AN INVESTIGATION OF DYSREGULATED NEUTROPHIL ACTIVITY IN
ALPHA-1 ANTITRYPSIN DEFICIENCY

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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 Medicine

Supervisors: Professor Noel G. McElvaney
Dr. Emer Reeves
July 2016
Candidate Thesis Declaration

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Signed ______________________

RCSI student number 14133563

Date ________________
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<td>ALI</td>
<td>acute lung injury</td>
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<td>ANCA</td>
<td>anti-neutrophil cytoplasmic antibody</td>
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<tr>
<td>ATP</td>
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<td>ATS</td>
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<td>AZD9668</td>
<td>synthetic neutrophil elastase inhibitor</td>
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<td>BCA</td>
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<td>C5a</td>
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<td>Ca^{2+}</td>
<td>calcium ion</td>
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<tr>
<td>CaCl₂</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CB</td>
<td>cytochalasin B</td>
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<tr>
<td>CDC</td>
<td>Centre for Disease Control</td>
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<tr>
<td>CF</td>
<td>cystic fibrosis</td>
<td></td>
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<tr>
<td>CFPE</td>
<td>combined pulmonary fibrosis and emphysema</td>
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<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
<td></td>
</tr>
<tr>
<td>CG</td>
<td>cathepsin G</td>
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<tr>
<td>Cl⁻</td>
<td>chloride anion</td>
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<tr>
<td>CO</td>
<td>carbon monoxide</td>
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<td>COPD</td>
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<td>CRAMP</td>
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<td>CXCR1</td>
<td>C-X-C motif chemokine receptor 1</td>
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<tr>
<td>delF508</td>
<td>deletion of phenylalanine at position 508 of cystic fibrosis transmembrane regulator gene</td>
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<tr>
<td>DFP</td>
<td>diisopropylfluorophosphate</td>
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<td>DLCO</td>
<td>diffusing capacity of the lungs for carbon monoxide</td>
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<td>DLD</td>
<td>diffuse lung disease</td>
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<td>DMF</td>
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<td>1, 4 dithiothreitol</td>
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<td>EGFR</td>
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<td>ELF</td>
<td>epithelial lining fluid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbant assays</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>European Respiratory Society</td>
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FDA
Food and Drug Administration
FEV<sub>1</sub>
forced expiratory volume in 1 second
fMLP
formyl-methionyl-leucyl-phenylalanine
FPR1
formyl peptide receptor 1
FPR2
formyl peptide receptor 2
FRC
functional residual capacity
FRET
fluorescence resonance energy transfer
g
gram
G<sub>551D</sub>
substitution of glycine for aspartic acid at position 551 of cystic fibrosis transmembrane regulator gene
GAG
glycosaminoglycans
GAP
guanosine triphosphatase activating protein
GDI
guanosine diphosphate dissociation inhibitor
GDP
guanosine diphosphate
GGTase-1
geranylglyceranyltransferase type I
GNEF
guanine nucleotide exchange factors
gp41
glycoprotein 41
GPA
granulomatosis with polyangiitis
GPI-anchored
glycosylphosphatidylinositol-anchored
GST-PAK-CRIB
glutathione sepharose-tagged-p21 activated kinase-cdc42/Rac interactive binding domain
GTP
guanosine triphosphate
GTPase
guanosine triphosphatase
h
hour
H₂O
water
H₂O₂
hydrogen peroxide
HC
healthy control
hCAP-18
human cathelicidin antimicrobial protein
HEPES
n-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)
HIV
human immunodeficiency virus
HIV-1
human immunodeficiency virus-1
HO-1
haem oxygenase-1
<table>
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<td>hypochlorous acid</td>
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<td>HRP</td>
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<td>ICU</td>
<td>intensive care unit</td>
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<td>interferon gamma</td>
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<td>immunoglobulin-G</td>
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<td>Mycobacterium tuberculosis</td>
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<td>M-alpha-1 antitrypsin</td>
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<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mg/kg</td>
<td>milligrams per kilogram</td>
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<td>mg/kg/week</td>
<td>milligrams per kilogram per week</td>
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<td>min</td>
<td>minute</td>
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<td>mM</td>
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<td>MM</td>
<td>homozygous for M phenotype with normal serum AAT levels</td>
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n
NaCl
NDPK
NE
NFκβ
NK
nm
nM
NO
NOXA-1
NP-40
NSAID
O₂⁻
°C
OmpA
P. aeruginosa
PAb
PAGE
PAK-1
PAR2
PBS
PBSG
PBST
PFT
PGP
phox
PI
PMA
PMSF
PR3

number
sodium chloride
nucleoside diphosphate kinase
neutrophil elastase
nuclear factor kappa-light-chain enhancer of activated B cells
natural killer
nanometre
nanomolar
nitric oxide
NADPH oxidase activator-1
nonyl phenoxypolyethoxyethanol-40
non-steroidal anti-inflammatory drug
superoxide
degrees Celcius
outer membrane protein A
Pseudomonas aeruginosa
polyclonal antibody
polyacrylamide gel electrophoresis
p21 activated kinase-1
protease activated receptor 2
phosphate buffered saline pH 7.4
phosphate buffered saline pH 7.4 containing 5mM glucose
phosphate buffered saline pH 7.4 containing 0.05% Tween®
pulmonary function test
Pro-Gly-Pro
phagocytic oxidase
protease inhibitor
phorbol-12-myristate-13-acetate
phenylmethylsulfonyl fluoride
proteinase 3
PREX-1 phosphatidylinositol 3,4,5-triphosphate-dependent Rac exchanger-1
PVDF polyvinylidene fluoride
RA rheumatoid arthritis
r-AAT recombinant-alpha-1 antitrypsin
Rab27a ras-related protein Rab27a
RabGDI Rab GDP dissociation factor
Rac2 Ras-related C3 botulinum toxin substrate 2
RBL rat basophilic leukaemia cells
RCL reactive centre loop
REP Rab escort protein
RIPA radioimmunoprecipitation assay buffer
RNA ribonucleic acid
ROS reactive oxygen species
rpm revolutions per minute
SB sample buffer
SD standard deviation
SDS sodium dodecyl sulfate
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec second
SEM standard error of the mean
serpin serine protease inhibitor
SERPINA 1 serine protease inhibitor family A member 1 gene
sIC soluble immune complex
SLE systemic lupus erythematosus
SLPI secretory leucoprotease inhibitor
SOD superoxide dismutase
SOD3 superoxide dismutase 3
t time
TACE tumour necrosis factor alpha converting enzyme
TEMED tetramethylethylenediamine
TIAM-1 T cell-lymphoma-invasion-and-metastasis-1
TIM-3 T cell Ig and mucin-domain-containing molecule-3
TLC  total lung capacity
TLCK  Nα-tosyl-L-lysine chloromethyl ketone hydrochloride
TLR-4  toll-like receptor-4
TNF-α  tumour necrosis factor-alpha
TNFR1  tumour necrosis factor receptor 1
TPE  tropical pulmonary eosinophilia
Tris  tris(hydroxymethyl)aminomethane
TRPV-4  transient receptor potential vanilloid-4
u  units
V  Volts
v/v  volume/volume
VAV-1  vav-1 guanine nucleotide exchange factor
VIRIP  virus-inhibitory peptide
vs.  versus
w/v  weight/volume
w/w  weight/weight
WB  Western blot
x g  times gravity
ZZ-AATD  alpha one antitrypsin deficiency homozygous for Z allele
µg  microgram
µl  microlitre
µM  micromolar
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Abstract

Alpha-1 antitrypsin (AAT) deficiency (AATD) is characterised by excessive neutrophil degranulation and a protease: anti-protease imbalance leading to premature emphysema. Current specialised treatment for AATD consists of once weekly infusion of plasma purified AAT. Neutrophil degranulation is under the control of small GTP-binding proteins, including Ras-related C3 botulinum toxin substrate 2 (Rac2). The molecular basis for aberrant neutrophil degranulation in AATD has not been elucidated to date. The aim of this study was to fully characterise neutrophil degranulation in AATD and to determine the effects of AAT augmentation therapy on the AATD neutrophil.

