influence of novel alpha-1 antitrypsin protein binding partners on inflammation and the clinical phenotype in alpha-1 antitrypsin deficiency

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The influence of novel alpha-1 antitrypsin protein binding partners on inflammation and the clinical phenotype in alpha-1 antitrypsin deficiency

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Department of Medicine
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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfillment of the degree of Doctor of Philosophy

Supervisors:
Dr. E.P. Reeves
Professor N.G McElvaney

January 2016
Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Student Number: 12140686
Date: 29/7/2015
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<td>alpha-1 antitrypsin select resin</td>
</tr>
<tr>
<td>AAT</td>
<td>alpha-1 antitrypsin</td>
</tr>
<tr>
<td>AATD</td>
<td>alpha-1 antitrypsin deficiency</td>
</tr>
<tr>
<td>AAT-ox</td>
<td>oxidised alpha-1 antitrypsin</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ADAM-17</td>
<td>a disintegrin and metalloprotease domain-17</td>
</tr>
<tr>
<td>AMBP</td>
<td>Alpha-1-microglobulin/bikunin precursor</td>
</tr>
<tr>
<td>ANCA</td>
<td>anti-neutrophil cytoplasm antibodies</td>
</tr>
<tr>
<td>ASN</td>
<td>asparagine</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>ATS</td>
<td>american thoracic society</td>
</tr>
<tr>
<td>ApoA1</td>
<td>Apolipoprotein A1</td>
</tr>
<tr>
<td>ApoB100</td>
<td>apolipoprotein B100</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchioalveolar lavage</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<tr>
<td>BLT</td>
<td>Leukotriene B4 receptor</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C3</td>
<td>complement component C3</td>
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<tr>
<td>C4</td>
<td>complement component C4</td>
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<tr>
<td>C5</td>
<td>complement component C5</td>
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<tr>
<td>Ca^{2+}</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>COPD assessment test</td>
</tr>
<tr>
<td>CathG</td>
<td>cathepsin G</td>
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</table>
CF cystic fibrosis
CFHR1 complement factor H-related 1
CFTR cystic fibrosis transmembrane conductance regulator
Cl confidence intervals
Cl⁻ chloride
COPD chronic obstructive pulmonary disease
CR complement receptor
CRIg complement receptor immunoglobulin
CES cigarette smoke exposure
CT computerised tomography
Cu²⁺ copper
CXCR c-x-c chemokine receptor
Cys cysteine
DAF delay accelerating factor
des arg desarginated
DLCO diffusion capacity for carbon monoxide (gas transfer)
DMSO dimethyl sulfoxide
DPBS Dulbecco's phosphate buffered saline
DTT dithiothreitol
EDTA ethylenediaminetetraacetic acid
ELISA enzyme-linked immunosorbent assay
e.g. for example
ER endoplasmic reticulum
ERK extracellular-signal regulated kinase
ERS European respiratory society
FA formic acid
FcγR-III B/Fc gamma receptor three B/CD16
FD factor D
FDA food and drug administration
Fe²⁺ ferrous iron
FEV1 forced expiratory volume in one second
<table>
<thead>
<tr>
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<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>FH</td>
<td>factor H</td>
</tr>
<tr>
<td>FHL1</td>
<td>factor H-like protein 1/reconectin</td>
</tr>
<tr>
<td>FI</td>
<td>factor I</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl-methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>GE</td>
<td>general electric</td>
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<tr>
<td>GlcNac</td>
<td>N-acetyl-glucosamine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate</td>
</tr>
<tr>
<td>GOLD</td>
<td>global initiative for obstructive lung disease</td>
</tr>
<tr>
<td>GPA</td>
<td>granulomatosis with polyangiitis</td>
</tr>
<tr>
<td>GRP-78</td>
<td>glucose-regulated protein 78</td>
</tr>
<tr>
<td>GRP-94</td>
<td>glucose-regulated protein 94</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HClO</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>ICAM</td>
<td>intracellular adhesion molecule</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin 10</td>
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<td>IL-13</td>
<td>interleukin 13</td>
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</tr>
<tr>
<td>IL-17</td>
<td>interleukin 17</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1 beta</td>
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IL-8  interleukin 8
ITIH  Inter-alpha-trypsin inhibitor heavy chain
LC  liquid chromatography
LDL  low density lipoprotein
LPS  lipopolysaccharide
LTB4  leukotriene B4
LTQ  linear trap quadrapole
Lys  lysine
LysC  lysine-sensitive aspartokinase 3
MAC  membrane attack complex
MASP  Mannan-binding lectin serine protease
MAP  mitogen-activated protein
MBL  mannose binding lectin
MCP  membrane cofactor protein
Met358  methionine 358
MFU  mean fluorescent units
Mg^2+  magnesium
MI  myocardial infarction
min  minutes
MMP  matrix metalloprotease
mMRC  modified medical research council
MPO  myeloperoxidase
MS  mass spectrometry
MSGN  mesangiocapillary glomerulonephritis
NADPH  nicotinamide adenine dinucleotide
NCT  national clinical trial
NE  neutrophil elastase
NETs  neutrophil extracellular traps
NFκβ  nuclear factor kappa beta
NHBLI  national heart, lung, and blood institute
NO  nitric oxide
PAGE  
polyacrylamide gel electrophoresis

PAMPs  
pathogen associated molecular patterns

PAS  
periodic acid Schiff

PBS  
phosphate buffered saline

phosphate  
phosphate

pI  
isoelectric point

PMA  
phorbol12-myristate 13-acetate

PPA2  
protein phosphatase 2A

PR3  
proteinase 3

PRM  
pathogen recognition molecule

PSA  
prostate specific antigen

PVDF  
polyvinylidene fluoride

Quantum-1  
quantitative chest CT unmasking emphysema progression in AATD

RA  
rheumatoid arthritis

rAAT  
recombinant alpha-1 antitrypsin

RCL  
reactive centre loop

RCT  
randomised controlled trial

ROS  
reactive oxygen species

RPC  
reverse phase chromatography

SDS  
sodium dodecyl sulphate

SEC  
serpin enzyme complex

Serpin  
serine protease inhibitor

SLE  
systemic lupus erythematosus

SLPI  
secretory leukoprotease inhibitor

TACE  Δ-convertase enzyme

TCA  
trichloroacetic acid

TCC  
terminal complement cascade

TEM  
transendothelial migration

TEMED  
tetramethylethylenediamine

TGF-β  
transforming growth factor beta
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>TLC</td>
<td>total lung capacity</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNFR1</td>
<td>TNF receptor 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>USA</td>
<td>united states of america</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>v</td>
<td>version</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WHO</td>
<td>world health organisation</td>
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<tr>
<td>Zn^{2+}</td>
<td>zinc</td>
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Presentations and publications

Prizes/awards

- European Alpha-1-Antitrypsin Laurell’s Training Award (eALTA) Recipient 2013
  - €50,000 bursary for a 1-Year Research Fellowship
- Winner Sheppard’s prize: Best oral presentation in the MD category, Beaumont Hospital, 2014.

Journal articles:

Book chapters:


Oral Presentations


Published abstracts

Summary

The findings of this thesis demonstrate that individuals with alpha-1 antitrypsin deficiency (AATD) and a more severe clinical phenotype, characterized by worsening pulmonary emphysema, have evidence of persistent inflammation despite clinical stability. The binding of alpha-1 antitrypsin (AAT) to proteins of various systems in the circulation is an important mechanism through which AAT mediates its anti-inflammatory properties. A key finding of this thesis is that AAT binds to complement component C3 and can prevent its dysregulated cleavage by neutrophil-derived proteases, in particular NE. Complement activation may be one mechanism through which the perpetuation of inflammation is mediated in AATD through an axis of neutrophil recruitment and activation, protease release, complement degradation, generation of chemotactic complement peptides, and consequently further neutrophilic inflammation.

The discovery of a relationship between the observed clinical phenotype in AATD individuals, homozygous for the Z-allele, and complement activation implicates this pathophysiological process in pulmonary disease progression. The ratio of the complement cleavage product C3d to total C3 may also serve as a biomarker of pulmonary disease severity. Furthermore, the role of C3d as an innate immune adjuvant to the adaptive immune response cannot be overlooked. This bridge between innate and adaptive immunity has implications for the pathogenesis of pulmonary emphysema, a mechanism that warrants further investigation in the future.
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There was a steep learning curve in relation to the basic scientific techniques employed in my initial experiments and I could have not hoped for a better team to aid me through that point. I am especially thankful to Nessa, Dave, Ciara, Michelle, Tomás, Kerstin, and Cormac for the scientific knowledge and techniques they imparted to me. I developed many friendships with in the lab during my time there, this maintained a great work environment; I would like to thank Cormac, Imran, Tomás, Ciara, Michelle, Kerstin, Noreen, Paul, Bojana, Chiara, Gillian, Sebastian, Niamh, Tidi, Killian, Irene, Eoghan, Catherine, and Laura.

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Dedication

I would like to dedicate this thesis to my wife, Eimear, who has always been a pillar of support in everything that I do. I could not have completed this work without her understanding and encouragement. I am grateful for all the sacrifices that were made over the past three years and I am immensely proud of Eimear’s own achievements in that time. We have welcomed two wonderful children, Fionn and Caoimhe, and I look forward to all the adventures that lie ahead for us as a family.
Chapter 1:

1.1 Introduction
1.1.1 Alpha-1 antitrypsin: Production

Alpha-1 antitrypsin (AAT) is a 52kDa plasma glycoprotein and is the canonical serine protease inhibitor encoded by the SERPINA1 gene on chromosome 14q32.1-32.2 (1). AAT is abundant in plasma where the normal concentration is 1.3 g/L (0.9–1.75 g/L) and it has a half-life of 4–5 days. Production of circulating AAT is almost entirely contributed to by hepatocytes, though it is also produced in smaller quantities by other cells such as monocytes, macrophages, pulmonary alveolar cells and intestinal epithelial cells (2-4). From plasma, 80% of AAT diffuses to interstitial tissues, and 0.5–10% reaches biological fluids, including alveolar fluid where local concentrations reach 0.1–0.3 g/L (5). AAT is an acute phase protein, its plasma concentration can rise between two and four fold during periods of inflammation through stimulation of production by the pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin 1β (IL-1β), and to some extent interleukin 8 (IL-8), transforming growth factor β (TGF-β) and interleukin 17 (IL-17) (6,7). These cytokines are produced by a range of cell types, most importantly antigen presenting cells, such as macrophages and monocytes, as well as epithelial cells leading to up-regulation of gene expression (8,9)
1.1.2 Alpha-1 antitrypsin: Genetics

The SERPINA1 gene has two alleles, which are transmitted from parents to their children by autosomal co-dominant Mendelian inheritance. The AAT protein product of SERPINA1 is polymorphic with over 120 alleles recognized to date. Mutations may result in altered production of the AAT protein, culminating in reduced circulating AAT levels. Mutations are classified by their phenotypic expression and electrophoretic mobility on isoelectric focussing; PiM (medium), PiS (slow), and PiZ (very slow) (10-12). The most severe mutation occurs in the Z protein that is a result of a point mutation at position 342 resulting in an amino acid change from glutamic acid to lysine (Glu342Lys). The estimated carrier frequency of the Z allele is 1:25, with a disease incidence of 1:1575 to 1:2100 in some western European populations (13,14). Homozygous ZZ individuals have a marked reduction in circulating plasma AAT levels to less than 10% of the normal protein concentration, additionally the Z-AAT protein is a less competent protease inhibitor than M-AAT and can take twice as long to inhibit a given concentration of neutrophil elastase (NE) (5,15). The net effect of reduced circulating protein and diminished anti-protease activity results in a marked protease/anti-protease imbalance.
1.1.3 Alpha-1 antitrypsin: Glycosylation

The AAT protein undergoes a process of co-translational N-glycosylation within the hepatocyte endoplasmic reticulum, resulting in the addition of three oligosaccharide residues contributing 12.5% to the resultant molecular weight of the protein. Comprehensive glycoproteomic analysis has identified these residues at positions Asn70, Asn107 and Asn271 (16), see Figure 1.1. Glycosylation of AAT is critical for its function through prolongation of its plasma half-life, conferring resistance to proteolytic degradation, modulating intermolecular interactions, and the prevention of protein aggregation. AAT circulates in a high energy metastable state, on docking with a target protease the induced conformational change results in energy release and a stable protease/anti-protease complex is formed (17). Unusually the glycosylation status of AAT does not appear to stabilize the native state of the protein, it may even cause disruption of its inhibitory function (18).

The predominant mechanism for AAT elimination, which is distinct to SERPIN: enzyme complex (SEC) receptor-mediated protease complex removal, is through the asialoglycoprotein receptor (19). This is expressed on hepatocytes and on recognition of terminal galactose residues it expediently removes the protein from the circulation. Sialic acid is a terminal monosaccharide found on complex type N-glycans, such as those on AAT, and is mainly responsible for the negative charge of glycans. The addition of sialic acid to terminal glycans shields the residues from receptor binding and thereby prolongs the half-life of AAT (20). To illustrate the relevance and clinical importance of this effect, recombinant AAT protein produced by bacteria and yeasts is not similarly glycosylated and is subject to rapid plasma clearance, it is therefore therapeutically ineffective compared to plasma-derived AAT for the purpose of intravenous augmentation therapy (21).
Figure 1.1: Structure of AAT with glycan residues

The protein backbone is represented in green. The reactive centre loop with the exposed Met358 residue is shown in red at the apex of the molecule. The glycans in blue are attached to the asparagine residues 70, 107 and 271 of the molecule during the process of glycosylation. McCarthy et al., 2014 (23).

Differential glycosylation of AAT permits phenotypic characterization of the circulating protein from peripheral blood. The protein isoforms have variations across each isoelectric point (pI) that are evident when separated using isoelectric focusing across a pH gradient (between pH 4 – 5) on an agarose gel, identification is enhanced by an added immunofixation step with specific anti-AAT antibody. In AAT deficiency (AATD), qualitative measurement of SERPINA1 mutations using this technique permits identification of various phenotypes based on their electrophoretic mobility and this is commonly employed for the clinical diagnosis of AATD as discussed earlier, see Figure 1.2.
The glycans of the M-AAT protein have been identified and well studied, with 9 different M glycoforms described, often classified as M0 to M8 (22). Increased core and outer arm fucosylation, including sialyl Lewis-X determinants, of the Z-AAT protein has recently been characterized (23). This finding may have implications for the role of Z-AAT as an immune modulatory protein and its effect upon leukocyte-mediated inflammation in AAT deficiency (AATD), irrespective of its reduced anti-protease activity.

Non-enzymatic glycosylation of AAT, through exposure to high plasma glucose levels, may reduce plasma AAT levels has been shown to impair anti-protease activity in diabetic subjects (24,25). In addition, rare disorders of carbohydrate metabolism have been associated with deficiency of AAT in adulthood (26).
Figure 1.2: Isoelectric focusing gel demonstrating AAT phenotypes

Characterization of various AAT phenotypes by isoelectric focusing using the Hydrasys electrophoresis platform (Sebia) with the Hydragel-18 A1AT Isofocusing kit (Sebia). The AAT phenotype is determined by its electrophoretic mobility in the gel; M=medium, S=slow, Z=very slow. Individuals with severe AATD will usually have a pattern consistent with PiSZ (lane 6) or PiZZ (lane 3).

This method allows detection of common SERPINA1 mutations based on their electrophoretic mobility/phenotype, note the reduced protein amount and cathodal shift of PiZZ (lane 3) compared to PiMM (lane 4).

Figure adapted from Carroll et al, Respir Res. 2011 (14).
1.1.4 Alpha-1 antitrypsin: Structure & function

The structure and functions of AAT as serine protease inhibitor are inextricably linked (27). The main function of AAT is as an anti-protease, primarily against NE though it has a wide range of protease inhibitory activity and contributes to 90% of the total serine protease inhibitory capacity of plasma. Following transcription the resultant AAT protein is comprised of three β-sheets, nine α-helices and a reactive central loop (RCL) at the C-terminal region (28). The reactive methionine residue at position 358 (Met358) is located on RCL, which extends out from the body of the protein and directs binding to the target protease. The reactivity of the Met358 residue is primarily responsible for the spectrum of protease binding, it has the highest affinity for the serine hydroxyl residue on NE to which it binds with an association constant of $k = 6.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and inhibits it in an equimolar ratio. A point mutation at position 358 can drastically alter the function of the AAT molecule by reducing or changing the specificity of this bond for its target protease; this has been best illustrated by the rare mutation of Met358 to arginine (AAT-Pittsburgh) resulting in greatly diminished anti-elastase activity and markedly increased antithrombin activity resulting in a fatal bleeding disorder (29).

Cleavage of the active Met358 by the protease establishes a covalent linkage between the carboxyl group of the serpin reactive site and the serine hydroxyl of the protease. This event triggers a major structural rearrangement that involves loosening of the β-sheets and an irreversible conformational change by incorporation of the RCL into the β-sheet region of the AAT protein. The translocation of the attached protease by 71Å from its initial position induces irreversible inactivation of the protease through distortion of the protease active binding site (30), see Figure 1.2. This mechanism is akin to the function of a mousetrap with the methionine residue serving as the ‘bait’ that lures the protease to its fateful end (31). The protease is thereby inactivated and the resultant AAT-protease complex is highly stable, it is removed expediently from the circulation through engagement of a newly exposed binding site with the
hepatocyte SEC receptor located (32). This interaction on the hepatocyte cell surface signals for increased gene expression of SERPINA1 in a positive feedback loop (33,34).

Figure 1.3: Binding of AAT with NE

This a representative figure of the binding of the reactive Met358 residue to the target carboxyl terminal that is exposed on NE. On protease binding, the RCL (yellow) is cleaved resulting in a conformational change in the AAT molecule with incorporation of the RCL into the β-sheet (red). This results in irreversible inhibition of the protease and the complex becomes a target for removal from the blood.


Met358; methionine 358, NE: neutrophil elastase; RCL, reactive centre loop, AAT; alpha-1 antitrypsin.
1.1.5 Alpha-1 antitrypsin: Inactivation by oxidation

The reactive Met358 is a surface exposed methionine residue that is readily oxidised by hydrogen peroxide in cigarette smoke and by oxidising agents released by leukocytes during inflammation (35). The Met351 and the thiol reactive cysteine-232 (Cys232) residues of AAT are also susceptible to oxidative inactivation (36,37). Oxidized AAT (AAT-ox) persists in a functionally inactive form in the circulation, whereby its protease binding capacity is markedly reduced, and does not stimulate further up-regulation of AAT production, this is due to the oxidative conversion of exposed galactose alcohol residues to aldehyde residues on the attached glycoproteins (38).

The resultant loss of anti-protease activity renders AAT-ox functionally deficient against NE. Under certain conditions oxidative inactivation may be physiologically favourable, and necessary for host protease defence, to enable in vivo function of NE within a local environment. This process is likely active in the immediate environment of de-granulating neutrophils to facilitate their activity, where reactive oxidative species released concomitantly with serine proteases accomplish oxidative inactivation of nearby protease inhibitors. Interestingly, AAT-ox has been shown to retain certain anti-inflammatory properties, despite losing its serum elastase inhibitory capacity, through the prevention of neutrophil recruitment to the lungs in a rat model of smoke-induced emphysema (39). The observed anti-inflammatory mechanism relates to tumour necrosis factor-α suppression and provided partial protection to the development of emphysema in this model.

Oxidative inactivation of AAT is of major importance in the pathogenesis of emphysematous lung destruction in smokers. The hypothesis of an acquired protease imbalance within the lung is a critical mechanism of emphysema in the general population and is further compounded in those with humoral AAT deficiency (40). Additionally, oxidation of the mutant Z-AAT by cigarette smoke induces polymerization that may further contribute to lung disease (41).
1.1.6 Alpha-1 antitrypsin: Z protein polymerization

In addition to the contribution of humoral AAT deficiency in the development of pulmonary emphysema though protease/anti-protease imbalance, a greater understanding of the pathogenesis of AATD was reached on discovery that polymerisation of the Z protein occurs at sites of production (4,42,43). In PiZZ homozygotes, the Glu342Lys mutation results in disruption of an intramolecular salt bridge in strand 5 of the five-stranded β-sheet and uncoiling of the upper part of helix F (44). This induces conformational instability of the protein which involves an initial zero-order conversion of AAT to an intermediary polymerogenic monomer termed M* (45). This leads to a slower concentration-dependent intermolecular association step resulting in polymerisation through the incorporation of the RCL from an adjacent molecule into the patent shutter region of the affected β-sheet (42,43,46).

Factors that favour polymerisation in vitro include increased temperature, higher Z-AAT concentration and acidosis, all of which can occur at sites of tissue inflammation in vivo. Consequently the misfolded protein accumulates within the endoplasmic reticulum (ER) and can be visualised as a 'beads on a string' appearance on periodic acid Schiff (PAS) stain of liver biopsy samples. There is a marked reduction of Z protein egress from the cell leading to ER stress, thereafter hepatocyte autophagy is overwhelmed and cellular decompensation ensues (47-49). Due to this mutant gain of function individuals with severe AATD are at risk of fulminant hepatic failure. This is not limited to individuals with the PiZZ phenotype, conformationally unstable AAT variants, which may occur in PiSZ and PiIZ individuals, may also lead to clinically relevant liver disease due to the development of AAT heteropolymers (50).

Furthermore, local Z-AAT polymer production may also provide an explanation for the progression of lung disease in PiZZ homozygotes after smoking cessation and despite the initiation of augmentation therapy (51). The formation of pro-
inflammatory Z polymers may contribute to an augmented systemic inflammatory response and influence the clinical phenotype of COPD in AATD (51,52). In non-smoking PiMZ heterozygotes, who maintain an adequate antiprotease buffer, this augmented inflammatory response may confer a selective advantage (53).
1.1.7 Alpha-1 antitrypsin: Inhibition of neutrophil derived proteases

The neutrophil is the key effector cell of the innate immune system and plays a critical role in the response to infection in the lung through recruitment to the pulmonary interstitium by transendothelial migration (TEM) and thence to the alveolar lining fluid (54).

The neutrophil has an armoury at its disposal to effect microbial killing. Firstly it can engulf an invading microbe through a process of phagocytosis though interaction with complement receptors expressed on its cell membrane (55-57). On ingestion the microbe undergoes degradation within the phagolysosome, which is mediated by the direct microbicidal effects of proteases, proteolysis, and oxidation (58). Secondly, neutrophil degranulation is a process that can be triggered by inflammatory stimuli, e.g. tumour necrosis factor alpha (TNFα), C3a, and C5a, and can be increased greatly by interaction with the extracellular matrix (59). The consequent release of granular products into the extracellular space mediates bacterial killing but at the expense of local tissue damage. The formation of neutrophil extracellular traps (NETs) is the third recognised mechanism whereby neutrophils extrude extracellular chromatin fibres that can entrap invading microbes, contain them physically, and expedite killing within a hostile local microenvironment created by granule products (60).

Neutrophil granules contain three serine proteases, neutrophil elastase (NE), cathepsin G (CG) and proteinase 3 (PR3), as well as microbicidal cationic peptides. Neutrophil derived proteases have both intra- and extracellular activities and play an important role in the inflammatory response to infection. Membrane bound proteases are also necessary for neutrophil adhesion and migration (61). The neutrophil respiratory burst is an acute mechanism whereby reactive oxygen species (ROS), including superoxide, ozone, and hydrogen peroxide, are generated in the phagolysosome through the action of NADPH oxidase (62). The enzymatic activity of myeloperoxidase catalyses the formation of hypochlorous acid (HClO) which is 50 times more potent at microbial killing than hydrogen peroxide alone (63). These ROS can interact non-specifically with
a wide array of targets leading to oxidative damage. The release of NE, CG and PR3 into the extracellular matrix, in the absence of sufficient protective inhibition, results in the degradation of structural tissues, which may culminate in the development of emphysematous lung changes. This paradigm of protease:anti-protease imbalance in the pathogenesis of emphysema has been well characterised for over fifty years using the papain model: This was first described by Gross et al. whereby the cysteine protease papain was instilled directly into the lungs of rats to create an experimental model of the disease (64).

The activity of neutrophil proteases is subject to tight homeostatic regulation in health. Tissue destruction can ensue at sites of excess inflammation due to a failure of protease inhibitory activity, which in the lung leads to elastolysis and breakdown of structural components of the extracellular matrix. Furthermore endothelial damage can occur through NE proteolysis of cadherins, this can affect the integrity of the lung microvasculature and lead to acute lung injury during sepsis or inflammation (65-67). The described protease imbalance in the acute respiratory distress syndrome (ARDS), a severe form of acute lung injury, has been found to be predictive of a poorer clinical outcome (68,69). NE also mediates important effects on neutrophil adhesion to the endothelial surface through integrin- and cadherin-dependent mechanisms to facilitate TEM (61,70). An alternative mechanism for this process includes the stimulation of proteinase activated receptors, leading to the activation of specific intracellular signalling pathways that culminate in epithelial apoptosis (71). Whether the actions of NE are required or simply facilitate neutrophil TEM remains controversial (72).

AAT is the archetypal serine protease inhibitor and its role in the neutralisation of neutrophil derived proteases has been well characterised (73). The discovery that individuals with AATD develop early onset pulmonary emphysema led to the development of the protease/anti-protease theory in the pathogenesis of this condition (40). Absence of AAT in the lung leads to free NE activity that promotes the production of the potent neutrophil chemoattractant leukotriene B4 (LTB4) from resident pulmonary macrophages, thereby perpetuating a cycle of inflammation (74). Excess protease activity also contributes to this process.
through up-regulation of interleukin-8 (IL-8) production via a toll-like receptor 4 (TLR4)-dependent mechanism (75) and the cleavage of tumour necrosis factor receptor 1 (TNF-R1) promoting neutrophil recruitment (76). This is further exacerbated by the effect of smoking tobacco (77-80). AAT also controls NE activity at the neutrophil surface membrane, this may limit peri-cellular proteolysis to zones of apposition between the neutrophil and target tissue where protease inhibitors cannot penetrate, in AATD this mechanism to keep neutrophil inflammation in check may be diminished. Furthermore, through this same mechanism AAT deficiency may have important effects on TEM resulting in enhanced neutrophil recruitment to the lung (81,82).
1.1.8 Alpha-1 antitrypsin: Anti-inflammatory effects beyond protease inhibition

Increasingly it has been recognised that AAT has pleiotropic functions that mediate a broad range of anti-inflammatory activities beyond protease inhibition (83,84), see Table 1.1. Recent investigations into alternative biological functions for AAT focused on the ability of AAT to regulate neutrophil chemotaxis by binding IL-8 and preventing its interaction with its cognate receptor CXC chemokine receptor 1 (CXCR1) on the neutrophil membrane. It was demonstrated that neutrophils migrate down a functional gradient of AAT in response to an increasing gradient of IL-8, and that glycosylation is critical for this mechanism. Furthermore, AAT prevented immune complex mediated neutrophil recruitment by inhibiting a disintegrin and metalloprotease domain-17 (ADAM-17) enzymatic activity and shedding of Fc gamma receptor three B (FcγRIIIb) (CD16) (85).

A recent fascinating discovery is that AAT can also mediate anti-inflammatory effects through TNFα signalling. AAT has been shown to inhibit TNFα–converting enzyme (TACE) activity resulting in up-regulation of TNF receptor 1 (TNF-R1) and reduced TNFα secretion. This causes an initial augmented response to inflammation in the acute phase followed by selective inhibition that contributes to the resolution of chronic inflammation (86). AAT can also modulate neutrophil activity by inducing protein phosphatase 2A (PP2A) activation to prevent the inflammatory and proteolytic responses triggered by TNFα stimulation in the lung (87). Another role for AAT includes its ability to inhibit TLR4 signalling and to modulate TNFα production by monocytes following stimulation with pro-inflammatory cytokines (88).

Neutrophil apoptosis is accelerated in individuals with AATD by mechanisms involving ER stress and aberrant TNFα signalling, this results in decreased bacterial killing though it can be ameliorated by augmentation therapy (89). Furthermore, observations that AAT can inhibit the apoptotic factors, caspase-3
and caspase-1, has widened our perspective on the role of AAT in the pathogenesis of emphysema (90,91). In addition, AAT has been shown to reduce structural alveolar cell apoptosis independent of elastolytic activity by inhibition of vascular endothelial growth factor (VEGF) receptors with resultant suppression of caspase-3 activation and oxidative stress (92).

These novel anti-inflammatory and anti-apoptotic functions of AAT may lead to fresh applications for AAT augmentation therapy in a range of conditions beyond the current therapeutic indication for pulmonary disease in AATD (93).
Table 1.1: Pleiotropic effects of alpha-1 antitrypsin

This table illustrates many of the effects of AAT that have been published, underscoring its role within diverse biological systems beyond the classic paradigm of protease inhibition.

<table>
<thead>
<tr>
<th>Role of AAT</th>
<th>Reference</th>
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<tr>
<td>Anti-protease effects</td>
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<tr>
<td>Apoptosis; inhibition of caspase-1, caspase-3, and calpain-1</td>
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</tr>
<tr>
<td>Oxidative stress inhibition</td>
<td>(36)</td>
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<tr>
<td>Repair, fibroblast proliferation, procollagen synthesis, and activation of MAP kinase pathways</td>
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<td>Signalling via ADAM-1</td>
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<td>Substrate for Metalloproteinase MMP-9 activity</td>
<td>(98)</td>
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<td>AAT-LDL complexes have been detected in human atherosclerotic lesions</td>
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<td>AAT inactivates the catalytic domain of matriptase in vitro and inhibits epithelial sodium transport in vitro and in vivo</td>
<td>(102)</td>
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<td>(104,105)</td>
</tr>
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<td>Induce vascular endothelial growth factor (VEGF) production and to prevent proteolytic degradation of VEGF.</td>
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<td>Biomarker of malignancy</td>
<td>(106,107)</td>
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1.2 Alpha-1 antitrypsin deficiency

1.2.1 Alpha-1 antitrypsin deficiency prevalence and detection

Alpha-1 antitrypsin deficiency is an autosomal co-dominant inherited condition that results in reduced circulating levels of AAT protein and predisposes affected individuals to early onset lung and liver disease. The association of a hereditary form of pulmonary emphysema and AATD was first reported by Laurell and Ericsson in 1963 (108). Subsequently the susceptibility of individuals with AAT liver disease was recognized (109,110). Despite its prevalence, AATD is a condition that is rarely diagnosed and historically it has accounted for 1-2% of patients with chronic obstructive pulmonary disease (COPD), though it accounts for a greater proportion of lung transplantation for emphysema (111). Detection rates in some countries are less than 10% of the at risk population (112,113), this may relate to low awareness of the condition among physicians that can often lead to significant delays before the diagnosis is reached (114).

Severe AATD is defined by a serum AAT level less than 35% of the mean expected value (11μM or 50mg/dL measured by nephelometry). It is usually associated with the PiZZ genotype, but is also encountered frequently with combinations of PiSZ, PiSS, and rare or null alleles. The majority of individuals encountered in clinical practice are PiZZ homozygotes and the disease may manifest clinically in a number of ways, see Figure 1.4 (115,116). The World Health Organisation (WHO), American Thoracic Society (ATS) and European Respiratory Society (ERS) advocate targeted testing for AATD in all individuals with COPD, non-responsive asthma, cryptogenic liver disease and first degree relatives of individuals with AATD (117). This approach has led to higher rates of detection in the populations most at risk of lung disease, though widespread under recognition of the condition remains (118). In Ireland 1:2104 individuals are PiZZ homozygotes, though this is less common in regions of North America and the Antipodes (119,120). Highlighting the effectiveness of the targeted detection approach, published data from our centre has shown that 1:71 tested were PiZZ
homozygotes (14). Neonatal screening can help determine the true prevalence within a population, indicate the natural history of liver and lung disease and permit early intervention focusing on lifestyle modification to prevent morbidity in later years (121,122). The benefit and cost effectiveness of newborn screening in AATD has yet to be determined.

In the absence of familial or population screening for AATD, the disease usually presents with clinical symptoms, often at a stage where significant morbidity from the condition is established. Early literature on patient outcomes in AATD originated from specialist centres with a patient population largely comprised of ex-smokers with severe impairments in lung function (17). These case based series demonstrated premature death at a median age below 50. The majority (>60-70%) of individuals attending specialised centres are severely deficient, i.e. PiZZ or PiSZ, and are detected due to symptomatic pulmonary disease related to smoke exposure (123-126). The remainder of deficient individuals are detected due to the presence of liver disease or through family screening of probands.

The detection of asymptomatic individuals through the family screening of probands is an opportunity to better understand the natural history of this condition as the majority of individuals with AATD have yet to be identified (127,128). Though the confounding effects of genetic modifiers and environmental factors should be taken into consideration, published data on non-index cases suggest they have a life expectancy approaching that of the general population (129). Determining the range of clinical phenotypes that occur in AATD is essential to develop a greater understanding of the underlying pathophysiology of the disease, correct stratification for research studies and therapy, and to prognosticate outcomes.
Figure 1.4: Clinical manifestations of AATD

AAT plays an important role in health by protecting the lung from damage by proteases such as neutrophil elastase, it may also modulate inflammation by controlling the influx of inflammatory cells like neutrophils in to the lung. In deficiency states, reduced local and circulating AAT results in excessive lung tissue damage. Additionally, the misfolded protein accumulates in the liver leading to lower plasma levels and can eventually cause cirrhosis. Rarely, AATD is associated with an inflammatory skin condition called panniculitis.


AAT; alpha-1 antitrypsin, AATD; alpha-1 antitrypsin deficiency, NE: neutrophil elastase.
1.2.2 Alpha-1 antitrypsin deficiency pulmonary disease

Adults with AATD are susceptible to the premature development of lung diseases such as emphysema, chronic bronchitis, bronchiectasis and asthma in addition to liver cirrhosis. Patients with AATD often present with exertional breathlessness, wheeze, cough, and frequent pulmonary exacerbations on a background history of smoking. Symptoms usually begin from the age of 30 and the clinical suspicion for underlying asthma or COPD prompts referral for spirometric assessment. Individuals with AATD who are exposed to cigarette smoke have progressive emphysematous lung destruction and an accelerated decline in lung function measurement indices; all patients should be counselled to stop smoking immediately. Occupational exposure to chemicals and pollutants is also independently associated with a decline in lung function in AATD and patients should be advised of using personal protective respiratory equipment where necessary (123,124).

The diagnosis of fixed airway obstruction, indicative of COPD, in AATD is often made at a much younger age (<40 years) than the general population, however screening for AATD is recommended for all adults with COPD or incompletely reversible asthma (117). The recent Global Obstructive Lung Disease (GOLD) guidelines regarding the grading of COPD severity now include symptomatic dyspnoea scores and exacerbation frequency in addition to spirometric impairment of lung function (130). The GOLD guidelines appear to correlate well with clinically relevant outcomes in AATD (131). The finding of reversibility on spirometry is common (affecting approximately 50% of individuals), it often belies concomitant asthma and emphysema, and it can be associated with a worse prognosis due in part to on-going airway inflammation (132,133).

The classic pathological finding of bibasal pan-acinar emphysema can be now visualised with the widespread availability of high-resolution CT imaging, the unexpected detection of these changes should prompt the clinician to screen for AATD (Figure 1.1A). CT imaging with lung densitometry measurement facilitates
monitoring of disease progression in AATD (134) and may be a superior outcome measure to change in FEV1 in clinical trials examining the effect of augmentation therapy in AATD (135,136). The classic COPD phenotype of chronic bronchitis is characterized by recurrent exacerbations and is associated with poorer health status, increased healthcare utilization and progression of lung disease (137). A genetic polymorphism within the promoter region of TNFα has been determined to influence the development of the phenotype of chronic bronchitis in AATD independent of plasma TNFα levels (138). During acute pulmonary exacerbations there is evidence of excessive bronchial inflammation and increased protease burden in the AATD airway (139). Individuals with AATD experience frequent pulmonary exacerbations, which can lead to a gradual decline in gas transfer (140). Risk factors for exacerbations include pack year smoking history, impaired FEV1, and medication usage (141). There is a measurable benefit for intravenous augmentation therapy in reducing exacerbation frequency and improving patient reported outcomes (142).

Bronchiectasis is a recognised complication of AATD, however its true prevalence and clinical significance in the AATD population remains poorly understood (143,144). There are individuals with severe bronchiectasis whose health status is significantly impacted by the disease and evaluation of the causes of this is an important area of research (145), see Figure 1.1B. An observational study of a non-CF bronchiectasis population did not demonstrate an increased prevalence of AATD implying that it is an uncommon cause of bronchiectasis (146). However this study was underpowered to detect an effect due to the low gene frequency in this study population. CT imaging readily permits the identification of bronchiectasis, which may or may not be clinically significant. The reported prevalence of bronchiectasis varies considerably but may occur independently of emphysema and can be severe (145). Symptoms of bronchiectasis are often difficult to distinguish from chronic bronchitis/COPD and the prevalence and impact of this airway disease in AATD may be underestimated.
Figure 1.5: Radiological evaluation of pulmonary disease in AATD

A. Reflecting the most common disease manifestation in AATD, this CT image (axial view) of a PiZZ individual* with obstructive lung disease demonstrates severe bilateral pan-acinar emphysema in a basal distribution (lower lobes).

B. There is heterogeneity in the clinical and radiological manifestations of AATD, the spectrum of AATD lung disease is not confined to emphysema, particularly in those who never smoked. This CT image (axial view) of another PiZZ individual* with obstructive lung disease despite never smoking demonstrates severe bilateral bronchiectasis in a basal distribution.

*CT images were obtained with consent from study participants.
1.2.3 Alpha-1 antitrypsin deficiency liver disease

The association of AATD with liver cirrhosis was first documented in 10 children in 1969 (109) and subsequently reported in adults (110). AATD is one of the commonest causes for neonatal hepatitis and can account for up to 29% of cases in some paediatric centres (147). However, only a small proportion of PiZZ homozygotes present with a neonatal hepatitis syndrome, usually within the first 3-4 months of life. A large infant screening study of 200,000 new-borns identified 120 with the PiZZ phenotype, of which 22 (18.3%) had evidence of a hepatic abnormality. Prolonged jaundice occurred in 14 (11.7%) and 9 (7.5%) had severe clinical liver disease (13). The SZ phenotype was also associated with some biochemical liver abnormalities in this study, but no clinical manifestations. A number of risk factors for AATD associated liver disease in childhood have been identified including male sex, renal or pulmonary complications, and a first degree relative with AATD-related liver disease (147,148). The reported outcomes of childhood liver disease are variable, with one study reporting a mortality of 20/74 (27%) and persistent cirrhosis in a similar number, though most studies report complete clinical and biochemical recovery of liver function in most cases.

In adulthood, a strong relation between AATD and cirrhosis has been reported (OR = 7.8; CI 2.4 to 24.7), as well as primary liver cancer (OR = 20; CI 3.5 to 114.3) particularly affecting men (149). The prevalence of liver cirrhosis increases with age and usually occurs in those who have never smoked, perhaps as a consequence of the prolonged survival in this group (117). Both genetic and environmental modifiers may play a role in the pathogenesis of liver disease in AATD, however no specific aetiology has been identified to date. The prognosis is usually poor with a median survival of 2 years once the diagnosis of liver disease is made. The natural history of those with fulminant AATD related liver disease has been altered by liver transplantation and excellent survival rates have been achieved (150,151).
1.2.4 Alpha-1 antitrypsin deficiency extra-pulmonary manifestations

The rare occurrence of recurrent panniculitis has been noted in individuals with AATD and is due to neutrophilic inflammation with proteolytic destruction of connective tissue and fat at the affected sites (152). A number of case reports have reported the panniculitis to be responsive to intravenous augmentation therapy (153-155).

AATD has been associated with a variety of other medical conditions, the best described being ANCA-associated vasculitis and in particular granulomatosis with polyangiitis (GPA). A recent genome wide association study identified the Z allele of SERPINA1 to be associated with PR3-ANCA positivity (156). PR3 is inhibited by AAT and some case reports of PR3-ANCA vasculitis in PiZZ homozygotes report a severe disease phenotype (157).

The role of AAT in diseases of the circulatory system is incompletely understood, oxidised AAT can bind to the apolipoprotein B100 component of low density lipoprotein (LDL) in the circulation and may contribute to atherogenesis (101), additionally the cleaved C36 peptide fragment of AAT has been found complexed within atherosclerotic plaque indicating a role in monocyte recruitment within the intima of arterial walls (158). AAT complexes with immunoglobulin A (IgA) are found in the joints and sera of patients with rheumatoid arthritis (159,160), though there appears to be no strong link between the two conditions (161). Additionally, the association of an increased risk of type 2 diabetes mellitus with low AAT levels has also been reported (162).
1.2.5 Alpha-1 antitrypsin deficiency outcomes

Exposure to cigarette smoke has the most significant impact on survival in AATD, the median life expectancy in PiZZ homozygotes is reduced dramatically from 69 years to 49 years in smokers compared to non-smokers, with baseline FEV1 serving as the single most important predictor of survival (127,129). In 1989 the National, Heart, Lung and Blood Institute (NHBLI) established a patient registry to characterise the natural history of severe AATD (AAT <11μM). The primary outcomes of this registry were to establish the yearly decline in FEV1 for those with an FEV1 >30% predicted and ascertain mortality data in those with an FEV1 <30% predicted. Secondary outcomes were to establish the prevalence of associated disease and yearly incidence along with characterisation of symptoms such as cough, wheeze, sputum production and dyspnoea scores (132). Results of this study established the overall 5-year mortality at 19% (95% confidence interval 16-21%) and established age and baseline FEV1 as significant predictors of mortality. The rate of FEV1 decline was -54mls/annum (95% C.I. 35-79mls/annum) and this rate was increased in current smokers, those aged 20-44, those with an FEV1 of 35-79% predicted, and those who had a positive bronchodilator response (163). The excess mortality in AATD was shown to be entirely ascribable to lung and liver disease (164).