In this study, we examined degranulation by AATD neutrophils by Western blotting. This revealed a 3-fold increase in levels of myeloperoxidase (MPO), human cathelicidin antimicrobial protein (hCAP-18) and matrix metalloprotease-9 (MMP-9), markers of primary, secondary and tertiary granules, respectively (p=0.023, p=0.036 and p=0.042, respectively). Excessive active Rac2 content of 239% was confirmed in AATD neutrophils using a Rac2 activation assay (p=0.016). In addition, fluorescence resonance energy transfer (FRET) and a cytochrome c reduction assay demonstrated elevated membrane-bound neutrophil elastase (NE) on AATD neutrophils (p=0.034) and a 107% increase in superoxide (O$_2^-$) production by NE-stimulated neutrophils (p=0.011), respectively. Furthermore, we have identified a novel pathway of neutrophil reactive oxygen species (ROS) production via activation of trans-membrane protease activated receptor 2 (PAR2) by NE and we have demonstrated the ability of exogenous AAT to correct O$_2^-$ release by NE-stimulated neutrophils in-vitro (63.3% reduction, p=0.008).

Most importantly, this study confirmed for the first time that AAT augmentation therapy not only normalised excessive degranulation patterns but also resulted in a 75% decrease in elevated Rac2 activity by AATD neutrophils assessed by Western blotting ex-vivo (p=0.03).

In conclusion, this study demonstrated the ability of AAT to regulate aberrant degranulation by AATD neutrophils, supporting our hypothesis of the ability
of AAT to restore protease: anti-protease balance and highlighted a further dimension to the benefits of AAT augmentation therapy for our AATD patients.
Presentations and Publications

Prizes/Awards:

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Oral Presentations:


Landers SA, Reeves EP, McElvaney NG. Excessive degranulation of primary, secondary and tertiary granules by circulating neutrophils in individuals with alpha-1 antitrypsin deficiency. Shortlisted for the Postgraduate Scholar’s Prize (First Year), Research Day, Royal College of Surgeons in Ireland, March 2015.

Published Abstracts:

**Excessive Rac2 activity in ZZ-alpha-1 antitrypsin deficient individuals and the beneficial effect of alpha-1 augmentation therapy on aberrant neutrophil degranulation.**


**Dysregulation of neutrophil degranulation in alpha-1 antitrypsin deficiency.**


**Alpha-1 augmentation therapy significantly decreases aberrant neutrophil degranulation in individuals with alpha-1 antitrypsin deficiency homozygous for the Z allele**


**Neutrophils of Individuals with Alpha-1 Antitrypsin Deficiency homozygous for the Z allele release enhanced levels of primary, secondary and tertiary granule components**

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Dedication

I dedicate this thesis to my kind and wonderful husband Killian, my resilient and supportive mother Breda and my beloved late father Sean. And of course to Little Wriggly.
Chapter 1
Introduction
1.1 Alpha-1 antitrypsin and alpha-1 antitrypsin deficiency

1.1.1 Alpha-1 antitrypsin

Alpha-1 antitrypsin (AAT) is a 52-kDa potent serine protease inhibitor belonging to the serpin superfamily. It is coded for on chromosome 14q3-32.1 (Billingsley et al., 1993). It is produced primarily by hepatocytes in the liver and is released into the circulation (Rogers et al., 1983). The AAT molecule is found in abundance in human plasma, with normal concentrations in the range of 20-53 μM and a half-life of 4-5 days (Laurell et al., 1977). Smaller quantities of AAT can also be produced by neutrophils (duBois et al., 1991), macrophages (van’t Wout et al., 2012), epithelial cells (Cichy et al., 1997), monocytes (Carroll et al., 2010), human pancreatic islet cells (Bosco et al., 2005) and cancer cells (Chen et al., 2010).

The main role of AAT in the lung is to inhibit a wide range of proteases released from degranulating neutrophils, in particular neutrophil elastase (NE) (Carrell et al., 1982). AAT has also been shown to exhibit anti-protease activity against cathepsin G (CG) and proteinase 3 (PR3) (Korkmaz et al., 2005), mast cell-derived tryptase and chymase (He et al., 2004), epithelial cell protease matriptase (Janciauskiene et al., 2008) and lymphocyte-derived granzyme B (Mahrus et al., 2004). Interestingly, Korkmaz et al. (2005) demonstrated that NE was the main target protease of AAT in bronchoalveolar lavage fluid (BALF) and that CG and PR3 were only inhibited once NE was saturated.

AAT consists of 3 β-sheets and an exposed 9 amino acid ‘reactive centre loop’ (RCL) which contains a methionine residue at position 358 onto which the target protease docks (Figure 1.1). Following docking, the protease cleaves a peptide bond on the loop, releasing it and inserting it into β-sheet A, thereby trapping and inactivating the protease (Lomas et al., 2004).

Taggart et al. (2000) highlighted the importance of methionine residues in AAT by demonstrating that oxidation of methionine-358 and methionine-351 residues by hydrogen peroxide (H₂O₂), a component of cigarette smoke,
Figure 1.1: Structure of AAT. AAT consists of β-sheets and an exposed 9-amino acid reactive centre loop containing a methionine residue at position 358 onto which the target protease docks (Adapted from Huntington et al, 2000).
resulted in loss of anti-NE activity by AAT. AAT is a single-chain glycoprotein that undergoes post-translational N-glycosylation in the endoplasmic reticulum (ER). This involves the addition of glycans, N-linked oligosaccharides, to the nitrogen side chain of an asparagine residue on the protein. Glycans in general are important for protein folding, however this has not been specifically demonstrated for AAT (Parodi et al, 2000). However, glycosylation does increase the flexibility of the AAT protein in its dynamic state (Sarkar et al, 2011) and the meta-stable state of AAT is essential to facilitate conformational change for its interaction with proteases (Whisstock et al, 2000). Glycosylation is critical for protecting proteins from proteolysis, and in AAT this extends the half-life of the protein and also prevents aggregation (Shental-Bechor et al, 2009). Following glycosylation, the fully processed AAT protein enters the circulation. The normal allele, referred to as ‘M’, produces normal circulating levels of AAT.

The critical role of AAT in maintaining the protease: anti-protease balance in the lung is demonstrated by the genetic condition AAT deficiency (AATD) in which patients are predisposed to the development of premature emphysema and liver cirrhosis. In ZZ-AAT deficiency states, the most severe form of deficiency, substitution of lysine for glutamic acid at position 342 widens the β-sheet allowing either polymerisation or formation of a latent conformation and thereby inhibiting the anti-protease function of AAT. Accumulation of the misfolded Z-AAT protein in the ER of hepatocytes (Greene et al, 2008) and other AAT-expressing cells is a hallmark of ZZ-AAT deficiency (Carroll et al, 2010).

In 2014, McCarthy et al. performed glycoanalysis of Z-AAT and demonstrated increased fucosylation on N-glycans of Z-AAT compared with M-AAT, indicative of ongoing inflammation in AATD individuals.

Carboxyl-terminal (C-terminal) AAT fragments (CAAP) also exhibit biological functions. The CAAP, referred to as virus-inhibitory peptide (VIRIP), binds glycoprotein 41 (gp41) fusion peptide of human immunodeficiency virus-1 (HIV-1), preventing viral entry into target cells, thereby inhibiting HIV-1 infection (Janciauskiene et al, 2011), while CAAP48 has been found to be elevated in septic patients, suggesting a role for CAAP48 as a diagnostic
AAT is also known as an acute phase protein (Huber et al, 1989) with levels becoming elevated within hours of inflammation or infection (Perlmutter et al, 2004), as well as following acute coronary syndromes (Correale et al, 2012) and in the post-operative setting (Voulgari et al, 1982).

1.1.2 A brief introduction to AATD

AATD is a clinically under-recognised condition. It was first described in 1963 by Dr. Carl Bertil Laurell and his medical resident Dr. Sten Eriksson following the discovery of the absence of the α1 band on protein electrophoresis in 5 of 1500 patient samples; 3 of these patients were found to have developed emphysema at a young age (Laurell et al, 1963).

In AATD, serine proteases are free to break down the connective tissue, elastin, in the lungs leading to destruction of the alveolar matrix and the development of emphysema (McElvaney et al, 1997, Guenter et al, 1981, Tobin et al, 1983). Emphysema is defined as an abnormal and permanent dilatation of the air spaces distal to the terminal bronchioles accompanied by destruction of the airspace walls leading to impaired gas transfer across the alveoli (Figure 1.2). AATD is the only proven genetic risk factor for emphysema.

AATD is also associated with increased risk of liver cirrhosis (Sharp et al, 1976, Erikson et al, 1986) and in rare cases panniculitis of the skin (Clark et al, 1982, Pittelkow et al, 1988). Necrotising panniculitis seen in AATD manifests as spontaneous necrotic and suppurative areas of the skin and subcutaneous fat and has a predilection for the trunk, arms and gluteal region. In 2015, Franciosi et al. reported the first case of successful induction of clinical remission of AATD-related panniculitis following a single 120 mg/kg dose of plasma-purified AAT.
Figure 1.2: Computed Tomography (CT) of thorax demonstrating bullous emphysema in AATD. Bullae indicated by arrows.
1.1.3 Genetics of AATD

AATD is inherited via autosomal co-dominant transmission with the most common variants, the Z and S mutations, associated with both lung and liver disease (Curiel et al, 1989, Lomas et al, 1992). AATD occurs due to mutations in the SERPINA 1 gene spanning 12.2 kb on chromosome 14. The SERPINA 1 gene is organised into 4 coding and 3 non-coding exons (Lee et al, 2000). To date, at least 120 alleles of AAT (SERPINA 1) have been identified, and each has a letter code based upon electrophoretic mobility of the protein produced. The normal allele, referred to as ‘M’, produces normal circulating levels of AAT (American Thoracic Society (ATS)/European Respiratory Society (ERS) statement, 2003). As AAT is a protease inhibitor, “PI” is used to designate the gene. “PI*MM” refers to homozygosity for the normal gene while “PI*ZZ” denotes homozygosity for the most common mutation in the SERPINA1 gene that leads to AATD.

The “Z” point mutation Glu342Lys is caused by a substitution of a lysine for a glutamic acid at position 342 of the AAT molecule. This occurs at the hinge region of the AAT molecule causing it to have an increased susceptibility to polymerisation and aggregation. The Z allele is the most common abnormal allele associated with a circulating deficiency of AAT ranging from 2.02 to 9.56 µmol/L (0.11 to 0.52 g/L) (DeMeo et al, 2004) and the ZZ phenotype is most strongly associated with development of premature emphysema. The Z allele is most common in northwestern Europe with approximately 1 in 1,700 individuals homozygous for the Z allele (Blanco et al, 2001). Interestingly, Ireland has one of the highest incidences of AATD in the world with an estimated prevalence of the severe ZZ-AATD phenotype in Ireland of 1/2,104. (Carroll et al, 2011).

A number of less severe variants, such as S and I, can also lead to accumulation of the mutated AAT protein within hepatocytes and are associated with lung and liver disease only when present in a compound heterozygote. The S mutation, for example, is characterised by a single amino acid substitution of a valine for a glutamic acid at position 264 and
PI*SZ heterozygotes are recognised as having an increased risk of emphysema (Green et al, 2015).

The class of SERPINA1 variants termed “null” mutations are characterised by the absence of circulating AAT due to a premature stop codon in the AAT gene sequence and to date three novel null mutations, Nullbolton, Nullcork and Nulldublin, have been reported in Ireland (Alpha One Foundation Annual Report 2015, Ireland). While extremely rare, homozygous null mutations confer a particularly high risk of emphysema, but interestingly are not associated with liver cirrhosis as these mutations do not cause polymerisation of AAT in the hepatocyte (Fregonese et al, 2008). In addition to the mutations commonly associated with individuals of Northern European descent, the Mmalton mutation is recognised as relatively common on the island of Sardinia in the Mediterranean and is associated with both lung and liver disease (Ferrarotti et al, 2005).

While the wealth of international research has focused on AATD individuals homozygous for the Z allele, Molloy et al. (2014) explored the impact of MZ heterozygosity on lung health and detected significantly more airflow obstruction (p=0.04) and COPD (p=0.02) in ever-smoking PiMZ heterozygotes compared with PiMM individuals.

Of note, for the purpose of this research, the term ‘AATD’ refers to study participants with AATD homozygous for the Z allele.

### 1.1.4 Diagnosis of AATD

The diagnosis of AATD is confirmed by identifying a serum level ≤ 11 µmol/L (0.597 g/L) in combination with a deficient phenotype determined by isoelectric focusing electrophoresis that identifies alleles with abnormal protein migration patterns.
Guidelines published by the ATS/ERS (2003) advocate a targeted screening approach for the detection of AATD and recommend the following patient groups for screening:

1. Adults with symptomatic emphysema or COPD, regardless of age or smoking history
2. Adults with severe refractory asthma with airflow obstruction that is incompletely reversed after aggressive bronchodilator therapy
3. Asymptomatic individuals with persistent obstruction on pulmonary function tests (PFT) and identifiable risk factors such as cigarette smoking or occupational exposure
4. Adults with a history of panniculitis
5. Individuals with cryptogenic liver disease, including neonates, children and adults
6. All first degree relatives of known AATD individuals

In 2004, the Alpha One Foundation Ireland launched the National AATD Targeted Detection Programme at the RCSI Education and Research Centre, Beaumont Hospital, Dublin. If the serum AAT level is below 18.4 µmol/L (1.0 g/L), an isoelectric focusing gel is performed on the HYDRASYS system to identify the AAT phenotype (Figure 1.3 (A)) (Carroll et al, 2011). This method is sensitive, accurate and can also identify rare AAT phenotypes (Zerimech et al, 2008). Genotyping is performed on a LightCycler 480 (Roche) with specific primers and probes designed for the Z and S mutations (Figure 1.3 (B)) (Rodriguez et al, 2002).

Similar to the Irish National AATD Targeted Detection Programme, the screening algorithm at the University of Marburg, Germany and the Biochemistry Laboratory at Vall d’Hebron, Barcelona, Spain, employ determination of the AAT level before genotyping, while the University of Pavia, Italy performs a simultaneous AAT level with genotyping (Miravitlles et al, 2010).
Figure 1.3: Methods employed for analysis of AAT mutation. (A) Typical isoelectric focusing gel used for identification of AAT phenotype with the most common phenotypes included. Bands are labeled for the M variant, but each variant has its own bands relative to its individual migration pattern (Greene et al, 2013). (B) Genotyping assay used to identify the Z mutation (Alpha One Foundation Annual Report 2012, Ireland).
Since 2004 the Irish National AATD Targeted Detection Programme has screened over 16,000 individuals with COPD, asthma, liver disease and asymptomatic first-degree relatives of known AATD individuals. To date, 281 individuals have been identified with the ZZ phenotype, as well as 213 SZ individuals who are also at risk of developing lung and liver disease. The highest prevalence of ZZ-AATD in Ireland has been identified in counties Dublin, Cork and Donegal, likely reflecting population density as well as higher screening rates and awareness of AATD (Figure 1.4). In addition, a large number of other clinically significant phenotypes have been detected including 2,375 MZ, 80 SS, 1,620 MS and 275 with rare phenotypes (e.g. IZ, Null, Mmalton, FZ, IS) (Carroll et al, 2015). In addition, the National AATD Targeted Detection Programme has been participating in the United Kingdom National External Quality Assessment Service (UKNEQAS) quality assurance scheme for AAT phenotyping since 2007 and has achieved 100% compliance to date (Alpha One Foundation Annual Report 2015, Ireland).
Figure 1.4: Geographical distribution of ZZ-AATD cases per county in Ireland detected by the National AATD Targeted Detection Programme. (Alpha One Foundation Annual Report 2015, Ireland).
1.1.5 Clinical Manifestations of AATD

The main clinical manifestations of AATD relate to three separate organs: the lung, the liver and much less frequently, the skin.

The clinical manifestations of emphysema due to AATD are very similar to non-genetic COPD and health care professionals must be vigilant to consider AATD as the possible predominant aetiology. Dyspnoea is the most common symptom with many individuals reporting cough, wheeze, sputum production and upper respiratory tract infections (Black et al, 1978, Janus et al, 1985).

As per the ATS/ERS guidelines (2003), patients with persistent airflow obstruction on spirometry should also be tested for AATD. Two distinctive features associated with a severe deficiency of AAT are younger age of onset (Larsson et al, 1978) and a basilar-predominant pattern of emphysema (Eriksson et al, 1964). Bronchiectasis has also been associated with severe deficiency of AAT, occurring most commonly in lobes with high emphysema scores (Cuvellier et al, 2000).

In addition, pneumothorax secondary to a ruptured emphysematous bulla may complicate this condition (Serapinas et al, 2013). CT findings amongst ZZ-AATD individuals on the Irish National Alpha-1 Registry are highlighted in Figure 1.5.

Extrapulmonary hepatic manifestations may include adult-onset chronic hepatitis, cirrhosis and hepatocellular carcinoma (Stoller et al, 2012).