The effect of augmentation therapy was also examined and was found to reduce mortality and reduce FEV1 decline for a subgroup of patients with an FEV1 of 35-49% (163). However this was not a population-based study and inference of benefit must be interpreted with caution when considering the wider population of individuals with AATD, particularly as the results may have been influenced by healthcare provision.
1.2.6 Alpha-1 antitrypsin deficiency: PiZ heterozygotes

There has been an increasing number of compound heterozygotes detected with AATD and PiSZ individuals now comprise a large proportion of the severely deficient AATD population (128). Until recently there was a paucity of data in relation to outcomes of PiSZ individuals, an early study indicated that the prevalence of lung disease appears to be lower and less severe compared to PiZZ individuals (165). A recent larger study of 126 PISZ individuals compared their outcomes to PiZZ and PiMM individuals with COPD, it found that PISZ individuals were less susceptible to the effects of cigarette smoke and their pattern of emphysema more closely resembled the pattern in usual PiMM COPD (166). The authors commented that plasma AAT concentration and not genotype may more important in determining phenotype in this population, in addition comparison of outcomes and survival in this study were confounded by selection and survival bias as well as the potential for comorbid conditions in the PiMM group.

Controversy remains regarding commencement of augmentation therapy in this group (167). Indeed, the origin of the protective threshold for augmentation therapy was set based on the plasma level observed in PiSZ heterozygotes, however those who are severely deficient (<11μM) may be offered augmentation therapy in some centres.

As outlined earlier in this chapter, the significant advances in our understanding of Z-AAT, as well as its effect on neutrophil function when compared to PiMM controls and not PiSZ controls, have been established. In a clinical context, the role of augmentation therapy in PiZZ individuals is also better understood than in PISZ individuals; further work to clarify the benefits, if any, of augmentation therapy in the PiSZ group would be valuable in the future.
Liver disease is thought to be less prevalent in PiSZ than in PiZZ individuals as S-AAT is less polymerogenic than the Z protein. However, it is evident from Italian and Spanish registry data that a significant proportion of PiSZ individuals (17.7%) were detected due to liver disease screening (128). This is an area that warrants further research given the increased risk of liver cirrhosis and hepatocellular carcinoma that exists in PiZZ individuals (168).

Identification of index cases with resultant family screening, as well as targeted detection, will invariably detect many PiMZ heterozygotes. Appropriate genetic counselling in this group is important to alleviate any unnecessary anxiety, though clarification of the true risk of lung disease in this population is important, particularly in smokers. The identification of PiMZ individuals either directly or through the identification of probands, has significantly improved our understanding of the risk of lung disease, it has recently been established that ever-smoking PiMZ individuals have an increased risk for COPD and this risk is attenuated in never-smokers (169,170).
1.2.7 Alpha-1 antitrypsin deficiency treatment

Patient education is a cornerstone in the management of AATD, with particular emphasis on immediate smoking cessation to prevent lung function decline. In accordance with the WHO guidelines for the management of patients with lung disease, individuals with AATD should be offered annual vaccination against influenza and the polyvalent pneumococcal vaccine on a five-yearly basis, this has been shown to be a highly effective method for preventing infective exacerbations (126).

AATD individuals with COPD should be offered inhaled bronchodilator and steroid therapies in accordance with the current ATS/ERS and GOLD guidelines for the management of COPD (137,171). The effectiveness of inhaled corticosteroid therapy is being re-evaluated in the treatment of COPD due to an increased risk of hospitalisation and pneumonia (172). More recently a greater emphasis in the management of COPD has been placed on bronchodilation, the reduction of pulmonary exacerbations, preventing hospitalisation, and improving patient-reported outcomes.

It is important that pulmonary exacerbations of AATD be treated aggressively to minimise the proteolytic lung damage that may ensue, however in accordance with the above guidelines antibiotic therapies should be reserved for the treatment of bacterial infections and short-term use of steroid therapies can be considered for relief of symptoms. A reduction in exacerbation frequency has been demonstrated in the general COPD population with regular administration of azithromycin (173). At present there is insufficient evidence to recommend routine use in AATD and this must be weighed against the possible adverse side effects (173-175). Augmentation therapy, consisting of weekly infusions of purified human-derived plasma AAT, is the only specific approved therapy for COPD/emphysema related to AATD.
1.2.8 Alpha-1 antitrypsin deficiency augmentation therapy

Efforts to restore normal circulating plasma levels of AAT culminated in the development of AAT augmentation therapy in the 1980s from pooled donor plasma (176). Initial studies demonstrated safe and effective delivery of the purified AAT protein to maintain levels above a putative protective threshold of 0.5g/dL (11μmol/L). This threshold was inferred from the lower limit of the circulating plasma level of PiSZ individuals as it was observed that they rarely develop emphysema, the other common AAT phenotypes are milder and the mean plasma AAT concentrations are also above this level, see Figure 1.5. No randomised controlled trials were performed prior to the United States Food and Drug Administration (FDA) approval in 1987 based on biochemical efficacy (177). A number of obstacles were evident in conducting a RCT at that time, firstly there were insufficient patients to power the study in individual centres and secondly, FEV1 proved to be a poor outcome measure. The advent of computerised tomography (CT) thoracic imaging which can visualise and objectively quantify the primary pathophysiological process in the lung, emphysema, has led to recommendations that it be used as the primary endpoint to evaluate the effect of augmentation therapy on disease stabilisation and progression (135).

The first randomised controlled trial of intravenous AAT augmentation therapy in severe AATD (serum concentration <11μmol/L) has only recently been published; the results demonstrate that intravenous AAT augmentation slows the progression of emphysema using CT-lung densitometry as the primary outcome measure (136). The annual rate of lung density loss at total lung capacity (TLC) was significantly less in patients in the treatment arm (−1·45 g/L per year) versus the placebo group (−2·19 g/L per year); absolute difference 0·74 g/L per year [95% confidence intervals (CI) 0·06–1·42], p=0·03. The authors conclude from this data that augmentation therapy may preserve lung parenchyma in individuals with emphysema secondary to severe AATD.
Another study evaluating changes in lung density using similar methods in individuals with severe AAT on augmentation therapy is also underway in the QUANTUM-1 trial (QUANTitative Chest Computed Tomography UnMasking Emphysema Progression in Alpha-1 Antitrypsin Deficiency, NCT00532805).

Figure 1.6: Plasma AAT levels in the Irish population

Plasma AAT levels, determined by nephelometry and correlation with clinical phenotype as assessed by isoelectric focusing. The dotted line indicates the putative protective threshold for AAT in blood at 0.5g/L (11μmol/L).

Adapted from Carrol et al. Respiratory Research 2011 (14).
Augmentation therapy has maintained an excellent safety profile, though concerns regarding cost-effectiveness persist (178). Efforts are underway to produce the AAT protein through recombinant techniques while preserving protein function (21). Other efforts to improve the efficacy of treatment include HDL enrichment of AAT augmentation preparations to potentiate the anti-elastase effect of AAT which may lead to improvements over standard augmentation therapy (179). In addition to improving the plasma half life of AAT, hyper-glycosylated (disialylated) AAT has increased activity against NE and increased affinity to E-selectin which can reduce neutrophil rolling and attachment to the endothelial wall (20).

The novel anti-inflammatory properties of AAT have led to speculation of the use of AAT augmentation therapy for a range of conditions (93). The observed beneficial effect of AAT on ischaemia-reperfusion injury in myocardial infarction, possibly by AAT mediated anti-apoptotic effects through downregulation of caspase-1 activity (94), has lead to the first clinical translational study of single dose augmentation therapy in acute ST elevation myocardial infarction (180). In this first clinical trial outside of AATD, augmentation therapy was safe and well tolerated with evidence of blunting of the acute inflammatory response. Promising results from murine models of pancreatic allograft transplantation (181,182), has led to more clinical trials in graft-versus-host disease (NCT01183468, NCT01523821, NCT01700036) and new onset type-I diabetes (NCT02093221).

Despite the emergence of a potentially novel anti-inflammatory role for AAT augmentation therapy, concern persists regarding the efficacy and dosing of currently existing AAT augmentation strategies for the treatment of deficiency (183,184). Randomised clinical trials are underway to establish the clinical benefit of higher dose delivery of AAT to maintain a higher protective threshold in AATD, NCT01669421 (185).
1.3 Conclusion

In the 50 years since the discovery of the clinical diseases associated with AATD there has been considerable advancement in our understanding of the mechanisms through which AAT mediates its effects in both health and disease. Protease inhibition remains the principal function of AAT, however the role of AAT in the modulation of neutrophil migration and activation, cytokine signalling and apoptosis has come increasingly under review.

Smoke exposure is a critical event for the development of lung disease in AATD and is the main determinant of the clinical phenotype. The anti-inflammatory properties of AAT remain incompletely understood and, given the diversity of systems through which AAT appears to exert its modulatory effects, it is likely that inflammation plays a central role in the determination of disease severity for those affected by AATD.

Some of the observed effects of AAT, beyond its protease inhibitory capacity, are mediated through its role as a binding protein. This is illustrated by the impact of glycosylation on the interaction of AAT with the pro-inflammatory cytokine IL-8, in addition to its effect upon the modulation of TNFα signalling. Plasma purified AAT has been established as a safe and effective specific therapy for those affected by AATD-related lung disease, however it is its potential anti-inflammatory properties that has led to its evaluation in other non-deficient conditions.

There is significant clinical heterogeneity in AATD, mounting evidence indicates that inflammation may play a key role in the development and progression of AATD-related lung disease. Furthermore, knowledge of the spectrum of AAT interactions in blood may uncover new effects of AAT and lead to novel therapeutic applications of augmentation therapy in AATD as well as other disease states.
The hypothesis of this thesis is that AAT modulates inflammation by binding to other blood constituents as it circulates in the body. The aim of this thesis is to elucidate the full range of binding partners to AAT in the circulation and correlate the findings with the established pulmonary disease phenotype in AATD. To fulfil this aim the specific objectives of this thesis were:

1. Determine a detailed clinical PiZZ phenotype from the Irish population of individuals with known AATD and correlate with levels of systemic inflammatory cytokines.
2. Investigate the physiochemical properties of AAT, with particular focus on the spectrum of protein binding partners in the circulation, to uncover potential new mechanisms by which AAT accomplishes its function.
3. Evaluate the physiological relevance of any AAT:protein interactions that are uncovered.
4. Explore the impact AAT interactions on inflammation and relate this to the clinical phenotype of pulmonary disease in AATD.
Chapter 2: Methods
2.1 Material suppliers

2.1.1 Chemicals and reagents

All chemicals and reagents used in this study were of the highest purity available and endotoxin free and were purchased from Sigma Aldrich (Dublin, Ireland) unless otherwise indicated.

2.1.2 Antibodies

The primary antibodies used for Western blot analysis and flow cytometry are listed in Table 2.1 and secondary antibodies in Table 2.2.

### Table 2.1: Primary antibodies employed in this study

Abbreviations: AAT: Alpha-1 antitrypsin, C3: Complement component C3, FITC= fluorescein isothiocyanate.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Reactivity</th>
<th>Manufacturer</th>
<th>Catalogue number</th>
<th>Working dilution</th>
<th>Size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Western Blot</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AAT</td>
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<td>Polyclonal</td>
<td>Abcam</td>
<td>Ab7633</td>
<td>1:5000</td>
<td>52</td>
</tr>
<tr>
<td>AAT</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Abcam</td>
<td>Ab9373</td>
<td>1:5000</td>
<td>52</td>
</tr>
<tr>
<td>C3</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>Novus</td>
<td>NBP1-32080</td>
<td>1:5000</td>
<td>190</td>
</tr>
<tr>
<td>C3c</td>
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<td>Abcam</td>
<td>Ab48611</td>
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<td><strong>Flow cytometry</strong></td>
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<tr>
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<td>19170</td>
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<tr>
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<td>Abcam</td>
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### Table 2.2: Secondary antibodies for Western blot analysis

Abbreviations: HRP = horseradish peroxidase, IgG = Immunoglobulin G.

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Catalogue number</th>
<th>Working dilution</th>
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<td>Santa Cruz</td>
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<tr>
<td>Rabbit IgG Isotype Control, FITC conjugate</td>
<td>Rabbit</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>Anti-rabbit IgG HRP-linked antibody</td>
<td>Goat</td>
<td>Cell Signalling</td>
<td>7074S</td>
<td>1:2000</td>
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2.2 Study design

2.2.1 Study Population

Healthy Individuals (n=20) were recruited to this study as controls; all were non-smokers, had no history of pulmonary disease, and were not taking any regular medication. The mean age was 30.7 ± 5.48 years with a measured FEV1 of 99.6 ± 7.95% predicted, see Table 2.3. All controls were confirmed as having a PiMM phenotype by isoelectric focusing and a serum AAT concentration within the normal range (25-50μM). AAT concentration was determined using a rate immune nephelometric method (Array 360 System; Beckman-Coulter). The phenotype was determined by Hydrasys electrophoresis platform (Sebia) with the Hydragel-18 AAT Isofocusing kit (Sebia, Evry, France) (186). AAT levels were measured by nephelometry (Siemens Dade- Behring BN II, Tarrytown, NY).

Table 2.3: Baseline demographics of PiMM healthy volunteers

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Male (%)</th>
<th>BMI</th>
<th>FVC%</th>
<th>FEV1%</th>
<th>FEV1/FVC</th>
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<tr>
<td>Mean 30.7</td>
<td>8 (40%)</td>
<td>24.5</td>
<td>105</td>
<td>99.6</td>
<td>80</td>
</tr>
<tr>
<td>SD 5.5</td>
<td>-</td>
<td>2.5</td>
<td>6.2</td>
<td>8</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Abbreviations: BMI: body mass index, FVC: forced vital capacity, FEV1: forced expired volume in 1 second, SD: standard deviation.

AATD individuals with a confirmed ZZ-AAT phenotype by isoelectric focusing were recruited from the Irish Alpha-One Antitrypsin Deficiency Registry. Individuals with AATD had been recruited to the registry at the time of their first outpatient clinic appointment in Beaumont hospital or shortly thereafter through personal interview, all those attending clinic were referred by health care professionals following a diagnosis of AATD due to clinical symptoms, or in a close family relative for the purpose of screening for the condition. All registry participants currently living in Ireland in January 2014 were identified for this study. Subjects who had undergone full clinical evaluation at the national referral
centre in Beaumont Hospital were selected and clinical demographics were recorded. PiZZ individuals on augmentation therapy (n=20 subjects) were receiving plasma purified AAT from CSL Behring (Zemaira®), given intravenously at a dosage of 60mg/kg body weight weekly. In the six weeks prior to obtaining blood samples, all subjects were exacerbation free. All participants provided written informed consent (included in appendix 1), which was approved by the Beaumont Hospital Institutional Review Board (reference 13/92). Subjects were classified as index cases if they were diagnosed with AATD after investigation of their symptoms and non-index if they were diagnosed through family screening. The AATD population captured by this recruitment strategy ensured that a representative population of symptomatic and asymptomatic individuals were recruited to the study. As Beaumont is a tertiary referral hospital for AATD in Ireland, and most are referred for pulmonary symptoms, the spectrum of severe COPD/emphysema may be overrepresented whereas liver disease may be underrepresented in the context of the general population with PiZZ AATD. The reasons for individuals to not participate in this study were; declined consent, lost to outpatient follow up, changed address and not contactable, or those who had recently died.

2.2.2 Pulmonary function testing

Pulmonary function testing was performed in all participants according to American Thoracic Society standards (187). Gas transfer (diffusing capacity of the lung for CO [DLCO]) was measured by the single breath carbon monoxide method. Pulmonary function data was recorded including FEV1 (forced expiratory volume at one second) percent predicted, FVC (forced vital capacity) to identify any relationships with clinical outcomes (188). The annualised rate of FEV1 decline was determined by linear regression analysis for those with sequential pulmonary function testing available for greater than one year or more.
2.2.3 Questionnaire

Cross sectional data was collected from the study participants via a self-reported questionnaire that was distributed by post. The questionnaires were coded and returned anonymously by stamped addressed envelope, following which the information was decoded and encrypted to match the clinical data. In addition, questionnaires were also distributed in the specialist AATD outpatient clinic. The clinical parameters that were recorded included; age of diagnosis, symptomatic detection (index) vs. family screening (non-index), smoking status, passive smoke exposure, number of pulmonary exacerbations, cough, sputum production, and oxygen use. As this was a retrospective study exacerbations were self-reported by respondents and not strictly defined by the Anthonisen criteria (189), in order to account for this antibiotic usage for exacerbations was recorded. Dyspnoea scores were calculated using the five point modified Medical Research Council score (mMRC) and health status in the previous week was determined using a visual analogue scale (VAS) scored between 0 and 100. Pack-year smoking history was determined by the function of number of cigarettes smoked per day and total years smoked.

2.2.4 Radiology

High Resolution computerized tomography (CT) images were obtained on a Siemens 16-slice scanner. All patients were imaged while supine and inspiratory images were obtained from the lung apices to the costophrenic angles. Scanning parameters were 120 kV and 90 mA. Images were reconstructed on mediastinal and lung windows.

A modified version of Bhalla’s scoring system for thin section CT in patients with AATD was applied to each scan (190,191). All lobes were individually assessed for purposes of evaluating severity of emphysema, bronchiectasis and peribronchial thickening. All criteria were scored on a scale of 0-3. The presence and location of bullae was also recorded. Once scores were assigned to each of the parameters, the overall score was calculated by summing the individual values.
to a maximum value of 21. Higher scores therefore indicate greater severity. Two radiologists, with a specialist interest in thoracic radiology, reviewed the CT imaging independently and subsequently a consensus opinion on the final score for each subject was determined. Both radiologists were blinded to the clinical severity of AATD when scoring. The presence of mediastinal lymphadenopathy (recorded when one or more lymph nodes with a short axis diameter (SAD) of greater than 1.0 cm were detected) was recorded. We did not assess for generations of bronchial divisions involved with bronchiectasis/plugging. Mucus plug attenuation was evaluated based on the degree of Hounsfield units detected within the plug. Mucus plugging was defined as tubular structures traced cephalad or caudal on adjacent scans confirming continuity with a bronchus (191). CT scores were compared to pulmonary function data including percent-predicted FEV1, FVC, and DLCO to identify any relationships.

### 2.2.5 Blood sample acquisition

Plasma samples were collected in Lithium-heparin bottles (Sarstedt) and centrifuged at 350g for 5min at room temperature, plasma was aliquoted for immediate use or stored at -80°C. Serum samples (Sarstedt) were collected and allowed to clot for 45min before centrifugation at 350g, samples were immediately aliquoted and stored at -80°C. Plasma samples were filtered through a 0.45μM filter to remove contaminants prior to analysis.

### 2.2.6 Bronchioalveolar fluid sample acquisition

All patients were undergoing bronchoscopy for clinical reasons. Full informed consent was obtained prior to the procedure in accordance with the institutional ethical approval. All procedures were performed in accordance with British Thoracic Society guidelines for bronchoscopy (192). Six individuals with AATD were recruited to the study and matched plasma samples were obtained on the morning of the bronchoscopy prior to the procedure. During fibreoptic
bronchoscopy in a standardised fashion, bronchioalveolar fluid (BAL) samples were obtained by instilling aliquots of 3 × 50 mL 0.9% saline into a division of the right middle lobe of the lung with immediate re-aspiration. The total volume returned was recorded. Samples were then stored on ice and transported for immediate processing. BAL fluid was then filtered through sterile gauze and centrifuged at 500g for 10min at 4°C. The supernatant was divided into aliquots and stored at −80°C. The urea concentration of was measured in each sample and compared to the plasma urea concentration (193); the most dilute sample was then chosen as the reference concentration and each subsequent sample was diluted to normalize the concentration for comparison of BAL contents between each individual.
2.3 Laboratory methods

2.3.1 Enzyme linked immunosorbent assay

A quantitative sandwich enzyme linked immunosorbent assay (ELISA) technique was used to measure IL-6, IL-8, IL-10, TNF-R1 (R&D systems), C3 (Abcam), C3a (Quidel), and C3d (Cusabio) levels. The generation of the standard curve required either the serial dilution method of a known concentration of substrate or by using the standards provided in the ELISA kit, see Figure 2.1. A standard curve was generated for each ELISA in accordance with the manufacturers’ instructions using GraphPad Prism v6.0, see Figure 2.2. Plasma samples were thawed rapidly and placed on ice prior to complete defrosting; each sample was diluted and loaded in duplicate onto a 96 well plate. Following incubation at 37°C for 1h with gentle agitation, sequential addition of capture and detection antibody to each well was performed with intermediate washing steps. The final colour generating step was stopped at a given time point (specific to the ELISA performed) prior to reading the plate in a standard microplate reader. The recorded values were subtracted from blank controls and the final concentration of each plasma constituent determined by comparison with the standard curve and multiplying by the concentration factor.

Figure 2.1: ELISA standard serial dilution method

This figure demonstrates the serial dilution method with high grade H2O utilised in the generation of standard curve for the ELISA detection of a target substrate.

Source: Product information leaflet; sTNF R1 Quantikine ELISA (R&D Sytems Inc. USA).
Figure 2.2: ELISA standard curves

This figure shows the standard curves generated for each ELISA performed in the course of this work. All standard curves were generated in accordance with the manufacturer’s instructions. Measured values were extrapolated from the curves using GraphPad Prism v6.0 and multiplying by the dilution factor of the tested
2.3.2 Gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) sample buffer (10x containing 0.2% (w/v) bromophenol blue, 50% (w/v) sucrose, 1% (w/v) SDS, 1% (w/v) dithiothreitol, 200mM EDTA, 3M Tris-HCL) was added to protein samples, which were then subjected to 12.5% (w/v) 1D SDS-PAGE under denaturing conditions. The resolving gel (42% (v/v) Protogel (National diagnostics, Atlanta, USA), 375mM Tris pH 8.9, 0.1% (w/v) SDS, 0.06% (w/v) ammonium persulphate (APS) and 0.025% (v/v) tetramethylethylenediamine (TEMED) was poured between 2 glass plates and allowed to set before the stacking gel (17% (w/v) Protogel, 65mM Tris HCL pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS and 0.1% (v/v) TEMED) was added on top with a comb placed inside. Gels were stained with Coomassie blue stain (10% (v/v) acetic acid, 45% (v/v) methanol, 45% (v/v) H₂O, 0.2% (w/v) Coomassie brilliant blue R250) for visualisation of protein banding patterns, or alternatively proteins were transferred onto polyvinylidene fluoride (PVDF) membrane by Western blotting.

2.3.3 Western blot analysis

Following electrophoresis the resolving gel was equilibrated for 1min in transfer buffer (39mM glycine, 48mM Tris, pH 8.3, (20% w/v) methanol and placed on top of a PVDF membrane. This membrane was sandwiched between Whatmann chromatography paper (Whatmann International Ltd, Maidstone, England) that was pre-soaked in transfer buffer. Proteins were transferred onto the membrane at 150mA for 60min using the semi-dry blotting apparatus. Following transfer the membrane was blocked in 5% (w/v) non-fat powdered milk in PBS containing 0.1% Tween-20 (PBST) for 1h at room temperature. The membranes were incubated with 1μg/ml polyclonal goat anti-AAT antibody or 1μg/ml polyclonal rabbit anti-C3 antibody for 1h at room temperature. Following intermediate wash steps with PBS-Tween, the membrane was blocked with 1% (w/v) Bovine Serum Albumin (BSA) for 1h. The secondary antibodies were thereafter applied, HRP-linked anti-rabbit or anti-goat, for 1h at room temperature and washed in
PBS-Tween again. All antibodies used are listed in Table 2.1 and 2.2. Visualisation of immunoreactive protein bands was achieved using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) in a dark room using film (UltraCruz™ Autoradiography Film, Santa Cruz) exposed to fixing and developing solution (Kodak, USA) with varying lengths of exposure from 10s to 10min for optimal development. Protein band mass was estimated using by comparison to molecular mass markers and subsequently densitometry was performed using the GeneSnap SynGene Programme (Synoptics).

2.3.4 Co-immunoprecipitation of AAT binding partners

Alpha-1 Select resin is a commercially available chromatography medium with a high selectivity for AAT. The resin is comprised of porous agarose particles with a covalently attached ligand that contain a Camelidae-derived single domain antibody fragment that is specific for a region on the AAT protein. Utilisation of this chromatography system allows purification and concentration of both the M-AAT and Z-AAT form of the protein from plasma. To isolate the candidate binding partners, freshly drawn plasma samples were obtained and processed within 30min at room temperature. Following dilution of plasma in a ratio of 1:1 with Dulbecco’s Phosphate Buffered Saline (DPBS), 100μL of plasma was incubated with AAT Select resin for 1h at room temperature. Following intermediate wash steps with gentle centrifugation at 8g to re-pellet the sample, the candidate binding partners were eluted from the resin using 500mM Mg$_2$Cl$_2$ initially. A second 2M Mg$_2$Cl$_2$ elution step was performed thereafter to remove any remaining bound AAT and complexed binding partners. The resultant elution samples were acetone precipitated overnight in a ratio of 5:1, and then pelleted at 550g for 10min at 4°C. The protein pellet desalted into PBS using NAP TM-10 desalting columns (GE Healthcare Life Sciences) and resuspended in reducing sample buffer for SDS-PAGE analysis.
2.3.5 Size exclusion permeation chromatography

Size exclusion permeation chromatography was performed using a Superdex 200GL 10/300 column (GE Healthcare) using the AKTA Prime plus (GE Healthcare) Fast Protein Liquid Chromatography (FPLC) system. The column was initially equilibrated with two column volumes of Dulbecco’s Phosphate Buffered Saline (DBPS). Calibration with known high molecular weight standards (GE Healthcare) was performed; Thyroglobulin (Mw 660kDa), Ferritin (Mw 440kDa), Aldolase (Mw 158kDa), Alpha-1 Antitrypsin (Athens research)(Mw 52kDa). UV absorbance at 280nm was measured using PrimeView 5.0 software, protein elution peaks were recorded and a standard elution curve constructed using GraphPad Prism v6.0.

2.3.6 FPLC to determine the elution profile of AAT in plasma

Fresh MM (n=6) or ZZ (n=3) plasma was filtered through a sterile 0.45μm PES membrane filter (GVS, USA). 100μL of start sample was injected onto the column with a buffer flow rate of 0.5ml/min. The eluent was collected in 0.5ml fractions and UV absorbance at 280nm was recorded using PrimeView software. To assess for electrostatic dissociation of potential high molecular weight binding partners the column was re-equilibrated with DPBS with 1M NaCl and each sample was repeated. The fractions corresponding to the UV elution profile were collected and analysed by denaturing SDS-PAGE (12.5%).
2.3.7 Purification of complexed binding partners to AAT at a high molecular mass.

Permeation chromatography was performed six times in DBPS buffer to generate a volume of 3mL of each fraction of MM (n=3) and ZZ (n=3) plasma. Fractions containing high molecular weight proteins were pooled to a volume of 12mL and incubated with 100μL AAT-Select Resin (GE Healthcare), a highly selective resin to purify AAT from the eluent. Following incubation for 1h at 25°C with agitation, the samples were centrifuged to remove any unbound supernatant. The resin was washed with DPBS six times and pelleted by centrifugation. A two-step elution process was performed to firstly remove any AAT-bound proteins and secondly to dissociate the AAT from the resin. In brief, the pellet was resuspended in 500mM MgCl₂ and following centrifugation this supernatant was removed. The pellet was then resuspended in 2M MgCl₂ and the supernatant was similarly removed.

The eluents were divided for mass spectrometry analysis; half the eluent underwent overnight acetone precipitation at 4°C to generate a protein pellet (in-solution), the second half was analysed by SDS-PAGE and silver staining to permit visualization of the purified protein profile (in-gel).

2.3.8 Mass spectrometry analysis

In-solution protein pellets were reduced and alkylated using DTT and iodoacetamide before being enzymatically digested with lysine-sensitive aspartokinase 3 (LysC) (6h) and Trypsin (overnight). The reaction was stopped using trifluoroacetic acid (TFA) and the peptides generated were separated using a 3h Reverse Phase Chromatography (RPC) separation into a LTQ Ion Trap Mass Spectrometer (MS).

In-gel digestion was carried out on the silver stained gels. Briefly, each lane was excised and dissected into bands. Each band was destained, reduced and alkylated before being digested overnight with 12.5ng of trypsin per band. Peptides generated were extracted from the gel slices before being evaporated
and resuspended in 2% Acetonitrile (ACN)/0.1% formic acid (FA) for liquid chromatography (LC) MS analysis over a 30min RPC gradient into a Linear Trap Quadrupole (LTQ) Orbitrap MS. The data generated was searched against the human subset of the UniProt/SwissProt database (Jan 2012 release) using SEQUEST search algorithm in BioworksBrowser 3.3.1. Carbamidomethylation of cysteine residues was selected as a fixed modification and oxidation of methionine was considered as a variable modification also allowing for two missed cleavages. The following SEQUEST filters were applied: for charge states 1, Xcorr >1.9; 2, Xcorr >2.2; 3, Xcorr >3.75 and Peptide Delta CN (Maximum delta Cn:0.1). The following MASCOT filters were also applied: MASCOT threshold score of 40 and MASCOT significance threshold of 0.05. For the Orbitrap MS an extra criterion of mass accuracy of 20ppm for the results was used.
2.4 Confirmation of AAT binding to C3

2.4.1 Co-immunoprecipitation of plasma C3 with AAT

In accordance with the manufacturers instructions 100μL Goat polyclonal anti-AAT antibody (10mg/ml) (Abcam) and 500μL control goat Ig (400μg/ml) (Abcam) in coupling buffer (0.2M NaHCO3, 0.5M NaCl, pH 8.3) were loaded onto HiTrap NHS-activated HP columns (GE Healthcare). Coupling efficiency was determined as 95.6% for the AAT-antibody column and 92% for the control column using a BCA assay.

PiMM plasma sample was diluted 50:50 with DPBS and passed through a 0.45µm filter. 1mL of plasma was injected and allowed incubate for 30min at 25°C in the control or AAT-antibody column. The column was then connected to an AKTA Prime plus FPLC and unbound sample was removed with five column volume washes of DPBS. Subsequently, 200μL fractions were collected by FPLC into prefilled eppendorfs with 50μL 1.5M Tris (pH 8.9) using 2mL of 100mM Glycine elution buffer (pH 2.3). The process was repeated a total of five times per plasma sample on each column. The fractions were acetone precipitated and resuspended in reducing sample buffer for SDS-PAGE western analysis.

2.4.2 Bicinchoninic Acid (BCA) assay

The efficiency of coupling of anti-AAT antibody to the HiTrap column was quantified by BCA assay (Thermo scientific, USA). The assay is based on the chelation of cuprous ions by BCA after copper binds to amino acids in alkaline conditions. The formed purple complex is very stable and can be measured spectrophotometrically. Reagents A and B were added to the samples and read on a microplate reader at 562nm as per the kit instructions. The concentration of the unknown samples were colourmetrically assessed based on a standard curve of BSA of known concentrations (0 to 3000µg/ml), the measurement was repeated in triplicate.
2.4.3 Native PAGE electrophoresis of AAT polymers to assess C3 binding.

NativePAGE Novex 4-16% Bis-Tris Protein Gels (Thermo Fisher scientific) were performed to examine for direct protein binding between AAT (CSL Behring) and C3. The AAT utilised in this experiment is highly concentrated (35mg/ml) and known to contain polymers. Following desalting through a PD-10 desalting column (GE Healthcare) equal protein concentrations of AAT (0.12µg/ml) and C3 (0.12µg/ml) were loaded in 10µL aliquots onto the native gel. Following co-incubation for 30mins at room temperature, the test sample of AAT and C3 at the same concentration (0.12µg/ml) was loaded into the third lane in a 10µL aliquot. The gel was run at a constant 90V for 3h in accordance with the manufacturers protocol with the addition of NativePAGE anode buffer and cathode buffer (GE Healthcare) to the respective compartments of the gel rig. The gel was stained with G250 Coomassie blue and imaged, then subsequently transferred to a PVDF membrane at a constant 30V for 1h in non-SDS transfer buffer (3.03g Tris base, 14.4g glycine, 800ml H2O, 200ml methanol). The membrane was subsequently probed for AAT and C3 with their respective antibodies.
2.4.4 FACS analysis of C3 binding to AAT-coated beads

Samples were analysed on a FACS calibur flow cytometer (Becton Dickinson, San Jose, CA, USA), which allows simultaneous acquisition of fluorescence, forward light scatter and side scatter. In this study, samples were excited at 488nm with an argon laser and the fluorescent channel FL1 (emission at 530nm) was employed for green fluorescence for FITC. AAT-coated beads were prepared as follows: 100μL of 10μm microsphere polystyrene beads (Polybeads®, Polysciences Europe GmbH, Germany) were incubated overnight with 120μg of AAT protein (Athens Research) at 4°C in 490μL of Voller’s Buffer (25.47mM Na2CO3). 50μL of sample was pelleted, washed and the supernatant removed. The control and AAT-coated pellet was resuspended with 5μg of native complement C3 (Abcam). Two further incubation steps with 4% (w/v) paraformaldehyde and 2% (w/v) BSA were performed to prevent non-specific binding to the beads. FACS analysis was performed on samples using rabbit anti-C3 FITC-labelled antibody or a nonspecific IgG1 FITC control (IsoAb) (Thermo Fisher Scientific). A total of 10,000 counts were acquired and the experiment repeated in triplicate. The data were analysed, and the mean fluorescence units (MFU) for each experiment determined, using BD CellQuest Pro software.

2.4.5 FACS analysis of competitive inhibition

To determine the impact of competitive inhibition of AAT protein interactions with C3, 5μg C3 was incubated with 50μg AAT for 1h with gentle agitation at 37°C. Additionally, to determine the degree of competitive inhibition, serial dilution of 5μg C3 in DPBS was performed to obtain a concentration of 5μg, 2.5μg, 1.25μg, 0.675μg and 0. FACS analysis was performed as described in 2.4.3.
2.4.6 Purification of AAT from human serum by fast protein liquid chromatography

Plasma from confirmed PiMM or PiZZ individuals was used to isolate M-AAT and Z-AAT respectively. AAT-Select resin was packed into a Tricorn Column (GE healthcare) and attached to the AKTA prime plus (GE healthcare). The plasma that was prepared by the addition of binding buffer (20mM Tris, 150mM NaCl, pH 7.4) in a ratio of 1:3 and passed through a 0.45µm filter. The sample was injected into the system at a flow rate of 0.5ml/min. This was repeated six times with peaks of protein concentration observed as the sample was added. The sample was eluted at a flow rate of 1.5ml/min over and increasing gradient of 2M MgCl₂ buffer from 0% to 100%. Fractions were collected during the elution process and the protein concentration of each fraction was determined based on UV absorbance at 280nm. Fractions containing AAT were concentrated using Amicon® Ultra centrifugal filters (Millipore) and desalted into PBS using NAP TM-10 desalting columns (GE Healthcare Life Sciences). The protein yield was quantified by BCA assay and the samples were analysed by SDS-PAGE and western blot to confirm the presence of AAT.

2.4.7 FACS analysis of M-AAT and Z-AAT binding to C3-beads.

To determine if C3 can bind to M-AAT and Z-AAT, 30µg C3 in 500µL coupling buffer (0.2M NaHCO₃, 0.5M NaCl, pH 8.3) was incubated for 1h with 100µL of HiTrap column beads (GE Healthcare) with gentle agitation. Control beads were created by incubating the beads for 1h with coupling buffer in the absence of C3. Once coupling was complete the control and C3-labelled beads were incubated with 10µg of either M-AAT or Z-AAT for 1h, and then washed with 1% (w/v) BSA. The beads were then suspended in sample buffer and analysed by SDS-PAGE and western blot immunoassay (n=3).
2.4.8 FACS analysis of non-glycosylated AAT binding to C3-beads.

The binding of C3 to plasma purified AAT (Athens Research) was compared to recombinant AAT (Prospec, USA). Recombinant AAT (rAAT) is produced in rice is a single, non-glycosylated polypeptide chain containing 384 amino acids and having a molecular mass of 43.1 kDa from the human SERPINA1 gene. Comparison of binding events between C3 and the glycosylated native protein and non-glycosylated recombinant protein may give insight into the importance of glycan residues for AAT binding to C3. AAT and rAAT were diluted to a standard concentration of 150µg/mL. 100µL of AAT or rAAT was incubated with 100µL C3 coated polystyrene beads (Polybeads®, Polysciences Europe GmbH, Germany) as described in 2.4.3. FACS analysis was performed on samples using goat anti-AAT FITC-labelled antibody or a nonspecific IgG1 FITC control (IsoAb) (Santa Cruz Biotechnology). A total of 10,000 counts were acquired and the experiment repeated in triplicate. As before, the data were analysed, and the mean fluorescence units (MFU) for each experiment determined, using BD CellQuest Pro software.
2.5 The interaction between AAT and C3 in AATD

2.5.1 Assessment of C3 cleavage in AATD BAL.

BAL fluid from AATD patients was defrosted at 37°C until nearly fully thawed, kept at 4°C to minimize complement activation, and immediately processed to prevent complement activation artefact in the samples. Urea levels were measured in BAL and matching plasma samples to determine the dilution factor. BAL fluid was suspended in non-reducing sample buffer and the presence of C3 in BAL fluid (n=7) was assessed by SDS-PAGE (12.5%) and western blot analysis. BAL fluid samples from PiMM individuals with cystic fibrosis (CF), a condition characterized by a high protease burden in the lungs, were utilized as an inflammatory control to evaluate if C3 is present in a cleaved state in BAL fluid. The CF BAL fluid samples were obtained and processed in an identical fashion to the AATD BAL samples.

2.5.2 Measurement of C3 and its activation products in plasma.

Plasma samples were defrosted at 37°C until nearly fully thawed, then kept at 4°C for immediate use to minimize complement activation in accordance the ELISA protocol. Samples had not previously been thawed. Total C3 was determined using a specific C3 ELISA (Abcam). Analysis of complement activation was determined by measuring C3d and C3a (desArg) levels in the same samples using the respective specific C3d (Cusabio), and C3a (desArg) ELISA (Quidel) as described in 2.3.1. Standard curves were generated for each experiment against the known standards provided in the ELISA kits in GraphPad Prism. The degree of complement activation within each sample was calculated by the ratio of the activation product to total plasma C3. The measurement of each sample was performed in duplicate and corrected for by the respective dilution factor of each ELISA.
2.5.3 Complement system screen for functional complement deficiency

Measurement of C3a and C3d as a ratio of total C3 provides important information regarding the degree of complement system activation and turnover, however it does not necessarily provide insight into function complement deficiency. Earlier experimental techniques that were employed to evaluate for complement deficiency included the time it take for haemolysis of sheep erythrocytes to assess the classic pathway (CH50) and alternative pathway (AH50). To evaluate the qualitative function of the Classic, Alternative and Lectin pathways in the complement system in AATD subjects an enzyme immunoassay was employed, the Complement System Screen (Wieslab, Eurodiagnostica, Sweden). Serum samples from AATD subjects (n=13) were added to the ELISA in duplicate and the degree of complement activation was determined as a percentage of the reference population.
2.5.4 Determination of proteolytically active NE in plasma AATD

Proteolytically active NE was determined spectrofluorometrically in each BAL sample by ortho-aminobenzoyl-peptidyl-N-(2,4-dinitrophenyl) ethylenediamine fluorescence resonance energy transfer (FRET) measurement on substrate cleavage by NE (194). Plasma samples were thawed rapidly and then placed on ice for immediate processing. 10µL of plasma was diluted in 40µL of buffer (0.1M HEPES, 0.5M NaCl, 0.1% Brij, PH 5.0) and loaded onto an opaque 96 well plate in duplicate. A standard curve of proteolytically active NE was prepared by sequential dilution of 34.5µM NE from 1:100 to 1:12800. On addition of 2mM FRET substrate the samples were immediately analysed on a standard spectrophotometer with a 470nm filter. The samples were analysed every 2min for 20min. The data points for each known concentration of NE were recorded and analysed by linear regression to create a standard for FRET cleavage. The amount of proteolytically active NE in each sample was determined using this standard curve as a reference.

2.5.5 FRET analysis of NE activity in BAL fluid from AATD subjects

Using the sample methods and FRET substrate employed in 2.5.4, the amount of proteolytically active NE was determined in the BAL fluid from the AATD subjects (n=7) and CF controls (n=6). In brief, 10µL of BAL fluid was diluted in 40µL of buffer (0.1M HEPES, 0.5M NaCl, 0.1% Brij, PH 5.0). Subsequently, 2mM of FRET substrate was added and the samples were immediately analysed spectrophotometrically and thereafter at 2min intervals for 20min. A standard curve was generated using serially diluted concentrations of NE as described in 2.5.4 and the amount of proteolytically active NE was determined in each sample.
2.6 Statistical Analysis

2.6.1 Data analysis

All results are expressed as mean ± SEM of biological replicates or independent experiments as stated in the figure legends. All univariate statistical analyses were performed using Microsoft Excel or GraphPad PRISM 6.0 (San Diego, USA). Student t-test was used where distribution was normal and when comparisons were being made between two groups. The Mann–Whitney U test was employed when data was not normally distributed. Statistical significance was determined as a two-tailed p-value <0.05. Results were significant (*) when P<0.05.

2.6.2 Linear regression modelling

Linear regression modelling was employed to examine the significance of any relationship within the clinical data or between the clinical data and the ELISAs performed in this study. Statistical significance was determined as a two-tailed p-value <0.05, the correlation (R-value) and goodness-of-fit (r²) was calculated using GraphPad PRISM 6.0 (San Diego, USA) and reported where available.