Additionally, panniculitis, a dermatological condition consisting of tender and painful neutrophil-driven inflammation of the subcutaneous fat, may occur in up to 0.1% of individuals with AATD (Warter et al, 1972). Higher dose infusional AAT (120 mg/kg/week) has been shown in a number of studies to reduce inflammation associated with severe panniculitis in PI*ZZ AATD individuals (Gross et al, 2009, Franciosi et al, 2015).
Figure 1.5: CT findings in ever-smoking (current/former smoker) and never-smoking ZZ-AATD individuals on the National Alpha-1 Registry (Alpha One Foundation Annual Report 2015, Ireland).
1.1.6 AAT augmentation therapy

The discovery of the structure and function of the AAT protein and its subsequent isolation and purification have led to the development of AAT replacement therapy known as “augmentation therapy” aimed at preventing progression of AATD-associated lung disease.


The main biochemical goal of AAT augmentation therapy is to raise AAT levels above the protective threshold ≥ 11 µmol/L (0.597 g/L) in the plasma and epithelial lining fluid (ELF) of the lung in order to restore the protease-anti-protease balance in the body (Stocks et al, 2006, Lieberman et al, 2006). This target concentration was derived from levels detected in PI*SZ AATD individuals, who if non-smokers, very rarely developed pulmonary disease (Crystal et al, 1990). The three key clinical goals of AAT augmentation therapy are to delay the progression of emphysema, reduce the frequency of exacerbations and improve health-related quality of life (Teschler et al, 2015).

One of the first observational clinical studies examining the effect of AAT augmentation therapy compared the rate of FEV₁ decline of 97 former smokers from a Danish AATD registry to a German group of 198 PI*ZZ AATD former smokers receiving AAT augmentation therapy (60 mg/kg/week) over a mean of 3.2 years. Overall, the rate of FEV₁ decline in the treated group was significantly lower than in the untreated group (-53 mL per yr versus -75 mL, p= 0.02) (Seersholm et al, 1997).

The National Heart, Lung and Blood Institute conducted a multi-centre, prospective cohort study of 1,129 patients with severe AATD and identified significantly slower rates of FEV₁ decline in a subgroup of patients with FEV₁ values between 39% and 49% receiving AAT augmentation therapy. The risk
ratio for death in augmentation therapy recipients was 0.64, significantly lower than non-recipients (p=0.02) (National Lung and Blood Institute, 1998).

The EXAcerbations and Computerised Tomography scan as Lung End points (EXACTLE) study was a multi-centre, randomised, placebo-controlled exploratory trial to evaluate the frequency of exacerbations and the progression of emphysema by means of multi-slice CT scans in patients with AATD receiving weekly AAT augmentation therapy over a 2-year period. While this demonstrated a trend towards efficacy of augmentation therapy in reducing loss of lung density, a post-hoc analysis showed that the treatment group had fewer severe exacerbations than the control group (Dirksen et al, 2009).

Importantly, the first prospective clinical trial using a radiological end-point to conclusively demonstrate clinical efficacy of AAT augmentation therapy followed (Chapman et al, 2015). The Randomised, placebo-controlled trial of augmentation therapy in Alpha-1 Proteinase Inhibitor Deficiency (RAPID) study was a multi-centre, randomised, placebo-controlled trial of AAT augmentation therapy (60 mg/kg/week Zemaira®, CSL Behring) in AATD patients that measured CT lung density at total lung capacity (TLC) and functional residual capacity (FRC) over a 24-month period. This identified that the annual rate of lung density loss at TLC alone was significantly less in patients receiving AAT augmentation therapy than in the placebo group (-1.45 g/L/yr vs. -0.74 g/L/yr, p= 0.03).

Recent data has also demonstrated that a reduction in respiratory exacerbations is directly associated with improved quality of life of individuals (Lieberman et al, 2000, Koczulla et al, 2008). Additional studies have shown biochemical efficacy with monthly AAT infusions at a dose of 250 mg/kg (Hubbard et al, 1988).

Intravenous AAT augmentation therapy is currently the most direct and efficient way of elevating AAT levels in the plasma and lung interstitium (Stoller et al, 2004, Sandhaus et al, 2004) and the ATS/ERS guidelines (2003) list the following indications for AAT augmentation therapy:
• High-risk AATD phenotype
• Plasma AAT level < 11 µmol/l
• Airflow obstruction by spirometry
• Likely compliance with the protocol
• Age ≥ 18 years
• Non-smoker or ex-smoker

Side effects with AAT infusion therapy have been uncommon and studies have shown that it is both safe and well tolerated (Lieberman et al, 2000). Low-grade fever and mild flu-like illness are infrequent and usually self-limiting. Although the current AAT treatment is a pooled human plasma derivative, no cases of human immunodeficiency virus (HIV) or hepatitis viral transmission in recipients has been reported (Stoller et al, 2003). Augmentation treatment for AATD is an expensive therapy, costing up to $150,000 (Silverman et al, 2009) with annual costs averaging $40,000 per individual patient (Mullins et al, 2001). Augmentation therapy is not recommended for patients with heterozygous phenotypes whose plasma AAT level exceeds 11µmol/L (Sandhaus et al, 2008).

1.1.7 Supportive and experimental therapies for AATD

Supportive therapies for AATD patients with airflow obstruction follows the guidelines for management of COPD and include smoking cessation (O’Brien et al, 2015), bronchodilator therapy (Campos et al, 2009), inhaled or oral glucocorticoids (Corda et al, 2008), prompt treatment of respiratory tract infections (Wilkinson et al, 2004), influenza and pneumococcal vaccinations (Varkey et al, 2009), oxygen therapy if indicated (Stoller et al, 2010) and pulmonary rehabilitation (Sahin et al, 2016). Experimental therapies for AATD include aerosolised AAT (Hubbard et al, 1990, Brand et al, 2009), recombinant AAT (Alkins et al, 2000) and gene therapy (Sandhaus et al, 2004).
Direct delivery of AAT to the lung by inhalation has been an attractive alternative to intravenous infusion due to the ease of delivery and the ability to directly target the site of clinical damage. Both human and animal studies have shown that pooled AAT can be aerosolised to a particle size sufficiently small to enter the lower respiratory tract and that the plasma and ELF levels were elevated above the protective threshold (Hubbard et al, 1990, Brand et al, 2009). A study by Geraghty et al. (2008) examined BALF from ZZ-AATD patients receiving aerosolised AAT therapy and demonstrated reduced cathepsin B and MMP-2 activity, and higher levels of lactoferrin and secretory leucoprotease inhibitor (SLPI).

An alternative to plasma-derived AAT is recombinant AAT using transgenic technology. However, this form of treatment is still under evaluation as immune-mediated toxic effects have been described due to the presence of animal-derived AAT and alpha-1 anti-chymotrypsin in these formulations (Spencer et al, 2005).

AATD has been an attractive target for the development of gene therapy because it is a single gene disorder. Gene therapy for AATD consists of the exogenous transfer of deoxyribonucleic acid (DNA) which codes for normal AAT to deficient human cells, allowing for ongoing endogenous production to augment deficient levels (Sandhaus et al, 2004). \textit{In-vivo} studies have been carried out using a novel plasmid-cationic liposome complex delivering the normal AAT gene to the respiratory epithelium of deficient patients (Brigham et al, 2000), as well as using intramuscular injection of a recombinant adeno-associated virus vector expressing normal AAT (Brantly et al, 2009).

However, these studies suggest that the duration of the gene expression may be finite, because exogenously induced genes are inactivated over time by host cell mechanisms that are not yet fully understood (Sandhaus et al, 2004). In 2013, Mueller et al. described the development of recombinant adeno-associated virus 1(AAV1)-AAT capable of producing sustained serum AAT levels at 3% of the target level after intramuscular administration in humans. As limb perfusion targets greater muscle mass, further increases in target AAT levels are anticipated, as well as future roles for intrapleural and airway delivery of rAAT and genome editing (Loring and Flotte, 2015).
1.1.8 The anti-inflammatory effects of AAT on immune and structural cells

There is now increasing evidence that AAT may also exhibit unique anti-inflammatory properties affecting several cell types and modulating inflammation caused by both host and microbial factors, independent of its anti-protease function (Figure 1.6) (Bergin et al, 2012, Bergin et al, 2010).

**Monocytes and macrophages**

Both monocytes and macrophages can produce small amounts of AAT (Carroll et al, 2010) and its production is up-regulated by IL-6, interleukin-1-β (IL-1-β), NE and TNF-α (Knoell et al, 1998, Perlmutter et al, 1988). In AATD, excessive levels of NE can induce secretion of leukotriene B₄ (LTB₄) in alveolar macrophages and airway epithelial cells (Hubbard et al, 1991).

Stockley et al. (2002) reported a significant reduction in plasma LTB₄ concentrations following four infusions of AAT (60 mg/kg) at weekly intervals (p=0.02) (Stockley et al, 2002). In addition, Churg et al. (2007) described the ability of AAT to suppress TNF-α and matrix metalloprotease-12 (MMP-12) production by cigarette smoke-stimulated alveolar macrophages in a murine model. The authors proposed that MMP-12 could regulate TNF-α release by cleaving biologically active TNF-α similar to other MMPs in leukocytes as reported by Gearing et al. (1994).

**T and B lymphocytes**

In addition to monocytes and macrophages, AAT has also been shown to interact with T and B lymphocytes of the adaptive immune response. Co-culture of peripheral blood lymphocytes with either T or B cell mitogens led to the appearance of lymphoblasts with membrane-associated AAT or increased binding capacity for AAT, respectively (Lohrisch et al, 1981).

Gupta et al. (2007) identified that COPD patients with AATD had significantly decreased numbers of CD4+ T lymphocytes (p<0.0009). AAT has also been
Figure 1.6: AAT modulates multiple cell types during inflammation. AAT maintains anti-inflammatory effects on several cell types and modulates inflammation caused by host and microbial factors. These cells include neutrophils by inhibiting chemotaxis, degranulation, NADPH oxidase and ANCA, macrophages by inhibiting cytokine release, eosinophils, mast cells by inhibiting histamine release, B lymphocyte proliferation, T lymphocyte proliferation and modulation of NFκB, erythrocytes during E. coli infection, endothelial cell apoptosis and epithelial cells via epidermal growth factor receptor (EGFR) and TLR. (Adapted from Bergin et al, 2012).
demonstrated to inhibit HIV replication in CD4+ T lymphocytes through altered IκBα ubiquitination (Zhou et al., 2011). Münch et al. (2007) reported the discovery of a virus inhibitory peptide corresponding to the C-proximal region of AAT that had the ability to interact with the gp41 fusion peptide inhibiting entry of a wide variety of HIV-1 strains including those that are multi-drug resistant, thus highlighting the potential for AAT to lead the way in a new class of anti-retroviral drugs.

In the case of B lymphocytes, Wingren et al. (2006) identified inhibition of Moraxella IgD binding protein (MID)-induced B lymphocyte proliferation and IL-6 production by native and polymerised forms of AAT. Human AAT has also been shown to modify B lymphocyte responses during skin allograft transplantation in mouse models, suggesting that AAT may be beneficial in pathologies that involve excessive B-cell responses, such as allograft rejection (Mizrahi et al, 2013).

Cytotoxic lymphocytes or natural killer (NK) cells are a third class of lymphocyte whose function is modulated by AAT. Okumura et al. (1985) demonstrated that AAT had inhibitory effects on NK activity for ‘fast target’ K562 cells. This is of particular importance in the context of Kawahara and colleagues’ study (2010) that demonstrated the critical role of NK cells in the rejection of human hepatocytes after xenotransplantation in an immunodeficient human chimeric mouse model, with high levels of AAT resulting in a lower percentage of hepatocyte graft failures.

**Eosinophils**

Eosinophils are a class of leucocyte involved in combating multicellular parasites, in addition to playing a key role in the immune response to allergy and asthma (Martin et al, 1996). In a study by Ray et al. (2011), patients treated with diethylcarbamazine for tropical pulmonary eosinophilia (TPE) had a significant rise in AAT levels with an associated fall in absolute eosinophil count following treatment, indicating that deficiency of AAT observed in TPE is probably acquired. Johansson et al. (2001) identified localisation of AAT to specific granules of eosinophils and suggested that by
releasing AAT, eosinophils may, in a microenvironment, play a role in counteracting the tissue damage caused by serine proteases released by neutrophils in inflammatory conditions.

**Alveolar cells**

Alveolar cells are also known to produce AAT, which may contribute to both anti-protease and anti-inflammatory effects within the healthy lung (Venembre *et al.*, 1994). Inflammatory mediators including oncostatin M, interleukin-1-β (IL-1-β) and dexamethasone therapy have been shown to increase this local production of AAT (Boutten *et al.*, 1998, Cichy *et al.*, 1997). While, incubation of human bronchial epithelial cells with NE results in an increase in interleukin-8 (IL-8), glycosylated AAT has the ability to bind IL-8 preventing its interaction with its chemokine receptor, C-X-C motif chemokine receptor 1 (CXCR1) (Bergin *et al.*, 2010).

Of note, neutrophils and the effect of AAT on neutrophil function will be addressed in detail in subsequent sections 1.2.1 to 1.2.5.

Collectively, this accumulating data indicate that AAT has significant anti-inflammatory properties affecting a wide range of inflammatory cells, leading to its potential use as an effective therapy for a broad number of inflammatory conditions.

**1.1.9 Potential alternative uses of AAT augmentation therapy**

In addition to its role in balancing the protease: anti-protease disequilibrium in AATD, AAT is also recognised as having significant anti-inflammatory properties. This has led to AAT being considered as a potential novel therapeutic agent for other inflammatory diseases.
Cystic fibrosis (CF)

CF is a genetic disorder caused by mutations of the CF trans-membrane conductance regulator (CFTR) gene resulting in defective chloride (Cl⁻) transport by epithelial cells. McElvaney et al. (1991) previously described the ability of aerosolised AAT to suppress the NE activity in respiratory ELF and also positively impact neutrophil-mediated killing of Pseudomonas aeruginosa (P. aeruginosa) in CF patients. Subsequent studies by Griese et al. (1997) demonstrated decreased activity of NE activity in sputum as well as reduced levels of TNF-α and IL-8 after 4 weeks of aerosolised AAT. A phase II clinical trial assessing the efficacy of recombinant aerosolised AAT showed a trend towards an improvement in time to first acute CF exacerbation compared with placebo (Martin et al, 2006). Further extended trials of aerosolised AAT in CF > 4 weeks are anticipated.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory polyarthritis characterised by progressive joint destruction. Synovial inflammation occurring in RA is caused by infiltration of immune cells including neutrophils (Mcllnnes et al, 2007). In addition to non-steroidal anti-inflammatory drugs (NSAIDs), treatment of RA involves the use of biological agents such as TNF-α-blockers and anti-interleukin-6 (IL-6) receptor antibody. These cytokines are inhibited by AAT at several levels, including both their release and function, suggesting that AAT may help to limit the positive inflammatory feedback loop that perpetuates RA. Notably in 2014, Bergin et al. demonstrated that AAT augmentation therapy decreased neutrophil membrane expression of TNF-α. Moreover, inhibition of NE by AAT in animal models of RA has been shown to interfere with disease progression (Kakimoto et al, 1995). Grimstein et al. (2011) recently reported that AAT protein and gene therapies for AAT decreased autoimmunity and delayed arthritis development in a mouse model of RA.
Anti-neutrophil cytoplasmic antibody-associated vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis is also associated with AATD (Elzouki et al, 1994). AAT is a major inhibitor of PR3, the main target antigen of ANCA seen in granulomatosis with polyangiitis (GPA), a potentially fatal systemic vasculitis. Case reports have described the successful use of AAT as a novel therapy for cutaneous vasculitis in individuals with AATD, but to date there has not been a large-scale study to assess the safety and efficacy of its use in systemic vasculitis (Dowd et al, 1995).

Diabetes mellitus

In addition to autoimmune rheumatological and vasculitic conditions, AAT has also been trialed in the treatment of type 1 diabetes mellitus, which results from autoimmune destruction of the pancreatic islet cells leading to an absolute insulin deficiency. Non-functioning circulating AAT has been identified in individuals with type 1 diabetes mellitus (Bristow et al, 1998, Hashemi et al, 2007). Importantly, AAT has been shown to prolong pancreatic islet allograft survival in a mouse model (Lewis et al, 2005). AAT is under evaluation for treatment of type 1 diabetes mellitus in multiple clinical trials with preliminary results suggesting that up to 50% of individuals displaying improved pancreatic islet cell function (Fleixo-Lima et al, 2014).