2.6.3 Multiple regression modelling

A large number of variables that were analysed in the course of this study, however a degree of multiple co-linearity was apparent in some of the univariate analyses due to overlap between the outcome variables (e.g. FEV1 and FEV1/FVC). To take this account multiple regression analysis was performed using StataMP (v13.0) statistical software package. The regression model was constructed utilising the demographic features that were determined to be statistically significant on univariate analysis and other categorical and dependent variables that may influence the clinical phenotype were included for analysis.
Chapter 3:

3.1 Determination of the spectrum of clinical phenotypes of pulmonary disease in alpha-1 antitrypsin deficiency
3.1.1 Introduction

Alpha-1 antitrypsin deficiency (AATD) is the only known heritable form of pulmonary emphysema; its discovery has led to many scientific advances and a major breakthrough in our understanding of the role of protease imbalance in the pathogenesis of emphysema in humans (40). The burden of pulmonary disease is the major cause for morbidity and mortality in AATD (195). However individuals with AATD are not only susceptible to the premature development of emphysema and chronic bronchitis, but also bronchiectasis, asthma, and the extra-pulmonary manifestations of liver cirrhosis, panniculitis and vasculitis (17).

There is increasing awareness that there are phenotypic differences in AATD characterised by variability in physiologic, radiologic and clinical characteristics of the population (196). Our knowledge of the many phenotypes and endotypes in airway disease has advanced considerably over the past decade and this has impacted our approach to the introduction of treatment in the broader context of pulmonary disease (197,198). For example, novel approaches to the management of asthma have led to the development of monoclonal antibodies that target and bind to IgE, furthermore the role of IL-13 as an inflammatory mediator of disease has also led to targeted therapy for this pathway (199). However, despite these advances the benefit of treatment is only seen in a small subset of people with this condition, usually those with a more severe clinical phenotype and frequent pulmonary exacerbations. The development of biomarkers, such as periostin, may help guide clinicians regarding who will benefit from treatment (200). In COPD, the role of inflammation in disease pathogenesis has been the subject of extensive study, initially through the modulation of protease and oxidant-induced injury and more recently in relation to molecular and genomic effects (201). The importance of inflammation is illustrated by the quantification of pro-inflammatory cytokines and chemokines (IL-1β, IL-6, IL-8, IL-13, IL-17, TNFα, LTB4, C5a) along with anti-inflammatory cytokines and cytokine gene expression (202,203).
In a murine model, over-expression of IL-13 led to the induction of MMPs and consequent emphysema (204), however TNFα knockout mice are relatively protected from developing these features after exposure to cigarette smoke (205). In humans, the detection of inflammatory cytokines in the circulation and from sputum analysis is associated with disease severity and inflammatory cell infiltration to the airway (206,207). IL-8 levels are elevated in the sputum of stable patients with COPD, indicative of a persistent inflammatory burden (208), and have been found to be higher in AATD (209). This has led to the development of specific anti-IL-8 therapies (210,211), though the clinical benefit is yet to be fully established in COPD (212).

The relationship between pulmonary and systemic inflammation is not yet clear, therefore the elucidation of a suitable biomarker or panel of biomarkers, may be an important advance to reflect the underlying pathological processes that give rise to different COPD phenotypes (213). Increasingly it is recognized that, in addition to the classic descriptions of emphysema and chronic bronchitis, an overlap of COPD with asthma exists alongside a frequent exacerbation phenotype where inflammation may dominate over the symptoms of hyperinflation (214,215). It is also unclear whether therapy aimed at optimizing pulmonary function in COPD results in a reduction of systemic inflammation in these patients or whether the direct targeting of systemic inflammation influences the natural history of COPD (216,217).

Predicting the clinical phenotype for the population with AATD remains difficult as the precise roles of environmental and genetic interactions have yet to be fully elucidated (218,219). The detection of asymptomatic individuals through the family screening of probands is an important opportunity to establish the natural history of this condition (220). The establishment of national registries and international collaboration between expert centres has contributed significantly to this evolving knowledge base (127,221). Familial studies illustrate significant heterogeneity in the clinical phenotype, in particular spirometric assessment of lung disease, despite common inherited SERPINA1 mutations and childhood environmental exposures (222).
It is firmly established that cigarette smoke exposure is the major determinant of an accelerated decline in lung function in AATD causing early death in this population (125,223). Occupational exposure to chemicals and pollutants is also independently associated with detrimental effects on lung function (123,124), the excess protease burden driven by inflammation during pulmonary exacerbations is implicated in the progression of disease (139-141). Pulmonary function testing remains a valid and reproducible measure of airflow obstruction in lung disease; evidence of bronchodilator responsiveness at any time has been associated with poorer outcomes in AATD (164). The measurement of carbon monoxide gas transfer provides some insight into the impairment of gas exchange within the diseased lung parenchyma (224), and more recent studies highlight the good predictive value of DLCO measurement in AATD as a marker for disease severity (196).

The guidelines from the GOLD initiative for the classification of COPD now incorporate symptomatic features of disease severity, including pulmonary exacerbations and breathlessness scores, in addition to the degree of spirometric impairment in lung function (130), see Figure 3.1. This new strategy performs well in the stratification of those at increased risk of poorer outcomes such as mortality, lung function decline and pulmonary exacerbation in AATD (131).
Figure 3.1: Updated GOLD classification 2011

When assessing risk, the highest risk according to GOLD grade or exacerbation history. One or more hospitalisations for COPD should be considered high risk (130)

mMRC = modified Medical Research Council dyspnoea score
CAT = COPD assessment test
GOLD = Global Initiative for Chronic Obstructive Lung Disease
The classic pathological finding in AATD lung disease is pan-acinar emphysema that can affect any region of the lung though it is distributed predominantly in the lung bases. Established bi-basal emphysema can be detected in clinical practice by chest x-ray imaging, the unexpected detection of which should prompt the clinician to screen for AATD, particularly at a younger age, see Figure 3.1A (224). The advent of computerised tomography (CT) has heralded a significant advance in the evaluation and interpretation of the emphysematous changes in AATD (190). High resolution CT imaging permits visualisation of the underlying pathophysiological process of pulmonary emphysema and is used to establish disease severity. The extent of emphysema can be established either by radiological severity scoring algorithms (190) or by lung densitometry of the CT images (225). Lung densitometry facilitates monitoring of disease progression in AATD (226) and may be a superior outcome measure to change in FEV1 in clinical trials examining the effect of augmentation therapy in AATD (135). In addition, CT imaging also permits the identification of other disease processes within the lung, particularly bronchiectasis, which may be clinically significant. The reported prevalence of bronchiectasis in AATD varies considerably but may occur independently of emphysema and can be severe (145). Symptoms of bronchiectasis are often difficult to distinguish from chronic bronchitis/COPD and the prevalence and impact of this airway disease in AATD may be underestimated (144).

AAT has been shown to be an endogenous inhibitor of pro-inflammatory cytokine production in blood, in particular IL-6, IL-8, TNFα and IL-1β but not the anti-inflammatory cytokine IL-10 (227). These cytokines are important signalling molecules in the acute phase response to infection and are also key modulators of neutrophil driven inflammation. The elastolytic proteases that are released during neutrophil recruitment and activation are the predominant cause for the pathological findings in AATD (73). The restoration of protease balance by intravenous augmentation therapy has been shown to normalise neutrophil chemotaxis (85) and correct accelerated apoptosis (89). We hypothesized that an alteration in the cytokine profile implicated in neutrophil recruitment may be
observed in the plasma of AATD individuals and serve as a biomarker of systemic inflammation. Furthermore, correlation of this cytokine profile with disease severity may be determined by a thorough evaluation of the clinical phenotype of study subjects with AATD, as assessed by spirometry, gas transfer, radiological disease severity, and exacerbation frequency. To date no study has demonstrated a correlation between systemic pro-inflammatory cytokines and clinical phenotype in AATD and the effect of intravenous augmentation therapy on plasma cytokine levels is not known.
3.2 Hypothesis

Integrative analysis of symptoms, environmental exposure, physiologic measurement, and radiological severity scoring may lead to a more complete determination of the clinical phenotype in AATD. The aim of this study was to determine the spectrum of the clinical phenotype in AATD and correlate this with candidate biomarkers of disease severity. In addition, the findings of this study may reveal new insights into the mechanisms by which AAT modulates inflammation in health and disease states. Identification of those at greatest risk of disease progression may facilitate the development of predictive models to determine who will benefit most from earlier initiation of augmentation therapy.

To fulfil this aim the specific objectives of this chapter were:

Establish the phenotype of each individual within the study population through a comprehensive clinical evaluation and study questionnaire.
Explore factors that may predict clinical phenotype, in particular by evaluating the impact of environmental smoke exposure exposures in AATD.
Investigate the role of inflammation by measurement of systemic cytokines and correlate with AATD disease activity in the established clinical phenotypes.
Results 3.3

3.3.1 Determination of PiZZ clinical phenotype through comprehensive clinical and radiological evaluation.

In order to ascertain the clinical phenotype of each individual with AATD in this study, the largest survey to date of PiZZ individuals from across the country of Ireland was undertaken. A questionnaire was designed to gather information on environmental smoke exposure, clinical symptoms, pulmonary exacerbations and medication usage (Appendix 1). A total of 204 PiZZ individuals were identified from the National AATD Registry maintained at Beaumont Hospital, Dublin. Questionnaires were distributed to 139 individuals who had attended Beaumont Hospital within the past five years. A response rate of 107/139 (77%) was obtained and clinical demographics were available for all respondents. 21/107 (19.6%) of respondents were currently receiving intravenous AAT augmentation therapy. There was CT thoracic imaging available in 72/107 (67.2%) of respondents; this underwent review by two radiologists with a special interest in thoracic radiology. Utilising the modified Bhalla scoring system (Appendix 2), each individual had emphysema and bronchiectasis severity determined. The radiological findings were analysed and correlated to the results of the study questionnaire and clinical parameter measurement.
3.3.2 Evaluation of the updated GOLD classification of COPD in PiZZ AATD.

Recent revisions to the GOLD document now incorporate dyspnoea scores and exacerbation frequency in addition to the spirometric assessment of airflow obstruction, this has been previously found to perform well in AATD lung disease (41,42). To determine if there was a strong relationship between GOLD grouping in the clinical phenotype of PiZZ AATD in our study population, respondents were classified according to the updated guidelines: 17 (16%) respondents had no spirometric evidence of obstructive airway disease and were asymptomatic; 7 (6.5%) were classified as GOLD group A, 4 (3.7%) group B, 33 (31%) group C and 46 (43%) as group D, see Figure 3.2A.

There was a large range (5-100) of patient reported health status within the preceding week, as determined by visual analogue scale, however this correlated strongly with FEV1 ($r=0.69$, $r^2=0.43$, $p<0.0001$), see Figure 3.1B. In addition, breathlessness as determined by the dyspnoea score (mMRC) increased with worsening impairment in DLCO ($r=-0.603$, $r^2=0.352$, $p<0.0001$), see Figure 3.2C. Though the relationship between dyspnoea and FEV1 was not as strong, it remained significant on univariate linear regression analysis ($r=-0.19$, $r^2=0.04$, $p=0.048$). Consistent with the chronic bronchitis phenotype of COPD, 48/107 (45%) of respondents reported a chronic productive cough and had greater than two pulmonary exacerbations in the past two years.

Respondents reported a mean frequency of pulmonary exacerbations of 2.1/annum in the past year and 1.82/annum over a two-year period.

To account for the self-reported nature of the exacerbation data, antibiotic usage over a one-year period was also captured; respondents reported an average of 2.1 courses of antibiotics per annum, which is very similar to the one-year exacerbation rate. Indeed, there was a significant correlation between reported exacerbation frequency and antibiotic prescription usage over a one year period, $r=0.88$, $r^2=0.78$, $p<0.0001$, indicating that the self reported exacerbations were of such severity to usually warrant antibiotic treatment. Inpatient hospitalisations
were not recorded. Increased frequency of pulmonary exacerbation within the preceding two years was associated with lower DLCO values, higher emphysema scores, and a more severe impairment in FEV1 (r= -0.5, r²=0.164, p<0.0001), Figure 3.2D.

The majority of respondents in this study had GOLD group moderate or severe COPD. Increased dyspnoea scores and pulmonary exacerbation frequency as indicators of clinical phenotype severity appears to be valid in AATD as reflected by the strong relationship between these parameters, patient reported symptoms, and objective measurements of disease severity including reduction in FEV1 and DLCO % predicted.
Figure 3.2: Correlation between symptoms and pulmonary function impairment

A. Study participants grouped according to the updated GOLD document: No COPD, n = 17; Group A, n = 7; Group B, n = 4; Group C n = 33; Group D, n = 46.

B. Patient reported health status; there was a significant correlation between the reported impairment in health status as assessed by a visual analogue scale (VAS, range 0-100) and the FEV1% predicted; r = 0.686, r^2 = 0.43, p < 0.0001.

C. Patient dyspnoea score: The modified Medical Research Council (mMRC) dyspnoea score correlated well with the measured impairment in DLCO% predicted; r = -0.603, r^2 = 0.352, p < 0.0001.

D. The frequency of pulmonary exacerbation over a two ear period correlated with the impairment in FEV1, r = -0.5, r^2 = 0.164, p < 0.0001.

a. All relationships were calculated using linear regression analysis in GraphPad Prism v6.0.
3.3.3 The impact of environmental smoke exposure on the clinical phenotype of PiZZ AATD.

Smoke exposure is known to be the single biggest influence on the determination of pulmonary disease severity in AATD, the biological effects of environmental smoke exposure on AAT inactivation have been well characterised (80,117). A detailed smoking history was obtained from the questionnaire and correlated with clinical indices of disease severity to evaluate the impact of smoke exposure in our study population.

The majority of respondents, 71/107 (66.3%), reported a history of smoking for more than one pack year. Most ever smokers, 37/71 (52%), had quit prior to their diagnosis of AATD. Of those who smoked at the time of diagnosis, 30/34 (88%) reported that a diagnosis of AAT helped them quit (median time of two weeks). On univariate analysis ever smokers had a marked reduction in FEV1, DLCO and degree of airflow obstruction, increased breathlessness, poorer health status, increased sputum production, and increased frequency of pulmonary exacerbations (Table 3.1). Multivariate regression analysis identified the following independent variables in ever smokers; lower DLCO values, increased emphysema, increased airflow obstruction and increased sputum production. This would be consistent with the classic phenotypes of emphysema and chronic bronchitis that is observed in COPD. Interestingly there was less bronchiectasis observed in the ever smokers, a finding that persisted after multivariate analysis (-2.5, 95% C.I. -0.8 to -4.9, p=0.047).
Table 3.1: Univariate analysis of ever smokers vs. never smokers

Data are presented as n (%), or mean ± standard deviation.
BMI: body mass index; FEV1: forced expiratory volume in 1s (% predicted); FVC: forced vital capacity; DCLO: diffusing capacity of the lung for CO. mMRC: modified Medical Research Council dyspnoea score; VAS: Visual Analogue Scale health status in the past week.
* Significant on multivariate analysis (p<0.05).

Abbreviations: yr=year, PS=passive smoke, exp=exposure, dec=decline

<table>
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<th>Total</th>
<th>Ever-Smoker</th>
<th>Never smoker</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>61/107 (57)</td>
<td>39/71 (55)</td>
<td>22/36 (61.1)</td>
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<tr>
<td>Age</td>
<td>52.91 ±12.12</td>
<td>52.94 ±10.66</td>
<td>52.87 ±14.76</td>
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<tr>
<td>Age at diagnosis</td>
<td>44.26 ±12.99</td>
<td>44.35 ±11.93</td>
<td>44.1 ±15.02</td>
<td>0.682</td>
</tr>
<tr>
<td>BMI</td>
<td>26.5 ±5.447</td>
<td>27.16 ±5.923</td>
<td>25.18 ±4.11</td>
<td>0.628</td>
</tr>
<tr>
<td>FEV1</td>
<td>63.1 ±31.97</td>
<td>52.56 ±29.08</td>
<td>83.86 ±27.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FVC</td>
<td>99.62 ±23.96</td>
<td>96.13 ±26.15</td>
<td>106.6 ±17.11</td>
<td>0.167</td>
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<td>FEV1/FVC</td>
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<td>0.443 ±0.172</td>
<td>0.6368 ±0.19</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>DLCO %</td>
<td>55.59 ±23.17</td>
<td>47.88 ±19.73</td>
<td>71 ±22.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Exacerbations 1yr</td>
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<td>2.42 ±2.87</td>
<td>1.472 ±1.89</td>
<td>0.039</td>
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<tr>
<td>2yr</td>
<td>3.632 ±4.178</td>
<td>4.443 ±4.77</td>
<td>2.056 ±1.93</td>
<td>0.004</td>
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<tr>
<td>mMRC</td>
<td>1.598 ±1.24</td>
<td>1.915 ±1.156</td>
<td>0.972 ±1.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pack yr smoking</td>
<td>-</td>
<td>20.46 ±12.46</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Cough</td>
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<td>33/71 (46.5)</td>
<td>14/36 (38.9)</td>
<td>0.58</td>
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<td>Sputum</td>
<td>60/107 (56.1)</td>
<td>47/71 (66.2)</td>
<td>13/36 (36.1)</td>
<td>0.004*</td>
</tr>
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<td>VAS health status</td>
<td>58.07 ±21.78</td>
<td>52.23 ±21.37</td>
<td>69.58 ±17.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Childhood PS exp</td>
<td>88/107 (82.2)</td>
<td>62/71 (87.3)</td>
<td>26/36 (72.2)</td>
<td>0.065</td>
</tr>
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<td>Annual FEV1 dec</td>
<td>-41.6 ±90.4</td>
<td>-48.95 ±80</td>
<td>-26.36 ±109.1</td>
<td>0.81</td>
</tr>
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<td>Emphysema</td>
<td>8.267 ±6.17</td>
<td>10.65 ±5.17</td>
<td>3.208 ±4.96</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>3.681 ±3.62</td>
<td>2.979 ±3.14</td>
<td>5.083 ±4.15</td>
<td>0.024</td>
</tr>
</tbody>
</table>
A clear relationship between pack year smoking history and impairments in both FEV1 and DLCO emerged ($r=-0.62$, $r^2=0.38$, $p<0.0001$ and $r=-0.52$, $r^2=0.28$, $p<0.0001$ respectively), see Figure 3.3A and B. In addition, DLCO correlated well with FEV1 ($r=0.73$, $r^2=0.53$ $p<0.0001$), also a DLCO value $<50\%$ predicted had a sensitivity of 96.7\% and specificity of 76.3\% for the requirement of O$_2$ usage (Figure 3.3C). This is not unexpected as increased O$_2$ requirements are associated with worsening impairment in gas transfer. Portable O$_2$ was used by 31/107 (29\%) and 17/107 (16\%) reported using long-term O$_2$ therapy (LTOT). The requirement for O$_2$ therapy is indicative of significant functional impairment and can have a major impact on quality of life indices. The prescription of O$_2$ therapy can ameliorate this and lead to measurable benefit in a COPD population (228). The finding of a DLCO $<50\%$ of predicted value had a 96.7\% sensitivity to predict the requirement for portable O$_2$ therapy indicative of a more severe clinical phenotype.
Figure 3.3: Correlation between smoking pack year history and pulmonary function impairment

A. There was a progressive decline in FEV1 % predicted with increasing lifetime pack year smoking history, \( r = -0.62, r^2 = 0.38, p < 0.0001 \).

B. Similarly, but not to the same extent as FEV1, a progressive decline in DLCO % predicted values was observed with increasing lifetime pack year smoking history, \( r = -0.52, r^2 = 0.28, p < 0.0001 \).

C. There was a strong correlation between DLCO with FEV1 % predicted; \( r = 0.73, r^2 = 0.53, p < 0.0001 \). A DCLO value <50% had a 96.7% sensitivity to predict the requirement for portable \( O_2 \) therapy.
3.3.4 The impact of occupational exposure on lung function in AATD

55/107 (51.4%) of respondents were currently in full time employment. 28/107 (26.2%) of respondents reported that they had to change job or retire as a result of AATD, this group had significant impairment in lung function compared to those who continued in employment (mean FEV1 39% vs. 71%, p=0.02). 47/107 (43.9%) of respondents reported occupational inhalational exposure during the course of their working life, the predominant exposure was to dust.

Of note, 48/88 (54.5%) of the respondents exposed to passive smoke were exposed in the workplace. Following the introduction of the workplace smoking ban 10 years earlier in Ireland, 39/48 (81%) of those with workplace exposure reported an overall decrease in passive smoke since that time compared to 22/40 (55%) who were not exposed in the workplace, see Figure 3.4A. This is of importance as never smokers with workplace passive smoke exposure (n=13) had impaired age adjusted lung function compared to never smokers not exposed (n=12) (FEV1 95.7% vs. 68.3%, p= 0.02), see Figure 3.4B. This indicates an association with passive smoke exposure in the workplace with poorer lung function in never smokers with AATD, though no causation can be inferred from this observational data.
Figure 3.4: Effect of workplace passive smoke exposure in AATD

A. The majority of respondents who were exposed to passive smoke in the workplace reported a decrease in passive smoke exposure as a result of the introduction of the workplace smoking ban, 48/88 (54.5%) vs. 22/40 (55%), Fisher exact test for difference p=0.0007.

B. In never smokers, a significant difference in age-adjusted FEV1 was noted between those with passive smoke exposure in the workplace (n=13) and those who did not (n=12), FEV1 percent predicted 95.7% vs. 68.3%, p= 0.02. Passive smoke exposure in the workplace may have important implications for the progression of lung disease in AATD in those who never smoked.
3.3.5 Never smokers have better age adjusted lung function but a similar rate of decline in FEV1 as ever smokers with AATD.

Evaluating the clinical phenotype in never smokers in this study population is an important aspect for prognosticating outcomes and delineating the effect of disease in these individuals. There were 36/107 (33.6%) never smokers in this study and they had a higher mean FEV1 value (83.8% vs. 52.6%) and DLCO (71% vs. 47.9%) compared to smokers (Table 3.1). Interestingly, there was no significant difference in the annualised rate of FEV1 decline between ever smokers and never smokers (Figure 3.5). This may be accounted for by a number of possible factors which include; all ever smokers were now ex-smokers, a lower initial FEV1 in the smoker group, the modifying effect of medication use, and the survivor effect of those with improved lung function.

![Graph showing annualised rate of FEV1 decline in smokers and never smokers](image)

**Figure 3.5: Annualised rate of FEV1 decline in smokers and never smokers**

Linear regression analysis revealed a similar rate of FEV1 decline between ever smokers (solid line) and never smokers (dashed line) despite initial lower FEV1 values in ever smokers (ever smokers -31mls/annum vs. never smokers -38mls/annum, p=0.97). The mean percentage decline in FEV1 %predicted per annum was also similar between the two groups: Ever smokers -1.4%/annum vs. -1.84%/annum in never smokers, p=0.52.
Subgroup analysis of never smokers revealed that symptomatic subjects (index cases) have lower age adjusted FEV1 (75% vs. 95%, p=0.042) and DLCO (63 are% vs. 83%, p=0.006) values compared to asymptomatic never smokers (Figure 3.5). Multivariate regression analysis confirmed the association for lower DLCO (-9.96%, 95% C.I -4.6 to -19.5%, p=0.041), indicative of a more severe phenotype in symptomatic individuals with AATD, see Figure 3.6. Our data implies that the natural history of AATD in never smokers is altered at some point in symptomatic individuals, by an unknown precipitant, to worsen their pulmonary function and bring them to medical attention.

Figure 3.6: Annualised rate of FEV1 decline in smokers and never smokers

A. In symptomatic (Index, n=23) never smokers the age adjusted FEV1 values are lower than in asymptomatic (Non-index, n=13) never smokers (mean FEV1 77% vs. 95%, p=0.042).
B. Age adjusted DLCO values in symptomatic (index, n=23) never smokers are lower than asymptomatic (non-index, n=13) never smokers (mean FEV1 64% vs. 83%, p=0.006). Multiple regression analysis confirmed significantly lower DCLO% predicted values in symptomatic compared to asymptomatic never smokers (-9.96%, 95% C.I -4.6 to -19.5%, p=0.04).
3.3.6 Passive smoke exposure

Passive smoke exposure in childhood is associated with a number of adverse health effects in later life including an increased cancer risk and increased risk from the development of COPD (229-231). A high proportion of respondents, 88/107 (82%), reported passive smoke exposure in childhood with the majority, 52/88 (59%), reporting parental passive smoke exposure. Parental smoke exposure did not emerge as an independent risk factor for poorer lung function in adulthood in our analysis. This may be due to the high prevalence of parental smoking overall and insufficient power to detect a statistically significant difference in lung function in our study population. Those who were exposed to passive smoke in childhood were more likely to smoke in adulthood, odds ratio 2.6 (95% confidence interval 0.9645 to 7.28, p= 0.06), and had a significantly higher mean pack year smoking history (17.25 vs. 9.84, p=0.0025).

The impact on the workplace-smoking ban in Ireland was assessed following its implementation 10 years ago; 63/107 (59%) reported that passive smoke exposure had reduced as a result of the ban, 44/107 (41%) reported no change, and no subjects reported increased smoke exposure.

The finding that children with AATD exposed to passive smoke are more likely to take up smoking and smoke more places them at increased risk for the development of lung disease in adult life. Though no causal relationship between passive smoke exposure and adult smoking behaviour can be inferred from this study, our findings are consistent with other studies in this area (232,233). This interesting observation is of particular relevance to this most vulnerable patient group and is being addressed by public health initiatives to limit childhood passive smoke exposure.
3.3.7 Radiological findings

Quantification of pulmonary disease severity in AATD is achieved by thoracic CT imaging, which permits direct visualisation of the primary pathophysiological process in the lung.

Subjects with available thoracic CT images were identified from the individuals recruited to this study, 72/107 (67%). Two radiologists with a special interest in thoracic imaging evaluated each thoracic CT study independently and then reached an agreed score using the modified Bhalla criteria (191).

The majority of respondents, 52/80 (65%), had radiological evidence of emphysema that was most severe in the lower lobes. The pattern of emphysema seen was similar in smokers compared to never smokers though it was more severe, mean emphysema score 10.7 vs. 3.2 respectively (p<0.0001) see Figure 3.7A. Utilising linear regression analysis to determine the stochastic difference in emphysema severity by age, by examining the slope elevation of each group using linear regression analysis, ever smokers had comparable emphysema severity twenty years earlier than those who had never smoked (p<0.0001), see Figure 3.7B. Emphysema correlated most strongly with DLCO% (r=0.83, p<0.0001), Figure 3.7C, further indicating the value of this measurement in addition to its predictive value for oxygen therapy.

Radiological evidence of bronchiectasis was evident in 58/72 (80.6%) of cases. The severity of bronchiectasis was generally mild and diffuse with greater than one lobe affected in 51/58 (88%). This diffuse process also affected most bronchopulmonary segments in any given lobe and no lobar preponderance seen, see Figure 3.8A. In addition, mucous plugging was uncommon with evidence in only 6/58 (10%), which is a distinguishing feature from other causes of bronchiectasis, such as cystic fibrosis and allergic bronchopulmonary aspergillosis. No saccular bronchiectasis was reported in this study population.
Figure 3.7: Analysis of radiological emphysema severity

A. The degree of pulmonary emphysema was increased in the lung bases, this was observed in both ever smokers and never smokers, however as expected emphysema was more severe as measured in each lobe in ever smokers, two-tailed T-test for each lobe, p<0.0001 (***)

B. The severity of emphysema is apparent at an earlier age in smokers, difference in slope elevation on linear regression analysis is 20 years, p<0.0001.

C. There was a significant correlation between emphysema severity and DLCO% predicted on linear regression analysis, r=0.83, p<0.0001.

Abbreviations: RUL = right upper lobe; RML = right middle lobe; RLL = right lower lobe; LUL = left upper lobe; LLL = left lower lobe.
Previous reports have linked the presence of emphysema with the occurrence of bronchiectasis, with some reports that bronchiectasis is a secondary phenomenon (146). The presence of bronchiectasis increased with age ($r=0.41$, $p=0.004$), see Figure 3.8B, and while there was a similar age-adjusted prevalence of bronchiectasis in smokers and never smokers, bronchiectasis severity was more significant in never smokers ($5.08$ vs. $2.98$, $p=0.02$), (Table 3.1). There was no correlation between the presence of emphysema and bronchiectasis in our study population, ($r=-0.1664$, $p=0.1625$), see Figure 3.8C.

The emergence of different radiological phenotypes based on smoking status was evident; the individuals with the most severe bronchiectasis had little or no evidence of emphysema and those with the most severe emphysema had mild bronchiectasis. Multivariate regression analysis identified increasing age as the most significant risk factor for the development of bronchiectasis independent of smoking history ($p<0.001$). Those with predominant radiological evidence of bronchiectasis reported less cough, sputum production and pulmonary exacerbations compared to those with evidence of emphysema indicating a milder clinical phenotype in this group. This is contrary to previous reports of severe bronchiectasis in AATD (143,144,234), however our findings demonstrate a spectrum of bronchiectasis disease severity that is consistent with the findings in a similarly sized study population (145).
Figure 3.8: Analysis of bronchiectasis severity

A. The severity of bronchiectasis was mild in the study population overall, there was no significant lobar predilection of bronchiectasis observed.

B. Increased CT detected bronchiectasis was evident with advancing age. There was no significant difference in the age-adjusted severity of smokers vs. never smokers using linear regression analysis, p=0.022.

C. On examination for correlation between the severity of CT detected bronchiectasis and emphysema, no significant relationship was observed, r=-0.1664 (p=0.163).
3.3.8 Index versus non-index cases

The majority of individuals with AATD that are attending specialist centres were detected on the basis of clinical symptoms. Previous studies have evaluated the method of ascertainment, index vs. non-index, on the resultant clinical phenotype in AATD (129,222).

Of the respondents, there were 72/107 (67.3%) index cases; the remaining 35 (32.7%) non-index cases were detected by family screening. Both groups were well matched in relation to symptoms of cough, sputum production, pulmonary exacerbations and health status measurement as both groups had a similar percentage of smokers and an equal pack year smoking history, see Table 3.2. However on univariate analysis index cases appeared to have significantly lower FEV1 values (58% vs. 74%, p=0.0273), DLCO values (51% vs. 64%, p=0.0126) and a greater degree of airflow obstruction (47% vs. 58%, p=0.01) despite similar rates of FEV1 decline (-36.4mls/annum vs. -51.2mls/annum, p=0.49), see Table 3.2.
Table 3.2: Univariate analysis of index vs. non-index cases

Data are presented as n (%), mean vs. non-index cases e stated. BMI: body mass index; FEV1: forced expiratory volume in 1s(% predicted); FVC: forced vital capacity; DLCO: diffusing capacity of the lung for CO; mMRC: modified Medical Research Council dyspnoea score; VAS: Visual Analogue Scale health statue in the past week.
*Significant on multivariate analysis: (p<0.01).

Abbreviations: yr=year, PS=passive smoke, exp=exposure, dec=decline

<table>
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<tr>
<th></th>
<th>Total</th>
<th>Index</th>
<th>Non-Index</th>
<th>p-Value</th>
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<tr>
<td>Male</td>
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<td>43/72 (59.7)</td>
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<td>FEV1</td>
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<tr>
<td>FEV1/FVC</td>
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<td>0.468 ±0.19</td>
<td>0.58 ±0.19</td>
<td>0.01</td>
</tr>
<tr>
<td>DLCO %</td>
<td>55.59 ±23.17</td>
<td>51.09 ±20.65</td>
<td>64.2 ±25.5</td>
<td>0.013*</td>
</tr>
<tr>
<td>Exacerbations: 1 yr</td>
<td>2.103 ±2.60</td>
<td>2.292 ±2.67</td>
<td>1.714 ±2.42</td>
<td>0.128</td>
</tr>
<tr>
<td></td>
<td>3.632 ±4.18</td>
<td>3.761 ±3.59</td>
<td>3.371 ±5.23</td>
<td>0.059</td>
</tr>
<tr>
<td>mMRC</td>
<td>1.598 ±1.235</td>
<td>1.611 ±1.193</td>
<td>1.57 ±1.34</td>
<td>0.799</td>
</tr>
<tr>
<td>Pack year smoking</td>
<td>13.58 ±14.03</td>
<td>13.36 ±12.76</td>
<td>14.04 ±16.54</td>
<td>0.886</td>
</tr>
<tr>
<td>Never smoker</td>
<td>36/107 (33.6)</td>
<td>23/72 (32)</td>
<td>13/35 (37.1)</td>
<td>0.665</td>
</tr>
<tr>
<td>Cough</td>
<td>47/107 (43.9)</td>
<td>32/72 (44.4)</td>
<td>15/35 (42.9)</td>
<td>1</td>
</tr>
<tr>
<td>Sputum</td>
<td>60/107 (56.1)</td>
<td>41/72 (57)</td>
<td>19/35 (54.2)</td>
<td>0.838</td>
</tr>
<tr>
<td>VAS health status</td>
<td>58.07 ±21.78</td>
<td>56.46 ±20.43</td>
<td>61.37 ±24.37</td>
<td>0.183</td>
</tr>
<tr>
<td>Childhood PS exp</td>
<td>88/107 (82.2)</td>
<td>61/72 (84.7)</td>
<td>27/35 (77.1)</td>
<td>0.42</td>
</tr>
<tr>
<td>Annual FEV1 dec</td>
<td>-41.61 ±90.4</td>
<td>-36.41 ±85.35</td>
<td>-51.28 ±100.0</td>
<td>0.486</td>
</tr>
<tr>
<td>Emphysema</td>
<td>8.267 ±6.174</td>
<td>9.574 ±5.51</td>
<td>5.52 ±6.18</td>
<td>0.011</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>3.681 ±3.622</td>
<td>4.298 ±4.09</td>
<td>2.52 ±2.16</td>
<td>0.13</td>
</tr>
</tbody>
</table>
In the univariate subgroup analysis of the thoracic CT data it was also found that index cases had higher mean emphysema scores (9.574 vs. 5.52, p=0.011). However, once age adjustment of the data was undertaken, there was no significant difference observed in FEV1, airflow obstruction or emphysema between the groups (P=0.51), see Figure 3.9. Since both index and non-index cases had similar lifetime smoke exposure, the observed reduction in lung function in the index cases is explained by an older age in this group. Our data supports previous reports of poor lung function status in matched individuals independent of pulmonary symptoms. As previously discussed in Section 3.3.4, non-smokers with pulmonary symptoms do appear to have some mild impairment in DLCO compared to asymptomatic individuals.

Figure 3.9: Analysis of index versus non-index cases

There is no difference detected in age-adjusted FEV1 values between index and non-index cases (p=0.51).
3.3.9 The effect of augmentation therapy on clinical symptoms

A significant proportion of subjects were receiving intravenous augmentation therapy for a minimum period of two years at the time of this study, 21/107 (19.6%). To evaluate the impact of augmentation therapy on the health status and pulmonary symptoms, the augmentation group (n=21) was matched to other respondents in this study based on indication for treatment in accordance with licensed approval (n=44). The groups are well match for age, sex, pack year smoking history, spirometric impairment in lung function and radiological disease severity, see Table 3.3.

There was a statistically significant difference between the augmentation group and the controls in the DLCO % predicted (48.6 vs. 40.5% respectively, p = 0.03) despite the augmentation group being of an older age. In addition there was a significant improvement in health status as measured by VAS in the augmentation group (55.8 vs. 44.95, p=0.024).

Those receiving augmentation therapy had a similar exacerbation frequency to the matched PiZZ controls (2.71/annum vs. 2.75/annum, p=0.96). The severity of symptomatic breathlessness was also similar in both groups. The interpretation of this observational result is limited due to the small size of the study population and the lack of longitudinal data. In addition, the patients on augmentation therapy were all previously recruited to clinical trials to receive therapy indicating that these individuals may have underlying characteristics that are inherently different to the matched controls, in particular healthcare utilisation. Pre-augmentation therapy exacerbation data was not captured, so the impact of augmentation therapy on exacerbation frequency in treated individuals could not be determined. No assessment of the impact of augmentation therapy on mortality was possible.
### Table 3.3: The impact of augmentation therapy on modulating AATD

The grouped analysis comparing those receiving augmentation and a control group matched for FEV1 demonstrates that despite an older age, patients on augmentation therapy have a higher DLCO % predicted compared to controls. In addition, the VAS score is increased 20% (mean difference 10.9) in the augmentation group indicative of an improved perceived health status. The groups are otherwise well matched for smoking history, exacerbation frequency, and radiological assessment scores.

Data are presented as n(%) or mean ± standard deviation. Statistical analysis between groups was performed using a two-tailed t-test.

BMI: body mass index; FEV1: forced expiratory volume in 1s(% predicted); FVC: forced vital capacity; DLCO: diffusing capacity of the lung for CO; mMRC: modified Medical Research Council dyspnoea score; VAS: Visual Analogue Scale health status in the past week; Exac = exacerbations

<table>
<thead>
<tr>
<th></th>
<th>Augmentation N=21</th>
<th>PIZZ Control N=44</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>12/21 (57.1)</td>
<td>27/44(61.3)</td>
<td>0.6</td>
</tr>
<tr>
<td>Age</td>
<td>57.88 ±8.0</td>
<td>54.22 ±8.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Age of Diagnosis</td>
<td>46.04 ±7.6</td>
<td>46.10 ±7.2</td>
<td>0.98</td>
</tr>
<tr>
<td>BMI</td>
<td>26.66 ±4.3</td>
<td>26.28 ±4.5</td>
<td>0.38</td>
</tr>
<tr>
<td>Pack Years</td>
<td>18.85 ±11.3</td>
<td>20.61 ±15.0</td>
<td>0.64</td>
</tr>
<tr>
<td>FEV1 % values</td>
<td>43.28 ±12.9</td>
<td>39.72 ±14.6</td>
<td>0.34</td>
</tr>
<tr>
<td>FVC%</td>
<td>90.96 ±20.2</td>
<td>91.18 ±26.3</td>
<td>0.75</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>0.39 ±0.1</td>
<td>0.37 ±0.1</td>
<td>0.45</td>
</tr>
<tr>
<td>FEV1 decline/year</td>
<td>-58.47 ±80.8</td>
<td>-34.60 ±77.8</td>
<td>0.26</td>
</tr>
<tr>
<td>DLCO %</td>
<td>48.66 ±16.7</td>
<td>40.48 ±12.8</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>mMRC value</td>
<td>1.90 ±1.1</td>
<td>2.27 ±1.0</td>
<td>0.18</td>
</tr>
<tr>
<td>VAS</td>
<td>55.81 ±19.2</td>
<td>44.95 ±16.9</td>
<td><strong>0.024</strong></td>
</tr>
<tr>
<td>Exac 1 year</td>
<td>2.71 ±2.0</td>
<td>2.75 ±3.2</td>
<td>0.96</td>
</tr>
<tr>
<td>Exac 2 year</td>
<td>5.19 ±3.8</td>
<td>4.79 ±5.2</td>
<td>0.31</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>2.4 ±2.0</td>
<td>3.37 ±2.5</td>
<td>0.13</td>
</tr>
<tr>
<td>Emphysema</td>
<td>12.13 ±4.5</td>
<td>12.07 ±2.7</td>
<td>0.95</td>
</tr>
</tbody>
</table>
3.3.10 Cytokine analysis

To assess markers of systemic inflammation in the circulation four candidate cytokines were selected based on their role in neutrophil recruitment and activation; IL-8, IL-6, TNF-R1, and IL-10. These cytokines were measured in the plasma of 6 healthy PiMM controls; 6 healthy PiZZ subjects without evidence of obstructive lung disease; 16 PiZZ subjects with obstructive lung disease with a range of impairment in FEV1; and 4 PiZZ subjects with obstructive lung disease both on day 0 and on day 2 following intravenous augmentation therapy.

There was no significant elevation of IL-10 or IL-6 detected in any of the study groups, see Figure 3.10. TNF-R1 levels were not increased in healthy ZZ individuals compared to healthy MM controls, however there was a 39% increase in ZZ subjects with obstructive lung disease (mean value 1257 vs. 1751 pg/mL, p=0.03). No change in TNF-R1 levels was seen following the administration of intravenous AAT augmentation therapy.
Figure 3.10: Analysis of cytokine profiles in study participants

A. There was no significant difference in IL-6 levels between the PiMM healthy controls, (n=6) healthy PiZZ subjects (n=6) and PiZZ subjects with obstructive lung disease (n=25).

B. There was minimal elevation of IL-10 in the study participants and no significant difference between the groups was detected. The measured values were at the lower limit of detection of the assay.

C. TNF-R1 was elevated in PiZZ subjects with obstructive lung disease compared to healthy MM controls, mean value 1257 vs. 1751 pg/mL, p=0.03. Statistical analysis was performed using a two-tailed t-test.
There was no detectable IL-8 found in the plasma of PiMM controls, however a small rise was found in the healthy PiZZ population (mean value 0 vs. 94.47 pg/mL, p<0.0022). A significant increase was seen in PiZZ subjects with obstructive lung disease compared to healthy controls (mean value 0 vs. 479.1 pg/mL, p<0.0001). Importantly, when the effect of AAT augmentation therapy was assessed, a significant reduction in plasma IL-8 levels was demonstrated on day two following treatment (mean value 1384 vs. 449 pg/mL, p=0.0106), though the levels did not normalise to healthy PiMM or PiZZ control levels, see Figure 3.11.

**Figure 3.10: IL-8 cytokine analysis**

There was no detectable IL-8 measured in healthy MM controls. Healthy ZZ subjects had elevated IL-8 levels compared to healthy MM controls (mean value 0 vs. 94.47 pg/mL, p<0.0001).

- An approximate five fold increase in plasma IL-8 levels was seen in ZZ subjects with obstructive lung disease compared to healthy ZZ subjects, however this was not statistically significant (94.47 vs 480.5 pg/mL, p=0.1291).
- A significant reduction in plasma IL-8 levels was demonstrated on day two following intravenous AAT augmentation treatment (mean value 1384 vs. 449 pg/mL, p=0.0106), though the levels did not normalise to healthy PiMM or PiZZ levels.