Overall these studies indicate that AAT holds significant potential as a safe and well-tolerated novel anti-inflammatory therapy for a range of respiratory, rheumatological and immunological diseases.
1.2 The role of neutrophils in AATD

1.2.1 Brief overview of neutrophil function

Neutrophils are the most abundant white blood cell with $10^{11}$ produced daily and provide the first line of host defense against both bacterial and fungal infections (Segal et al, 2008). They are the first cells to be recruited to sites of infection and inflammation. Neutrophils migrate through the bloodstream to the site of tissue injury in response to cytokines and chemokines released from these sites (Segal et al, 2005). Invading pathogens are opsonised by innate and acquired immune processes, such as fixation of complement C3 fragments and immunoglobulin G (IgG) (Ricklin et al, 2010). Upon reaching the site of infection, neutrophils phagocytose invading micro-organisms through receptor recognition. To aid bacterial killing, neutrophils contain a potent antimicrobial arsenal within three types of intracellular granules called primary, secondary and tertiary granules and through a process called degranulation, neutrophil granules sequentially release their proteases (Faurshou et al, 2003). The non-oxidative method of bacterial killing therefore involves fusion of the neutrophil granules with the phagosome, killing bacteria within seconds of ingestion (Döring et al, 1994).

In contrast, the oxidative pathway involves activation of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) system on the phagosome wall (Hampton et al, 1998). NADPH is responsible for the respiratory burst and the production of $O_2^-$ radicals within the phagosome. $O_2^-$ dismutates to $H_2O_2$, which in turn is converted to hypochlorous acid (HOCl), which facilitates bacterial killing by oxidation (Segal et al, 2005).
1.2.2 Neutrophil granules

This research study aims to fully characterise neutrophil degranulation patterns in AATD and it is therefore important to turn our attention to neutrophil granules. Three types of neutrophil granules filled with antimicrobial mediators are formed sequentially during their maturation. These are primary (azurophilic) granules containing the neutrophil serine proteases (NE, PR3, CG) as well as myeloperoxidase (MPO), secondary (specific) granules, which contain lactoferrin and human cathelicidin antimicrobial protein (hCAP-18), and tertiary (gelatinase) granules, which contain matrix metalloproteinase-9 (MMP-9) (Kolaczkowska et al, 2013) (Figure 1.7).

Primary granules

MPO is the most abundant peroxidase in the neutrophil, constituting about 25% of primary granule content, achieving a concentration of 100 mg/mL in the vacuole (Segal et al, 2005). MPO is essential for oxygen-dependent killing of pathogens by catalysing the conversion of H₂O₂ and Cl⁻ ion to cytotoxic HOCl during the neutrophil's respiratory burst (Hampton et al, 1998). Stockley et al. (2001) demonstrated that graduation of sputum colour correlated directly with sputum MPO content (p < 0.003) and suggested this may be a useful clinical tool for monitoring chronic airways disease and response to treatment. In vitro studies reveal that MPO-deficient neutrophils are markedly less efficient than normal neutrophils in killing Candida albicans (Diamond et al, 1980) and hyphal forms of Aspergillus fumigatus (Rex et al, 1990). However, excessive levels of MPO have previously been shown to positively correlate with airflow obstruction and sputum production (p=0.001) in homozygous delF508 CF patients (Garner et al, 2004), while Schindhelm et al. (2009) demonstrated that elevated MPO levels are also associated with high oxidative stress in atherosclerosis.
Figure 1.7: Neutrophil granules and their contents.
Also contained in neutrophil primary granules is the serine protease NE, that can be released from the neutrophil to the extracellular environment and may also rebind and become expressed on the cell surface (Owen et al, 1997). NE plays an important role in protecting the lung by catalysing the breakdown of microorganisms that can lead to infection in the lung and in the clearance of necrotic lung tissue (Grammes et al, 2011). The positive surface charge of NE mediates strong binding to bacterial membranes, which alone may inhibit bacterial protein synthesis and induce membrane depolarisation and disruption (Zasloff et al, 2002). NE is known to degrade the outer membrane protein A (OmpA) of Escherichia coli (E.coli) (Belaouaj et al, 2000) as well as preferentially cleaving virulence factors of Shigella, Salmonella and Yersinia (Weinrauch et al, 2002).

NE also plays a role in several immune processes that drive inflammation. NE cleaves an essential component of basement membranes called laminin-332 generating laminin fragments that are chemotactic to neutrophils (Mydel et al, 2008). The bioactive tri-peptide, Pro-Gly-Pro (PGP), produced as a result of collagen degradation by NE, activates neutrophils through chemokine CXCR receptors (Weathington et al, 2006). Previous studies have also shown that NE-deficient mice are more susceptible to infection by Gram-negative and -positive bacteria (Reeves et al, 2002).

The physiological balance between proteases and anti-proteases is crucial in maintenance of the lung’s connective tissue and an imbalance in favour of proteases results in lung injury (Tetley et al, 1993). Excess levels of serine proteases are found in airway secretions of patients with inflammatory lung conditions (Stockley et al, 1994). If left unopposed, serine proteases can damage a broad range of extracellular matrix proteins including elastin (Shapiro et al, 1991), collagen (Borregaard et al, 1997), fibronectin (Vissers et al, 1991) and laminin (Heck et al, 1990). This can generate pro-inflammatory peptides such as the neutrophil chemoattractant N-acetyl PGP (Weathington et al, 2006), as well as leading to alveolar matrix damage of the lung resulting in emphysema (Guenter et al, 1981). NE has been shown to up-regulate expression of the potent chemokine IL-8 via toll-like receptor-4 (TLR-4) in bronchial epithelium of CF airways (Devaney et al, 2003). NE also
possesses the ability to up-regulate other destructive enzymes such as the MMPs and cathepsins that are released from neutrophils (Greene et al., 2009). The MMPs are a family of calcium and zinc-dependent proteases that degrade extracellular matrix. Of note, MMP-2 and MMP-9 are elastinolytic and are found in high concentrations in BALF of patients with emphysema (Tetley et al., 2002). Hubbard et al. (1991) demonstrated that NE could induce secretion of LTB₄ in airway epithelial cells and macrophages in AATD (Hubbard et al., 1991). Excess NE has also been shown to cleave and inactivate the complement 5a (C5a) receptor in BALF of CF patients, contributing to suboptimal local neutrophil priming and bacterial clearance (van den Berg et al., 2014). Further data suggests that NE may activate protease activator receptor 2 (PAR2) (Zhao et al., 2015), in turn causing release of ROS. Therefore, in addition to its physiological function as a powerful host defense-molecule, NE released in excessive amounts can drive pathophysiological processes resulting in alveolar matrix damage as seen in AATD.

Neutrophil primary granules also contain the serine proteases CG and PR3. CG can cleave alveolar matrix proteins including elastin (Reilly et al., 1980), collagen (Starkey et al., 1977) and fibronectin (Vartio et al., 1981), as well as inactivate both TNF-α (Scuderi et al., 1991) and IL-8 (Padrine et al., 1994). In addition, extracellular CG can degrade leukotoxin, a membrane pore-forming virulence factor of Actinobacillus actinomycetemcomitans, which can lyse neutrophils (Johansson et al., 2000) and cleave the pro-inflammatory bacterial virulence factor, flagellin (López-Boado et al., 2004). Reeves et al. (2002) showed that mice deficient in CG were susceptible to staphylococcal and candidal infections. PR3, released from primary granules, can also degrade a number of extracellular matrix proteins including elastin, collagen and laminin (Rao et al., 1991). It also has the ability to cleave and activate the precursor forms of TNF-α (Coeshott et al., 1999) and IL-8 (Padrine et al., 1994). PR3 is the main antigenic target of ANCA and individuals with ANCA directed against PR3 are more susceptible to protracted staphylococcal and streptococcal infections (Bonaci-Nikolic et al., 2010).
Secondary granules

The human cathelicidin antimicrobial protein-18 (hCAP-18) is a cationic antibacterial protein found in neutrophil secondary granules (Sørensen et al., 1997). Proteolytic cleavage of hCAP-18 by PR3 liberates the active C-terminal peptide, human cathelicidin LL-37 (LL-37) (Sørensen et al., 2001), found in epithelial cells of the respiratory and gastrointestinal tract (Doss et al., 2010). LL-37 disrupts the integrity of bacterial membranes and can neutralise bacterial endotoxins of Gram-negative and Gram-positive bacteria (Gennaro et al., 2000, Dürr et al., 2006). Inactivation of LL-37 in CF BALF is due in part to its association with glycosaminoglycans (GAGs) and interestingly hypertonic saline promotes the antimicrobial effects of LL-37 by disrupting GAG-LL-37 complexes (Bergsson et al., 2009). In 2010, Alalwani et al. demonstrated that mice deficient in cathelin related antimicrobial peptide (CRAMP), the murine homologue of LL-37/hCAP-18, displayed decreased antimicrobial activity compared with wild-type mice. Excessive production of LL-37 has been shown to have a role in inflammatory dermatological conditions such as psoriasis and rosacea (Reinholz et al., 2012). More recently, Sun et al. (2014) reported that increased levels of LL-37 in airway epithelium following cigarette smoke exposure could stimulate collagen production in underlying lung fibroblasts contributing to the pathogenesis of small airway remodeling in COPD.

hCAP-18 was found to co-localise and co-mobilise with lactoferrin in neutrophil secondary granules (Sørensen et al., 1997). Lactoferrin is an iron binding protein and thus removes an essential substrate required for bacterial growth and also binds to lipopolysaccharide of bacterial walls affecting membrane permeability resulting in cell lysis (Farnaud et al., 2003).