Key: * = p<0.05  ** = p<0.01  *** = p<0.001
3.4 Discussion

In this chapter detailed clinical phenotyping of individuals with AATD has been performed. The results are congruous with previous studies in the population of AATD individuals attending specialized centres with symptomatic disease (17,177). Determination of clinical phenotype in AATD is essential for a greater understanding of the underlying pathophysiology of the disease, the correct stratification for research studies and therapy, and to prognosticate outcomes. The majority of respondents in this study had GOLD group D COPD reflecting the prevalence of a more severe phenotype within this AATD population attending a tertiary referral centre. The results may also be influenced by ascertainment and reporting bias where those with less severe disease were less likely to respond.

In this study, pulmonary emphysema was the predominant radiological finding primarily in ever smokers. Two thirds of the study population had radiological evidence of emphysema predominantly in the lower zones and this correlated well with pulmonary function measurements. DLCO was proven to be a useful measurement of disease status, as impairments in DLCO correlated most significantly with higher emphysema scores. A DLCO value below 50% predicted was found to be highly predictive of portable oxygen requirement, and lower DLCO values correlated strongly with worsening dyspnoea and health status measurement outcomes. This implies that patients with DLCO cut-off values below 50% predicted may benefit from assessment regarding portable oxygen requirement irrespective of pulmonary symptoms at the time of assessment. Recent recommendations regarding clinical trials in AATD suggest that serial CT densitometry be used as the primary endpoint to demonstrate stabilisation and prevention of progression (135), however in clinical practice this is rarely feasible and our data would suggest that DLCO may be employed as a surrogate determinant of disease status.

There remains a significant proportion of smokers with AATD that have yet to be identified, and this may relate to widespread under recognition of the disorder in the medical profession (114). In this study, no difference was observed between
index and non-index cases in relation to pulmonary symptoms, measurements of lung function, and severity of emphysema. This is likely explained by the equivalent smoke exposure in both groups. Smoke exposure is the single biggest determinant for progression of emphysema in AATD. Our analysis of the influence of smoke exposure in AATD demonstrates the presence of emphysema, chronic bronchitis and resultant airflow obstruction with the resultant classic phenotypes of COPD presenting at a young age.

All respondents had stopped smoking at the time of this study. Individuals with AATD who quit smoking had similar rates of FEV1 decline compared to never smokers, this finding supports previous studies in similar populations of AATD individuals and may be due to measurements late in the disease process with lower initial FEV1 values. This is a matter of great encouragement to people newly diagnosed with AATD and who may wish to quit smoking.

It has been ten years since Ireland became the first country in the world to introduce a ban on smoking in the workplace. Passive smoke exposure is a known risk factor for the development of emphysema and COPD, individuals with AATD are particularly at risk and have most to benefit from public health initiatives that reduce cumulative lifetime smoke exposure. The fact that no individual reported an increase, and the majority reported a decrease, in passive smoke exposure speaks to the success of this programme pioneered in Ireland and emulated worldwide. Most respondents with passive smoke exposure in the workplace reported a decrease since the ban. This is of particular interest, as never smokers in this group were observed to have lower lung function compared to never smokers who did not have occupational exposure. Apart from increasing the cumulative lifetime exposure to tobacco smoke, the results of this study indicate that passive smoke exposure in childhood, in particular parental smoking, influence smoking habits in adulthood by increasing the likelihood of ever smoking and the total pack year cigarette consumption. Further efforts to reduce passive smoke exposure in automobiles are welcome.
initiatives that are undergoing legislative implementation in some countries at present (237).

The clinical phenotype in asymptomatic never smokers was analysed in order to better understand the natural history of AATD. The observation that lung function is normal, and preserved beyond middle age, is an important finding that it is consistent with published data suggesting they have a life expectancy approaching that of the general population (127). Never smokers with symptomatic lung disease appear to have lower FEV1 and DLCO values indicating a more severe clinical phenotype than asymptomatic never smokers, though the confounding effects of genetic modifiers and environmental factors not measured on our study should be taken into consideration. Other research into this area has suggested that recurrent infections may play a significant role in this respect, however longitudinal data is required to replicate these findings in our study cohort (238).

Bronchiectasis is a recognised pulmonary complication of AATD, however the true prevalence and clinical significance of bronchiectasis in the AATD population remains poorly understood. Observational studies in populations of non-CF bronchiectasis have not demonstrated an increased prevalence of the condition implying that AATD as a cause of bronchiectasis is uncommon and that it occurs as a result of co-existent emphysema (146). Our data would contradict these findings, demonstrating that radiologically detected bronchiectasis is common in AATD and has no dependent relationship with emphysema or indeed to prior smoking history. This is supported by another large study on bronchiectasis in AATD, and by registry data from Spain and Italy, showing a high prevalence of bronchiectasis in the AATD population (128,145). The majority of subjects in our study had evidence of mild radiological bronchiectasis with minimal associated clinical symptoms, such as increased frequency of cough or pulmonary exacerbation compared to those with emphysema. There was a subgroup with severe bronchiectasis as the predominant lung pathology and they did report
significantly poorer health status outcomes. Determining why this occurs is an important area of research.

Individuals with AATD who were receiving augmentation therapy had very similar objective and demographic characteristics compared to matched controls. Quality of life measurements were increased in the augmentation therapy group, however interpretation of this data is limited due to a number of factors including healthcare utilisation and prior clinical trial recruitment introducing a number of biases.

AAT has been shown to increase cAMP synthesis and IL-10, an anti-inflammatory cytokine, production in human monocytes in vitro (239,240). No significant difference in IL-10 levels was detected in the plasma of AATD individuals compared to healthy controls, Figure 3.6. Similarly there was no significant difference in IL-6 levels observed. IL-6 is a pro-inflammatory cytokine that can upregulate the gene expression of AAT that contributes to the acute phase protein response.

AAT has been shown to inhibit ADAMS-17 enzymatic activity, resulting in the modulation of TNFα driven inflammation (85). Tumour necrosis factors are pleiotropic cytokines that are considered primary modifiers of the inflammatory and immune reactions in response to injury or infection. TNF-R1 is a soluble receptor for TNFα that is shed from the cell surface, there is a correlation between increased TNFα levels and soluble receptor levels, suggesting that stimuli that cause TNFα levels to rise also induce shedding of TNF receptors. TNF R1 is more stable than TNFα and is it therefore preferable to measure. The elevated levels found in in the more severe phenotype of AATD in our study indicate that there is ongoing TNFα-mediated inflammation.

IL-8 is a pro-inflammatory cytokine that is a major neutrophil chemoattractant, Recent work in our laboratory has shown that AAT plays a pivotal anti-inflammatory role by binding to IL-8 through its glycan residues and thereby
attenuate neutrophil chemotaxis (116). In AATD where the circulating levels of AAT are markedly reduced, homeostatic regulation of IL-8 activity may be perturbed and thereby perpetuate on-going inflammation. In addition, AAT has been shown to inhibit NF-KB activation, which is involved in the up-regulation if IL-8 gene expression (241,242). IL-8 levels were markedly elevated in PiZZ subjects with obstructive lung disease, the finding that IL-8 levels are reduced on day two following intravenous augmentation therapy is proof of the concept of the role of AAT as an immunomodulatory agent.

Together these results indicate that those with a more severe AATD clinical phenotype have higher detectable levels of pro-inflammatory mediators, including circulating IL-8 levels. As AAT has previously been shown to bind and modulate the IL-8 signalling axes (85), we hypothesized that AAT binds an array of mediators of the immune system within the circulation, thereby modulating their biological functions. With this in mind ensuing experiments were designed to fully understand the therapeutic potential of AAT by identifying inflammatory mediators that interact with this molecule. Indeed, no study has attempted to fully evaluate the binding profile of AAT as it circulates in plasma and the results of such studies are described in ensuing chapters.
3.5 Conclusion

Evaluating the natural history and progression of pulmonary disease in AATD contributes to our understanding of the interaction between genetic susceptibility and environmental exposure in this population. The detrimental effect of smoke exposure on the clinical phenotype of AATD is clear, and becomes apparent at a young age. Individuals with AATD who have ever smoked have a similar COPD clinical phenotype irrespective of their method of diagnosis (index vs. non-index), this is characterised by the development of emphysema and chronic bronchitis. Symptomatic never smokers have some impairment in lung function that has brought them to medical attention, though they have a milder clinical phenotype compared to those who ever smoked. Importantly, our data would support the premise that asymptomatic never smokers have normal lung function and are healthy. Public health initiatives to reduce smoking uptake, promote smoking cessation and reduce passive smoke exposure are likely to be of most benefit to individuals with AATD that have yet to be diagnosed.

The identification of elevated inflammatory cytokines in the plasma of individuals with a severe clinical phenotype of AATD is important. The effect of AAT on the modulation of IL-8 and TNFα inflammatory signalling through glycan-dependent binding is novel and points to the immunomodulatory role of AAT as a carrier protein or buffer for the homeostasis of neutrophil driven inflammation (85,243).

The role of inflammation in the development and progression of emphysema in usual COPD is only beginning to be appreciated, with consequent important pathophysiological and therapeutic implications (207). There is also much left to be understood in relation to the impact of inflammation on disease specific outcomes and mortality in COPD (244). Though COPD controls were not utilised in the data presented, it is possible that the systemic inflammation observed in AATD is similarly active in COPD with the common feature of emphysema as the underlying disease process. AAT may have a role in modulating the response to
this inflammation in usual emphysema/COPD and further research in this area could lead to important new discoveries.

The full binding profile of AAT in plasma is not known, whether this binding profile influences the development of disease has not been established to date. Knowledge of the full range of binding partners to AAT may have important implications for the utilisation of augmentation therapy in AATD and other conditions with an altered inflammatory response (93). In the Chapter 4 we set out to elucidate the binding profile of AAT in healthy individuals and in AATD. This may lead to the discovery of new mechanisms by which AAT mediates its anti-inflammatory effects and lead to a greater understanding of the pathophysiological processes underlying AATD.
Chapter 4

4.1 The binding profile of alpha-1 antitrypsin in plasma
4.1.1 Introduction

Serine protease inhibitors diverged from a common precursor molecule approximately 500 million years ago, they share similar gene structures and have common protein homology (245). AAT is the canonical SERPIN, and retains its primary function as an extracellular protease inhibitor, with the resultant pulmonary disease phenotype arising in deficiency states. It has a key role in innate immune defence and during the acute phase protein response its plasma concentration increases two to four fold from a mean level of 1.3 g/L (0.9-1.75 g/L) in health. However, AAT can reach higher concentrations in tissues, particularly during inflammation, where its ability to bind proteins, peptides, and cytokines as well as interact with cell surfaces may have important implications for the regulation of inflammation (246). The primary function of other extracellular, non-inhibitory, serine proteases as carrier proteins in plasma is well established, particularly in the case of SERPINA6 (cortisol-binding globulin) and SERPINA7 (thyroxine-binding globulin) (247,248). The potential role of AAT as a carrier protein in plasma has been eluded to in studies of its three dimensional structure, principally in relation to its corticosteroid binding domain (27), and also through investigation of candidate binding partners, summarized in Table 4.1. However the relative importance of AAT as a carrier protein in the circulation or its binding function at sites of inflammation is incompletely understood at present. To understand how AAT interacts with other components of the systemic circulation, an overview of AAT protein binding mechanisms is described.
Table 4.1: Known binding partners to AAT

All previously known binding partners to AAT are listed from the published literature has been assembled for the purpose of this study. The binding partners are categorised by biological compartment. The involvement of the respective protein and disease process is provided where applicable and whether this association has been determined from in vivo or in vitro studies.

Glossary: IgA = Immunoglobulin A. BPH = benign prostatic hypertrophy. OA = osteoarthritis.

**Table 4.1: Known binding partners to AAT**

<table>
<thead>
<tr>
<th>Protein</th>
<th>kDa</th>
<th>Location</th>
<th>Disease process</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein B-100</td>
<td>550</td>
<td><em>in vivo</em></td>
<td>Atheroma</td>
<td>Mashiba et al. 2001</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>340</td>
<td><em>in vivo</em></td>
<td>Healthy</td>
<td>Laurell et al. 1975</td>
</tr>
<tr>
<td>IgK light chains</td>
<td>12</td>
<td><em>in vivo</em></td>
<td>Myeloma</td>
<td>Laurell et al. 1974</td>
</tr>
<tr>
<td>HSP 70</td>
<td>70</td>
<td><em>in vivo</em></td>
<td>Diabetes mellitus</td>
<td>Finotti et al. 2004</td>
</tr>
<tr>
<td>Grp94</td>
<td>98</td>
<td><em>in vivo</em></td>
<td>Diabetes mellitus</td>
<td>Pagetta et al. 2003</td>
</tr>
<tr>
<td>Psa/kallikrein 3</td>
<td>29</td>
<td><em>in vivo</em></td>
<td>BPH, prostate cancer</td>
<td>Zhang et al. 1999</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-</td>
<td>*in vitro</td>
<td>Not specified</td>
<td>Janciauskiene et al. 1993</td>
</tr>
<tr>
<td>Heme</td>
<td>0.62</td>
<td>*in vitro</td>
<td>Not specified</td>
<td>Karnaukhova et al. 2012</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>54</td>
<td>*in vitro</td>
<td>Emphysema</td>
<td>Karnaukhova et al. 2010</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>32</td>
<td><em>in vivo</em></td>
<td>Apoptosis, emphysema</td>
<td>Petrache:2006</td>
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<tr>
<td>Calpain 1</td>
<td>78</td>
<td>*in vitro</td>
<td>Neutrophil activation</td>
<td>ALOmari et al. 2011</td>
</tr>
<tr>
<td>IgA complexes</td>
<td>150*</td>
<td><em>in vivo</em></td>
<td>Synovial fluid/rheumatoid arthritis</td>
<td>Laurell et al. 1975, Tomasi et al. 1974.</td>
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<tr>
<td>Surfactant protein A</td>
<td>36</td>
<td><em>in vitro</em></td>
<td>Airway surface liquid</td>
<td>Gorrini et al. 2005</td>
</tr>
<tr>
<td>Aggrecanase 1</td>
<td>83</td>
<td><em>in vivo</em></td>
<td>Synovial tissue/OA</td>
<td>Yoshida et al. 2005</td>
</tr>
<tr>
<td>Bile acids</td>
<td>-</td>
<td><em>in vitro</em></td>
<td>Bile</td>
<td>Janciauskiene et al. 1994</td>
</tr>
</tbody>
</table>
4.1.2 AAT binding mechanisms

The function of many proteins is determined by its interactions in blood (fluid phase), the extracellular matrix, at the cell surface, and at target tissues such as the alveolar lining fluid. AAT may interact with both structural and immune molecules through a number of described mechanisms, see Table 4.2. As previously outlined, some of the recent observations on the anti-inflammatory effects of AAT are independent of its specific anti-protease activity. Knowledge of the mechanisms through which AAT binds beyond protease inhibition, permits us to explore the extent of its interactions in this chapter.

Table 4.2: Potential binding mechanisms of AAT

In addition to the primary protease inhibitory function of AAT, that is mediated through binding of the Met 358 residue to the carboxyl residue on a target protease, protein interactions can occur through a number of different interactions outlined in this table.

Glossary: Met358 = methionine residue at position 358.

<table>
<thead>
<tr>
<th>Method of AAT protein insertions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive Met358 residue</td>
</tr>
<tr>
<td>Carbohydrate binding domains</td>
</tr>
<tr>
<td>Hydrophobic binding site</td>
</tr>
<tr>
<td>Heparin binding motif</td>
</tr>
<tr>
<td>Ionic/Hydrogen binding interactions</td>
</tr>
<tr>
<td>Intermolecular (Van der Waal’s) forces</td>
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</tbody>
</table>
The affinity between the surfaces of two complex proteins within a biological system is often divided between specific and non-specific interactions (249). Many of the interactions involving AAT that have been described are specific, uniform contacts that result in a molecular structural change that is often irreversible. Non-specific interactions occur across the surface of the molecule and generally do not result in a structural of either molecule within the macromolecular complex. Instead, they are driven by superimposition of three or four intermolecular interactions (e.g. Van der Waal’s forces, electrostatic, steric, and hydrophobicity) and a multiplicity of structurally dependent weak interactions (250,251).

4.1.3 AAT Protease binding

The primary anti-protease binding activity of AAT has been well characterised. The formation of denaturant-stable complexes between AAT and its target serine proteinase results in a conformational change that is kinetically irreversible. Serpins have evolved in parallel with their cognate proteases: e.g. antithrombin with thrombin, C1-inhibitor with C1-esterase, and antiplasmin with plasmin. The major function of AAT is the inhibition of its cognate enzyme NE, to which it binds avidly with an association constant of $K = 6.5 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$, one of the highest binding constants found in nature. AAT has been shown to inhibit a wide range of other serine proteases including Cath-G (252), PR-3 (253), and Factor Xla (254,255), summarised in Table 4.2. The delay time of inhibition is an important factor regarding the functional effect of AAT in vivo; if it is too long, the protease may not have enough time to reach its substrate, thus rendering the inhibitor inefficient. In addition, the target enzyme could inactivate the inhibitor by proteolytic attack at a site remote from the active site (252). In an environment where multiple proteases are active, such as sites of inflammation in vivo, AAT will bind preferentially to NE, e.g. should PR-3 and NE be liberated at the same time and in equal concentrations, 89% of AAT would be bound to NE and 11% bound to PR-3 (256). As outlined earlier the binding of AAT to these
proteases is mediated through its reactive methionine residue at position 358, oxidation of this critical amino acid reduces the binding association by a factor of 2000 (see Table 4.2). Oxidative inactivation of AAT either through smoke exposure, or through the oxidative burst of leucocytes is a mechanism for the inactivation of AAT binding at sites of inflammation (37).

Cleavage of the RCL at Met358 results in a conformational change in the AAT protein, which exposes a new binding pentapeptide domain in the carboxyl terminal fragment. This can then engage with the SERPIN: enzyme complex (SEC) receptor expressed on hepatocytes, with consequent internalisation of the complex and lysosomal degradation of the protease/anti-protease complex (32). In addition, this structurally rearranged AAT molecule possesses chemoattractant properties and can mediate increased AAT synthesis by mononuclear phagocytes and hepatocytes (33).

In health, there is minimal or no detectable neutrophil protease activity in plasma. The broad spectrum of AAT protease binding raises the possibility these interactions play a role in the homeostasis of other systems that involve serine protease cleavage, such as the coagulation pathway and complement cascade. Furthermore, this balance may be perturbed in AATD states.
Table 4.3: Known serine proteases that bind and are inhibited by AAT

Serine proteases that are inhibited by AAT collated from the published literature. The list is presented in order of decreased association avidity, and where possible data regarding the interaction between oxidized AAT and the serine protease is presented. It should be noted that, despite a relatively low association constant between AAT and a given serine protease, the relative abundance of AAT in plasma may account for important protease inhibitory effects, e.g. Activated Protein C. Additionally, it is likely that AAT exerts some inhibitory effect on many other serine proteases that have not been studied to date.

Glossary; $K = $ association constant, units (M$^{-1}$s$^{-1}$). AAT-ox = oxidised alpha-1 antitrypsin. kDa = kiloDalton. *homodimer

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>$K$ (AAT)</th>
<th>$K$ (AAT-ox)</th>
<th>kDa</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil Elastase</td>
<td>6.5± 4.0 x 10$^7$</td>
<td>3.1± 0.2 x 10$^4$</td>
<td>28.5</td>
<td>Beatty et al. 1980</td>
</tr>
<tr>
<td>Proteinase 3</td>
<td>8.1 x 10$^6$</td>
<td>-</td>
<td>27.8</td>
<td>Duranton et al. 2003</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>4.1± 0.6 x 10$^5$</td>
<td>6.5± 0.3 x 10$^2$</td>
<td>28.8</td>
<td>Beatty et al. 1980</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>5.4± 0.6 x 10$^6$</td>
<td>1.0± 0.4 x 10$^6$</td>
<td>29.4</td>
<td>Beatty et al. 1980</td>
</tr>
<tr>
<td>Trypsin 2 (Anionic)</td>
<td>7.3± 1.8 x 10$^4$</td>
<td>3.2± 0.1 x 10$^4$</td>
<td>26.4</td>
<td>Beatty et al. 1980</td>
</tr>
<tr>
<td>Trypsin 1 (Cationic)</td>
<td>1.1± 0.2 x 10$^4$</td>
<td>3.0± 1.1 x 10$^3$</td>
<td>26.5</td>
<td>Beatty et al. 1980</td>
</tr>
<tr>
<td>Factor XIa</td>
<td>1.3 x 10$^6$</td>
<td>-</td>
<td>160*</td>
<td>Scott et al. 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Janciauskiene et al. 2008</td>
</tr>
<tr>
<td>Matriptase</td>
<td>3.1 x 10$^2$</td>
<td>0</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Plasmin</td>
<td>1.9± 0.1 x 10$^2$</td>
<td>0</td>
<td>90.5</td>
<td>Levi et al. 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fredenburgh et al. 2001</td>
</tr>
<tr>
<td>Thrombin</td>
<td>4.8± 0.5 x 10$^1$</td>
<td>0</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Activated Protein C</td>
<td>1.1 x 10$^4$</td>
<td>-</td>
<td>60</td>
<td>Heeb et al. 1988</td>
</tr>
</tbody>
</table>
4.1.4 AAT glycan binding potential

The three oligosaccharide residues attached to AAT contribute approximately 12% of the resultant circulating molecular weight of the protein. The addition of glycan residues to a protein generally confers greater conformational stability, however AAT is an unusual exception as glycosylation does not appear to affect the native metastable state (18).

The glycan residues, and their resultant electrostatic charge, may modulate intermolecular interactions of AAT in the circulation; they may bind to other protein glycans (carbohydrate:carbohydrate interactions), to the amino acid backbone of the other proteins (carbohydrate:amino-acid interactions), or affect cell receptor interaction (257). The glycosylation of AAT also protects the protein from glycolysis, prevents protein aggregation, is less polymerogenic, prolongs its plasma half life, and importantly it does not interfere with its anti-proteinase activity (258-261).

In the context of AAT augmentation therapy the significance of this co-translational modification becomes apparent; intravenous recombinant AAT (rAAT) lacks efficacy compared to human purified AAT (hp-AAT) due to the impact of glycosylation (183). The use of nebulised rAAT overcomes some of the shortcomings of intravenous therapy and permits delivery to the local site of inflammation (262) and the ability of rAAT to neutralize NE is preserved using this approach (263), however the modulation of markers of inflammation has only been observed using hp-AAT to date (264). It appears that AAT glycans may be necessary for some of its observed immunomodulatory properties but not its anti-elastase effects, moreover the glycans are also important for prolonging the plasma half-life of the protein. This is further illustrated in the studies of our laboratory that demonstrate the normalization of neutrophil chemotaxis through the glycan-dependent binding of IL-8, and successful modulation of TNFα signalling by hp-AAT augmentation therapy (85,246).
4.1.5 AAT cysteine binding potential

AAT has a single cysteinyl residue (Cys-232) that is protected in a crevice due to the close proximity of three lysine residues (see Figure 4.1); this unique structural environment provides the thiolate stabilization required for a high degree of reactivity across a broad pH range (36). Cys-232 is reactive under physiological conditions with proteins and small molecules such as cysteine, glutathione, myeloma immunoglobulin light chains, immunoglobulin A and nitric oxide (NO) (265-268). It has been demonstrated that AAT forms a disulphide bond with the penultimate C-terminal cysteine on the alpha chain of IgA (269), while still retaining its anti-protease activity (270).

It has been shown that the AAT can undergo S-nitrosation, through the interaction of Cys232 with NO formed at the sites of tissue ischaemia or by the action of endothelial or inducible NO synthases at sites of inflammation (268,271). The resultant S-NO-AAT molecule is bacteriostatic, can induce vasorelaxation, inhibit platelet aggregation and inhibit neutrophil adhesion to the endothelial surface (84,268). AAT thereby may act as a NO reservoir and mediate cytoprotective effects through the attenuation of ischemia-reperfusion injury by maintaining tissue blood flow (272-274). This property has led to clinical trials in humans utilising AAT augmentation therapy in ST-segment myocardial infarction (180)
Figure 4.1: Surface properties of the 3D model of alpha-1 antitrypsin

A. The active methionine residue at position 358 (yellow) is attached to the RCL (black).
B. The expanded structural region displays the active cysteine residue at position 232 (yellow), within a favourable binding pocket surrounded by three lysine residues. Adapted from Griffiths et al. 2002 (36).
C. An expanded view of a favourable hydrophobic pocket is displayed with a model LTB₄ molecule docked within it. Adapted from O’Dwyer et al. 2015 (283).
D. The composite hydrophobicity of the AAT molecule is presented in this figure, the axis and orientation is the same as that depicted in A). The hydropathy of AAT has been determined using a sliding window of 15 amino acid residues compared to a table of hydrophobic properties. The hydrophobic regions are depicted (red), these include the RCL at the M358 end of the molecule (arrow). The hydrophilic regions are depicted (blue) and surface Van der Waal’s forces indicate the accessible surface of the molecule (orange).
4.1.6 Heparin binding motif of AAT

The effect of heparin binding to serine proteinase inhibitors is illustrated by the potentiation of antithrombin III activity, a property that is exploited in clinical practise with the use of unfractionated heparin and low molecular weight heparin for the purpose of anticoagulation (275). Binding of heparin is mediated by ionic interactions between its sulphate and carboxylate groups and positively charged side chains of the target protein. AAT contains a heparin binding motif, the function of which in the presence of heparin may be to enhance the binding affinity of the reactive Met358 compared to native forms of the AAT protein (276). However, contrary to this, it has been previously demonstrated that the binding affinity of AAT to NE is reduced in the presence of heparin due to the formation of heparin:elastase complexes, in this study heparin was not found to bind AAT (277). Further work is warranted in this area before any definitive conclusion regarding the significance of heparin binding to AAT can be made.

4.1.7 Electrostatic interaction with AAT

The secondary structure of AAT has been well characterized; there are three beta sheets and 8-9 alpha helices, a property that it is well conserved between other members of the SERPIN superfamily (278,279). The total accessible surface area AAT \((2.34 \times 10^4 \text{Å}^2)\) is largely hydrophilic in nature surrounding a hydrophobic core, all hydrogen bonds are satisfied on the surface mainly by interactions with main-chain atoms (27). The surface of AAT has a dipolar character with the positive pole at the S359 end and the negative pole at the M358 end. The isoelectric point (pI) of AAT is 5.37 and therefore it carries a negative charge at physiologic pH. In a study of the surface interactions of AAT with an array of small peptides, AAT bound preferentially to longer, more hydrophobic peptides (251), this may relate to its relatively small size minimizing the effect of Columbic interactions over a shorter inter-protein range and thereby favouring hydrophobic binding. The influence of divalent cations, such as Mg\(^{2+}\), Ca\(^{2+}\), Cu\(^{2+}\),
Zn$^{2+}$, and Fe$^{2+}$, are important in modulating protein binding (280) and levels can change during the acute phase response that may potentially alter the bound protein profile of AAT in inflammatory states (6).

4.1.8 Hydrophobic binding of AAT

Approximately 13% (3.2x10$^3$ Å$^2$) of the accessible surface area of AAT is hydrophobic in nature, with five possible hydrophobic pockets identified; one central hydrophobic core has been located that is filled on protease cleavage of the RCL or during polymer formation (281). This site has become a target for drug delivery to prevent loop sheet polymerization without abolishing the function of AAT (282). This location is also a potential binding site for other small hydrophobic molecules, such as the potent neutrophil chemoattractant LT$B_4$. In our laboratory we have demonstrated that AAT can bind LT$B_4$ and that AAT-LT$B_4$ complex formation modulates BLT1 engagement and downstream signalling involved in neutrophil adhesion and degranulation (283). The ability of AAT to bind LT$B_4$ contributes to the growing evidence regarding the anti-inflammatory properties of AAT and furthermore uncovers another mechanism for the beneficial effects of AAT augmentation therapy.
4.1.9 Biological effects of AAT binding

Much interest has arisen in recent years regarding the alternative biological effects of AAT augmentation therapy as a result of scientific developments in the field of AAT research, in particular its potential anti-inflammatory and anti-apoptotic properties (93). Intravenous AAT augmentation therapy has been evaluated in a range of conditions apart from AATD, such as diabetes mellitus (NCT02093221), the prevention of graft rejection in pancreatic islet cell transplantation, and post myocardial infarction (180,284). The effect of AAT on inflammation and apoptosis is not dependent on its anti-protease effects, but rather it may be mediated by a variety of different pathways including the modulation of TNFα signalling, S-nitrosation, and its interaction with intracellular caspases (85,89,90,92,246,268,285). Knowledge of the full binding profile of AAT as it circulates in health and in deficiency states may lead to a deeper understanding of its effects and uncover novel mechanisms of action. This may ultimately lead to innovative therapeutic applications for AAT augmentation therapy in a host of other disease conditions.
4.2 Aims

We hypothesized that AAT may bind to an array of abundant proteins within the circulation that are involved in the immune response, thereby modulating or facilitating their biological activity. Utilising the immunoprecipitation properties of a novel AAT protein isolation resin, combined with mass spectrometry analysis, we sought to identify proteins that interact with AAT as it circulates in the body. The importance of this project is outlined by the emerging therapeutic applications of AAT augmentation therapy in a diverse range of conditions.

To fulfil this aim the specific objectives of this chapter were:

1. Establish the profile of plasma proteins that bind to AAT in the circulation.
2. Explore any differences in the profile of plasma proteins that bind to M-AAT and Z-AAT.
3. Identify all plasma proteins that bind to AAT in the circulation.
4.3 Results:

4.3.1 Co-immunoprecipitation of binding partners to AAT from plasma

This is the first study to assess the profile of AAT binding to other abundant proteins in the circulation. Recent observations in our laboratory have detected impurities in the preparation of purified AAT from plasma, corresponding to high molecular weight protein bands Coomassie stained SDS PAGE gels, these impurities were confirmed by western blot to be AAT, see Figure 4.2. Based on the hypothesis that the binding properties of AAT may in part mediate its observed anti-inflammatory properties, we utilised a novel commercially available affinity chromatography medium, AAT Select Resin (GE Healthcare), to co-immunoprecipitate and visualise any bound proteins to circulating M-AAT and Z-AAT. Firstly, 7.5% SDS PAGE gels were used in this experiment as they provide better resolution of high molecular weight bands that may contain AAT compared to the 12.5% SDS PAGE gels initially employed. Co-eluted proteins to M-AAT from 1% PiMM (n=3) plasma were visualised on reducing 7.5% SDS PAGE gels using Coomassie blue stain and western blot (WB) with goat anti-AAT antibody (Abcam) (Figure 4.3A). Following multiple wash steps a number of high molecular weight bands were seen in both the 500mM and 2M MgCl₂ elution, a greater number of bands were visualised in the 500mM elution indicative of more loosely associated proteins that may be associated with the bound AAT. On WB analysis, a number of distinct bands of AAT were detected above the predicted molecular with of 52kDa, at approximately 120kDa and at 250kDa.
Figure 4.2: Identification of a high molecular weight AAT complex in plasma

A. Panel A depicts a 12.5% SDS PAGE gel stained with Coomassie blue.
   1. The first lane shows the profile of 10μL 20% plasma in DPBS from a healthy PiMM individual.
   2. Lane 2 shows the profile of unbound 10μL plasma following incubation of 50μL AAT Select Resin (GE Healthcare) in 500μL 20% plasma.
   3. Lane 3 depicts the bound AAT purified from 20% plasma following an incubation step. A prominent band is evident at approximately 52kDa corresponding to the expected molecular weight of AAT. Two other high molecular weight bands are evident at 130kDa and above 250kDa.

B. A Western blot was performed on an identical gel to that in panel A using goat polyclonal anti-AAT antibody to visualise if the high molecular weight bands contain AAT.
   1. In lane 1 there are two AAT immunobands identified in plasma, indicating that AAT is present in plasma above its predicted molecular weight, potentially in a complexed form at approximately 130 - 140kDa.
   2. A faint AAT signal is detected in the unbound plasma fraction due to the successful removal of most AAT in the plasma sample.
   3. Two strong AAT immunobands are seen in the concentrated and purified M-AAT sample, confirming that the bands seen on Coomassie blue stain gel correspond to AAT at 52kDa and 130kDa on this 12.5% SDS PAGE gel.

AAT: alpha-1 antitrypsin, DBPS: Dulbecco’s phosphate buffered saline
The plasma concentration of circulating Z-AAT is approximately one tenth of the M-AAT, in addition the elastase inhibitory potential is markedly reduced. However, recent evaluation of the glycosylation status of Z-AAT compared to M-AAT indicate many similarities with some subtle differences in outer arm and core glycosylation (57). Any differences in potential binding partners co-eluted during the purification of Z-AAT were assessed from 1% PiZZ plasma (n=3). A similar protein profile is seen on Coommassie blue stain, though the high molecular weight bands stain more intensely compared to M-AAT purification, see Figure 4.3B. Despite a smaller amount of Z-AAT purified when assessed by WB, as would be expected, high molecular weight bands of Z-AAT above the predicted weight of 52kDa were still detected at approximately 120kDa and faintly at 250kDa. Upon visualisation of potential binding partners to Null-AAT co-eluted from 1% Null/Null plasma, see Figure 4.3C. A similar protein profile was seen on Coomassie blue stain compared to M-AAT and Z-AAT purification. A small amount of truncated Null AAT is purified when assessed by WB, however no significant high molecular weight bands were detected of Null-AAT.
Figure 4.3: Co-immunoprecipitation of binding partners to AAT from plasma

A. Purification of M-AAT from 1% PiMM plasma using AAT Select Resin (GE Healthcare). Co-eluted proteins were visualised on reducing 7.5% SDS PAGE gels using Coommassie blue stain and western blot (WB) with goat anti-AAT antibody (Abcam). Following multiple wash steps a number of high molecular weight bands are seen in the 500mM and 2M MgCl₂ elution with distinct bands of AAT detected above the predicted molecular with of 52kDa.

B. Visualisation of potential binding partners to Z-AAT co-eluted from 1% PiZZ plasma. A similar protein profile is seen on Coommassie blue stain, the high molecular weight bands stain more intensely compared to M-AAT purification. A smaller amount of Z-AAT is purified when assessed by WB, however high molecular weight bands of Z-AAT were also detected.

C. Visualisation of potential binding partners to Null-AAT co-eluted from 1% Null/Null plasma. A similar protein profile is seen on Coommassie blue stain, the high molecular weight bands stain more intensely compared to M-AAT and Z-AAT purification. A small amount of truncated Null AAT is purified when assessed by WB, however no significant high molecular weight bands are detected.
4.3.2 Plasma separation by molecular mass to identify the elution profile of M-AAT

In order to define the range of binding partners to AAT in plasma, size exclusion gel permeation chromatography was performed on plasma from healthy PiMM individuals (n=6). Utilizing a Superdex 200GL 10/300 column (GE Healthcare) with an optimum separation range of 10kDa to 600kDa the elution profile of proteins in plasma was determined employing the AKTA Prime plus (GE Healthcare) Fast Protein Liquid Chromatography (FPLC) system. Due to the properties of the cross-linked agarose and dextran medium of this column, high molecular weight proteins elute from the column at an earlier time to low molecular weight proteins. The elution profile was visualised by UV spectrometry and the corresponding separated protein samples were collected in 500μL fractions in physiological buffer at pH 7.4, see Figure 4.4A. The complete protein profile of plasma was demonstrated on Coomassie blue stained SDS PAGE gels, see Figure 4.4B. The elution profile of AAT by western blot immunoassay (anti-AAT antibody) identified two distinct high molecular weight peaks; the first peak was at 298kDa (range 250-600kDa) (peak 1) and the second at 50kDa (peak 2). This indicates that AAT circulates in plasma both at its predicted molecular weight and also complexed to other protein(s) with an estimated increased molecular weight of 250kDa (range 200 – 550kDa), see Figure 4.4C. Area under the curve analysis of the plasma elution profile measured by densitometry of AAT immunobands determined that 13% of AAT is in a high molecular mass complexed form in peak 1.
Figure 4.4: Purification of M-AAT to identify complexed binding partners

A. Size exclusion gel permeation chromatography of MM plasma (n=6) in DPBS running buffer. The elution chromatogram was measured at 280nm using the AKTA Prime+ FPLC.

B. Coomassie Blue stained SDS-PAGE gels (12.5%) of the protein profile of fractions 14 – 35.

C. SDS-PAGE and western blot analysis of the corresponding fractions. Two distinct elution peaks were detected, firstly at a predicted molecular weight of 298kDa (fraction 23) and the second at 50kDa (fractions 33). AUC analysis determined 13% eluted in the high molecular weight fraction, with the remaining 87% eluting at its predicted molecular weight.

D. Western blot analysis of MM plasma eluted in DPBS buffer with 1M NaCl indicating a single AAT elution peak at fraction 33.

E. Densitometry of SDS-PAGE anti-AAT western analysis of MM plasma eluted in DPBS (n=6) demonstrating two molecular weight peaks, each point represents a mean value ± SEM.
4.3.3 Electrostatic dissociation of protein binding partners to M-AAT.

In order to evaluate the nature of the interaction between the AAT complexes found in plasma, repeat size exclusion gel permeation chromatography was performed once the column was equilibrated with 1M NaCl added to the column running buffer. The purpose of the high salt concentration in the buffer was to disrupt electrostatic interactions between AAT and its binding partners. The elution profile of AAT was again determined using western blot immunoassay, see Figure 4.4D. The visualised profile for AAT in the presence of 1M NaCl demonstrates a single peak at the predicted molecular weight of 50kDa. The results of these combined experiments indicate that under physiological conditions a significant portion of AAT is complexed at a high molecular mass to other binding partner(s) in plasma. Furthermore this experiment has demonstrated that this interaction is reversible and relates to electrostatic interactions between the bound proteins.

Importantly, the results of these experiments do not demonstrate the occurrence of covalent bound proteins to AAT or circulating polymer formation; covalent bonds are not affected by electrostatic charge and therefore do not account for the high molecular weight elution peak of AAT. Furthermore AAT:protease complexes are not degraded under reducing conditions and are readily demonstrable as a second band on SDS PAGE gels. As the primary function of AAT is related to the formation of protease:anti-protease complexes through an irreversible conformational change in the molecule, the observed electrostatic interaction of AAT in plasma points to a separate role of AAT independent of its protease inhibitory function.
4.3.4 Purification of M-AAT high molecular weight binding partners.

Prompted by the novel finding of a significant proportion of AAT complexes in the circulation within peak 1, we next purified AAT from this high molecular mass elution fraction. Repeat FPLC of PIMM plasma (n=3) was performed six times to obtain a larger volume of each fraction and to obtain a sufficient amount of AAT protein from peak 1. Utilizing AAT Select resin (GE Healthcare) we exploited the properties of this chromatography medium to co-elute AAT-bound proteins. We proceeded to co-immunoprecipitate proteins bound to AAT on the resin using 500mM MgCl₂ and 2M MgCl₂ elution buffer. The 500mM elution was of particular interest in this analysis as the disruption of the electrostatic interactions permitted release of the AAT-linked proteins but not the resin-bound AAT, as would be expected with the 2M MgCl₂ elution. Acetone precipitation of the resultant pooled samples was performed and the precipitated purified binding partners were directly visualized on silver stained SDS PAGE gels. In addition, the corresponding precipitated protein pellets from this process were stored for in-solution mass spectrometry analysis.

The result of this two stage purification process is shown in Figure 4.5: Lane 1 represents the start plasma from peak 1; lane 2 illustrates the unbound plasma following incubation with the AAT Select resin; lane 3 contains the binding partners to AAT following 500mM MgCl₂ elution; lane 4 contains the remainder of the AAT protein and associated binding partners that were still bound and subsequently eluted with 2M MgCl₂. On inspection of lane 3, there is a small band at 52kDa that corresponds to AAT. At least a further 8 bands were visible above the 50kDa molecular weight marker. The SDS PAGE gels were analysed under reducing conditions, therefore a number of smaller protein fragments are expected from larger proteins that contain disulphide bonds, which have been cleaved by DTT in the sample buffer.
Figure 4.5. Visualisation of binding partners to purified M-AAT from the high molecular weight elution peak following size exclusion gel chromatography.

SDS-PAGE (12.5%) silver-stained gel of pooled fractions containing complexed binding partners to M-AAT, corresponding to following the performance of a second purification step using Alpha-1 Select resin (GE Healthcare).

Lane 1) Start sample from pooled high molecular weight FPLC fractions.
Lane 2) Unbound sample following incubation with AAT-Select resin for one hour at 25°C.
Lane 3) Protein profile following 500mM MgCl₂ elution from AAT resin.
Lane 4) Protein profile of remaining bound proteins to AAT following 2M MgCl₂ elution step, AAT is indicated by the arrow.
4.3.5 Mass spectrometry identification of M-AAT high molecular weight binding partners following in-solution and in-gel digestion.

Visualisation of the high molecular weight binding partners by SDS PAGE is useful, however to definitely establish what each protein band represents requires further scrutiny. Mass spectrometry analysis is a highly sensitive and specific technique to determine what peptides are present in a given sample and furthermore determine if they correspond to known proteins from a reference source.

The high molecular weight binding proteins seen from the 500mM elution profile were excised from the gel (lane 3) and dissected into bands, see Figure 4.5. Each band was de-stained, reduced and alkylated before undergoing overnight trypsin digestion. Peptides generated were extracted from the gel slices, evaporated and resuspended in solution for reverse phase chromatography separation through a linear ion trap Mass Spectrometer.

The in-solution protein pellets were analysed in a similar manner as an additional step to corroborate the findings from in-gel digestion. The peptides generated underwent MS as described above. The data generated was searched against the human subset of the UniProt/SwissProt database (Jan 2012 release) using SEQUEST search algorithm in BioworksBrowser 3.3.1. The proteins detected by in-gel digestion of the high molecular weight binding partners to M-AAT are shown in Table 4.3, the results are presented in order of confidence score based on statistical analysis and the number of peptides detected.