Tertiary granules

Neutrophil tertiary granules release matrix metalloprotease-9 (MMP-9), a 92 kDa type IV collagenase involved in the degradation of the extracellular matrix in normal physiological processes. MMP-9 is secreted in the inactive pro-form and activated when cleaved by extracellular proteases, such as NE
(Ohnishi et al, 1998). MMP-9 is critical for efficient phagocytosis and \( O_2^- \) production by neutrophils (Hong et al, 2011). However, excessive levels of plasma MMP-9 have been reported in intensive care unit (ICU) patients with acute respiratory distress syndrome (ARDS) and have shown a significant correlation with a decrease in oxygenation (\( P_aO_2/FiO_2 \)) (Hsu et al, 2015). In addition, higher exhaled MMP-9 concentrations have also been detected in the breath samples of severe asthmatics compared to mild to moderate cases, suggesting that measurement of exhaled MMP-9 could help to monitor ongoing airway remodeling as well as recognize severe stages of asthma and guide appropriate therapy (Barbaro et al, 2014). A population-based cohort study by Linder et al. (2015) demonstrated that median MMP-9 levels were significantly higher in COPD patients than non-obstructed individuals and that a decreasing FEV\(_1\) correlated significantly with increased MMP-9 levels in individuals with COPD (p=0.004).

Nevertheless, further longitudinal studies are crucial to understand the full role of MMP-9 in the pathogenesis of different COPD phenotypes.

1.2.3 Neutrophil chemotaxis and AAT

Circulating neutrophils have been shown to have the ability to produce RNA transcripts of AAT and the mature AAT protein (du Bois et al, 1991). Bergin et al (2010) identified detectable levels of both the Z-AAT and M-AAT protein localised predominantly to the outer surface of the neutrophil plasma membrane in a ZZ-AATD liver transplant recipient suggesting that both serum- and neutrophil-derived AAT are associated with neutrophils. Neutrophil chemotaxis involves neutrophil migration to the sites of tissue inflammation or infection along a chemical gradient in response to cytokines, including IL-8 (Muhaida et al, 2000), formyl-methionyl-leucyl-phenylalanine (fMLP) (Wu et al, 2004) and LTB\(_4\) (Nauseef et al, 2014).

In 2010, Bergin et al. demonstrated that AAT can modulate both IL-8- and soluble immune complex (sIC)-induced chemotaxis by divergent pathways. This study identified that neutrophil chemotaxis is dependent on opposing
gradient concentrations of both IL-8 and glycosylated AAT and that the AAT: IL-8 complex formation prevented interaction between IL-8 and its chemokine receptor CXCR1. In addition, AAT also modulates neutrophil chemotaxis by controlling membrane expression and release of glycosylphosphatidylinositol-anchored (GPI-anchored) Fc receptor FcγRIIib by inhibiting ADAM metallopeptidase domain-17 (ADAM-17). Following AAT augmentation therapy, Bergin et al. (2010) reported return of chemotactic responses of AATD neutrophils to that of normal levels.

Animal models of smoke-induced emphysema have described the ability of M-AAT to significantly reduce the number of infiltrating neutrophils to the site of injury (Churg et al, 2003). Conversely, due to polymerisation of the Z-protein in the lung, Mulgrew et al. (2004) identified potent chemoattractant properties of the ZZ-AAT protein in the airways, comparable to that of IL-8. AATD individuals homozygous for the Z allele are also characterised by increased neutrophil adhesion in response to LTB₄. Interestingly, O'Dwyer et al. (2015) demonstrated that AAT could bind to and inhibit LTB₄ activity via a protein-lipid hydrophobic interaction that modulated BLT1 receptor engagement and subsequent downstream signaling events, including 1,4,5-triphosphate production and Ca²⁺ flux, without affecting the anti-elastase function of AAT. The authors concluded that this study illustrated the ability of AAT augmentation therapy to modulate LTB₄/BLT1-mediated inflammatory pathways in human neutrophils.

1.2.4 Neutrophil degranulation and AAT

Dysregulated neutrophil degranulation is recognised as an important event in inflammatory conditions including asthma and acute lung injury (Skubitz et al, 1999). In 2014, Bergin et al. identified increased plasma concentrations of neutrophil-released secondary and tertiary granules proteins from ZZ-AATD neutrophils in response to TNF-α as well as an increase in autoantibodies directed against degranulated lactoferrin, leading to an enhanced rate of
neutrophil ROS production. O’Dwyer et al. (2015) demonstrated a 50% and 33% increase in MPO and hCAP-18 release by ZZ-AATD neutrophils, respectively, compared to MM controls in response to LTB₄ (100 nM). Importantly, the addition of exogenous AAT (27.5 µM) significantly inhibited the degranulation of ZZ-AATD neutrophil primary and secondary granules induced by LTB₄ (p=0.01 and p=0.03, respectively) with a comparable trend observed in tertiary granules (O’Dwyer et al, 2015). More recently, Shaharom et al. (2015) identified ER stress in ZZ-AATD neutrophils and reported that inducing ER stress with thapsigargin, a non-competitive inhibitor of ER Ca²⁺ ATPase, in MM neutrophils resulted in a four-fold and ten-fold increase in the release of hCAP-18 and MMP-9 from secondary and tertiary granules, respectively, when compared to untreated cells (Shaharom et al, 2015). However, degranulation patterns of neutrophil primary granules in AATD in response to TNF-α in the presence of other important inflammatory mediators such as fMLP have not been characterised to date.

1.2.5 Neutrophil apoptosis and AAT

Neutrophil apoptosis, the process of programmed cell death, is a tightly regulated process that limits the destructive capacity of neutrophil cytotoxic products to surrounding tissue (Fox et al, 2010). Subsequent phagocytosis of apoptotic cells by phagocytic cells such as macrophages is central to the successful resolution of an inflammatory response.

However, in AATD, neutrophil apoptosis has been shown to be accelerated due to accumulation of the misfolded Z-protein in the ER causing ER stress and increasing the expression of the pro-apoptotic cytokine TNF-α (Hurley et al, 2014). AAT is known to possess anti-apoptotic properties and this has previously been demonstrated in alveolar cells (Petrache et al, 2006) and pancreatic β cells (Zhang et al, 2007). In 2014, Hurley et al. identified that treatment of AATD individuals with AAT augmentation therapy decreased ADAM-17 activity resulting in decreased shedding of soluble TNF-α and
therefore normalised neutrophil apoptosis in vivo. Of particular significance, Hurley et al. (2014) also demonstrated that neutrophils isolated from AATD individuals two days post-AAT augmentation therapy successfully reduced survival of P. aeruginosa to 60%, similar to bacterial survival observed for healthy MM control cells (p=0.003).

Collectively, these studies highlight the role of AAT in the maintenance and resolution of neutrophil-associated inflammation in health and disease.