The findings of in-solution digestion (n=3) are shown in Table 4.4. When the in-gel and in-solution digestion are compared, the profile of identified proteins appears similar, however the in-solution results provide a more detailed picture of the spectrum of potential linker proteins to M-AAT. Broadly, the binding partners detected from this two-stage purification process, and MS analysis,
indicate that M-AAT interacts with plasma proteins involved in lipid transport, the coagulation system, the complement system and immunoglobulin system.

Table 4.4: High molecular binding partners to M-AAT following in-gel trypsin digestion of the early elution peak and mass spectrometry analysis (n=3)

Following two-stage M-AAT purification, the pooled high molecular weight fractions underwent SDS PAGE analysis; all visualised bands from the 500mM MgCl₂ elution (lane 3) were excised and labeled. In-gel trypsin digestion of the bands was performed followed by LC/MS analysis over a 30min reverse phase chromatography by a LTQ Orbitrap mass spectrometer. The data generated was searched against the human subset of the UniProt/SwissProt database (Jan 2012 release) using SEQUEST search algorithm in BioworksBrowser 3.3.1. The proteins found are listed in descending order of confidence score and peptide count.

<table>
<thead>
<tr>
<th>M-AAT binding partner</th>
<th>P-value</th>
<th>Confidence Score</th>
<th>kDa</th>
<th>Peptide count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoprotein B-100</td>
<td>$1.51 \times 10^7$</td>
<td>190.20</td>
<td>515.28</td>
<td>19</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>$3.87 \times 10^{13}$</td>
<td>120.23</td>
<td>36.13</td>
<td>12</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>$2.95 \times 10^8$</td>
<td>110.23</td>
<td>69.32</td>
<td>11</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>$1.11 \times 10^{15}$</td>
<td>100.28</td>
<td>262.46</td>
<td>10</td>
</tr>
<tr>
<td>Fibrinogen alpha chain</td>
<td>$3.33 \times 10^{15}$</td>
<td>80.19</td>
<td>94.91</td>
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</tr>
<tr>
<td>Fibrinogen gamma chain</td>
<td>$2.44 \times 10^8$</td>
<td>80.17</td>
<td>51.48</td>
<td>8</td>
</tr>
<tr>
<td>Complement factor H</td>
<td>$8.66 \times 10^9$</td>
<td>60.17</td>
<td>139.00</td>
<td>6</td>
</tr>
<tr>
<td>Fibrinogen beta chain</td>
<td>$4.46 \times 10^9$</td>
<td>50.20</td>
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<td>$2.98 \times 10^7$</td>
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<td>52.57</td>
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</table>
Table 4.5: Binding partners to M-AAT following in-solution trypsin digestion of 500mM elution fraction and mass spectrometry analysis

Following permeation chromatography and co-immunoprecipitation of M-AAT to isolate complexed binding partners, the pooled fractions underwent acetone precipitation (n=3). The 500mM MgCl₂ elution protein pellet underwent reduction for 6 hours and overnight trypsin digestion. In-solution samples then underwent MS analysis as previously described. The data generated was searched against the human subset of the UniProt/SwissProt database (Jan 2012 release) using SEQUEST search algorithm in BioworksBrowser 3.3.1. The proteins found are listed in descending order of confidence score and peptide count.

ITIH = Inter-alpha-trypsin inhibitor heavy chain

<table>
<thead>
<tr>
<th>M-AAT binding partner</th>
<th>P-value</th>
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<th>kDa</th>
<th>Peptide count</th>
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<td>69.32</td>
<td>19</td>
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<tr>
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<td>54.31</td>
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<tr>
<td>Alpha-2-macroglobulin</td>
<td>1.33 x 10^{14}</td>
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<tr>
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4.3.6 Plasma separation by molecular mass to identify the elution profile of Z-AAT

In AATD, particularly in PiZZ individuals, there is marked deficiency of circulating AAT. The abnormal structure of the Z-AAT protein, coupled with severe humoral deficiency, and existent pulmonary or liver disease processes in some individuals, may influence the binding profile of Z-AAT compared to M-AAT. To investigate the binding profile of the Z-AAT, repeat size exclusion gel chromatography was performed under the same experimental conditions as was performed for the M-AAT protein, see Figure 4.6. The range of AAT protein elution was evaluated from the plasma of healthy PiZZ individuals (n=3) under physiological buffer conditions and in the presence of 1M NaCl. Z-AAT eluted at two distinct molecular peaks, the first (peak 1) at 570kDa and the second (peak 2) at the predicted molecular weight of 50kDa. Area under the curve analysis determined 33% of Z-AAT eluted in peak 1, with the remaining 66% eluting at its predicted molecular weight in peak 2.

In the presence of 1M NaCl in the elution buffer there was a shift of peak 1 to 160kDa though it was not fully removed, the 50kDa elution peak was again demonstrated. This is consistent with electrostatic binding of Z-AAT to proteins in that molecular weight range, however there is also evidence of covalent binding occurring with the formation of an AAT:protease complex. In support of AAT:protease complex formation in these healthy PiZZ individuals, two AAT immunobands are demonstrated within peak 1; one at 50kDa and the second at 64kDa on SDS-PAGE, see Figure 4.6D. This larger AAT:protease complex may account for the observed electrostatic shift in Z-AAT binding of peak 1 from 570kDa to 160kDa but not complete dissociation as was observed in the M-AAT protein elution. The interpretation of the results from these experiments, evaluating the high molecular weight elution peak of Z-AAT, indicate that both the native form of the protein and AAT:protease complexes can participate in electrostatic interactions in plasma with other proteins.
Figure 4.6: Purification of high molecular weight binding partners to Z-AAT (n=3).

A. Size exclusion gel permeation chromatography of PiZZ Plasma using DPBS buffer. The protein elution chromatogram was measured at 280nm using the AKTA Prime+ FPLC system.

B. Coomassie Blue stained SDS-PAGE gels (12.5%) of the protein profile of fractions 14 DS-P.

C. SDS-PAGE and western blot analysis of the corresponding fractions probed with anti-AAT antibody. Two distinct elution peaks were detected, firstly at a predicted molecular weight of 570kDa (fraction 20) and the second at 52kDa (fractions 32).

D. Western blot analysis of PiZZ plasma eluted in DPBS with 1M NaCl buffer solution showing a shift of the elution profile to a lower molecular weight peak at 160kDa (fraction 25), with loss of the high molecular weight elution fraction of AAT and a single elution peak at approximately 50kDa (fraction 32).

E. Densitometry of SDS-PAGE anti-AAT western analysis of PiZZ plasma eluted in DPBS (n=3) demonstrating two molecular weight peaks, each point represents a mean value ±SEM. There is a rightward shift in the molecular mass of the eluted proteins in the presence of 1M NaCl, however there is incomplete dissociation. The presence of two immunobands within a single lane indicates that AAT is complexed to a protease such as NE.
4.3.7 Purification of Z-AAT high molecular weight binding partners and mass spectrometry identification following in-solution and in-gel digestion.

The incomplete dissociation of Z-AAT in 1M NaCl buffer, and the presence of two bands in some high molecular weight fractions on SDS PAGE western blot analysis, is indicative of the difference in the binding relationships between M-AAT and Z-AAT. However, despite the apparent disparity between the two forms of AAT under investigation, it is not known if the range of binding partners is different.

To elucidate the binding partners complexed to Z-AAT, it was necessary to repeat the same experimental design as was performed in the M-AAT analysis. The plasma of healthy PiZZ individuals (n=3) was obtained and gel permeation chromatography was performed six times to acquire a sufficient concentration of Z-AAT binding partners. Following incubation with AAT Select resin and a two-stage elution process with 500mM MgCl₂ and 2M MgCl₂, as outlined before. The potential binding partners to Z-AAT were visualised on silver stained SDS PAGE gels, Figure 4.7. The total plasma Z-AAT concentration is <10% of circulating M-AAT values, therefore the amount of resin-bound Z-AAT was less than that seen in the M-AAT analysis. As the process of affinity chromatography involves co-elution of the binding partners along with Z-AAT, fewer bands are visualised despite sensitive silver staining (lane 3). Following this observation, we proceeded to perform in-gel and in-solution digestion of the proteins purified from the Z-AAT elution peak 1.

The results of in-gel digestion from the 500mM elution (Figure 4.7, lane 3) are shown in Table 4.6. In-solution digestion of the Z-AAT binding partners revealed a similar profile to that observed following in-gel digestion (Table 4.7). The results are shown with a lower confidence score cut off value of 40. A similar spectrum of proteins was identified in the analysis of Z-AAT compared to M-AAT; it was demonstrated that Z-AAT interacts with plasma proteins involved in lipid
transport, the coagulation system, the complement system and immunoglobulin system. Interestingly, despite the presence of protease complexes on western blot analysis, there were no protease fragments detected in the MS analysis. The digestion of the protease into peptide fragments below the confidence limits of the MS analysis may account for this during sample preparation.

Figure 4.7. Visualisation of high molecular binding partners to Z-AAT from the high molecular weight elution peak.

SDS-PAGE (12.5%) silver-stained gel of pooled fractions containing complexed binding partners to Z-AAT, corresponding to following the performance of a second purification step using Alpha-1 Select resin (GE Healthcare).

Lane 1: Start sample from pooled high molecular weight fractions.
Lane 2: Unbound sample following incubation in AAT-Select resin for 1h at 25°C.
Lane 3: Profile of proteins following 500mM MgCl$_2$ elution from AAT resin.
Lane 4: Protein profile of remaining bound proteins following 2M MgCl$_2$ elution step.
Table 4.6: Identified binding partners to Z-AAT following in-gel trypsin digestion of the early elution peak and mass spectrometry analysis

Following two-stage Z-AAT purification, the pooled high molecular weight fractions (n=3) underwent SDS PAGE analysis; all visualised bands from the 500mM MgCl₂ elution (lane 3) were excised and labeled. In-gel trypsin digestion of the bands was performed followed by MS analysis. The data generated was searched against the human subset of the UniProt/SwissProt database (Jan 2012 release) using SEQUEST search algorithm in BioworksBrowser 3.3.1.

ITIH = Inter-alpha-trypsin inhibitor heavy chain

<table>
<thead>
<tr>
<th>Z-AAT binding partner</th>
<th>p-value</th>
<th>Confidence score</th>
<th>kDa</th>
<th>Peptide count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement C3</td>
<td>4.44 x 10^{15}</td>
<td>490.30</td>
<td>187.0</td>
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<td>Apolipoprotein B-100</td>
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<td>515.3</td>
<td>49</td>
</tr>
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<td>163.2</td>
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</tr>
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<td>251.5</td>
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<td>Fibrinogen beta chain</td>
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<tr>
<td>Fibrinogen gamma chain</td>
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<td>Fibrinogen alpha chain</td>
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Table 4.7: Identified binding partners to Z-AAT following in-solution trypsin digestion of the early elution peak and mass spectrometry analysis

Following two-stage Z-AAT purification, the pooled high molecular weight fractions (n=3) underwent acetone precipitation; the 500mM MgCl₂ elution protein pellet underwent reduction for 6 hours and overnight trypsin digestion. In-solution samples then underwent MS analysis. The data generated was searched against the human subset of the UniProt/SwissProt database (Jan 2012 release) using SEQUEST search algorithm in BioworksBrowser 3.3.1.

ITIH = Inter-alpha-trypsin inhibitor heavy chain

<table>
<thead>
<tr>
<th>Z-AAT binding partner</th>
<th>p-value</th>
<th>Confidence score</th>
<th>kDa</th>
<th>Peptide count</th>
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4.3.8 String analysis of protein system interactions for M-AAT.

A considerable amount of data has been generated in the experiments described above. Whilst the importance of the individual protein interactions may be relevant in the healthy state, it is also important to examine the interaction of AAT and its binding partners within the biological systems where it may have a functional effect.

To facilitate the analysis of this data and aid in the interpretation of the importance of any new interaction that has been described, String v9.0 was employed to depict any known associations with AAT and the described proteins found on MS analysis of the in-solution digestion process.

String analysis gathers information from a range of sources, including the published scientific literature to determine the interaction of candidate proteins with other protein networks (286). Cluster analysis (stage 4) was performed to group proteins according to function to assist in the interpretation of the presented data, only significant interactions between the analysed proteins were depicted, see Figure 4.8. Based on this analysis, different protein clusters were apparent: Coagulation in green, complement in red, protease inhibition (including SERPINA1) and lipid transport in blue, and immunoglobulin function and protease inhibition in yellow.

M-AAT is shown to interact with coagulation factor 5, kininogen, fibronectin, clusterin, and albumin, with a moderate binding association with fibrinogen and alpha-2 macroglobulin (dotted blue lines). In conclusion, this illustrates the diverse range of protein systems where M-AAT may have an established role. Interestingly there is no association depicted between M-AAT and complement proteins. Given the importance of the complement system in regulating the innate and adaptive immune response, this novel description of an interaction between AAT and C3 may be relevant to the biology of these two systems in health and deficiency states.
Figure 4.8. Visualisation of the network of M-AAT complexed binding partners

Proteins were analyzed using String v9.0 to depict any known interactions. Stage 4 cluster analysis was performed to organise proteins according to function to assist with the interpretation of the tabulated data. Significant interactions between the analysed proteins are depicted here; known binding interactions are depicted with a blue line; catalytic reactions are shown with a purple line; and known reactions are shown with a black line. Interactions that are not relevant in humans or do not relate binding have been faded out. Based on this analysis, different protein clusters become apparent: Coagulation in green, complement in red, protease inhibition (including SERPINA1) and lipid transport in blue, and immunoglobulin function and protease inhibition in yellow. See abbreviation section for a full list of protein names used in this figure.

SERPINA1 = Alpha-1 antitrypsin
4.3.9 String analysis of protein system interactions for Z-AAT.

String v9.0 was employed to analyse the spectrum of high molecular weight binding partners to Z-AAT following MS analysis of the in-solution digestion process, as described in the above section. In addition to depicting the network of binding partners to Z-AAT, this interactive protein map permits easier comparison of the associations found between M-AAT and Z-AAT with the identified binding partners.

Following stage 4 cluster analysis significant interactions between the analysed proteins and Z-AAT were depicted, see Figure 4.9. Once again this analysis illustrates the different protein clusters that are identified from MS experimental data: Coagulation in blue, complement in red, protease inhibition (including SERPINA1) and lipid transport in green, and immunoglobulin function and protease inhibition in blue and yellow.

The range of proteins found to bind either M-AAT or Z-AAT are quite similar apart from some subtle differences, it is apparent that the protein systems involved in binding are similar for the two molecules. Indeed, no other class of protein interaction with Z-AAT is found, despite the observation that a greater proportion of protein binding occurs in peak 1 as described in earlier experiments. This result indicates that the role of M-AAT and Z-AAT binding appears similar in healthy individuals, with quantitative differences in circulating AAT protein leading to an increased proportion of Z-AAT bound to potential binding partners in the circulation. Restoration of circulating AAT through the administration of intravenous augmentation therapy may result in restoration of the plasma binding profile, however this was not examined.
Figure 4.9. Visualisation of the network of Z-AAT complexed binding partners

Proteins were analyzed using String v9.0 to depict any known interactions. Stage 4 cluster analysis was performed to group proteins according to function in assist in the interpretation of the presented data.

Significant interactions between the analysed proteins are depicted here; known binding interactions are shown by a blue line; catalytic reactions are shown with a purple line; and known reactions are shown with a black line. Interactions that are not relevant in humans or do not relate binding have been faded out.

Based on this analysis, different protein clusters become apparent: Coagulation is depicted by the blue circles, complement in red, protease inhibition (including SERPINA1) and lipid transport in green, and immunoglobulin function and protease inhibition in blue and yellow.

See abbreviation section for a full list of protein names used in this figure.

SERPINA1 = Alpha-1 antitrypsin
4.4.1 Discussion

Within Chapter 4, experiments focused on the characterization of the range of binding partners to AAT in the plasma of healthy PiMM and PiZZ individuals. Employing novel methodologies to purify and identify AAT in plasma we have determined that a significant proportion, approximately 13%, of total circulating M-AAT is complexed to other proteins in a healthy state. The absence of detectable specific covalent binding events in PiMM plasma indicates that protease inactivation does not contribute significantly to the circulating bound fraction of AAT in healthy subjects. The binding of M-AAT to candidate binding partners was shown to be electrostatic in nature.

Despite the absence of overt inflammation, AAT:protease complexes were detected in the plasma of healthy PiZZ individuals and were not seen in healthy PiMM subjects under physiological buffer conditions. Despite lower plasma concentrations a larger proportion of Z-AAT complexed to other proteins in the circulation than M-AAT (33% vs. 13%). This observed difference in the binding profile of the M-AAT and Z-AAT protein may be accounted for by an excess of protease activity, most likely NE, due to on-going neutrophil mediated inflammation in AATD as discussed in Chapter 1. In addition the abnormal protein structure of the Z-AAT, differences in glycosylation status, circulating Z-AAT polymers and severe humoral deficiency may affect the relative proportion of binding partners. There was partial but incomplete electrostatic dissociation of the Z-AAT protein complexes from peak 1 in the presence of 1M NaCl largely due to the presence of covalent protease complexes. However, it is interesting to observe that these AAT:protease complexes do appear to participate in protein binding in the circulation.

The non-specific electrostatic protein interactions observed between AAT and its binding partners are likely dependent on the attached carbohydrate residues or the hydrophilic/hydrophobic surface charge on the AAT protein. Despite a similar tertiary protein structure, the increased proportion of circulating Z-AAT bound to
other proteins may relate to slight changes in glycosylation status with a resultant 0.05 to 0.13 cathodal shift in the isoelectric point of all glycoforms of Z-AAT through the presence of increased outer and core fucosylated glycans (23). This is consistent with earlier reports examining the binding fraction of AAT to fibrinogen that found a similar amount of bound Z-AAT and M-AAT when co-immunoprecipitated from plasma despite far lower plasma AAT concentrations in PiZZ individuals (287). This is congruent with the experiments in this chapter where we observed a greater proportion of Z-AAT bound to other proteins compared to M-AAT, though quantification of the absolute AAT concentration within peak 1 was not undertaken for the respective separation experiments. Furthermore, the impact of intrinsic inflammation on binding due to the polymerogenic nature of Z-AAT should be considered, this may be a contributory factor to the systemic inflammatory state independent of pulmonary or liver disease status in the healthy subjects recruited in this study (43,51,52).

The interaction of AAT with potential high molecular weight binding partners was electrostatic in nature, demonstrated by the loss of the high molecular elution peak in the presence a high molar salt concentration. Complete dissociation of the macromolecular protein aggregates through electrostatic disruption indicates that the observed protein-binding partners were reversibly associated rather than covalently attached. In relation to AAT plasma binding partners, the electrostatic binding to other proteins likely relates to non-specific interactions that are dependent on the charge on the accessible surface of AAT, the glycosylation status, or hydrophobic interactions.

It is apparent from the experiments in this chapter that AAT has an important role as a carrier protein in plasma; this is supported by its abundance, structural similarities to other lipophilic serine protease carrier proteins, its documented hydrophobic binding domain (5), and the relative specificity for binding hydrophobic proteins compared to other plasma glycoproteins (63). This is illustrated by recent novel observations in our laboratory that AAT binding to LTB₄ and IL-8 inhibits its interaction with cognate receptors BLT1 and CXCR1.
respectively, thereby mediating anti-inflammatory effects through down-regulation of neutrophil recruitment and activation (85,283).

4.4.2 AAT in the coagulation system

In this chapter with have demonstrated the association of AAT with fibrinogen, antithrombin III, and coagulation factors V, XI and XII. The coagulation cascade is a tightly regulated process and many of the activated coagulation factors are serine proteases. It has been shown previously that AAT accounts for the majority of the plasma inhibition of Factor Xla (254). However, this has been challenged by the role of other serine protease inhibitors such as C1-inhibitor and α2-antiplasmin through the measurement of Factor Xla:protease inhibitor complexes in blood (255). Nevertheless, the fact that AAT can bind to fibrinogen in blood (265,287), and is found in significant proportions within formed clot samples (289), indicates that there is a function for AAT not only in the inhibition of clot propagation through control of the coagulation cascade but also fibrinolysis and the homeostasis of thrombosis (290).

It has been demonstrated that there is reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases (291). Thrombosis is an important facet of the innate immune response as an additional mechanism to prevent the propagation of microbial invasion (292). Local AAT inactivation may lead to a microenvironment that favours thrombosis, which in the setting of localized infection may be favourable however uncontrolled thrombosis within the circulation is deleterious to health. Disseminated intravascular coagulation is a fulminant disease process with a high mortality and previous studies have eluded to the important role that AAT may exert in the humoral response to this devastating complication (290).

It has been postulated that localized fibrin formation may contribute to the pathogenesis of pulmonary emphysema due to increased platelet aggregation
potentiating thrombosis leading to a favourable microenvironment for neutrophil attachment (293,294). Of particular interest is the role that AAT may play in the protection of fibrinogen form dysregulated proteolysis, particularly by neutrophil derived proteases (295,296). A recent study evaluating a specific NE cleavage point in fibrinogen (Aα-Val360) demonstrated that increased fibrinogen cleavage correlated with disease severity in AATD and may be a useful surrogate marker of disease activity in patients with early disease in whom therapeutic intervention may be indicated (297).

The results in this chapter demonstrating AAT binding to fibrinogen is supported by early published literature in this area, the biological plausibility that AAT functions as an anti-protease buffer to protect fibrinogen from dysregulated proteolysis is supported by clinical studies in AAT deficient individuals (297). The study of the interaction of AAT with fibrinogen points to an important biological mechanism of the role that AAT may play as a circulating binding partner to other proteins identified in this chapter.
4.4.3 AAT in the lipoprotein system

In this study we have identified apolipoprotein B-100 (ApoB100), a major protein component of LDL and VLDL, as a binding partner to AAT. Lipoproteome analysis of the process of VLDL to LDL conversion has demonstrated that AAT is acquired from plasma, other lipoprotein classes, or peripheral cells (298).

Our data is in concordance with previously published data where an estimated 1% of plasma AAT has been shown to co-elute with ApoB100, furthermore this fraction was found to be complexed within LDL. From a pathophysiological perspective, the impact of oxidation of AAT on the modulation of binding was illustrated by the formation of AAT:LDL complexes which have been demonstrated in atherosclerotic plaque within the arterial wall implicating a role for oxidized AAT in atherogenesis (101).

Furthermore the finding in this chapter that apolipoprotein A-1 (ApoA1) and apolipoprotein-E (ApoE), other major constituent proteins in lipoprotein particles, co-eluted with AAT supports its role in lipid metabolism. This may have a particular effect during the acute inflammatory response which is characterized by changes in apolipoprotein synthesis (6,299). Moreover, it has been recently reported that enrichment of AAT with HDL afforded better protection against elastase-induced pulmonary emphysema in a murine model than AAT augmentation therapy alone (179). This synergistic effect, which is not explained sufficiently by anti-protease activity alone, may underscore a physiological reason for this association and could have implications for the design and administration of AAT augmentation therapy in the future.
4.4.4 AAT in the immune response

In this study we have identified the immunoglobulin heavy chains as a major binding partner to AAT. It has previously been reported that AAT has a strong affinity for monomeric light chain thiolate ions and this may constitute a mechanism for the linkage and transport of peptides with reactive thiols or disulphides released into plasma and extracellular fluids. It has been demonstrated that in vivo complexes between immunoglobulin-κ chains can occur without affecting protease inhibitory capacity (266). In myeloma patients, between 3-25% of AAT in plasma has been shown to co-elute with IgA (270). In addition, there has been significant interest in the role of AAT in the inflammation associated with rheumatoid arthritis, particularly in relation to the formation of IgA:AAT complexes in plasma (300), and in the inflammatory milieu of the synovial fluid of individuals with inflammatory arthritis (159,301). This association may relate to the glycosylation status of AAT in plasma during the acute phase response in inflammatory arthritis (302). However there is insufficient evidence to date to indicate that AAT levels correlate with disease activity (303), or indeed that AATD is an independent risk factor for inflammatory arthritis.

4.4.5 AAT and tissue repair healing

Fibronectin was found to be a binding partner to AAT in this study; fibronectins have a broad in vivo activity in relation to cell adhesion, cell motility, opsonisation, maintenance of cell shape, and wound healing. The association of AAT with fibronectin has previously been investigated; it has been shown that AAT protects fibronectin from enzymatic degradation in wound tissues and is necessary for wound healing, this is due to the presence of proteolytically active NE in chronic wounds but not healing wounds (304,305).
4.4.6 AAT in the complement system

The finding in this chapter that AAT can bind to proteins of the complement system is of particular interest, specifically the possibility that AAT can bind to complement component C3. The cleavage of C3 into the effector molecules of the innate immune response, C3b and the C3a anaphylatoxin, is the central point of convergence of the complement cascade.

Products of complement activation, C3a and C5a, are important neutrophil chemoattractants and complement activation products have been found to be elevated in emphysema (306). There is a paucity of published literature in relation to the role of the complement system and AATD. A more detailed review of AAT and the complement system will be the subject of further experimental evaluation and discussion in the forthcoming chapters.
4.4.7 Important points for consideration:

There is no evidence that the experimental techniques employed in this study led to the induction or reduction of AAT binding to other proteins, however there are a number of important points to consider in the interpretation of the results from this study.

Co-purification of non-target proteins is a recognized drawback of affinity-based protein purification methods, however it remains a robust and selective isolation technique (307). Multiple wash steps were performed during the experiments to reduce contamination and assist in the purification of the bound AAT to the resin, however aggressive washing was avoided to reduce the risk of inadvertently removing any candidate binding partners. It is possible that contact activation of the coagulation, immunoglobulin, and complement systems may have occurred by the addition of AAT select resin to plasma, with resultant non-specific binding to the immunoglobulin ligand of the resin occurring in vitro. However, in mitigation against this, the experimental methods employed in this chapter support the previously published literature on this topic as discussed above. The purification of AAT from plasma has the effect of concentrating the protein to the resin surface to an extent far greater than what can occur naturally in vivo. Non-specific binding events are therefore more likely to occur at the surface of the concentrated AAT protein and the bound protein profile may not reflect what occurs in the native state within the circulation.

Nevertheless, our aim was to determine the spectrum of abundant protein binding partners that can bind to AAT in the circulation and this was achieved. Both in-solution and in-gel MS analysis was performed on the M- and Z-AAT protein as an additional step to further resolve the protein profile. There was significant concordance between these results. In support of the methodologies employed in this work, some of the proteins determined to bind AAT have been previously described as outlined in the discussion above, in particular fibrinogen, apolipoprotein B-100 and albumin (101,265,308) (see Table 4.1).
Mass spectrometry is a highly sensitive technique, it permits in-depth characterization of the protein components and is an excellent tool for hypothesis generation (309). Often MS techniques lead to unexpected protein associations and functional characterization within complex biological systems; the data obtained from this analysis indicates what proteins are present in the purified samples with a high degree of confidence though it does not demonstrate actual binding events occurring between the candidate proteins. Complementary methodologies are required to confirm if any inferred protein interactions with AAT based on mass spectrometry analysis are indeed occurring, in the case of the novel interaction between AAT and C3, additional experimental evaluation was performed to confirm this interaction and furthermore to determine what the biological significance of this interaction may be. This will be the subject of experimental evaluation in the forthcoming chapter.
4.5 Conclusion:

In summary, the experiments in this chapter have advanced our current understanding of the role of AAT in plasma; we have established that a significant proportion of AAT is bound to other abundant plasma proteins in the circulation and that this interaction is electrostatic in nature. Despite markedly lower circulating plasma AAT levels in deficiency states, a greater proportion of Z-AAT protein is bound to other abundant proteins in plasma, however the profile of binding partners is similar to M-AAT. Utilizing novel methodologies to purify both the M-AAT and Z-AAT proteins, we have established that AAT may interact with proteins belonging to multiple pathways in plasma: the coagulation system; lipid transport; immunoglobulin system; and the complement system.

Determination of the full spectrum of protein binding partners is an important advance in our knowledge of the many facets through which AAT exerts its effects in health. Perturbation of this binding function, through alterations in the protein structure, glycosylation status, or oxidation, may in part account impaired host homeostatic mechanisms for the resolution of inflammation and contribute to the pathogenesis of the pulmonary disease phenotype, particularly in deficiency states.

The results of this chapter lead us to examine the function of AAT in a number of different plasma protein systems. Importantly this work may uncover a novel mechanism for the pathogenesis of emphysema in AATD and point to new ways regarding how AAT augmentation therapy exerts anti-inflammatory effects that may be applicable in a myriad of disease processes beyond protease inhibition. In particular these results present us with the fascinating hypothesis that AAT may bind to C3, this interaction may have an important effect in the inhibition of neutrophil-derived proteolysis of C3 and protect against dysregulated activation of the complement cascade. The interaction of AAT with C3 and the consequent implications for AATD will be the subject of the work in the forthcoming chapters.
Chapter 5

5.1 The interaction of AAT with complement component C3
5.1.1 Introduction

The finding in Chapter 4 that a significant proportion of AAT circulates within a high molecular weight fraction bound to a diverse range of abundant plasma proteins provides new insight into the function of AAT in plasma. Importantly with regard to the role of AAT in the innate immune response, the revelation by mass spectrometry that AAT may bind to complement component C3 requires confirmation.

5.1.2 Complement component C3

Complement component C3 has a molecular weight of 187kDa and is the most abundant complement protein in plasma (mean 1.2 mg/ml). C3 belongs to the α2-macroglobulin family of proteins and was first recognized and characterized in 1960 (310). C3 contains three potential N-linked sites, though only two oligomannose residues are attached to the protein at Asn 917 and Asn 63, which contribute to 1.5% of the final molecular weight (311). The restricted repertoire of oligomannose structures on C3 suggests that the glycosylation sites are relatively inaccessible. Indeed, the glycosylation status of C3 plays a key role in the differentiation of the protein from other complement proteins such as C5, which share similar structural homology. The interaction of C3 and its activated products with target surfaces can be modulated by the presence or absence of glycan residues on regulatory proteins such as Factor H (FH) (312).

The production and regulation of C3 mirrors that of AAT as C3 is mainly synthesized by hepatocytes (313), in addition to macrophages (314) and vascular endothelial cells (315). In addition to resident macrophages in the lung, C3 is produced by type II pulmonary alveolar cells (316,317) and by respiratory epithelial cells (318), which contribute significantly to the local host pulmonary immune response prior to plasma exudation. C3 is an acute phase protein with levels rising two to four fold during an acute inflammatory response whereby its
synthesis is up-regulated by pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNFα but not IFNγ (6,318).

The structure of human C3 and its fragments has been solved (Figure 5.1) and this has provided insight into the function of the protein during complement activation (319). The electrostatic surface charge of C3 has been determined to be bipolar in nature (320). C3 is comprised of an α-chain and β-chain that are linked by non-covalent forces and a disulphide bond. Together these chains form 13 domains which under cleavage and structural rearrangement during proteolysis (Figure 5.2). Close to the surface of the C3 there is a highly reactive thioester that is protected within a pocket of the intact molecule from spontaneous reactions with water and other nucleophiles (321). The functions of C3 are dependent upon the exposure within C3 of binding sites for other serum proteins or for membrane C3 receptors. In addition to steric inaccessibility of the oligosaccharide residues, none of the internal binding sites are available on native C3 and it is relatively inert in blood.
Figure 5.1 Representative structure of C3

A. Structure of native C3 with domains coloured by α-chain (purple) and β-chain (green).
B. Ribbon representation of native C3 (13 domains), the intact thioester (red spheres and arrow), anchor region (grey) and a’ N-terminal (black) are also shown.
C. Electrostatic surface potentials (red for negative, blue for positive) shown between −12 and +12 kT as measures of thermodynamic entropy. Figure was prepared with GRASP (Graphical Representation and Analysis of Surface Properties)11.

Figure adapted from Janssen BJC, et al. Nature 2006 (319).
Figure 5.2 Representative structures of C3 during activation and surface binding

A. Domain sequence and arrangements in C3. The colour scheme matches in the primary and tertiary structures. Shown are the thioester site (white triangle – dashed line) within the TED domain, disulphide bridges, glycan positions (ASN63 and ASN 917) and multiple cleavage sites. The cleaved products of the C3 molecule are depicted below the domain sequence in respect to their structural relationship to the native molecule.

B. Shown schematically are the four stages: C3, C3b with C3a, iC3b with C3f and C3dg with C3c. On complement activation the thioester site becomes exposed and attached to the target surface (red circle - black arrow). These conformational changes determine the binding affinities towards soluble proteins (for example, factor B, properdin and factor H) and cell-surface receptors (for example, CR1-4, CR1g, DAF and MCP) that underlie their biological activity.


CR, complement receptor. CR1g, complement receptor immunoglobulin. DAF, decay accelerating factor. MCP, membrane cofactor protein.
5.1.3 Biological effects of the complement system

C3 occupies a central position in the complement-activation process and its cleavage products are the key effector molecules of the complement system and are critical for any downstream activation of the terminal complement cascade and membrane attack complex formation to protect the host against invading pathogens or altered self-structures. Cleavage of C3 is mediated by enzyme complexes (C3 convertases), which initially generate two major protein fragments C3b (177kDa) and C3a (9kDa).

C3a mediates its effects through binding to the G protein–coupled chemoattractant receptor C3aR on a wide range of inflammatory cells including mast cells, eosinophils, basophils and lymphocytes, as well as smooth muscle cells (322). It is often referred to as an anaphylatoxin due to its ability to stimulate mast cell degranulation, histamine release and stimulate smooth muscle contraction. In addition, direct antimicrobial properties of C3a have been uncovered due to its amphipaticity, cationicity, and helical structure (323) and it has been shown to efficiently kill gram positive and gram negative bacterial species (324).

Nascent C3b is able to bind covalently to cell and other target surfaces via the exposed thioester, this thioester bond (Cys 988) undergoes transformation into a free thiolate anion, which reacts readily with available hydroxyl groups and to a lesser extent amino groups (325). The metastable C3b is promiscuous in that it is unable to differentiate self from non-self structures. Complement activation results in the indiscriminate deposition of C3b with only 10% reaching target tissues with a half-life of approximately 60-90μs (326). It is rapidly inactivated in the fluid phase by water molecules, non-specific protein interactions, and by FH family proteins (325,327). The carbohydrate environment of the surface on which C3b fragments are deposited, determines the relative affinity of C3b for proenzymes Factor B (FB) or FH. On the host cell surface that is abundant in polyanions such as sialic acid, FH binds to C3b with higher affinity than does FB.
and halts the formation of C3 convertase thereby terminating complement activation (328-330). Most pathogenic microbes have a carbohydrate-rich cell wall surfaces that favours FB binding to C3b with a greater affinity than FH. This leads to amplification of the complement cascade and enhanced C3b opsonisation of microbial target surfaces, this is further catalysed by Factor D and inhibited by Factor I (331). The opsonized microbe is now a target for phagocytosis through the interaction with membrane bound complement receptors on host immune cells.

Excessive local activation of complement in the presence of the catalytic subunit of FB, Bb, results in the formation of the C5 convertase (C3bBbC3b and C4bC2aC3b) which cleaves C5 into C5a, a structurally similar but more potent anaphylatoxin and leucocyte chemoattractant than C3a (332). Formation of C5b is the key enzymatic event required for the assembly of the membrane attack complex, which disrupts the phospholipid bilayer of target cells resulting in cell lysis and death as part of the terminal complement cascade.

The importance of the complement system is not restricted to innate immunity; C3 opsonisation is necessary for the adaptive immune response to viral illness such as influenza (333), whilst C5aR has been shown to be essential for the modulating effect of complement on T-cell immunity in various models (334). Products of complement activation serve an important role in modulating the adaptive immune response, in particular C3d is molecular adjuvant of innate immunity that may bridge the innate and adaptive immune response (335,336).
5.1.4 Interaction of C3 with AAT

Given the pro-inflammatory properties of C3 activation byproducts and the role of AAT in counterbalancing neutrophil-driven inflammation it is not unexpected that these two abundant plasma proteins may interact. A recent abstract publication has indicated that C3a may bind to AAT, which may have implications for downregulation of complement-mediated inflammation (337). It has also recently been shown that C3b interacts with a range of plasma proteins including AAT, vitamin D binding protein, and α1-acid glycoprotein, by forming high molecular weight aggregates through covalent interactions in complement activated serum and plasma (338). The function of these aggregates is not fully understood at this time and whether they occur in blood in the presence of erythrocytes is not known. It is postulated that role of C3b binding to denatured protein fragments is to remove them from the circulation without inducing inflammation through clearance by apoptotic pathways (339).
5.2 Hypothesis

The experiments conducted in this chapter set out to verify the ability of AAT to bind C3 and explore the potential functional implications of this finding in plasma.

The aims of this chapter were:

1. Determine if an interaction between AAT and C3 can be detected in plasma of healthy PiMM individuals.
2. Confirm if both the M-AAT protein and Z-AAT protein can bind to C3.
3. Assess if the glycosylation status of AAT influences C3 binding.
4. Determine a functional effect of AAT binding to C3 in health and deficiency states.
5.3 Results

5.3.1 Determination of the elution profile of C3 in plasma

The elution profile of AAT determined in Chapter 4 demonstrated two distinct elution peaks at approximately 300kDa and at the predicted molecular weight of 50kDa. C3 (187kDa) was found to bind AAT in the high molecular weight elution fraction by mass spectrometry. To evaluate the elution profile of C3, size exclusion gel permeation chromatography was utilized. The eluted fractions were visualised on 12.5% SDS PAGE gels by Coomassie staining and by western blot using rabbit anti-C3 antibody, see Figure 5.3D. Under reducing conditions the disulphide bond between the α and β chain of C3 is removed yielding two bands, the first at 115kDa (α-chain) and second at 70kDa (β-chain). Densitometry of C3 immunobands reveals an elution peak at 250kDa (n=6). The co-elution profile of C3 in plasma demonstrates overlap with AAT between fractions 21 and 28, corresponding to a predicted molecular weight range of 323kDa and 190kDa, and provides further evidence that these two proteins may interact in plasma, see Figure 5.3E.
Figure 5.3: Visualisation of the elution profile of M-AAT and C3.

A. Size exclusion gel permeation chromatography of PiMM plasma using Dulbecco’s PBS running buffer. The elution chromatogram is shown at 280nm using the AKTA Prime+ FPLC system.

B. Coomassie Blue stained SDS-PAGE gels (12.5%) showing the elution profile of fractions 14 – 35.

C. SDS-PAGE and western blot analysis of the corresponding fractions probed with goat polyclonal anti-AAT antibody (1:5000 dilution). Two distinct elution peaks were detected at 298kDa (fraction 23) and at 50kDa (fractions 33) as described in Chapter 4.

D. Visualisation by western blot analysis of the elution profile of C3 using rabbit anti-C3 antibody (1:2500) on the corresponding fractions. C3 eluted at a single elution peak at 234kDa (fraction 24), two immunbands were seen at each fraction corresponding to the α- and β-chain of C3.

E. Densitometry of SDS-PAGE anti-AAT and anti-C3 western blot analysis of MM plasma eluted in DPBS (n=6) demonstrating co-elution of the protein profiles supporting the presence of AAT complexed with C3. Each point represents a mean value ±SEM.
5.3.2 Co-immunoprecipitation of complement C3 with AAT from PiMM plasma.

The initial finding of C3 binding to AAT was detected using an affinity chromatography medium (AAT Select), the properties of which were exploited to co-immunoprecipitate bound proteins to AAT. To confirm the binding of C3 to AAT, a second co-immunoprecipitation experiment utilising an alternative methodology was performed. An AAT immunoprecipitation column was made by coupling goat polyclonal anti-AAT to HiTrap NHS-activated HP columns (GE Healthcare). Another immunoprecipitation column was made by coupling goat IgG antibody to a second HiTrap column to act as an isotype control. Plasma from healthy PiMM individuals (n=3) was allowed to flow into each column bed and then incubated for 1h at 37°C. Following a number of wash steps, glycine elution was performed and the eluted fractions were collected through an AKTA Prime+ system. Each fraction was then visualised on SDS-PAGE analysed by western blot for AAT and C3, see Figure 5.4. Utilising densitometry measurement of immunobands, a significantly greater amount of C3 co-eluted with AAT from the AAT immunoprecipitation column compared to control, two-tailed p-value = 0.0313 (n=3).
Figure 5.4: Co-immunoprecipitation of complement C3 with AAT from PiMM plasma.

Goat polyclonal anti-AAT antibody and Goat Ig Control were coupled to HiTrap NHS-activated HP columns (GE Healthcare). PiMM plasma (1mL) was then loaded onto the column bed (n=3) and incubated at 37°C for 30min. Glycine elution (pH 2.3) of bound proteins was performed and samples collected for SDS-PAGE and western analysis. Upper panel depicts increased elution from the anti-AAT column vs. the control column by western analysis (rabbit polyclonal anti-AAT antibody 1:5000). The lower panel and bar graph depict corresponding increased co-immunoprecipitation of C3 with AAT by western analysis (rabbit polyclonal anti-C3 antibody 1:2500) indicative of C3:AAT binding in plasma. The bar chart is representative of the densitometry values of the experiment in triplicate, two-tailed p-value = 0.0313.
5.2.3 Binding of AAT polymers to complement C3.

It is recognised that immunoprecipitation of AAT results in an increased local concentration of the protein, which can promote the formation of polymers (31). The increasing concentration of AAT as it binds to the AASR, may result in the formation of AAT polymers on the resin surface. This induced conformational change of AAT could potentially account for increased binding events to C3 as a result of AAT polymer formations. To investigate if C3 binding to AAT was a result of an interaction with AAT polymers, native PAGE electrophoresis was performed. There was no evidence of C3 binding to M-AAT polymers, see Figure 5.5.
Fig. 5.5. AAT polymers and C3 binding assessed by native gel electrophoresis

- NativePAGE gel stained with G250 Coommassie blue demonstrating the native profile of AAT in lane 1, C3 in lane 2 and the combined profile of AAT+C3 in lane 3 following a 30min incubation period at room temperature.
- Western blot of the same native gel using rabbit polyclonal antibody to C3 (Abcam) 1:5000, which demonstrates a C3 monomer in lane 2 without any evidence of C3 polymerisation. There is no evidence of C3 overlapping with AAT polymers in lane 3.
- Western blot using goat polyclonal antibody to AAT (Abcam) demonstrating multiple M-AAT polymers in lane 1, with an identical profile is seen in lane 3. This experiment illustrates that there is overlap between the monomeric form of AAT and C3, but not AAT polymers.
5.2.4 Binding of AAT to complement C3.

To further investigate the binding properties of AAT to C3, flow cytometry (FACS) analysis was employed as another experimental methodology. Firstly, 100μL of 10μm microsphere polystyrene beads (Polybeads®) were incubated overnight with 120μg of AAT (Athens Research) at 4°C in Voller’s Buffer. Secondly, 5μg of C3 (Abcam) was incubated with AAT-bound beads or control beads for 1h at room temperature, then washed and blocked for 1h in 1% (w/v) BSA. Following incubation with anti-C3c FITC labelled antibody (Abcam) for 30min, fluorescent detection of the labelled beads was performed by FACS analysis employing 10,000 counts in triplicate, see Figure 5.6. The results of this experiment indicated a marked increase in the fluorescence detection of C3 binding to the AAT-bead compared to the control bead. The binding of AAT to C3 was thus confirmed by flow cytometry in addition to the two previous immunoprecipitation experiments.

![Graph showing binding of AAT to C3](image)

**Figure 5.6: Binding of AAT to complement C3**

Flow cytometry (FACS) analysis of C3 binding AAT-bound beads (n=3); Fluorescent detection was performed with anti-C3c FITC labeled antibody by FACS up to 10,000 counts (in triplicate). The purple peak is the isotype control, the grey line indicates control bead binding events, and the green line indicates 5μcontrol, the grey line indicates control fluorescence detection of C3 binding to the AAT-bead compared to the control bead.

FITC = Fluorescein isothiocyanate.
5.3.4 Dose response and competitive inhibition of C3 binding to AAT-beads

To further evaluate the effect of incremental amounts of C3 binding to AAT-beads, repeat FACS analysis was performed. 100μL of 10μm microsphere polystyrene beads (Polybeads®) were incubated overnight with 120μg of AAT (Athens Research) at 4°C in Voller’s Buffer. Incremental doses, from 0 to 5μg, of C3 (Abcam) were incubated with AAT-bound beads or control beads. Fluorescent detection was performed with anti-C3c FITC labelled antibody by FACS employing 10,000 counts (in triplicate), Figure 5.7A. As indicated in Figure 5.6B, the results demonstrate a stepwise increase in the amount of C3 bound to the AAT-coated beads.

In a subset of experiments, a pre-incubation step was performed by incubating 50μg AAT and 5μg C3 (10:1) for 1h at room temperature in DBPS. The solution was then incubated with the AAT and control beads as previously described and the mean fluorescence of the bound C3 was measured by FACS analysis. A significant reduction in the binding of C3 to the AAT-bead to approximately one tenth of control 5μg fluorescence was observed, see Figure 5.7B. These results confirm competitive inhibition of C3 binding due to pre-formed AAT:C3 complexes within the solution.
Figure 5.7: Binding of AAT to complement C3

A. Flow cytometry (FACS) analysis of C3 binding AAT-bound beads (n=3). 100μL of 10μm microsphere polystyrene beads (Polybeads®) were incubated overnight with 120μg of AAT (Athens Research) at 4°C in Voller’s Buffer. Incremental concentrations of C3 (up to 5μg) (Abcam) was incubated with AAT-coated beads or control beads, which were not coated with AAT but were treated in an identical fashion. Fluorescent detection was performed with anti-C3c FITC labeled antibody by FACS employing 10,000 counts (n=3). The purple line indicates control bead events without AAT coating, the green line indicates 5μg C3. Pink line represents detection of 5μg C3 following a pre-incubation step with 50μg AAT indicative of competitive inhibition of C3 binding to the AAT-labelled beads.

B. Bar chart representation of the FACS data showing a dose response relationship between fluorescent detection and C3 concentration bound to the AAT-coated beads. Pre-incubation of 5μg C3 with AAT results in a marked competitive inhibition of binding event to the AAT-coated beads.
Figure 5.3.5 Plasma purified M-AAT and Z-AAT bind to C3.

The results of the mass spectrometry analysis in Chapter 3 identified C3 as a potential binding partner to AAT purified from both PiMM and PiZZ individuals. Any potential difference in the binding of C3 to either form of the AAT protein may have implications for the role of any interaction between AAT and C3. To assess the binding of plasma purified M-AAT and Z-AAT to complement component C3, 30μg of C3 (Sigma) was coupled to 50μL of HiTrap NHS activated beads (GE Healthcare). Processing and fixing the same volume of HiTrap beads without the addition of protein generated the control beads for this experiment. 10μg of plasma purified M-AAT or Z-AAT was incubated with C3-bound or control beads for 1 hour at 25°C with agitation (in triplicate). Unbound protein was removed during multiple wash steps. The beads were then resuspended in reducing sample buffer and analysed by SDS PAGE western blot analysis with polyclonal anti-AAT antibody. Representative densitometry values of the experiment in triplicate are shown in Figure 5.8, and there was significantly increased binding of both M-AAT and Z-AAT to the C3-labelled beads (two-tailed p-value for M-AAT = 0.04 and Z-AAT = 0.03). There was no significant difference between the ability of M-AAT and Z-AAT to bind the C3-labelled beads.
Figure 5.8: Plasma purified M-AAT and Z-AAT bind to C3.

Complement C3 (30μg, Sigma) was coupled to 50μL of HiTrap NHS activated beads (GE Healthcare). Plasma purified M-AAT or Z-AAT (10μg) was incubated with C3-bound or control beads, that were not coated with C3, for 1 hour at 25°C (n=3). Unbound protein was removed with multiple wash steps. The beads were then resuspended in reducing sample buffer for SDS PAGE and western analysis. The upper panel demonstrates detection of AAT bound to C3 and control beads (goat polyclonal anti-AAT antibody 1:5000). The bar chart is representative of the densitometry values of the experiment in triplicate, two-tailed p-value for M-AAT = 0.04 and Z-AAT = 0.03. There was no significant difference between M-AAT and Z-AAT binding to C3-labelled beads.
5.3.6 Comparative binding of C3 to purified M-AAT and rAAT

The role of glycosylation has been discussed earlier in Chapter 3, in particular the role of glycan residues in effecting protein-protein interactions through binding to other glycan residues, binding to the amino acid backbone, or though electrostatic interactions. Recombinant AAT (rAAT) lacks the three carbohydrate residues that are found on both M-AAT and Z-AAT. To evaluate if the binding interaction of C3 with AAT is mediated through the glycosylation status of AAT, we performed FACS analysis of C3 binding to plasma purified glycosylated M-AAT beads compared to commercially available non-glycosylated rAAT. Microsphere polystyrene beads (100μL of 10μm Polybeads®) were incubated overnight with 120μg of M-AAT or rAAT at 4°C in Voller’s Buffer. Firstly, C3 (Abcam), 5μg, was incubated with M-AAT beads or rAAT beads. Then fluorescent detection by FACS was facilitated by the use of anti-C3c FITC labelled antibody employing 10,000 counts (in triplicate). The results of this experiment, shown in Figure 5.9, demonstrate a 50% reduction in the measured fluorescence of bound C3 to rAAT compared to M-AAT (n=3, p<0.001). This indicates that the glycosylation status of AAT accounts for a significant proportion of C3 binding, however the absence of the glycan residues did not obliterate the occurrence of binding.
Figure 5.9: Comparative binding of C3 to purified M-AAT and rAAT.

A. Flow cytometry (FACS) analysis of C3 binding AAT-bound beads. 100μL of 10μm microsphere polystyrene beads (Polybeads®) were incubated overnight with 120μg of AAT (Athens Research) at 4°C in Voller’s Buffer. 5μg C3 (Abcam) was incubated with AAT-bound beads or control beads. Fluorescent detection was performed with anti-C3c FITC labeled antibody by FACS up to 10,000 counts (in triplicate). Purple line indicates control bead events, green line indicates 5μg C3 bound to M-AAT and redline indicates 5μg C3 bound to rAAT. There was a 50% increase in C3 binding to M-AAT compared to rAAT as assessed by immunofluorescence.

B. Bar chart representation of the FACS data showing increased binding of C3 to plasma purified M-AAT compared to rAAT labelled beads.
5.3.7 Complement proteolysis by NE

The experiments performed in this chapter have confirmed the binding of C3 to AAT; however the significance of this interaction has not yet been elucidated. AAT is the principal inhibitor of neutrophil derived proteases, in particular NE. C3 is subject to dysregulated proteolytic cleavage during inflammation and coagulation, in addition the degradation products of NE cleavage of C3 have been well characterized (340,341). The potential role of AAT binding to C3 may be to buffer C3 from dysregulated proteolysis by neutrophil derived proteases released during inflammation.

Firstly, the degradation products of C3 proteolysis were visualised in the presence of NE. Figure 5.10A depicts a silver stain of a non-reducing 12.5% SDS PAGE gel demonstrating C3 in its native form in lane 1. The digestion products of C3 proteolysis are depicted in lane 2 following incubation with NE for one hour at 37°C. A number of proteolytic bands are seen, the first at 148kDa corresponding to a C3c containing fragment. A second band at 33kDa corresponds to a C3d fragment. A band at approximately 9kDa is also seen which corresponds to the C3a analogue peptide.

AATD provides an excellent in vivo model to evaluate the effects of marked deficiency of circulating AAT protein. Exogenous NE was added to 1% plasma from healthy PiMM and PiZZ individuals (n=3) and incubated for one hour at 37°C to evaluate C3 proteolysis. Western blot of non-reducing 12.5% SDS PAGE gel was performed using polyclonal rabbit anti-C3 antibody. There was no formation of C3 proteolytic fragments detectable in control PiMM plasma with DBPS or in PiMM plasma with the addition of 10μg/mL NE, see Figure 5.10B. There were no bands of C3 proteolysis detectable in control PiZZ plasma, however with the addition of 10μg/mL NE to the PiZZ plasma a number of products of C3 proteolysis were detected (Figure 5.9B) that correspond to the fragments detected in Figure 5.9A. This indicates that the plasma of PiZZ has limited buffering capacity against the proteolytic actions of NE compared to
PiMM control plasma. These results indicate that the protease inhibitory capacity of AAT may play an important role in the protection of C3 from dysregulated proteolysis.

Figure 5.10: Complement proteolysis by NE

A. Silver stain of a 12.5% non-reducing SDS PAGE gel demonstrating C3 in its native form in lane 1. The digestion products of limited C3 proteolysis are depicted in lane 2 following incubation with NE for one hour at 37 °C. A number of proteolytic bands are seen; one at 148kDa corresponding to a C3c containing fragment. A second band at 33kDa corresponds to a C3d fragment. A band at approximately 9kDa corresponds to the cleaved C3a-des arg.

B. Western blot of 12.5% non-reducing SDS PAGE gel of using polyclonal rabbit anti-C3 antibody. This demonstrates no formation of C3 fragments between in control PiMM plasma (lane 1) or with the addition of 10μg/mL NE (lane 2). There are no detectable bands of C3 proteolysis in control PiZZ plasma (lane 3), however with the addition of 10μg/mL NE the products of C3 proteolysis are detected (lane 4). This indicates that the plasma of PiZZ has limited buffering capacity against the proteolytic actions of NE compared to PiMM control.
5.4 Discussion:

In summary, this body of work forms a comprehensive evaluation of the binding of AAT to native C3. Co-elution of AAT and C3 in healthy PiMM plasma was demonstrated and C3 was shown to co-immunoprecipitate with AAT from PiMM plasma. Furthermore, the binding of C3 to AAT occurs in a dose dependent manner and is subject to competitive inhibition in the fluid phase. Equal binding of C3 to plasma purified M-AAT and Z-AAT was observed, however the absence of AAT glycan residues partially abrogated the degree of binding. In the presence of similar concentrations of exogenous NE, C3 underwent proteolysis in PiZZ but not PiMM plasma underscoring a potential role for AAT binding to protect against dysregulated C3 proteolysis.

The primary aim of this chapter was to firmly establish the binding of AAT to C3 based on earlier mass spectrometry findings in this thesis. Secondly the role of this binding interaction was evaluated.

There is very little published on the interaction of AAT with proteins of the complement system. It is interesting to note that many of the properties of the complement system, in particular the role of products of C3 cleavage as key effector mechanisms of the innate immune response, contrast to the anti-inflammatory properties of AAT that have been documented in recent years.

Early studies demonstrated that AAT could inhibit complement-mediated erythrocyte rosette formation mediated through its carbohydrate moieties, though a direct interaction between AAT and C3 was not demonstrated using radiolabelled products (342). Furthermore, AAT induced a dose dependent inhibition of complement-mediated phagocytosis of opsonized yeast particles mediated through its glycan residues (343). Importantly these authors made an early and astute observation regarding a role for AAT distinct from its anti-protease activity. However, given the limited understanding of the regulation of complement at that time, the relevance of the interaction of C3b with the many
complement control proteins that exist was not recognised. In particular, the findings are consistent with non-specific steric inhibition by AAT carbohydrate residues that is a feature of other glycoproteins that were not studied. More recent studies contradict the conclusion that AAT may impair the complement-mediated phagocytosis drawn from these *in vitro* experiments. Within a complex *in vivo* environment AAT plays a protective role during tissue inflammation in the lung by offsetting the effect of abundant neutrophil-derived proteases which can severely impair C3-mediated microbe phagocytosis (344).

The interaction of AAT with C3, and other proteins of the complement system, appears to be multifaceted and relate to inflammatory factors with the host microenvironment:

- At sites of inflammation AAT may prevent dysregulated proteolysis of C3 and preserve host complement function.
- AAT may prevent the cleavage of complement receptors from the surface of host phagocytes thereby protecting against ‘opsonin-receptor mismatch’.
- In the presence of excess complement activation within plasma, AAT may function as a non-specific humoral inhibitor of C3 activation fragments in the fluid phase. AAT may provide important functional redundancy to the complement system despite the presence of other primary inhibitors of C3 activation. The high circulating plasma concentration of AAT in health and increased tissue concentrations during inflammation may prevent the potentially harmful humoral expansion of complement activation.
- Increased sialylation of AAT, particularly with the increased expression of the Sialyl Lewis X determinant during inflammation (23), may affect the interaction of AAT with C3b by enhancing complex formation with FH and arresting C3 cleavage.
- Furthermore, upward humoral inhibition of complement activation is known to focus complement deposition downward at the surface of target microbes thereby enhancing opsonisation (345).
5.4.1 Proteolytic degradation of C3 by neutrophil-derived proteases

C3 can be cleaved by neutrophil derived proteases, including NE, CathG and PR3, to activate complement and by the generation of fragments with C3 convertase activity, though the full extent of biological activity is not maintained (340). Proteolysis of C3 into smaller fragments by neutrophil elastase was first demonstrated 40 years ago (346). Characterization of the structural and functional properties of the proteolytic fragments of C3 reveals that in the presence of relatively low molar concentrations, C3 undergoes limited proteolysis resulting in the release of a 9kDa fragment and a second 148kDa fragment comprised of the β-chain and a number of fragments from the α-chain analogous to C3c (340). In the presence of higher molar concentrations the 148kDa fragment is cleaved at a further four sites resulting in the production of a number of smaller fragments. Amino acid sequencing of the C3 9kDa fragment demonstrates structural similarity to C3a, however it lacks any demonstrable anaphylatoxin properties. This is due in particular to the absence of the COOH-terminal position arginyl residue, this residue is essential for C3a anaphylatoxin activity (347). Endogenous plasma carboxypeptidase B activity targets the arginyl residue at this site and is the mechanism responsible for C3a inactivation in plasma (347). The resultant C3a-desarg molecule is also known as the acylation stimulating protein and is an important adipokine that is involved in the regulation of triglyceride synthesis (348).

The second 148kDa fragment is structurally similar to iC3b/C3c; it has opsonic activity and can contribute to further complement activation, though it retains approximately 15% of its functional properties compared to the C3b molecule formed by C3 convertase activity (340).

It has been demonstrated that cleavage of membrane bound C3bi by neutrophil proteases can occur on surfaces of the microbial target (349,350). To compound this effect, excessive protease activity in the lung can also affect complement opsonin function by cleavage of elastase-sensitive CD11b (CR1) from the neutrophil membrane with resultant impaired opsonophagocytosis. The concept
of an ‘opsonin-receptor mismatch’ was identified as a pathogenic mechanism in cystic fibrosis airway disease due to the high protease burden that contributed to impaired opsonophagocytosis of bacterial *Pseudomonas aeruginosa* (344). The cleavage of surface-bound C3bi and phagocyte CR1 significantly impaired host defence mechanisms against the invading pseudomonas bacteria. Restoration of the protease balance in the airway via aerosolized AAT is an attractive mechanism that may normalize complement-mediated neutrophil function that has undergone clinical trials in individuals with cystic fibrosis and AATD (262,351).
5.4 Conclusion

Confirmation of the association between AAT and C3 in plasma emphasizes an important novel role for AAT within the complement system. Furthermore, given that individuals with AATD have a reduced humoral protective concentration of AAT, complement activation may be a hitherto unexplored mechanism for the development of clinical disease in AATD. Based on the experimental observations in this chapter, it is possible that individuals with AATD would have impaired complement function due to dysregulated C3 proteolysis that may lead to functional complement deficiency, particularly at the site of neutrophil-dominated inflammation. Restoration of the humoral protease balance with AAT augmentation therapy may alter the degree of complement activation in AATD; this may represent a novel mechanism whereby augmentation therapy mediates beneficial effects in this condition.

The combined results of Chapters 3 and 4 indicate that AAT may function as a chaperone molecule within the circulation, loosely associated through non-covalent bonds to preserve anti-protease function. The humoral deficiency of AAT anti-protease activity may predispose to dysregulated proteolysis of C3, particularly during acute inflammation where the effects of abundant neutrophil proteases are dominant. Complement activation in AATD will be examined in detail in Chapter 5.
Chapter 6:

6.1 Complement activation in alpha-1 antitrypsin deficiency
6.1.1 Introduction

The results of experiments in Chapter 5 point to a fascinating role for AAT in the modulation of complement activation. The impact of complement activation in AATD is incompletely understood; in particular the function of AAT to inhibit dysregulated complement activation in the presence of high protease concentrations at the site of tissue inflammation warrants further investigation. Additionally, the implications of complement activation on the clinical phenotype in AATD warrant assessment.

The complement system is the major humoral component of innate immunity comprised of more than thirty proteins in various tissue fluids and on the surface of cells, including proteins of the enzymatic cascade, regulators and receptors (327). It was first recognised in the late 19th century and is so-named as its actions were seen to ‘complement’ the antibody-mediated immune activity of blood. Human plasma has an excellent capacity to opsonize invading microorganisms; this is largely mediated by activation of the complement cascade.

The complement system comprises a group of circulating precursor proteins, the cleavage of which results in the activation of a proteolytic cascade culminating in the production of opsonins and chemotactic factors at an exponential rate in response to an inflammatory or infective stimulus. The products of complement activation are involved in inflammation, leucocyte chemoattraction and immune cell activation (see Figure 6.1). Ultimately, progressive complement activation leads to the terminal complement cascade (TCC), culminating in the assembly of the membrane attack complex (MAC) that forms pores in target cell membranes causing cell death by osmotic lysis (327).
Figure 6.1: The varied effects of complement activation

Complement activation results in varied innate and adaptive immune responses in addition to the traditional functions of opsonisation, leucocyte chemotaxis and membrane attack formation. These include immune cell activation, clearance of apoptotic cells, autoimmunity, and modulating inflammation.

Adapted from Ricklin et al 2010 (352).

MAC: Membrane attack complex, TLR: Toll like receptor, FcγR: Fc gamma receptor
There are three distinct pathways involved in complement activation; all three pathways converge to produce C3 convertase (C3bBb or C4bC2a) that cleaves the reactive thioester bond of C3 resulting in the production of the two bioactive fragments, C3a and C3b, see Figure 6.2. Proteins of the classical pathway are designated C1 through C9, while the components of the alternative pathway are called factors, followed by a letter (e.g. factor B, factor H). In a rapid sequence of coordinated events C3b formation catalyses C3 convertase (C3bBb or C4bC2a) formation and amplification of C3b opsonisation of microbial targets the site of local invasion. With sufficient concentrations of C3b the C3 convertase in the presence of the catalytic subunit of factor B (FB), Bb, changes its specificity from C3 to C5, hence becoming a C5 convertase (C3bBbC3b or C4bC2aC3b). The action of C5 convertase on C5 results in the production of C5a, a more potent anaphylatoxin than C3a, and C5b. This is the key enzymatic event for the assembly of the MAC that disrupts the phospholipid bilayer of target cells resulting in cell lysis.

The classic (antibody-mediated) pathway was the first pathway to be recognised and is activated by the deposition of immunoglobulins, IgM and IgG, on the surface of invading microbes. The classic pathway is initiated by C1q recognition of surface bound IgM, IgG, C reactive protein (CRP) or pathogen-associated molecular patterns (PAMPs). As part of the C1 complex (C1qr2s2) the serine proteases C1r and C1s are consecutively activated following surface binding of C1q, leading to downstream generation of a C3 convertase (C4bC2b) and opsonisation (352). Complement (C1q) can be activated in response to PAMPS but also in response to damage-associated molecular pattern molecules (DAMPs) and may plays an important role in the clearance of apoptotic cells (353).

The lectin pathway is functionally similar to the classical pathway and is mediated by the pathogen recognition molecule (PRM) activity of Mannose Binding Lectin (MBL), and other ficolins. MBL can bind to specific non-mammalian carbohydrate residues on the surface of bacteria, fungi, parasitic protozoans, and viruses such as mannose, N-acetyl-d-glucosamine (GlcNAc), N-
acetyl-mannosamine, fucose, and glucose. Carbohydrates that are found on mammalian glycoproteins, such as D-galactose and sialic acid, have no affinity for MBL thereby avoiding unwanted self-recognition. Once bound to the surface each PRM can assemble with MBL-associated serine proteases (MASP-1 and MASP-2), which are then able to cleave C2 and C4 to form C3 convertase (C4bC2b).

The alternative pathway is constitutively activated through spontaneous hydrolysis ‘tick-over’ of its internal thioester bond thereby exposing new binding sites and resulting in the production of a small fraction of circulating C3_h2o. The serine protease Factor B can then bind C3_h2o, which is subsequently cleaved by factor D to form C3 convertase (C3_h2oBb). This alternative mechanism facilitates constant immune surveillance, probing for invading microbes to initiate early complement activation without the need for amplification from the precursor complement pathways. The alternative pathway may account for 80-90% of total complement activation.
Figure 6.2: Complement activation pathways

An overview of the three pathways of complement activation leading to the generation of C3 convertase*. All three pathways converge to cleave C3 which can then mediate downstream effects through opsonization (C3b), cell wall lysis (MAC formation) and inflammation (C3a).

Adapted from Dunkelberger et al. 2010 (334).
6.1.2 Regulation of complement activation

As production of these complement factors may result in harmful effects upon normal host tissue, controlling the cleavage of C3 and the initiation of complement cascade is tightly regulated by a number of serine protease inhibitors (354). The most studied of these control proteins is C1 inhibitor (C1-inh) that is encoded on the SERPING1 gene (355,356). Both C1-inh and AAT are canonical serine protease inhibitors and they share many of the same structural and functional characteristics, however C1-inh has two reactive residues, arginine (Arg P₁) and residue (Ala P₂). In addition, C1-inh is more highly glycosylated with N- and O-glycosylation contributing to 49% of the total molecular mass of the protein. C1-inh has reactivity against C1s, C1r, MASP1, MASP2, kallikrein, factor XII, and chymotrypsin (352,357). Another abundant serine protease inhibitor, α2-macroglobulin, can inactivate the MASP enzymes only, therefore its role is limited to inhibition of the lectin pathway (358).

AAT is not an effective inhibitor of the classic and lectin pathways of complement activation. To illustrate the inability of AAT to inhibit complement proteases, a mutated AAT protein engineered to mimic C1-inh function, with identical P₁ and P₂ reactive sites within the reactive centre loop, was not an effective inhibitor of the complement system (359). This diminished activity of mutant AAT forms against proteases of the complement system indicates that the glycosylation status of C1-inh is critical for the orientation of the protein to permit effective binding of target proteases and effective inhibition (360).
6.1.3 Control of complement activation

The catalytic process of complement activation is rapidly terminated away from the site of inflammation by a number of proteins that can accelerate decay and halt complement activation either in the fluid phase or at the cell surface, these are summarized in Table 6.1. The complement control protein Factor I (FI) is a proteolytic enzyme that binds C4b and C3b, generating iC4b and iC3b, which then leads to further proteolysis and complement inactivation (361,362). Factor H is a cofactor for FI and can displace Bb in the C3 convertase to promote C3b inactivation (330,331). Complement control proteins have a higher plasma concentration than the proteins involved in complement activation in order to prevent fluid phase propagation of the complement cascade in plasma. Additionally, the presence of a concentration gradient of complement control proteins may cause paradoxical enhancement of C3b opsonin deposition on target tissues due to preferential inhibition of C3 cleaving enzymes in the fluid phase directing activation to the site of inflammation (345).

On cell surfaces C3 and C5 convertase activity is regulated by three integral membrane proteins: Decay-accelerating factor (DAF), destabilizes the C3 convertase by promoting the release of factor Bb; Membrane Cofactor Protein (MCP), expressed on leukocytes and platelets, favours the dissociation of FB and promotes C3b association with FI; and complement receptor type 1 (CR1) which behaves like FH and through displacement of Bb from the C3 convertase and facilitates inactivation by FI (363). CD59 and the C8-binding protein are found on erythrocytes and most host cells where they bind to proteins of the terminal complement cascade and thereby inhibit C9 polymerization and formation of the membrane attack complex (364).

As described in Chapter 5, neutrophil-derived proteases such as NE, CathG and PR3 can cleave C3 and contribute to the dysregulated activation of complement. As the primary humoral inhibitor of these proteases, AAT may prevent dysregulated proteolysis of C3 during inflammation despite the absence of
documented activity against the proteases involved in the control of complement activation. At the cell surface AAT has been shown to be the predominant inhibitor of a complement cleaving protease, p57, on the erythrocyte membrane and may prevent elastase mediated cleavage of CR1 (365,366). Complement activation may be dysregulated in AATD due to the protease/anti-protease imbalance that exists in this condition; this has not been previously examined as a pathological mechanism of pulmonary disease in AATD.

Table 6.1: Regulation of complement activation

The proteases and protease inhibitors that are involved in the initiation of complement activation are summarized below. Control of complement activation requires a number of cofactors and control proteins that are located in plasma or at the cell surface.

<table>
<thead>
<tr>
<th>Activating protease</th>
<th>Protease inhibitors</th>
<th>Regulatory proteins</th>
<th>Humoral</th>
<th>Cell membrane</th>
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</thead>
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<td>Factor B</td>
<td>DAF</td>
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<td>Alpha-2</td>
<td>Factor H</td>
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<td>Factor I</td>
<td>CD59 (protectin)</td>
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<td>sMAP</td>
<td>CFHr-1</td>
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<tr>
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<td>MAP-1</td>
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<td>Vitronectin</td>
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<td>Carboxypeptidase-N</td>
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6.1.4 Hypothesis

The results of the experiments in Chapter 5 indicate that AAT may protect against dysregulated proteolytic cleavage of C3 in the context of neutrophil-mediated inflammation with excess protease activity. We hypothesized that complement activation is dysregulated in AATD due to protease/anti-protease imbalance and that this contributes to the manifestation of the pulmonary disease phenotype in this condition.

To investigate this hypothesis, the aim of this chapter was to assess if complement activation, particularly C3 proteolysis, occurs in AATD.

To fulfill this aim the following three objectives were set:

1. Examine the integrity of the pathways of complement activation to assess if AATD contributes to humoral complement deficiency.
2. Evaluate endogenous NE activity as potential mechanism for complement activation in AATD.
3. Assess the effect of complement activation on the clinical phenotype of AATD and determine if correlates with measurements of disease activity.
6.2 Results

6.2.1 Total C3 is within the normal range in Z-AATD

As an initial assessment of the complement system in AATD, plasma from PiZZ individuals (n=50) with a range of clinically stable disease was obtained along with healthy PiMM control plasma (n=15). Total complement component C3 was measured using a C3 ELISA (Abcam) that detects intact C3 and C3 breakdown products. Each sample was measured in duplicate by immunofluorescence at 450nm and the plasma concentration was determined using a standard curve. There was no difference in mean C3 concentration between all PiZZ subjects compared to PiMM controls (556.1 vs. 660.7μg/ml, p=0.25). When PiZZ subjects were divided into those with evidence of COPD (FEV1/FVC <70% predicted and FEV1 <80% predicted), there was no difference seen between PiMM controls and non-obstructed PiZZ subjects (556.1 vs. 531.4μg/ml, p=0.61), see Figure 6.3. Despite no statistical difference in the mean C3 concentration between obstructed PiZZ subjects and those without obstruction (696.2 vs. 531.4μg/ml, p=0.55) and healthy PiMM controls (696.2 vs. 556.1μg/ml, p=0.21), there appears to be a subgroup within the AATD population with obstructed airway disease that had a marked elevation in C3. C3 is an acute phase protein and its levels can rise in response to inflammatory stimuli before they begin to fall in cases of excess complement activation. It is possible that low grade inflammation leading to complement turnover and stimulation of C3 production may account for the elevated levels observed in those with AATD and evidence of obstructive lung disease compared to healthy controls.
Figure 6.3: Measurement of total C3 concentration in AATD

Total complement component C3 was measured in plasma of obstructed PiZZ individuals (FEV1/FVC <70% and FEV1 <80% predicted), non-obstructed PiZZ subjects (n=11), and healthy PiMM controls (n=17) using a C3 ELISA (Abcam). There was no statistical difference in the mean C3 concentration between any of the three groups using non-parametric analysis (Mann-Whitney U-test): Mean plasma C3 in PiMM subjects was 556.1μg/ml; PiZZ (non-obstructed) 531.4μg/ml; and PiZZ (obstructed) 696.2μg/ml. The skewed distribution of C3 in obstructed PiZZ subjects indicates that this is not a homogenous population; C3 is elevated in some individuals but not others.

FEV1; forced expiratory volume in 1s, FVC; forced vital capacity
6.2.2 Complement turnover is increased in Z-AATD

The measurement of total C3 is a crude indicator of the status of the complement system as all C3 products are measured by the assay, whereas quantification of complement activation products can provide further insight into the turnover of C3. C3a is a direct product of C3 cleavage and it fulfils important biological functions as outlined earlier. C3a is rapidly inactivated in plasma by the action of carboxypeptidases, however the resultant C3a-desarg is stable with a longer plasma half-life permitting it to be more accurately quantified. Spontaneous hydrolysis of the internal thioester bond of C3 occurs regularly in plasma (complement tick over), therefore the generation of C3a can be detected in healthy individuals and corresponds to approximately 1% of total C3 turnover in health (352).

C3a-desarg (C3a) levels were measured in plasma of PiZZ individuals (n=51) and healthy PiMM controls (n=15) using a specific C3a ELISA (Quidel). Each sample was measured in duplicate by immunofluorescence at 405nm and the plasma concentration was determined using a standard curve. The mean C3a concentration was increased by 50% in the plasma of PiZZ individuals compared to PiMM controls (406.0 vs. 270.2ng/ml, p=0.0119). When analysed with respect to the presence of airway obstruction, there was no difference observed between the PiMM control group and non-obstructed PiZZ subjects (270 vs. 307.4ng/ml, p=0.38), see Figure 6.4A. However when PiMM controls were compared to PiZZ subjects with airflow obstruction there was a significant increase in plasma C3a observed (270.2 vs. 459.5ng/ml, p=0.02), see Figure 6.4A.

As C3 production is related to total C3 turnover, an accurate degree of complement activation can be determined by the ratio of plasma C3a to total C3 (C3a:C3%). The C3a:C3 ratio was increased in PiZZ individuals compared to PiMM controls (0.079 vs. 0.048%, p=0.044). There was no difference observed between the PiMM control group and non-obstructed PiZZ subjects (C3a:C3% 0.048 vs. 0.059%, p=0.38). When PiMM controls were compared to PiZZ subjects with
airflow obstruction complement turnover was observed to be significantly increased by 76% (C3a:C3% 0.048 vs. 0.085 p=0.034, see Figure 6.4B. Expressed in relation to the percentage of the molecular mass, this corresponds to an expected C3 breakdown of 1.01% in PiMM controls vs. 1.79% in PiZZ individuals with airflow obstruction. Therefore complement turnover is increased significantly by approximately 78% in individuals with AATD and a more severe pulmonary phenotype.
Figure 6.4: Measurement of C3 turnover in AATD

A. C3a-desarg levels were measured in plasma of PiZZ individuals (n=51) and healthy PiMM controls (n=15) using a C3a ELISA (Quidel). There was no significant difference observed between PiMM controls and non-obstructed PiZZ subjects (n=11), 270 vs. 307.4ng/ml, p=0.38. However when PiMM controls (n15) were compared to PiZZ subjects with airflow obstruction (n=40) there was a significant increase in plasma C3a observed, 270.2 vs. 459.5ng/ml, p=0.02.

B. To reflect a more accurate degree of complement activation, the ratio of C3a:C3 was determined as C3a production is related to total C3 turnover. C3a:C3 (%) is expressed as a fraction of the measured complement products in ng/ml. Complement turnover (C3a:C3%) was increased by 76% in obstructed PiZZ subjects compared to the PiMM controls (0.048 vs. 0.085 p=0.034), indicating that complement activation is occurring in AATD and may relate to the severity of the pulmonary phenotype in AATD.
6.2.3 Complement activation is increased in Z-AATD

To further investigate the extent of complement activation in AATD, a more specific indicator of this process, C3d, was quantified. C3d is a domain and cleavage product of C3, it also contains the site of the reactive thioester that is involved in C3b binding as well as functioning as an immune adjuvant with phagocytes and antigen presenting cell interactions with the adaptive immune response (336). Unlike C3a, C3d is not spontaneously produced by C3 hydrolysis in plasma; it is produced during complement activation through the actions of FI on iC3b though it can also be produced by the dysregulated C3 proteolysis, such as with NE. Therefore C3d in plasma and in tissue samples serves an excellent biomarker of complement activation.

Quantification of C3d was performed in plasma of PiZZ individuals (n=25) with a range of clinically stable disease and healthy PiMM controls (n=7). A specific ELISA for a neo-epitope exposed only on C3d (Cusabio) was employed. Each sample was measured in duplicate by immunofluorescence at 405nm and the plasma concentration was determined using a standard curve. The mean C3d concentration was increased in PiZZ individuals and barely detectable in PiMM controls (20.1 vs. 0.037μg/ml respectively, p<0.0001), confirming that complement activation is occurring in AATD, see Figure 6.5A.

The degree of complement activation was assessed by determining the ratio of C3d to intact C3 (C3d:C3%). Consistent with the findings of increased complement turnover, complement activation (C3d:C3%) was elevated in PiZZ individuals compared to healthy PiMM controls (0.124 vs. 4.69%, p=0.0002), see Figure 6.5B. Approximately 5% of total C3 within the circulation had undergone activation, indicating that this process may reflect a potential role for the complement system in the immunopathology of AATD.
A. C3d levels were measured in plasma of PiZZ individuals with a variable degree of airway obstruction (n=25) and healthy PiMM controls (n=7) using a C3d specific ELISA (Cusabio). The mean C3d concentration was increased in PiZZ individuals and barely detectable in PiMM controls (20.1 vs. 0.037µg/ml respectively, p<0.0001), confirming that complement activation is occurring in AATD.

B. The degree of complement activation was assessed by determining the C3d:C3 ratio. C3d:C3 was elevated in PiZZ compared to PiMM (0.124 vs. 4.69% respectively, p=0.0002). This indicates that approximately 5% of total circulating C3 has been activated in AATD, reflecting the finding of increased C3 turnover in the previous experiment.
6.2.4 Complement activation correlates with the pulmonary clinical phenotype in AATD

In order to determine the importance of complement activation in AATD, C3d:C3% was evaluated in PiZZ individuals who are well and also in those with a range of clinically stable disease. In Chapter 3, a detailed clinical phenotype was determined for each individual with AATD recruited to our study. Firstly C3d:C3% was evaluated in those with and without evidence of obstructive airway disease (FEV1/FVC <70%). Individuals with AATD and airway obstruction had greater complement activation (C3d:C3%) compared to those without (2.58 vs. 5.96%, p = 0.0127), see Figure 6.6A.

Linear regression analysis was performed to further assess the relationship between complement activation and airflow obstruction; C3d:C3% (n=25) had a significant inverse correlation with the degree of impairment in FEV1 (r = -0.4211, r² = 0.177, p = 0.041), see Figure 6.6B. This finding indicates that complement activation in AATD may relate to the disease process in the lungs of individuals with more severe airway obstruction. Though higher in those with a more severe clinical phenotype, complement activation was also found to be an active process in those with a milder clinical phenotype despite being clinically well.
Figure 6.6: Correlation of the C3 activation product, C3d, with clinical phenotype in AATD

A. Complement activation as measured by the C3d:C3 ratio was assessed by comparing PiZZ individuals with and without evidence of airway obstruction, defined as FEV1/FVC ration of <70%. PiZZ individuals with airway obstruction had greater complement activation compared to those without airway obstruction, C3d:C3 2.58 vs. 5.96%, p = 0.0127. This may be indicative of ongoing inflammation in the more severe PiZZ phenotype.

B. To assess the correlation between complement activation and FEV1, linear regression analysis was employed. The C3d:C3% was found to inversely correlate with the degree of impairment in FEV1 (r = -0.4211, r^2 = 0.177, p = 0.041). Complement activation in AATD may relate to inflammatory processes that perpetuate in the lungs of individuals with more severe airway obstruction.
6.2.5 Complement activation correlates with emphysema but not bronchiectasis in AATD

The current gold standard for the evaluation of lung disease in AATD is thoracic CT imaging and this enables the most direct visualization of the pulmonary disease process in this condition. Utilizing the data generated from Chapter 3, a linear regression analysis was performed between emphysema severity and C3d:C3% (n=25). There was a strong correlation detected between emphysema and complement activation (r = 0.61, r^2 = 0.37, p = 0.0012), see Figure 6.7A. This result indicates that a significant proportion of complement activation observed in AATD relates directly to the primary pathological manifestation of the condition, pulmonary emphysema.

Interestingly there was no relationship detected between C3d:C3% and CT determined bronchiectasis (r=0.04, p=0.8636), see Figure 6.7B. Intuitively one would expect to find some relationship, given the link between complement deficiency, in particular C3 deficiency, and the clinical manifestations of recurrent infection and bronchiectasis. However this was not the case.
Figure 6.7: Correlation of the C3 activation product, C3d, with clinical phenotype in AATD

A. Complement activation strongly correlated with the degree of CT determined emphysema by linear regression analysis ($r = 0.61$, $r^2 = 0.37$, $p = 0.0012$). This indicates that a significant proportion of the complement activation observed in AATD may relate directly to the primary pathological manifestation of the condition, pulmonary emphysema.

B. There was no correlation with the degree of CT determined bronchiectasis and complement activation ($r=0.12$, $r^2 = 0.15$, $p = 0.43$).
6.2.6 Z-AATD does not result in functional hypocomplementaemia

The detection of increased C3 turnover may lead to excessive consumption of proteins of the complement system thereby leading to humoral complement deficiency. It is not known if this occurs in AATD. Recently, an enzyme immunoassay for the qualitative determination of functional classical, alternative, and lectin (MBL) complement pathways has been developed utilizing an immunoassay based on the principle of complement mediated haemolysis (CH50). Serum samples were collected from AATD individuals (n=13) and immediately frozen. The samples were thawed to 37°C and diluted for analysis with the Complement System Screen (Wieslab®). Each sample was evaluated in duplicate and the absorbance measured at 405nm. The percentage of complement activity was calculated based on the positive and negative control in accordance with the manufacturer’s instructions. There was no deficiency of the classic or alternative pathway of complement activation detected in the AATD samples obtained from those with a range of clinical disease. 5/13 (38%) of the AATD samples demonstrated <50% activity and two individuals had true deficiency (<50%) of the lectin pathway, see Figure 6.8. This is consistent with what is observed in the general population where 20-30% have true MBL deficiency. Therefore, despite indications of increased complement activation in the order of 5% in AATD, there is no indication of excess consumption that can result functional hypocomplementaemia.
To investigate if increased turnover of C3 led to humoral complement deficiency in AATD, an enzyme immunoassay for the qualitative determination of functional classical, alternative, and lectin complement pathways was performed using the Complement System Screen (Weislab). The percentage of complement activity in each serum sample was calculated based on the population control provided with the test.

- The results showed that there was no humoral deficiency of the classic and alternative pathway in all AATD samples tested (n=13).

- 5/13 (38%) demonstrated <50% activity and two individuals had true deficiency (<10%) of the lectin pathway, this is consistent with what is observed in the general population where 20-30% have true MBL deficiency.

Figure 6.8: Assessment of complement pathway deficiency in AATD
6.2.7 Neutrophil elastase activity in Z-AATD plasma is low and there is no observable difference when compared to PiMM controls

AAT is the main humoral inhibitor of NE in blood and protease imbalance is the major determinant for the development of pulmonary emphysema in AATD (40). In Chapter 5, exogenous NE activity was shown to cause dysregulated proteolysis of C3, this effect was more pronounced in the plasma of PiZZ individuals compared to PiMM individuals due to reduced protease buffering capacity of plasma. It is possible that circulating endogenously active NE in plasma contributes to dysregulated activation of C3; to date there has been no study that has directly evaluated NE activity in plasma of AATD individuals.

Utilising a NE-specific substrate N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (MSAAPVCK) (R&D systems, Minneapolis, MN, USA), the total amount of active NE was determined in plasma of PiZZ individuals (n=26) and healthy PiMM controls (n=6) by fluorescence resonance energy transfer (FRET) analysis. Reactions commenced with the addition of 3μl of the 1mM working substrate solution and fluorescence was recorded at excitation 320nm and emission 420nm for 1-40 min. The slope of known concentrations of NE was determined at each time point and regression analysis performed to create a standard curve of NE activity. Fluorescence was measured in duplicate and subtracted from control plasma then plotted against the standard curve to determine the NE activity of each sample.

There was no increased NE activity detected in the plasma of PiZZ individuals compared to PiMM controls (mean 0.23 vs. 0.37μMol·L⁻¹·min⁻¹, p=0.34), see Figure 6.9A. The actual amount of proteolytically active NE was very low and approached the limit of detection in most cases.

To evaluate if any relationship exists between proteolytically active NE and the clinical phenotype of Z-AATD, plasma NE activity was plotted against clinical measurement parameters including FEV1, DLCO and CT-determined emphysema in the AATD study participants, see Figure 6.9(B-D). There was no correlation
detected between active NE in PiZZ plasma and FEV1% predicted, \( p=0.71 \) (B), DLCO, \( p=0.67 \) (C) or CT determined emphysema, \( p=0.83 \) (D). This result is not unexpected given the large volume of distribution within the plasma, clinical stability at the acquisition of the sample, distance from the potential active site of inflammation (i.e. the lung), and the remaining circulating protease inhibitory activity of AAT and other serine protease inhibitors.
Figure 6.9: Neutrophil elastase activity in plasma does not correlate with clinical phenotype

A. The total amount of active NE was found to be low in the plasma of PiZZ individuals, interestingly there was no difference seen with the activity of NE in PiMM plasma (0.023 vs. 0.037 μMol L⁻¹ min⁻¹, p=0.34). All individuals were clinically stable at the time of recruitment.

B. There was no correlation with active NE in PiZZ plasma with FEV1% predicted, p=0.71.

C. There was no correlation with active NE in PiZZ plasma with DLCO, p=0.67.

D. Finally, there was no correlation with active NE in PiZZ plasma with CT determined emphysema, p=0.83.
6.2.8 Proteolytically active neutrophil elastase is not related to complement activation in the Z-AATD plasma.

Dysregulated C3 proteolysis by NE can cleave C3 and activate complement as previously outlined. AAT is the main humoral inhibitor of NE in blood. To examine the possibility of the generation of C3a and by endogenous NE activity in plasma, linear regression analysis was performed between C3a:C3% and active NE in plasma (n=23). The C3a analogue peptide produced by C3 cleavage by NE is immunologically indistinct from C3a. There was no relationship seen between active NE in plasma and complement turnover (\( r^2 = 0.01, p = 0.72 \)), see Figure 6.10. This experiment shows that complement activation is not related to plasma NE activity in clinically stable individual with AATD.

The lack of correlation between NE and the C3a:C3% is not unexpected: Firstly, the concentration of NE in plasma was very low, indeed it was undetectable in some cases where C3a:C3% was quite elevated. Secondly, any free NE in the circulation is likely to react with the circulating Z-AAT, particularly during periods of clinical stability where there is unlikely to be a significant burden of NE release. Thirdly, the observation that NE is present at similar levels in PiMM controls to PiZZ subjects, and the absence of correlation with any clinical biomarker of PiZZ pulmonary disease, indicates that it is not a good systemic biomarker of pulmonary disease activity. This finding supports the hypothesis of the lung as the site of inflammation as opposed to a systemic process of NE-mediated complement activation. This prompted an evaluation for the presence of endogenous NE and complement degradation products in the BAL fluid of PiZZ subjects.
Figure 6.10: Comparison of neutrophil elastase activity and complement activation in the plasma of PiZZ individuals.

In Chapter 5, exogenous NE activity was shown to cause dysregulated proteolysis of C3, this effect was more pronounced in the plasma of PiZZ individuals compared to PiMM individuals due to reduced protease buffering capacity of plasma.

In this experiment the relationship between the total amount of endogenous active NE and complement activation (C3a:C3%) was evaluated. There was no relationship seen between active NE in plasma and complement turnover ($r^2 = 0.01$, $p=0.72$).
6.2.9 Neutrophil elastase activity in Z-AATD is elevated in bronchioalveolar fluid of Z-AATD with a more severe clinical phenotype

As there was minimal proteolytically active NE detectable in the plasma of AATD subjects, and in light of the correlation between C3 activation products and emphysema, we evaluated if NE activity was detectable in the lung. Prior studies in COPD-related airway disease have demonstrated a correlation with complement activation products with the presence disease (306). BAL fluid and corresponding plasma was obtained from six PiZZ individuals undergoing bronchoscopy. The clinical phenotype was determined in all subjects; n=3 were well and did not have underlying obstructive lung disease, and n=3 had obstructive airways disease and previous pulmonary exacerbations but were clinically stable for one month prior to the procedure.

The samples were placed on ice immediately following collection and processed within 1h to prevent degradation. The urea concentration was determined for each BAL specimen and plasma sample and a standardized concentration of BAL was determined to permit uniform analysis.

The slope of known concentrations of NE was determined at each time point and regression analysis performed to create a standard curve of NE activity. Fluorescence was measured in duplicate and subtracted from control BAL, then plotted against the standard curve to determine the NE activity within each sample. In two individuals with obstructive lung disease, a mean value of 1.7μM of proteolytically active NE was detected. There was no NE activity detected in the remaining five samples.
6.2.10 Products of C3 proteolysis are detectable in BAL fluid of Z-AATD and correlate with NE activity

The degradation of C3 was evaluated by western blot analysis of the BAL fluid samples obtained from the Z-AATD individuals (n=6). Standardisation of urea concentration between blood and BAL fluid was performed in order to permit equal loading of each lane, this was confirmed by BCA analysis. 10μL of standardized BAL fluid was loaded onto a 12.5% non-reducing SDS PAGE gel and western blot analysis performed using polyclonal rabbit anti-C3 antibody.

In PiZZ individuals with a mild clinical phenotype (no airflow obstruction), there was no C3 detectable (Figure 6.11, Lane 1). In those with a more severe clinical phenotype (airflow obstruction) and no detectable proteolytically active NE, a single C3 band was detected at the predicted molecular mass of 190kDa (Figure 6.11, Lane 2). In the PiZZ individual with the NE concentration of 1.7μM, a number of distinct immunobands are seen corresponding to C3 proteolytic fragments, which are not present in those with no detectable active NE (Figure 6.11, Lane 3).

Complement proteolysis has been described in BAL fluid from individuals with CF bronchiectasis. BAL fluid from individuals with CF (n=3) was also examined as an inflammatory control; in Figure 6.11, lane 4 depicts C3 proteolysis in BAL from an individual with cystic fibrosis lung disease and >1μM proteolytically active NE. It has previously been documented that AAT:protease complexes are increased in CF BAL fluid, this results in degradation of the protease inhibitory shield (262,367).

This indicates that in the lungs of Z-AATD individuals, C3 production is upregulated in those with airflow obstruction indicative of a more severe clinical phenotype. C3 proteolysis was only detected in the individual with proteolytically active NE. C3 proteolysis was observed in CF inflammatory controls corresponding to neutrophil-mediated inflammation and the release of proteolytically active NE.
C3 production is increased and proteolysis can occur in the lungs of individuals with high local NE activity. Given the earlier findings that complement activation occurs in Z-AATD and correlates strongly with pulmonary emphysema, the finding of C3 proteolysis in the presence of proteolytically active NE is consistent with dysregulated activation of the complement pathway due to pulmonary disease in AATD.
Figure 6.11: Detection of complement proteolysis in BAL sample of PiZZ individuals by western blot analysis.

BAL fluid was obtained from PiZZ subjects undergoing bronchoscopy (n=7). BAL fluid from PiMM individuals with cystic fibrosis (CF) as an inflammatory control. Total proteolytically active NE was measured in all BAL fluid samples following standardization of urea concentration between blood and BAL fluid.

Western blot analysis of each sample on 12.5% non-reducing SDS PAGE gel using polyclonal rabbit anti-C3 antibody was performed.

1. Lane 1: There is no C3 or proteolytically active NE detectable in well ZZ individuals without evidence of airflow obstruction.
2. Lane 2 demonstrates a single C3 band detected in a clinically stable ZZ individual with evidence of airflow obstruction in the absence of detectable NE activity.
3. Lane 3 depicts multiple bands corresponding to C3 fragments indicative of C3 proteolysis in the BAL fluid in the presence of a mean value of proteolytically active NE of 1.7μM. This is detected despite a period of clinically stability ZZ individual with airflow obstruction.
4. Lane 4 is an inflammatory control which also depicts C3 proteolysis in a representative individual with cystic fibrosis lung disease and high proteolytically active NE (mean 4.1μM).
6.3 Discussion

The results of the experiments conducted in this chapter contribute to the continued expansion of our knowledge on the biological role of AAT in health and in deficiency states. C3 proteolysis by NE (in addition to CathG and PR3) indicates that this mechanism may have implications for complement activation under conditions of protease/anti-protease imbalance, particularly in AATD.

This is the first study to evaluate complement activation in the pulmonary disease phenotype of Z-AATD. The results of this chapter demonstrate a significant novel finding in the context of AATD, that is complement activation is a biomarker of disease activity in Z-AATD. Complement activation involves tightly regulated proteolysis that maintains a host innate immune defence balanced against the deleterious effects of excessive inflammation and host cell injury. Perturbation of this balance may lead to worsening of clinical disease in AATD through a cycle of leucocyte recruitment with increased protease burden, background inflammation, adaptive immune cell activation and impaired opsonophagocytosis. From a pathophysiological perspective there is sufficient evidence to localize the activation of complement to the lung in individuals with pulmonary disease due to the correlation of C3d:C3% with FEV1 and radiological determined emphysema.

In support of this observation, despite humoral AAT deficiency, there is minimal detectable proteolytic active NE in the plasma of clinically stable PiZZ individuals irrespective of their clinical phenotype. Based on the interpretation of the final experiments in this chapter, excess NE proteolytic activity localized to within the lung, and importantly not the plasma of these individuals. Excess NE activity correlates with the detection of complement breakdown products indicative of complement activation. This finding provides an intriguing insight into the pathophysiology of complement activation in the lungs of individuals with AATD. At sites of tissue inflammation particularly where there is abundant neutrophil-derived protease activity, protease excess may lead to ineffective
opsonophagocytosis through dysregulated consumption of complement pathway proteins, cleavage of surface bound C3b from target microbes, and cleavage of elastase-sensitive complement receptors (CR1) from phagocyte cell membranes (344). This process is termed ‘opsonin-receptor mismatch’ and may have important implications for host immune function. Despite utilising the most modern ascertainment methodologies, it was not possible to determine whether complement activation was related to the initiation of one of precursor complement pathways or due to dysregulated NE proteolysis, as C3a and C3d are immunologically indistinct regardless of the cleavage pathway.

The clinical relevance of AATD on complement activation was assessed by determining the ratio of the complement products C3a and C3d, which are produced in equimolar amounts due to C3 convertase activity. The degree of C3 activation correlated with the severity of obstructive airways disease and emphysema on CT imaging in clinically stable AATD individuals. C3 proteolysis may contribute to the pathogenesis of pulmonary disease in AATD and measurement of complement activation products may be a useful biomarker of disease activity in this condition. Measurement of complement activation has been used as a biomarker of disease activity in other inflammatory conditions such as ALI and ARDS (368). In addition the activation of complement on the surface of biomaterials during cardiac bypass and dialysis has been shown to contribute to thrombosis and perpetuation of inflammation through leucocyte activation (363).

Another important finding of this study was that complement activation correlated with disease activity but did not result in functional hypocomplementaemia. Approximately 5% of C3 in the circulation had been activated during a period of clinical stability, however compensatory mechanisms increased overall C3 turnover but did not result in C3 deficiency at this rate. The maintenance of an adequate plasma C3 concentration is essential to provide critical humoral complement protection from invading microbial infection (322). This is congruent with the observed clinical findings in AATD, where individuals are prone to pulmonary exacerbations related to their COPD
but do not show evidence of immunodeficiency, characterized by susceptibility to opportunistic infection. Whether this compensatory mechanism persists during times of active inflammation, e.g. during a pulmonary exacerbation or in the context of significant hepatic impairment, was not evaluated.
6.3.1 Role of complement activation in AATD

To date only one previous study has assessed complement activation in AATD; increased complement activation was demonstrated in paediatric patients with liver disease and was found to correlate with the severity of liver cirrhosis (369). The finding that C3 activation correlated with liver disease in children is interesting due the different mechanisms that are classically described between liver and lung disease in AATD. In recent years the role of Z-AAT polymerization outside of the liver has garnered much interest in relation to their chemotactic properties, induction of ER stress, pro-inflammatory cytokine signaling, and their potential role in apoptosis (43,51,52). Thus Z-AAT polymers contribute to a pro-inflammatory phenotype in the lung and liver, which may potentiate inflammation in these tissues and contribute to complement activation beyond oxidative stress and protease imbalance.

Complement activation has been found in pathological samples of AATD-associated panniculitis, a case series of AATD-related panniculitis revealed C3 deposition in the blood vessels of the affected area in all patients (152,153). The authors posited that the complement activation observed in panniculitis represented a phenomenon of secondary vasculitis in the affected tissue.

Complement activation is implicated directly in the pathogenesis of a spectrum of disease states, the best studied of which is Systemic Lupus Erythematosus (SLE) (370). In SLE, widespread complement activation occurs resulting in humoral deficiency of C3 and C4 with C3d found deposited along the basement membrane in SLE-associated nephritis. Humoral hypocomplementaemia due to complement activation is observed classically in SLE and mesangiocapillary glomerulonephritis (MSGN) (371,372). Indeed it is an interesting observation that the rare reports of renal disease in AATD, usually associated with liver cirrhosis, have similar histologically characteristics of C3d deposition to that described in MSGN indicating a potential pathological role for complement activation in AAT renal disease (117). Importantly, the findings of this chapter indicate that humoral complement activity is preserved in AATD.
6.4 Conclusion

To conclude, our data reports for the first time that complement activation occurs in individuals with PiZZ AATD. Furthermore we have demonstrated that the degree of complement activation correlates with the severity of emphysema in this condition as ascertained by radiological scoring of disease and also by spirometric measurements of airflow obstruction. This raises the possibility that complement activation, in particular C3d:C3%, may be a useful plasma biomarker for the determination of clinical phenotype in this condition. Additionally, through the use of novel FRET analysis methodologies, we have demonstrated that there is minimal proteolytically active NE detectable in the plasma of clinically stable individuals with AATD. However, our analysis of BAL fluid samples indicates that at the site of disease, in the lung, proteolytically active NE is detectable in some individuals despite being clinical stability and there is corresponding evidence of complement degradation at this site of inflammation. Finally, the pathological relevance of complement activation in the lungs of PiZZ individuals is that it may be a previously undescribed mechanism of disease in this condition.
Chapter 7:

7.1 General Discussion
7.1.1 Discussion

In line with the aims and objectives of this study, a detailed clinical phenotype of each severely AAT deficient subject recruited to this study was established in Chapter 3. The impact of cigarette smoke was apparent in this population, with greater impairment in lung function and an increased severity of pulmonary emphysema seen in those with more exposure. These measurements correlated with worsening self-reported health status, higher dyspnoea scores, and increased frequency of pulmonary exacerbations indicative of a more severe clinical phenotype of pulmonary disease. Furthermore, pro-inflammatory cytokines were elevated in the systemic circulation of some with more severe pulmonary disease. The administration of augmentation therapy resulted in lower IL-8 levels on day two of treatment compared to pre-infusion levels, indicative of the anti-inflammatory treatment effect of augmentation therapy that has recently been described (116). Despite elevated systemic cytokines levels not being a sensitive predictor of disease status, the results of Chapter 3 provide evidence of ongoing systemic inflammation in those with a more severe clinical phenotype of AATD despite clinical stability. Given that both IL-8 and TNF-R1 are involved in neutrophil recruitment, and neutrophil derived proteases are implicated in the pathogenesis of emphysema, the ability of augmentation therapy to modulate the activity of these cytokines through binding raises the possibility of other important interactions of AAT in the circulation.

Chapter 4 determined for the first time the full spectrum of binding partners to AAT and illustrated that AAT interacts with a diverse range of proteins in plasma involved in the complement, coagulation, lipid transport and fibrinolytic systems in vivo. In support of the purification and mass spectrometry methodologies employed in this work, many previously documented interactions were confirmed such as Apo-B100, fibrinogen and immunoglobulins (101,265). The uncovering of such a diverse range of potential binding partners belies the biological complexity of AAT interactions within the circulation. High plasma and tissue concentrations of AAT maintain the serine protease inhibitory shield,
which rises during the acute phase protein response. However increasingly the diverse effects of AAT beyond protease inhibition are coming to the fore, knowledge of the spectrum of protein binding partners provides insight into alternative functions in health and disease.

In Chapter 5, the novel interaction of AAT with C3 was confirmed. The physiological relevance of this relationship was explored with particular reference to NE-mediated proteolysis of C3 in vitro. C3 is able to bind both the M and Z forms of AAT protein. However the binding to rAAT was significantly reduced indicating that the glycosylation of AAT is important for this interaction. To investigate the reason for AAT associating with C3, we identified an increased susceptibility of PiZZ plasma to C3 degradation in the presence of exogenous NE. AAT may bind to C3 to prevent dysregulated complement activation and degradation of C3 activation products; this may have importance in both the fluid phase and also at the cell surface.

The establishment of clinical phenotypes in Chapter 3 provided a sound framework for evaluation of the clinical relevance of complement activation within this AATD study population. In Chapter 6, to explore the effect of the interaction of complement with AAT in severe AATD, we have described the novel finding of complement activation related to pulmonary emphysema in this population. The byproducts of complement activation, which were elevated in AATD, are pro-inflammatory and can contribute to neutrophil recruitment, thereby influencing the pathogenesis of pulmonary disease. Cleavage of C3 and dysregulated activation of the complement cascade occurs in the presence of NE and other serine proteases. Intact C3 was detected in BAL fluid from AATD subjects with emphysematous lung disease, however C3 cleavage products were seen in those with detectable levels of proteolytically active NE. This evidence of C3 cleavage due to NE, in clinically stable individuals with severe AATD and emphysema, indicates that the presence of AATD lung disease may be an inflammatory state with the lung serving as the site of continual complement
activation. Furthermore, the measurement of complement activation within the systemic circulation correlated significantly with the severity of pulmonary emphysema (C3d:C3) and may be a biomarker of disease activity. However, despite evidence from BAL fluid analysis, there was no increase in proteolytically active NE levels detected from the plasma of clinically stable PiZZ study subjects compared to healthy controls. In addition, there was no correlation between the amount of NE and parameters used to determine clinical phenotype severity.

The results from these combined experiments indicate that a perpetual inflammatory state exists in AATD. The influence of AAT binding to a variety of plasma proteins may protect against, or modulate the effects of, neutrophil protease release. The interaction of AAT with C3 represents an important link between two key protein systems in the blood, which may have implications for the pathogenesis of disease in AATD. Complement activation in clinically stable individuals with emphysema may contribute in part to pulmonary disease progression. This is evidenced from our experiments that point to lung as the site of complement activation in this study, with more pronounced elevation of systemic biomarkers of complement activation in those with a severe clinical phenotype of pulmonary emphysema.
7.1.2 The role of complement in lung disease

The role of the complement system, beyond its established function in innate immunity, has garnered much interest in a range of lung diseases in recent years (373). Research in this area supports the role of complement activation in a similar spectrum of disease processes that affect those with AATD; this includes asthma, emphysema, and lung infection.

Many of the characteristics of asthma, such as smooth muscle contraction, mucus hypersecretion and inflammatory cell recruitment, are consistent with the biological features of the complement anaphylatoxins, C3a and C5a. The release of C3a, via either direct cleavage by bacterial products or activation of complement pathways, leads to triggering of the C3a Receptor (C3aR), stimulation of cytokine release, IL-4 production and eosinophil recruitment (332,374). A murine model of allergic airway disease supports the role of C3a in asthma; a C3aR knockout model prevented pathophysiological changes in the lung following allergen challenge. Furthermore, human asthmatics demonstrated increased levels of C3a following intra-pulmonary allergen deposition (375). C3a has also been found to stimulate airway hyper-reactivity, eosinophil infiltration. Whilst another study identified a novel C3aR–dependent mechanism in the development of airway epithelial goblet cells and the regulation of Muc5ac production (376). C5aR signaling receptor sensitization may regulate inhalational allergen tolerance at the dendritic cell/regulatory T cell interface (377). Expression of C3aR and C5aR on bronchial epithelium is up-regulated in fatal asthma compared to controls with asthma who died of a non-pulmonary cause (378). Complement activation is thereby implicated as a major mediator of airway obstruction in asthma, whether this process contributes to reversible airway obstruction in AATD has yet to be investigated.

There is a mounting scientific evidence of the role of complement activation in the pathogenesis of pulmonary emphysema. In particular, it has been demonstrated that cigarette smoke can directly activate the alternative
complement pathway, modify the binding capacity of C3 for fI and fH, and cleave the internal thioester bond of C3(379,380). Cigarette smoke exposure (CSE) has also been shown to increase the chemotactic activity of serum samples, through the cleavage of C3 and the complement regulatory protein properdin factor B and generation of C5a (381). Should this occur in vivo, the increased C5a produced by CSE would be a potent stimulus for neutrophil and monocyte recruitment to the lung leading to increased tissue damage. This was explored in a C5 deficient knockout mouse model, where it was shown that neutrophil chemotactic activity to the lung following CSE was complement dependent (382).

In more recent experimental models of CSE, C3d deposition on the membrane of smoke exposed human respiratory cell cultures was demonstrated (383). In this study two knockout mouse models of complement deficiency were examined, C3−/− and fB−/− (alternative pathway). Interestingly, both complement knockout models were protective of respiratory mucosal damage following CSE. These authors went on to examine the effect of CES on the lung parenchyma in the same mouse model, which showed a reduction in inflammatory cell infiltration, cytokine production, and a concomitant recution in lung injury compared to complement sufficient wild type control mice (384). This indicates that complement activation is a central effector mechanism for lung inflammation following CSE, possibly through C3aR and, to a lesser extent, C5aR mediated signalling (385). Another group has also elucidated the critical role for C3a through autocrine/paracrine induction of C3aR-mediated inflammation in the pathogenesis of cigarette smoke induced emphysema (386).

The link between complement activation and pulmonary emphysema is less well established in human studies. One study has evaluated intact C4 as a systemic plasma biomarker of disease severity in COPD, where diminishing plasma C4 levels were found to correlate well with respiratory infections, emphysema, and hyperinflation (387). However, this study did not evaluate byproducts of complement activation or establish complement turnover. As C3 was not measured, no association with plasma C3 and the measured outcomes was
reported. Another study has evaluated the potential role for complement activation in the pathogenesis of pulmonary emphysema through the detection of complement anaphylatoxins in induced sputa of non-AATD individuals (306). Increased concentrations of C5a were found to correlate with degree of lung function impairment (DLCO% predicted), which is of relevance to the findings of this thesis. Furthermore, the byproducts of complement activation were localized to the lung supporting the hypothesis that the site of complement activation in emphysema is the lung.

The physiological relevance of complement activation in the emphysematous lung is likely multifactorial, and may be driven intrinsically by neutrophil activity in COPD or by other phagocytic cells (388), in addition to the classic, alternative and lectin pathways that occurs in the presence of microbial infection. NE activity appears to be required for the deleterious effects of complement activity to take effect in models of lung inflammation (66). Pulmonary exacerbations can contribute to the disease process and those with severe AATD are susceptible to frequent exacerbations (2.1/annum in this study population) that become more common with worsening obstructive airway disease (140). The excess protease burden during these exacerbations and the ensuing neutrophil influx may trigger a cycle of inflammation characterized by infection, tissue injury, excess protease activity, failure of resolution, and pulmonary function decline. Complement activation during exacerbations may be a contributory factor to this process. Some further insight may be gained from the CF airway model, where host and bacterial derived proteases overwhelm the protease inhibitory capacity of the alveolar lining fluid causing severe progressive bronchiectasis due to protease/anti-protease imbalance (9,389). This is compounded by bacterial colonization of the airway, host innate immune cell dysfunction, and a variety of intrinsic epithelial factors leading to persistent pro-inflammatory signaling (390-394). A minority of individuals with severe AATD develop progressive bronchiectasis, in the context of this subgroup of individuals the model of CF airway disease can be translated to explain these findings (145). However the results of Chapter 3 indicate that bronchiectasis is radiologically mild and
asymptomatic for the majority, with the severity of pulmonary emphysema correlating most strongly with complement activation and poorer health status measurements. The role of the pulmonary microbiome is an area of intense research at present, changes in the distribution of bacterial colonization during treatment for pulmonary exacerbations have been demonstrated in the general population with COPD/emphysema (395-397). Whether perturbation of the pulmonary microbiome in AATD has relevance to the clinical phenotype of pulmonary disease has not yet been elucidated.

Polymerization of Z-AAT in the liver and in the lung epithelium provides one explanation for persistent inflammation in AATD, with consequent effects on ER stress responses and activation of inflammatory pathways (4,43,51). However, the detection of Z-AAT polymers remains technically difficult \textit{in vivo} and to date there has been no correlation performed between the extent of Z-AAT polymerization and the clinical phenotype of AATD pulmonary disease. Preventing polymerization of Z-AAT is an enticing prospect to treat AATD lung and liver disease and may be achieved in the future through basic scientific advances and with a deeper understanding of the pathologic mechanism. The development of small molecules to target the hydrophobic pocket of AAT and thereby prevent loop-sheet polymerization have been evaluated (398,399). Should these drugs prove effective in clinical trials, it would be a major advance for the prevention and treatment of AATD. Whether any beneficial effect on the inflammatory state in AATD accrues from their use will be an interesting area of research. Furthermore, targeting loop sheet polymerization may have important therapeutic implications for a number of other conformational diseases, such as Alzheimer’s dementia and neurodegenerative prion disease (400).
7.1.3 The role of complement in apoptosis

An important function of complement activation includes the removal of modified/apoptotic host cells without inflammation due to the acquisition of soluble regulators C1q and Factor H (401). Tagging of damaged host tissue in the lung will lead to transport of affected particles to the follicular dendritic cells in lymph node tissue may lead to B-cell priming for T-cell mediated adaptive immune response. A recent study supporting the immunological hypothesis for the pathogenesis of emphysema in AATD was alluded to with the finding of follicular hyperplasia in the lungs of AATD and non-deficient individuals with emphysema (402). This is a process that may be augmented by the generation of C3d, which can promote and enhance IL-4 and IFN-γ responses, as well as functioning as a co-stimulatory immune adjuvant and general immunostimulatory agent (403). In addition, through modulation of cytokines involved in T cell signaling, C3a has been shown to alter memory T cell responses and affect regulatory T cell function (404) indicative of a broader role for complement in the regulation of adaptive immunity (322,405).

The apparent complementary role of C3 protein fragments and AAT in promoting apoptosis, albeit through different mechanisms, is intriguing (92,353). Erythrocytes are the main cell type within the circulation with significant turnover occurring on a daily basis. It has been postulated that erythrocytes and the CR1 receptor participate strongly in the removal of immune complexes and products of apoptosis are removed from the blood through the reticuloendothelial system without inflammation via a complement dependent mechanism (406,407). Interestingly, the erythrocyte membrane contains a serine protease, p57, which is known to cleave C3 (408). AAT has been identified as the principal endogenous p57 C3-cleaving serine proteinase inhibitor, it has been postulated that AAT may prevent the activity of p57 on the erythrocyte surface during transportation of C3b or iC3b bearing immune complexes or alternatively that AAT could prevent the cleavage of C3 in situ by membrane associated p57 in circulation (365). A finding supported by a recent study examining the function
of complement mediated-removal of denatured protein fragments from blood via an erythrocyte CR1 clearance mechanism (339). It is possible that humoral AATD may result in enhanced proteolytic cleavage of C3 with complement activation on the erythrocyte membrane at sites of inflammation.
7.1.4 Implications for complement activation in AATD

Humoral deficiency of AAT alone was not associated with complement activation in this study and healthy PIZZ individuals had a similar degree of complement activation/turnover compared to healthy controls. Despite clinical stability in the subjects enrolled in this study, the relationship between C3 activation and pulmonary emphysema points to a chronic inflammatory process in the lungs of AATD individuals. This may relate to an inflammatory burden in the lung that perpetuates an inflammatory cycle as discussed above. Local complement proteolysis in a protease rich environment, such as during a pulmonary exacerbation, may also lead to an ‘opsonin-receptor mismatch’ with resultant ineffective complement opsoninophagocytosis (344). It has previously been shown that AAT can cause steric inhibition of complement dependent monocyte phagocytosis through its carbohydrate residues (342,343). Though this process was due to the effects of glycan residues and not specific to AAT, other similar glycoproteins could potentially mediate similar effects. AAT has been exploited therapeutically in clinical trials to restore protease/anti-protease balance in the CF lung as well as in AATD (262,351). Our evolving understanding of the role of complement in lung disease may uncover a more complex interplay between host innate immune defence mechanisms and adaptive immunity in AATD. As previously discussed, it has been shown that cigarette smoke can directly lead to complement activation and a number of studies indicate the critical role of complement anaphylatoxins, C3a and C5a, in leucocyte recruitment and signalling through their respective receptors in the pathogenesis of emphysema. It is plausible that individuals with AATD have an increased susceptibility to emphysema mediated by NE proteolysis, excess complement activation, inflammatory cell recruitment, development of autoimmunity and failure to reestablish homeostasis. Drug development targeting complement activation may have a benefit in the prevention of emphysema progression and aid in the resolution of inflammation in this population.
7.2 Summary:

A tremendous amount of scientific endeavor over the past number of decades has led to a much deeper understanding of the role of AAT in health and disease (84,409). There is a growing body of evidence that documents the anti-inflammatory properties of AAT, which include the modulation of inflammatory cytokine signaling, reduced neutrophil recruitment and migration, inhibition of apoptosis, and vascular endothelial effects (85,89,92,116,410). This has led to the evaluation of augmentation therapy in a variety of non-deficient states with clinical trials underway (183). The neutrophil is the central effector cell of the innate immune system and the contents of its granules, which are so potent for microbial killing, have devastating consequences on bystander host tissue, particularly in the lung. Indeed the role of neutrophil-derived proteases is increasingly recognized in inflammatory states, through the activation of lymphocytes and modulating the biological activity of chemokines, cytokines and growth factors (411). The protease/anti-protease theory of pulmonary emphysema has long stood as the most influential factor for the development and progression of lung disease in AATD (40). Moreover, oxidative inactivation of AAT provides an elegant mechanism to explain the development of emphysema in smokers without AATD due to an acquired inadequate protease inhibitory shield (35,37). The provenance of this inflammatory cycle appears to relate to cigarette smoke exposure with consequent triggering of neutrophil influx and connective tissue breakdown, though the role of macrophages and metalloproteinase activity cannot be overlooked (80). This model provides important insight into the pathophysiology of alveolar destruction, however it fails to explain why progressive lung function decline ensues, albeit at a slower rate, despite smoking cessation. The main determinant for this persistent inflammatory state in AATD is subject to debate.

The findings of this thesis demonstrate that those with a more severe pulmonary clinical phenotype, characterized by worsening emphysema, have evidence of persistent inflammation despite clinical stability. The binding of AAT to various
proteins within the circulation alludes to a potential important mechanism through which AAT mediates its anti-inflammatory properties. Complement activation may be one mechanism whereby perpetuation of inflammation is mediated through an axis of neutrophil recruitment, activation, protease release, complement degradation, and generation of chemotactic complement peptides with consequent further neutrophil recruitment.

The finding of a relationship between complement activation in PiZZ individuals and the observed pulmonary clinical phenotype implicates this pathophysiological process in pulmonary disease progression and may serve as a biomarker of disease severity. Furthermore, the role of C3d as an innate immune adjuvant to the adaptive immune response cannot be overlooked. This bridge between innate and adaptive immunity provides has implications for the pathogenesis of pulmonary emphysema, a mechanism that warrants further investigation in the future.

AAT augmentation therapy may have a multifaceted role within this disease system through down-regulatory effects on neutrophil recruitment and activation, inhibition of complement deposition and subsequent phagocytic activity, protection of intact C3 through binding and inhibition of proteolysis and potentially binding and neutralizing cationic complement peptides C3a and C5a.
7.3 Future directions:

Since the initial discovery of alpha-1 antitrypsin deficiency the scientific community has looked to this disorder to better understand the pathophysiology of pulmonary emphysema. The determination of an accurate clinical phenotype of pulmonary disease in AATD may permit better selection and characterisation of patient populations for clinical trials; incorporating subjective and objective outcome measurements as employed in Chapter 3 can permit a more detailed evaluation of therapeutic interventions.

The elucidation numerous binding partners to AAT complexed at a high molecular weight illustrates the broad spectrum of interactions between AAT and other abundant plasma proteins. While the focus of this thesis concentrated on the interaction of AAT with the complement system, a number of other exciting binding partners were also identified including a number of proteins of the lipid transport system (e.g. ApoB100, ApoA1) and the coagulation system (e.g. fibrinogen, antithrombin III). Though some of these protein interactions have previously been characterized, many interactions have not. Our understanding of the importance of these interactions is far from complete, though what is apparent is that these seemingly separate plasma protein systems do not exist in isolation and that many complex interactions are occurring to maintain homeostasis within a diverse biological system. The interplay between serine protease inhibition, coagulation, lipid transport, and the complement system would be an intriguing area of future research with important implications for our understanding of innate immunology.

The experimental techniques employed in the discovery phase of Chapter 4 did not evaluate for less abundant proteins, such as cytokines and chemokines whose molecular mass is below the resolution of the permeation chromatography system employed. There may be other important plasma interactions that were not encountered, such as the documented ability of AAT to bind pro-inflammatory cytokines such as IL-8 through electrostatic
interactions with AAT glycan residues. A relevant future direction of research area may be to complete the evaluation of AAT binding to proteins, cytokines, and peptides of low abundance in the regulation of inflammation, in addition to its anti-protease effects. Understanding the roles and consequences of such protein interactions is fundamental to the knowledge of systems biology as well as uncovering novel therapeutic mechanisms for AAT augmentation therapy.

Many questions remain in relation to the activation and perpetuation of pro-inflammatory pathways, in recent years this has led to an unparalleled and extraordinary rate of development of novel monoclonal antibodies to target specific epitopes associated with inflammation and disease (e.g. anti-TNF therapies in rheumatologic disease; infliximab, etanercept). However despite the specificities of these biological agents, there are persistent concerns in relation to immunosuppression and increased rates of mycobacterial infection (412). Due our better understanding of the many anti-inflammatory effects of AAT augmentation therapy, and its excellent safety profile, the study of its use in inflammatory disorders presents a great opportunity. The results of this study indicate that inflammation is an active proves in stable AATD individuals with pulmonary emphysema; a fascinating future research project may examine the modulatory effects of AAT augmentation therapy on inflammatory signalling, cytokine expression, and complement activation in non-AATD pulmonary emphysema and a host of inflammatory diseases.

Complement activation is a biomarker of emphysema in AATD, however it was not evaluated in usual COPD/emphysema in this study, complement activation in emphysema in non AAT-deficient individuals remains a largely unexplored area with only a few human studies performed to date as outlined earlier. Some interesting animal models of complement deficiency have been developed with some emerging evidence that indicates that complement activation is required for the pathogenesis of emphysema. The findings of this thesis indicate that complement activation occurs in AAT pulmonary emphysema, it would be interesting to explore a more direct relationship with complement activation and
the progression of emphysema over time and assess if a relationship exists between plasma levels and CT determined emphysema densitometry. Should this be the case, then complement activation products may be used as a biomarker for pulmonary disease progression. Elucidation of a biomarker in conjunction with current clinical parameter measurements would significantly advance the management of AATD in a number of ways:

1. Contribute to our knowledge of the pathogenesis of emphysema
2. Establish the presence and activity of disease
3. Assess or monitor the effectiveness of AAT augmentation therapy or other novel therapies in AATD or non-AATD pulmonary emphysema.

Furthermore, targeting the inhibition of complement activation or blocking the signalling of effector molecules such as C3a and C5a may have important therapeutic implications for the management of emphysema. In addition, acute exacerbations of COPD can be particularly challenging to manage, and novel therapies directed at complement effector mechanisms may prove useful in clinical practice. Eculizumab is a monoclonal antibody that binds to C5 and prevents its cleavage into its active fragments; it is the first drug targeting the complement system that has been shown to be effective for the treatment of a disease, i.e. paroxysmal nocturnal haemoglobinuria. At present, the cost of eculizumab is prohibitive however future drug development strategies to inhibit the activity of bioactive complement fragments, either through binding or inhibiting their interaction with cognate receptors, could be explored in the treatment of emphysema.

The identification of increased circulating C3d is of particular interest, given its role as an innate immune adjuvant through its interaction with APCs and T-cells. The effect of complement activation of Fc gamma receptor signalling may prove important in the pathogenesis of autoimmunity which is increasingly being recognized as a pathological entity in COPD and AATD (246,413). Future studies may lead to an better understanding of the facilitatory role of complement products on autoimmunity in COPD, and their contribution to the persistence of
inflammation in the lung and as well as other organs. C3d itself may also be a promising drug target, whereby inhibition of C3d activity may downregulate immune activation and induce immune tolerance for damaged lung tissue to prevent progression of disease. An attractive option would be to perform an evaluation of AAT augmentation therapy on complement system activation and the induction of auto-immunity. This could be explored initially *in vitro* or in a cell-line model, with extension to animal studies possible within a short period of time.
Chapter 8

8.1 References


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Appendix 1:
You are being invited to take part in a clinical research study to be carried out at Beaumont Hospital.

Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP (doctor). Take time to ask questions – don’t feel rushed and don’t feel under pressure to make a quick decision.

You should clearly understand the risks and benefits of taking part in this study so that you can make a decision that is right for you. This process is known as ‘Informed Consent’.

You don’t have to take part in this study and a decision not to take part will not effect on your future medical care.

You can change your mind about taking part in the study any time you like. Even if the study has started, you can still opt out. You don’t have to give us a reason. If you do opt out, rest assured it won’t affect the quality of treatment you get in the future.
Alpha-1 antitrypsin deficiency is a hereditary disorder. Patients with alpha-1 antitrypsin deficiency have an abnormal gene resulting in lower than normal levels of alpha-1 antitrypsin in the blood. Alpha-1 antitrypsin is a protein produced by the liver which travels in the bloodstream and protects your lungs. The normal alpha-1 gene is called the M gene. The most common abnormal genes in Ireland are the S and Z genes. Patient’s suspected of having alpha-1 antitrypsin deficiency are characterised based on the presence of these genes as below:

1. Normal (MM)
2. Carry one alpha-1 gene (MZ) or
3. Carry two alpha-1 genes (SZ or ZZ).

Patients with the S or Z genes have less alpha-1 antitrypsin in their blood and lungs and this may lead to lung or liver disease.

While we are certain that patients who carry two abnormal genes (SZ or ZZ) have an increased risk of lung disease (emphysema), there still remains a large degree of uncertainty if patients who carry one abnormal (Z) gene are at increased risk of disease. We believe the immune response is altered in alpha-1 antitrypsin deficiency and we will be investigating this proposition in great detail.

This study is being carried out by the Department of Respiratory Medicine in Beaumont Hospital under the supervision of Professor N.G. McElvaney (Professor of Medicine, Consultant Respiratory physician) and Dr. Emer Reeves (Chief Scientist). This study will be funded by the Royal College of Surgeons in Ireland. This research will be included as part of an MD thesis for Dr Emmet O’ Brien and Dr Cormac McCarthy.

You are being asked to take part in this study because you have been evaluated for alpha-1 antitrypsin deficiency.

You and about 150 other people are being asked to provide a blood sample in the outpatients or in the Education and Research Centre of Beaumont Hospital. If you are having a diagnostic test called a bronchoscopy performed, you may also be asked to provide a sample of lung fluid during that procedure.

If you agree to take part in this study you may be asked to provide a small amount of blood (10 teaspoons) taken through a ‘regular needle’ by the doctor. You may be asked some simple questions about your physical condition to establish if you have a ‘flare up’ or exacerbation of
your lung disease. You will also be provided with a confidential health questionnaire that aims to establish your current health status and risk factors for lung disease, this questionnaire should take less than ten minutes to complete. By agreeing to participate in this study you give the study team permission to review your medical records and collect information about your medical history. We will also evaluate any radiological imaging you have had performed recently, such as a CAT scan or ultrasound. All information about your medical history and treatment will be kept completely private. Your care in the respiratory outpatients department is unaffected.

What other treatments are available to me?

You may be currently receiving treatment for your alpha-1 antitrypsin deficiency from your respiratory consultant (inhalers and tablets). Taking part in this study will not affect this treatment but your doctor will be able to review your individual needs at the study visit or later when you are seen again in the respiratory outpatients. If you do not take part in this study your treatment will not be affected in any way.

What are the benefits?

You may not benefit directly from taking part in this study, however, it is expected the scientific knowledge gained will add to our understanding of alpha-1 antitrypsin deficiency and lead to better treatments.

What are the risks?

Having blood drawn from a vein involves very little risk, other than mild discomfort, bruising, feeling faint or fainting or very rarely, infection at the blood collection site.

Having a sample of ‘broncholalveolar’ (BAL) fluid removed from the lung is a routine procedure for those undergoing bronchoscopy as part of their routine clinical care, it involves ‘washing’ a small volume of sterile water into the lung while you are sedated for the procedure. It is not painful and you will not feel anything, most people do not remember the procedure being performed. It is very safe, the most common side effect of this procedure is a self limiting temperature within 24 hours of the test being performed.

What if something goes wrong when I’m taking part in this study?

In the very unlikely event that you are harmed in any way, the researchers in this study are qualified doctors with many years experience and should be able to deal with any problems, concerns or questions. They are also covered by insurance through the clinical indemnity scheme and the doctors have current medical malpractice insurance cover. This insurance will cover you if you are injured as a result of taking part in this study.

Will it cost me anything to take part?

There are no costs to you for participating in the study. You will not be charged for any study related testing.
Is the study confidential?

Any information collected will be anonymous—that is data that does not identify you by name—and will have coded identities only available to the above-named investigators. Only medical staff employed by the Royal College of Surgeons in Ireland will have direct access to your personal medical records. Your information and samples will be stored for up to 10 years. Although we do plan to publish the study in scientific journals, it will not be possible to identify you in any published information. All researchers and medical staff are obliged to maintain confidentiality at all times. Your GP will be not be notified of your participation in the study unless you request otherwise.

Where can I get further information?

If you have any further questions about the study or if you want to opt out of the study, you can rest assured it would not affect the quality of treatment you get in the future.

If you need any further information now or at any time in the future, please contact:

Name Dr Emmet O’Brien
Address Education & Research Centre,
Royal College of Surgeons,
Beaumont Hospital,
Beaumont
Dublin 9.

Phone No 01 809 3796 (office hours only)
**Patient Consent Form**

**Study title:** Phenotypic Variance in Alpha-1 Antitrypsin Deficiency: Environmental Influences and the Immune Response.

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<th>I have read and understood the Information Leaflet about this research project. The information has been fully explained to me and I have been able to ask questions, all of which have been answered to my satisfaction.</th>
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<td>I understand that I don’t have to take part in this study and that I can opt out at any time. I understand that I don’t have to give a reason for opting out and I understand that opting out won’t affect my future medical care.</td>
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<td>I am aware of the potential risks of this research study.</td>
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<td>I give permission for researchers to look at my medical records to get information. I have been assured that information about me will be kept private and confidential.</td>
<td>Yes</td>
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<td>I have been given a copy of the Information Leaflet and this completed consent form for my records.</td>
<td>Yes</td>
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<td><strong>Storage and future use of information:</strong> I give my permission for information collected about me to be stored or electronically processed for the purpose of scientific research and to be used in related studies or other studies in the future but only if the research is approved by a Research Ethics Committee.</td>
<td>Yes</td>
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<td>I agree to give a blood and/or bronchoalveolar fluid sample for this research project. I understand that giving a blood and/or bronchoalveolar fluid sample for this research is my own decision.</td>
<td>Yes</td>
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<td><strong>Storage and future use of biological material:</strong> I give permission for my samples and information collected about me to be stored for possible future research studies but only if the research is approved by a Research Ethics Committee.</td>
<td>Yes</td>
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| Patient Name (Block Capitals) | Patient Signature | Date |

**Version 1.1**  **Date 21/10/13**  **Page 2**
To be completed by the Principal Investigator or nominee.

I, the undersigned, have taken the time to fully explain to the above patient the nature and purpose of this study in a way that they could understand. I have explained the risks involved as well as the possible benefits. I have invited them to ask questions on any aspect of the study that concerned them.

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3 copies to be made: 1 for patient, 1 for PI and 1 for hospital records.
Appendix 2:
Alpha-1 Antitrypsin Deficiency Health Status Questionnaire

Please fill out all sections below to the best of your knowledge. Please tick all relevant boxes ☑️.
Any additional details are welcome.
All information is strictly confidential.

Q1. Diagnosis:
   a) What age were you diagnosed with alpha-1? □ ☐ Yes ☐ No
   b) Were you diagnosed through family screening? ☐ Yes ☐ No
   c) Do you know what alpha-1 gene you carry? ☐ Yes ☐ No
      If so, what is it? (e.g. MZ, SZ, ZZ)
   d) Have you been diagnosed with any of the following conditions?
      □ COPD □ Emphysema □ Bronchitis □ Asthma □ Bronchiectasis □ Liver Disease
      □ High Blood Pressure □ High Cholesterol □ Angina/Heart Attack

Q2. Occupation:
   a) Current: ______________________________________________________________
   b) Previous: ______________________________________________________________
   c) Have you had to change your job due to being diagnosed with alpha-1? ☐ Yes ☐ No
   d) Have you had any exposure to any chemical fumes or dusts in the course of your employment?
      ☐ Yes ☐ No
      i. If so, please explain __________________________________________________
      __________________________________________________
      ii. Were you provided with protective breathing equipment? ☐ Yes ☐ No

   e) How many years have you spent living in each of these environments?
      Urban/Town: ________ Suburban: ________ Countryside: ________
Q3. Smoking Status:

☐ Never smoker (may skip to Q4)

☐ Current smoker Cigarettes/day: ____________

☐ Ex-smoker (or tobacco equivalent:_________

Years smoking: ____________

a) Have you had passive smoke exposure? ☐ Yes ☐ No

When did this occur? ☐ Childhood ☐ Adulthood

☐ Parents ☐ Partner ☐ Other, (please specify) ____________

☐ Open fire at home ☐ Workplace

b) Has the workplace-smoking ban in Ireland affected your passive smoke exposure? ☐ Increased ☐ Decreased ☐ No change

c) Did a diagnosis of alpha-1 motivate you to quit smoking? ☐ Yes ☐ No

Was this attempt(s) successful? ☐ Yes ☐ No

If yes, how long after a diagnosis of Alpha-1 did you quit? ____________

If no, did you reduce the amount smoked? ☐ Yes ☐ No

If so, by how much? ____________ Cigs/day

d) Have you sought help to quit smoking? ☐ Yes ☐ No

e) Have you used any therapies to help you quit smoking?

☐ None ☐ Zyban (Bupropion)

☐ Nicotine patch ☐ E-cigarette

☐ Nicotine inhaler ☐ Smoking cessation programme

☐ Champix (Varenicline) ☐ Online (internet) support service

Other , please specify (e.g. hypnotherapy) ________________

Q4. Vaccinations:

a) Have you received the flu vaccine recently, if so please tick all years that apply.

☐ No recent vaccines ☐ 2013 ☐ 2012 ☐ 2011 ☐ 2010

b) Do you plan to receive the flu vaccine in future? ☐ Yes ☐ No

c) Have you received the pneumonia vaccine in the last 5 years? ☐ Yes ☐ No
Q5. Baseline Health Status

a) Please rate your current health status by marking (X) on the scale below:

```
0 10 20 30 40 50 60 70 80 90 100

Worst imaginable health state  Best imaginable health state
```

b) Please describe your usual level of breathlessness: (please tick one only)

- I only get breathless with strenuous exercise
- I get short of breath when hurrying on level ground or walking up a slight hill.
- On level ground, I walk slower than people of the same age because of breathlessness, or have to stop for breath when walking at my own pace.
- I stop for breath after walking about 100 yards or after a few minutes on level ground.
- I am too breathless to leave the house or I am breathless when dressing.

\[\square\] Yes  \[\square\] No

If yes; how long have you had it? __________________

When does the cough most affect you?

- Morning  - Evening  - Daytime  - Night-time  - All day

\[\square\] Morning  \[\square\] Evening  \[\square\] Daytime  \[\square\] Night-time  \[\square\] All day

\[\square\] Yes  \[\square\] No

d) Do you produce sputum/phlegm?

- How would you describe it?

  - \[\square\] Clear  \[\square\] Dirty  \[\square\] Dirty only with a chest infection

  - \[\square\] Yes  \[\square\] No

e) What volume of sputum do you produce in a day?

- Teaspoon (5mls)  - Tablespoon (10mls)
- Egg cup (15mls)  - >15mls, please specify ______

\[\square\] Teaspoon (5mls)  \[\square\] Tablespoon (10mls)

\[\square\] Egg cup (15mls)  \[\square\] >15mls, please specify ______

\[\square\] Yes  \[\square\] No

f) Do you suffer from sinus congestion?

\[\square\] Yes  \[\square\] No

g) Do you consume alcohol?

How many units of alcohol do you consume per week?

\[\square\] Yes  \[\square\] No

How many units of alcohol do you consume per week?

(1 unit = half pint of beer or small measure of spirits. 1.5 units = small glass of wine)
Q6. Exacerbations:

a) Have you had a chest infection in the past 12 months? □ Yes □ No
   If yes, how many?
   How many courses of antibiotics were prescribed?

b) Have you had a chest infection in the past 2 years? □ Yes □ No
   If yes, how many?

Q7. Medications:

a) Do you currently use portable oxygen? □ Yes □ No
b) Do you currently use overnight oxygen or at rest? □ Yes □ No
c) Are you receiving Alpha-1 replacement therapy as an extension of a previous clinical research trial (e.g. intravenous/nebulised)? □ Yes □ No

d) Do you use an inhaler? □ Yes □ No
   If yes, which one? (please tick all that apply)
   □ Ventolin (blue) □ Spiriva
   □ Budesonide (brown) □ Seebri
   □ Seretide (purple) □ Onbreeze
   □ Symbicort (white & red) □ Other, please specify ______
   □ Unsure

e) Do you use a nebulizer? □ Yes □ No
   If yes, which one? (please tick all that apply)
   □ Ventolin
   □ Atrovent
   □ Combivent
   □ Pulmicort
   □ Unsure

f) Do you take steroid tablets daily (e.g. prednisolone)? □ Yes □ No
   If yes, what is the dose?
   If no, have you had a course in the past 12 months? □ Yes □ No
   How many courses?
g) Have you received an organ transplant as a result of alpha-1 antitrypsin deficiency?  
☐ Yes  ☐ No

If yes:  ☐ Lung  ☐ Liver

h) Are you currently on an organ transplant waiting list?  
☐ Yes  ☐ No

If yes:  ☐ Lung  ☐ Liver

Please provide any additional details or comments below:
Appendix 3:
**Modified Bhalla Score for α-1-Antitrypsin Disorder:**

1. Severity of bronchiectasis was graded on a scale of 0-3
   a. 0 = absent
   b. 1 = mild (bronchiole lumen diameter > than that of the adjacent blood vessel)
   c. 2 = moderate (bronchiole lumen diameter is 2-3 times that of the adjacent blood vessel)
   d. 3 = severe (bronchiole lumen diameter > 3 times that of the adjacent blood vessel)

2. Peribronchial thickening was graded on a scale of 0-3
   a. 0 = absent
   b. 1 = mild (bronchiole wall thickness = diameter of the adjacent blood vessel)
   c. 2 = moderate (bronchiole wall thickness ≥ twice the diameter of the adjacent blood vessel)
   d. 3 = severe (bronchiole wall thickness > twice the diameter of the adjacent blood vessel)

3. Extent of bronchiectasis was graded on the number of bronchopulmonary segments involved in each lobe

4. Presence of bullae

5. Emphysema was graded on a scale of 0-3
   a. 0 = absent
   b. 1 = mild (small/patchy regions of decreased lung density with predominantly normal looking lung parenchyma)
   c. 2 = moderate
   d. 3 = severe (predominantly abnormal looking lung)
Peer reviewed publication
The impact of smoke exposure on the clinical phenotype of alpha-1 antitrypsin deficiency in Ireland: exploiting a national registry to understand a rare disease.

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Abstract

Individuals with Alpha-1 antitrypsin deficiency (AATD) have mutations in the SERPINA1 gene causing genetic susceptibility to early onset lung and liver disease that may result in premature death. Environmental interactions have a significant impact in determining the disease phenotype and outcome in AATD. The aim of this study was to assess the impact of smoke exposure on the clinical phenotype of AATD in Ireland.

Clinical demographics and available thoracic computerised tomography (CT) imaging were evaluated from 139 PiZZ individuals identified from the Irish National AATD Registry. Clinical information was collected by questionnaire. Data was analysed to assess AATD disease severity and evaluate predictors of clinical phenotype.

Questionnaires were collected from 107/139 (77%) and thoracic CT evaluation was available in 72/107 (67.2%). 74% of respondents had severe Chronic Obstructive Pulmonary Disease (COPD) (GOLD stage C or D). Cigarette smoking was the greatest predictor of impairment in FEV1 and DLCO (%predicted) and the extent of emphysema correlated most significantly with DLCO. Interestingly the rate of FEV1 decline was similar in ex-smokers when compared to never smokers. Passive smoke exposure in childhood resulted in a greater total pack year smoking history. Radiological evidence of bronchiectasis was a common finding and associated with increasing age.

The Irish National AATD Registry facilitates clinical and basic science research of this condition in Ireland. This study illustrates the detrimental effect of smoke exposure on the clinical phenotype of AATD in Ireland and the benefit of immediate smoking cessation at any stage of lung disease.
Introduction

Alpha-1 antitrypsin deficiency (AATD) is an autosomal co-dominant inherited condition that results in reduced circulating levels of AAT protein and predisposes affected individuals to early onset lung and liver disease. It was first described by Laurell and Eriksson in 1963(1) and has since been recognised as one of the most common genetic conditions affecting people of Western European descent.

Despite its prevalence AATD is a condition that is rarely diagnosed. Detection rates in some countries are as less than 10% of the at risk population(2), this may relate to low awareness of AATD among physicians which can often lead to significant delays before the diagnosis is reached(3). The WHO, ATS and ERS advocate targeted testing for AATD in all individuals with COPD, non-responsive asthma, cryptogenic liver disease and first degree relatives of individuals with AATD(4). This approach has led to higher rates of detection in the populations most at risk of lung disease, though widespread under recognition of the condition remains (5,6).

The development of national registries for individuals with AATD can address many of the shortcomings in our knowledge of the disorder; facilitate understanding of the natural history of AATD; promote patient education and dissemination of information; aid recruitment for clinical research studies; and assist international collaboration with colleagues through research initiatives (7,8). In cystic fibrosis, a disease with a similar prevalence, the establishment of registries has led to significant improvements in patient related outcomes (9).

Irish registry experience

The establishment of the Irish National AATD Targeted Detection Programme in 2004 facilitated an increased detection rate of AATD, and in 2007 the National AAT Registry was created. The registry is maintained by the Irish Alpha One Foundation. The national referral centre for AATD in Ireland is based in Beaumont Hospital, Dublin. Referrals are received from across the island of Ireland covering a population of 6.38 million people. Given the relatively small geographic size of the country, all individuals with AATD can potentially be assessed in a single centre.

Our programme has detected over 12,000 individuals, leading to the diagnosis of 250 PiZZ and 185 PiSZ individuals. In addition to the common AATD alleles, we have identified a number of rare mutations including two newly described null mutations, a Null/Null homozygote and M_{Malton}/M_{Malton} homozygote (10). In the past year our registry information technology systems have been upgraded to enable more efficient data entry and extraction. In addition our website has been updated to be more user friendly and expand the resources and information available for individuals and family members of those affected by AATD (www.alpha1.ie).
**AATD epidemiology in Ireland**

The gene frequency for the Z protein is most prevalent in northern and western European countries with severe deficiency (PiZZ) affecting up to 1:1500 (2), though it is less common in countries of predominantly Western European descent such as regions of North America and the Antipodes (11,12). In Ireland 1:2104 individuals are PiZZ homozygotes, though in our targeted detection program 1:71 tested were PiZZ homozygotes highlighting the effectiveness of the targeted detection approach (5).

The detection of asymptomatic individuals (non-index) through the family screening of probands is an opportunity to better understand the natural history of AATD (13,14). Targeted detection will invariably detect many MZ heterozygotes and clarification of the true risk of lung disease in this population is important, particularly in smokers. Utilising index cases identified from the Irish Registry, it has been established that ever-smoking PiMZ individuals have an increased risk for COPD and this risk is attenuated in never-smokers (15).

**Clinical trial research**

Augmentation therapy for AATD was approved for clinical use in Ireland in 2007, however it remains inaccessible for patient use due to concerns about the significance of its clinical efficacy, the high cost of treatment, and lack of reimbursement for treatment by the Health Service in Ireland. The Irish National AATD Registry has proven to be an excellent resource for the recruitment of Irish patients with severe AATD into clinical trials. Our centre continues to participate in a number of international randomised controlled clinical trials, including intravenous AAT augmentation therapy (NCT00261833, NCT00670007) and nebulised AAT therapy (NCT01217671). The facilitation of clinical trial research into AATD in Ireland raises awareness of AATD, advances scientific knowledge, and importantly facilitates earlier access to augmentation therapy for patients affected by the condition.
Original Research:

Aim

To assess the impact of smoke exposure on the clinical phenotype of AATD in Ireland.

Methods

Study population

The National AATD registry was used to identify all individuals with a confirmed PiZZ phenotype currently living in Ireland in January 2014. Registry participants who had undergone full clinical evaluation within the past five years at the national referral centre in Beaumont Hospital were selected for this study and clinical demographics were recorded. All participants provided written informed consent, which was approved by the Beaumont Hospital Research Ethics Committee.

Pulmonary function testing was performed in all participants according to American Thoracic Society standards (16); post bronchodilator FEV1, FVC percent predicted, and gas transfer (DLCO) measured by the single breath carbon monoxide method were recorded. Pulmonary function test results from the preceding five years were recorded; the annualised rate of FEV1 decline was determined by regression analysis in those with three or more sequential results over a time period of one year or more.

Questionnaire

Cross-sectional data was collected from the study participants via a self-reported questionnaire that was distributed by post, questionnaires were also distributed in the outpatient clinic. Each returned questionnaire was coded to enable matching to the relevant individual’s clinical and radiological information and subsequently anonymised for analysis. Clinical parameters were recorded; age of diagnosis, symptomatic detection (index) vs. family screening (non-index), smoking status, passive smoke exposure, occupational exposure, frequency of pulmonary exacerbations, cough, sputum production, and oxygen use.

Dyspnoea scores were calculated using the five point modified Medical Research Council score (mMRC) and health status in the previous week was determined using a visual analogue scale (VAS) scored between 0 and 100. Pack-year smoking history was determined by the function of number of cigarettes smoked per day and total years smoked
Radiology

High Resolution CT images were obtained on a Siemens 16-slice scanner. All patients were imaged while supine and inspiratory images were obtained from the lung apices to the costophrenic angles. Scanning parameters were 120 kV and 90 mA. A modified version of Bhalla’s scoring system for thin section CT in patients with AATD was applied to each scan (17,18). All lobes were individually assessed for purposes of evaluating severity of emphysema, bronchiectasis and peribronchial thickening. All criteria were scored on a scale of 0-3. Once scores were assigned to each of the parameters, they were added to the patient’s individual score to a maximum of 21. Higher scores therefore indicate greater severity. Two radiologists with a specialist interest in thoracic radiology reviewed all scans independently; a consensus opinion was then determined. Both radiologists were blinded to the clinical severity of AATD when scoring.

Statistical analysis

Data was analysed using GraphPad Prism v7.0 and STATA v13.0 was employed for stepwise multiple regression modelling. Statistical significance was determined as a two-tailed p-value <0.05. Characteristics of the respondents were summarised using number and percentage of participants in each category. Pearson’s correlation coefficients were used to identify significant bivariate relationships. To determine factors that influence the severity of disease, significant relationships identified on univariate analysis were entered as independent variables into a stepwise multiple regression analysis with ascertainment (index vs. non-index) and smoking status assessed separately as the dependent variables.
Results

Response

A total of 204 PiZZ individuals were identified from the National AATD Registry. Seven individuals (3.4%) died within the preceding twelve months. Questionnaires were distributed to 139 individuals who had attended Beaumont Hospital within the past five years. The response rate was 107/139 (77%) and clinical demographics were available for all respondents. Thoracic CT imaging was available in 72/107 (67.2%) and this was analysed separately. 21/107 (19.6%) of respondents were currently receiving intravenous AAT augmentation therapy.

GOLD classification

Respondents were classified according to the updated GOLD document (19). 17 (16%) of respondents had no spirometric evidence of obstructive airway disease and were asymptomatic. 7(6.5%) were classified as GOLD group A, 4 (3.7%) group B, 33 (31%) group C and 46 (43%) as group D. 83/107 (77.6%) of respondents reported usage of a combined corticosteroid/LABA inhaler, with 58/107 (54.2%) reporting inhaled LAMA usage. DLCO correlated well with FEV1 (r=0.73, p<0.0001), and emphysema (r=0.83, p<0.001), (Figures 1A and 1B). 31/107 (29%) of individuals used portable O2 and 17 of these were reported using long term O2 therapy (LTOT). A DLCO value of <50% had a sensitivity of 96.7% and specificity of 76.3% to predict the requirement for portable O2 usage (Figure 1A).

Exacerbations

Respondents reported a mean frequency of pulmonary exacerbations of 2.1/annum in the past year and 1.82/annum over a two-year period. Increased frequency of pulmonary exacerbation was associated with more severe impairment in FEV1, lower DLCO values and higher emphysema scores. Interestingly there was no association found with pulmonary exacerbations or radiologically assessed bronchiectasis, though some individuals with a more severe bronchiectatic phenotype did have a high number of exacerbations in the past two years.

The compliance rate with annual influenza vaccination programmes was high and increased on an annual basis over the preceding four years from 62/107 (58%) to 81/107 (75.7%). Uptake of the pneumococcal vaccine was also high at 76/107 (71%).

Radiological findings
A subgroup analysis of respondents was performed on those with available thoracic CT imaging in 72/107 respondents. The majority of respondents, 52/80 (65%), had radiological evidence of emphysema that was most severe in the lower lobes; smokers had more severe emphysema at an earlier age compared to never smokers (Figure 1C). Radiological evidence of bronchiectasis was evident in 58/72 (80.6%) of respondents, this was mild in the majority of cases and there was no lobar preponderance. There was no correlation between the presence of emphysema and bronchiectasis, \((r=-0.1664, p=0.1625)\), (Figure 1D). Bronchiectasis increased with age \((r=0.41, p=0.004)\), and while there was a similar age-adjusted prevalence of bronchiectasis in smokers and never smokers, it was more significant in never smokers \((5.08 \text{ vs. } 2.98, p=0.02)\), (Figure 1E).

The emergence of different radiological phenotypes was evident; the individuals with the most severe bronchiectasis had little or no evidence of emphysema and those with the most severe emphysema had mild bronchiectasis. Multivariate regression analysis identified increasing age as the most significant risk factor for the development of bronchiectasis independent of smoking history \((p<0.001)\). Those with predominant radiological evidence of bronchiectasis reported less cough, sputum production and pulmonary exacerbations compared to those with evidence of emphysema indicating a milder clinical phenotype in this group.

**Index versus non-index cases**

Of the respondents 72/107 (67.3%) were index cases and the remaining 35 (32.7%) non-index cases were detected by family screening. Both groups were well matched in relation to symptoms of cough, sputum production, pulmonary exacerbations and health status measurement as both groups had a similar percentage of smokers and equal pack year smoking history (see Supplementary Table). However on univariate analysis index cases appeared to have significantly lower FEV1 values \((58\% \text{ vs. } 74\%, p=0.0273)\), DLCO values \((51\% \text{ vs. } 64\%, p=0.0126)\) and a greater degree of airflow obstruction \((47\% \text{ vs. } 58\%, p=0.01)\) despite similar rates of FEV1 decline \((-36.4\text{mls/annum vs. }-51.2\text{mls/annum, } p=0.49)\) (Supplementary Table). In the univariate subgroup analysis of the thoracic CT data it was also found that index cases had higher mean emphysema scores \((9.574 \text{ vs. } 5.52, p=0.011)\). However on adjustment for age, there was no significant difference observed in FEV1, airflow obstruction or emphysema between the groups \((P=0.51)\) (Supplementary Figure A). As both index and non-index cases had similar lifetime smoke exposure, the observed reduction in lung function in the index cases is explained by an older age in this group.
The effect of smoking

71/107 (66.3%) reported a history of smoking for more than one pack year. The majority of ever smokers had quit prior to their diagnosis of AATD 37/71 (52%). 30/34 (88%) people reported that a diagnosis of AAT helped them quit within a median time of two weeks. On univariate analysis ever smokers had a marked reduction in FEV1, DLCO and degree of airflow obstruction, increased breathlessness, poorer health status, increased sputum production, and increased frequency of pulmonary exacerbations (Table 1). Multivariate regression analysis identified the following independent variables in ever smokers; increased emphysema, lower DLCO values, increased airflow obstruction and increased sputum production. This would be consistent with the classic phenotypes of emphysema and chronic bronchitis seen in COPD. Interestingly there was less bronchiectasis observed in the ever smokers, a finding that persisted after multivariate analysis (-2.5, 95% C.I. -0.8 to -4.9, p=0.047).

There were 36/107 (33.6%) never smokers in this study with a mean FEV1 value of 83.8% and DLCO of 71%. There was no significant difference in the annualised rate of FEV1 decline between ever smokers and never smokers (Supplementary Figure B). This may be accounted for by a number of possible factors; all ever smokers were now ex-smokers, there was a lower initial FEV1 in the smoker group, the modifying effect of medication use, and the survivor effect.

Subgroup analysis of never smokers revealed that index cases have lower age adjusted FEV1 (75 vs. 95%, p=0.042) and DLCO (63 vs. 83%, p=0.006) values compared to asymptomatic never smokers (Supplementary Figure C). Multivariate regression analysis confirmed the association for lower DLCO (-9.96%, 95% C.I -4.6 to -19.5%, p=0.041), indicative of a more severe phenotype in symptomatic individuals with AATD (Supplementary Figure D).

88/107 (82%) of respondents reported passive smoke exposure in childhood with a large proportion of these, 52/88 (59%), reporting parental passive smoke exposure. Parental smoke exposure did not emerge as an independent risk factor for poorer lung function in adulthood. This may be due to the high prevalence of parental smoking overall and insufficient power to detect a statistically significant difference in lung function in our study population. Those who were exposed to passive smoke in childhood were more likely to smoke in adulthood, OR 2.650 (95% confidence interval 0.9645 to 7.279, p=0.065), and had a significantly higher mean pack year smoking history (17.25 vs. 9.84, p=0.0025), see Figure 1F. The impact on the workplace-smoking ban was assessed; 63/107 (59%) reported that passive smoke exposure had reduced as a result of the ban, 44/107 (41%) reported no change, while no subjects reported increased smoke exposure.

This data indicates that individuals with AATD who smoked have a similar clinical phenotype of COPD irrespective of their method of diagnosis (index vs. non-index).
Symptomatic never smokers have some mild impairment in pulmonary function that has brought them to medical attention, though they have a much milder clinical phenotype compared to those who ever smoked. Importantly, our data shows that asymptomatic never smokers have effectively normal lung function and no impairment in health status.

**Occupational exposure**

55/107 (51.4%) of respondents were currently in full time employment. 28/107 (26.2%) of respondents reported that they had to change job or retire as a result of AATD, this group had significant impairment in lung function compared to those who continued in employment (mean FEV1 39% vs. 71%, p=0.02). 47/107 (43.9%) of respondents reported occupational inhalational exposure during the course of their working life, the predominant exposure was to dust.
Discussion

Determina&on of &h&nomic phenotype in AATD is essential for a greater understanding of the underlying pathophysiology of the disease, the correct stratification for research studies and therapy, and to prog&sticate outcomes. The majority of respondents in this study had GOLD group D COPD reflecng the prevalence of a more severe phenotype within this AATD registry population. Obje&ve quantifica&on of pulmonary disease by thoracic CT imaging enables determination of the relationships between the primary pathophysiological process in AATD and health outcomes. In our study, pulmonary emphysema was the predominant radiological finding and it was primarily found in ever smokers. Regarding pulmonary function measurement, impairments in DLCO correlated most signiﬁcantly with higher emphysema scores. A DLCO value below 50% predicted was found to be highly predictive of portable oxygen requirement and lower DLCO values correlated strongly with worsening dyspnoea and health status measurement outcomes. This implies that patients with DLCO values below 50% predicted may beneﬁt from assessment regarding portable oxygen requirement. Recent recommendations regarding clinical trials in AATD suggest that serial CT densitometry be used as the primary endpoint to demonstrate stabilisation and prevention of progression (20), however in clinical practice this is rarely feasible and our data would suggest that DLCO may be employed as a surrogate determinant of disease status.

There remains a significant proportion of smokers with AATD that have yet to be identiﬁed, and this may relate to widespread under recognition of the disorder in the medical profession (3). In this study, no difference was observed between index and non-index cases in relation to pulmonary symptoms, measurements of lung function, and severity of emphysema. This is likely explained by the equivalent smoke exposure in both groups. Smoke exposure is the single biggest determinant for progression of emphysema in AATD. Our analysis of the inﬂuence of smoke exposure in AATD demonstrates the presence of emphysema, chronic bronchitis and resultant airﬂow obstruction with the resultant classic phenotypes of COPD presenting at a young age.

All respondents had stopped smoking at the time of this study. Individuals with AATD who quit smoking had similar rates of FEV1 decline compared to never smokers despite initial lower FEV1 values. This is a matter of great encouragement to people with AATD who wish to give up smoking and reﬂects what is seen in non-hereditary emphysema.

To better understand the natural history of AATD in never smokers, analysis of our registry revealed that lung function is normal and preserved beyond middle age in asymptomatic never smokers. This is an important observation and it is consistent with published data suggesting they have a life expectancy approaching that of the general population (13,21). Though the conﬁounding effects of genetic modiﬁers and environmental factors should be taken into consideration, never smokers with symptomatic lung disease appear to have lower FEV1 and DLCO values indicating a more severe clinical phenotype than asymptomatic never smokers. Our data implies that the
natural history of AATD in never smokers is altered at some point in symptomatic individuals, by an unknown precipitant, to worsen their condition and bring them to medical attention.

Bronchiectasis was recognised early to be a pulmonary complication of AATD, however the true prevalence and clinical significance of bronchiectasis in the AATD population remains poorly understood. Observational studies in populations of non-CF bronchiectasis have not demonstrated an increased prevalence of the condition implying that AATD as a cause of bronchiectasis is uncommon and that it occurs as a result of co-existent emphysema (22). Our data would contradict these findings, demonstrating that radiologically detected bronchiectasis is both common in AATD and that there is no dependent relationship to emphysema or indeed a history of smoking. This is supported by similar studies on bronchiectasis and emphysema in AATD and also by registry data showing a high prevalence of bronchiectasis in the AATD population in Spain and Italy (14,23). Most people in our study had evidence of mild radiological bronchiectasis with minimal associated clinical symptoms such as increased frequency of cough or pulmonary exacerbation compared to those with emphysema, however those with more severe bronchiectasis did report significantly poorer health status outcomes. Determining why this occurs is an important area of research.

It has been ten years since Ireland became the first country in the world to introduce a ban on smoking in the workplace(24). Passive smoke exposure is a known risk factor for the development of emphysema and COPD, individuals with AATD are particularly at risk and have most to benefit from public health initiatives that reduce cumulative lifetime smoke exposure (25). The fact that no individual reported an increase, and the majority reported a decrease, in passive smoke exposure speaks to the success of this programme pioneered in Ireland and emulated worldwide (24). Apart from increasing the cumulative lifetime exposure to tobacco smoke, the results of this study indicate that passive smoke exposure in childhood, in particular parental smoking, influence smoking habits in adulthood by increasing the likelihood of ever smoking and the total pack year cigarette consumption. Further efforts to reduce passive smoke exposure in automobiles are welcome initiatives that are undergoing legislative implementation in some countries at present (26).

The study of PiMZ individuals identified from probands in the Irish National AATD Registry has significantly improved our understanding of the risk of lung disease in this large population group (15). A significant proportion of the severely deficient AATD population in the Irish registry are compound heterozygote PiSZ individuals, a finding reflected in reports from other national registries (14). Despite a growing number of known PiSZ individuals there is a paucity of data in relation to outcomes of this AAT-deficient phenotype (4). Airflow obstruction appears to be milder and less common compared to PiZZ individuals, however the PiSZ phenotype is associated with significant risk of COPD in smokers (27).
Inclusion of rare SERPINA1 mutations in national registry databases permits epidemiological study regarding allele frequency and facilitates research collaboration to gain a deeper understanding of the effects of abnormal AAT protein production. Centres with expertise in genetic sequencing techniques have included increasing numbers of rare mutations identified through targeted detection programs (28). In the Irish National Targeted Detection Programme, approximately 1.5% of AATD cases detected possess a rare SERPINA1 mutation.

Conclusion

National registries play an important role in evaluating the natural history and progression of pulmonary and liver disease in AATD and contribute to our understanding of the interaction between genetic susceptibility and environmental exposure in this population. This registry study illustrates the detrimental effect of smoke exposure on the clinical phenotype of AATD in Ireland and the benefit of immediate smoking cessation at any stage of lung disease. Our data would support the premise that asymptomatic individuals with AATD who are not exposed to smoke are likely to be unaffected by pulmonary complications in their lifetime. Public health initiatives to reduce smoking uptake, promote smoking cessation and reduce passive smoke exposure are likely to be of most benefit to individuals with AATD that have yet to be diagnosed.
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Declaration of interest

MEOB received the eALTA award 2013 from Grifols. The other authors declare they have no competing interests to disclose. The authors are responsible for the content and writing of this paper.
References:


Figure 1:
A) Correlation of DLCO with FEV1 (r=0.73, p<0.0001) and a DCLO value <50% had a 96.7% sensitivity for the requirement for portable O₂. B) Significant correlation between emphysema severity and DLCO% predicted, r=0.83, p<0.0001. C) Increased severity of emphysema detected at an earlier age is observed in smokers, milder emphysema severity is observed in never smokers occurring at a later age. D) There was no significant relationship between severity of CT detected bronchiectasis and emphysema, r=-0.1664 (p=0.1625). E) Increased CT detected bronchiectasis with advancing age, no significant difference in ever-smokers vs. never smokers is observed. F) Increased total pack year smoking history in AATD individuals exposed to passive smoke in childhood (17.25 vs. 9.84 pack years smoking, p=0.003).

Supplementary Figure:
A) Similar age-adjusted FEV1 values between index and non-index cases (p=0.51). B) Linear regression analysis reveals similar rates of FEV1 decline between ever smokers and never smokers despite initial lower FEV1 values in ever smokers (-49mls/annum vs. -26mls/annum, p=0.81). C) Age adjusted FEV1 values in symptomatic (index) never smokers are lower than asymptomatic (non-index) never smokers (mean FEV1 77% vs. 95%, p=0.042). D) Age adjusted DLCO values in symptomatic (index) never smokers are lower than asymptomatic (non-index) never smokers (mean FEV1 64% vs. 83%, p=0.006). Multiple regression analysis confirmed significantly lower DCLO % predicted values in symptomatic vs. asymptomatic never smokers (-9.96%, 95% C.I -4.6 to -19.5%, p=0.04).
<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Ever Smoker</th>
<th>Never Smoker</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>61/107 (57)</td>
<td>39/71 (55)</td>
<td>22/36 (61.1)</td>
<td>ns</td>
</tr>
<tr>
<td>Age</td>
<td>53 ±12</td>
<td>53 ±11</td>
<td>53 ±15</td>
<td>ns</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>44 ±13</td>
<td>44 ±12</td>
<td>44 ±15</td>
<td>ns</td>
</tr>
<tr>
<td>BMI</td>
<td>26.5 ±5.5</td>
<td>27.16 ±5.9</td>
<td>25.2 ±4.1</td>
<td>ns</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>63 ±32</td>
<td>53 ±29</td>
<td>84 ±27</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>100 ±24</td>
<td>96 ±26</td>
<td>107 ±17</td>
<td>ns</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>0.50 ±0.2</td>
<td>0.44 ±0.17</td>
<td>0.64 ±0.19</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>DLCO (% predicted)</td>
<td>55.6 ±23.2</td>
<td>47.9 ±20</td>
<td>71 ±22.5</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Exacerbations: Past year 2 years</td>
<td>2.1 ±2.6</td>
<td>2.4 ±2.9</td>
<td>1.5 ±1.9</td>
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<tr>
<td></td>
<td>3.6 ±4.2</td>
<td>4.4 ±4.8</td>
<td>2.1 ±1.9</td>
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<tr>
<td>mMRC</td>
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<td>1.9 ±1.16</td>
<td>0.9 ±1.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pack year smoking</td>
<td>13.6 ±14</td>
<td>20.5 ±12</td>
<td>-</td>
<td>N/A</td>
</tr>
<tr>
<td>Cough</td>
<td>47/107 (43.9)</td>
<td>33/71 (46.5)</td>
<td>14/36 (38.9)</td>
<td>ns</td>
</tr>
<tr>
<td>Sputum production</td>
<td>60/107 (56.1)</td>
<td>47/71 (66.2)</td>
<td>13/36 (36.1)</td>
<td>0.004*</td>
</tr>
<tr>
<td>VAS health status</td>
<td>58 ±21.8</td>
<td>52.2 ±21.4</td>
<td>69.6 ±17.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Childhood passive smoke exposure</td>
<td>88/107 (82.2)</td>
<td>62/71 (87.3)</td>
<td>26/36 (72.2)</td>
<td>0.0646</td>
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<tr>
<td>Annual FEV1 decline</td>
<td>-41.6 ±90.4</td>
<td>-48.9 ±80</td>
<td>-26.4 ±109.1</td>
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</tr>
<tr>
<td>Emphysema</td>
<td>8.3 ±6.2</td>
<td>10.6 ±5.2</td>
<td>3.2 ±5.0</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>3.7 ±3.6</td>
<td>3.0 ±3.1</td>
<td>5.1 ±4.1</td>
<td>0.02*</td>
</tr>
</tbody>
</table>

Table 1: Univariate analysis of ever smokers vs. never smokers
Data are presented as n (%), mean ± SD unless otherwise stated.
BMI: body mass index; FEV1: forced expiratory volume in 1s (% predicted); FVC: forced vital capacity; DCLO: diffusing capacity of the lung for CO.
mMRC: modified Medical Research Council dyspnoea score; VAS: Visual Analogue Scale health stature in the past week.
* Significant on multivariate analysis (p<0.05).
<table>
<thead>
<tr>
<th></th>
<th>Total (n)</th>
<th>Index (n)</th>
<th>Non-index (n)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>61/107</td>
<td>43/72</td>
<td>18/35</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>53 ±12</td>
<td>54 ±13</td>
<td>51 ±11</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Age at diagnosis</strong></td>
<td>44 ±13</td>
<td>45.3 ±14</td>
<td>42 ±12</td>
<td>ns</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>26.5 ±5.4</td>
<td>26.2 ±5.7</td>
<td>27.2 ±5.0</td>
<td>ns</td>
</tr>
<tr>
<td><strong>FEV1 (%) predicted</strong></td>
<td>63 ±32</td>
<td>58 ±30</td>
<td>74 ±34</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>FVC (%) predicted</strong></td>
<td>100 ±24</td>
<td>98 ±23.</td>
<td>102 ±25</td>
<td>ns</td>
</tr>
<tr>
<td><strong>FEV1/FVC</strong></td>
<td>0.51 ±0.2</td>
<td>0.47 ±0.2</td>
<td>0.58 ±0.2</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>DLCO (%) predicted</strong></td>
<td>55.6 ±23.2</td>
<td>51.1 ±20.7</td>
<td>64.2 ±25.5</td>
<td>0.01*</td>
</tr>
<tr>
<td><strong>Exacerbations: Past year</strong></td>
<td>2.1 ±2.6</td>
<td>2.3 ±2.7</td>
<td>1.7 ±2.4</td>
<td>ns</td>
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<tr>
<td></td>
<td>3.6 ±4.2</td>
<td>3.8 ±3.6</td>
<td>3.4 ±5.2</td>
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<tr>
<td><strong>mMRC</strong></td>
<td>1.6±1.2</td>
<td>1.6 ±1.2</td>
<td>1.6 ±1.3</td>
<td>ns</td>
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<tr>
<td><strong>Never smoker</strong></td>
<td>88/107</td>
<td>61/72</td>
<td>27/35</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Cough</strong></td>
<td>47/107</td>
<td>32/72</td>
<td>15/35</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Sputum production</strong></td>
<td>60/107</td>
<td>41/72</td>
<td>19/35</td>
<td>ns</td>
</tr>
<tr>
<td><strong>VAS health status</strong></td>
<td>58.1 ±21.8</td>
<td>56.5 ±20.4</td>
<td>61.4 ±24.4</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Childhood passive smoke exposure</strong></td>
<td>88/107</td>
<td>61/72</td>
<td>27/35</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Annual FEV1 decline</strong></td>
<td>-41.6 ±90.4</td>
<td>-36.4 ±85.4</td>
<td>-51.3 ±100.0</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Emphysema</strong></td>
<td>8.3 ±6.2</td>
<td>9.6 ±5.5</td>
<td>5.5 ±6.2</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>Bronchiectasis</strong></td>
<td>3.7 ±3.6</td>
<td>4.23 ±4.1</td>
<td>2.5 ±2.2</td>
<td>ns</td>
</tr>
</tbody>
</table>

**Supplementary Table: Univariate analysis of index vs. non-index cases**
Data are presented as n (%), mean ± SD unless otherwise stated. BMI: body mass index; FEV1: forced expiratory volume in 1s(% predicted); FVC: forced vital capacity; DLCO: diffusing capacity of the lung for CO; mMRC: modified Medical Research Council dyspnoea score; VAS: Visual Analogue Scale health status in the past week.
* Significant on multivariate analysis: (p<0.01).
Figure 1
Supplementary Figure

A

B

C

D

Delta FEV1 (mL/annum)

FEV1 (% predicted)

Age (years)

FEV1 (% predicted)

Age (years)

FEV1 (% predicted)

Age (years)

Delta DLCO (mL/min/mmHg)

DLCO (% predicted)

Age

Ever smoker

Never smoker

Index

Non index

Index

Non index