1.3 Neutrophil granule trafficking

1.3.1 The role of Rab proteins and their activation

Neutrophil degranulation is a tightly regulated process under the control of small guanosine triphosphatases (GTPases) of the Ras superfamily (Lundquist et al, 2006). The Rab proteins are one subgroup regulating intracellular vesicular transport. To date, over sixty Rab proteins have been identified. Rab proteins regulate transport by cycling between an inactive guanosine diphosphate (GDP)-bound and active guanosine triphosphate (GTP)-bound state (Figure 1.8). They exert their function by interacting with specific effector molecules. Rab proteins become attached to the vesicle membrane in order to direct vesicle trafficking. Rab escort proteins (REPs) also known as Rab geranylgeranyltransferases, bind Rab proteins and facilitate prenylation by adding two hydrophobic geranylgeranyl groups to the C-terminus thereby anchoring the protein to the membrane (Alexandrov et al, 1994). Once the Rab is linked to the vesicular membrane, it is activated by guanine nucleotide exchange factors (GNEFs) that facilitate the exchange of bound GDP to GTP. Rab proteins are active in the GTP-bound state. Active Rab is then recycled from the membrane by Rab GDP dissociation inhibitor (GDI) and becomes available for the transport of other vesicles (Cherfils et al, 2013).
Figure 1.8: Activation of Rab proteins. Small GTPases serve as molecular switches and regulate many aspects of intracellular signalling. Activation of small G proteins is mediated by GDP/GTP nucleotide exchange factors (GNEFs) and deactivation by GTPase activating proteins (GAPs). GTP-bound proteins bind to effector molecules and a signal is propagated. Small G proteins are brought into the cytosolic form when inactive by binding to GDP dissociation inhibitors (GDIs). (Adapted from Cherfils et al (2013)).
1.3.2 The role of Rab27a protein and its activation

Rab proteins interact with motor and cytoskeletal proteins such as myosin and actin, along with tethering factors that dock vesicles to their target membranes to facilitate membrane fusion. Of these Rab proteins, Rab27a, is a specific protein highly expressed in blood cells and melanocytes involved in neutrophil secondary and tertiary granule trafficking (Herrero-Turrión et al, 2008). Rab27a remained uncharacterised until 2000 when it was discovered that Griscelli syndrome is predominantly caused by mutations in the RAB27A gene (Ménasché et al, 2000). Griscelli syndrome is a rare autosomal recessive syndrome characterised by an accumulation of melanosomes, melanin-containing vesicles, within the cell. This results in pigmented dilution of the skin and hair. Importantly, most patients with Griscelli syndrome also develop an uncontrolled T-lymphocyte and macrophage activation syndrome, known as haemophagocytic syndrome, leading to death in the absence of bone marrow transplantation (Ménasché et al, 2000). Activation of Rab27a is regulated by REPs, GNEFs, GTPase activating proteins (GAPs) and GDIs. Rab escort protein-1 (REP1) and Rab escort protein-2 (REP2) have been shown to bind Rab27a in vitro, while only the Rab27a-REP complex shows high affinity for the geranylgeranyltransferase (Köhne et al, 2013). Pohl et al. (2014) demonstrated that CF blood neutrophils released less secondary and tertiary granule components compared with HC neutrophils due to defective activation of the GTP-binding protein Rab27a involved in granule trafficking.

1.3.3 The role of Rac2 protein and its activation

As a major focus of this study is degranulation of primary granules in AATD, our attention turns to the Ras-related C3 botulinum toxin substrate 2 (Rac2). Rac2 is a small (~ 21kDa) signaling G protein and member of the Rho family of guanosine triphosphatases (GTPases) that is restricted to cells of the
haematopoietic system. Rac2 regulates a diverse array of cellular events, including the control of vesicle transport, cell division, oxidase activity and nuclear assembly (Grill et al., 2002). It is encoded by the gene RAC2 (Reibel et al., 1991).

Activation of Rac proteins is also mediated by GNEFs. The main GNEFs for Rac2 are T-cell-lymphoma-invasion-and-metastasis-1 (TIAM-1) (Haeusler et al., 2003), phosphatidylinositol 3,4,5-triphosphate-dependent Rac exchanger-1 (PREX-1) (Welch et al., 2002), and vav-1 guanine nucleotide exchange factor (VAV-1) (Ming et al., 2007) which is most efficient in inducing oxidase activity of Rac2 (Hordik et al., 2006). Activation of Rac2 requires isoprenylation at the C-terminus by geranylgeranyltransferase type I (GGTase-1) to anchor the protein to the vesicle membrane (Ming et al., 2006). Activated Rac2 interacts with several effectors that promote activation of important cellular pathways, including Rac2 interaction with nitric oxide synthase 2A that stimulates nitric oxide production (NO) (Kone et al., 2000).

Rac2 activity is inhibited by GAPs, including Rac GTPase activating protein-1 (RacGAP-1) (Tonozuka et al., 2004).

1.3.4 Rac2 and neutrophil primary granules

Neutrophils contain primary, secondary and tertiary granules that exhibit a hierarchy of release in response to intracellular Ca\(^{2+}\) spikes (Borregaard et al., 1997). The final step of granule fusion with the neutrophil membrane is reliant on GTP and Ca\(^{2+}\) (Barrowman et al., 1986). The exocytosis of primary granules is highly regulated since they contain highly reactive proteolytic enzymes and the peroxidase MPO. Clostridium difficile toxin B, a Rac2 inhibitor, has previously been shown to block degranulation from rat basophilic leukaemia (RBL) cells (Djouder et al., 2001).

A study by Abdel-Latif et al. (2004) that involved harvesting of peritoneal exudate and bone marrow neutrophils from Rac2\(^{-}\) mice demonstrated the
absence of neutrophil primary granule protein release (MPO and NE) in response to cytochalasin B (CB)/fMLP and CB/LTB₄, with intact secondary and tertiary granule exocytosis, therefore confirming that Rac2 is critical for neutrophil primary granule exocytosis. Addition of the potent priming cytokine TNF-α did not rescue the degranulation defect in Rac2⁻⁻ neutrophils in response to CB/fMLP.

Eitzen et al. (2011) performed proteomic analysis on CB/fMLP-stimulated bone marrow neutrophils that were isolated from wild-type and Rac2⁻⁻ mice and identified an accumulation of azurophilic CD63+ granule staining at the cell periphery as well as a reduction in the abundance of several granule proteins in wild-type mice that did not occur in Rac2⁻⁻ neutrophils (p<0.001), therefore confirming the requirement for Rac2 in degranulation of neutrophil primary granules. In addition, Eitzen and colleagues (2011) detected elevated levels of several isoforms of the actin remodeling protein, coronin-1A, in wild-type, but not Rac2⁻⁻ mice, following stimulation with CB/fMLP, suggesting that the control of Rac2-mediated degranulation in neutrophils likely functions through actin remodeling via activation of several actin-binding proteins.

Of note, the molecular mechanism underlying degranulation of neutrophil primary granules in AATD has not been elucidated to date.

1.3.5 Rac2 and reactive oxygen species

In addition to its role in neutrophil primary granule exocytosis, Rac2 is also required for the activation of the NADPH oxidase system. The NADPH oxidase system is one of the predominant cellular sources of ROS (Figure 1.9). NADPH oxidase is a multi-subunit complex comprising of Rac2 and 5 phagocytic oxidase (phox) units. The phox units gp91phox and p22phox make up the membrane-associated component of the enzyme (also known as cytochrome b558), while p40phox, p47phox and p67phox form a cytosolic complex in the resting cell. The cytoskeletal proteins ezrin and radixin bind to
Figure 1.9: Diagrammatic representation of the resting and activated forms of NADPH oxidase. The subunits gp91\textsubscript{phox} and p22\textsubscript{phox} make up the membrane-associated (cytochrome b558) component of NADPH oxidase, while p40\textsubscript{phox}, p47\textsubscript{phox} and p67\textsubscript{phox} form the cytosolic complex in the resting cell. Upon activation, the cytosolic complex translocates to the membrane, docking with cytochrome b558. GDP-bound Rac2 is stabilised by GDI in the resting state, but upon activation, GTP-bound Rac2 also translocates to the membrane. When fully assembled, the NADPH oxidase generates the ROS, O\textsuperscript{2-}, by accepting electrons from cytoplasmic NADPH and donating them to molecular O\textsubscript{2}, which dismutates to H\textsubscript{2}O\textsubscript{2}. MPO then catalyses the conversion of H\textsubscript{2}O\textsubscript{2} and Cl\textsuperscript{-} to cytotoxic HOCl (Adapted from McCann et al, 2013).
GDP dissociation inhibitor (GDI) and prevent it from binding with Rac2 in cytosol, thus promoting binding of Rac2 to p67\(^{\text{phox}}\) and translocation to the plasma membrane (Bokoch et al, 2006). Rac2 also binds to NADPH oxidase activator-1 (NOXA-1), which stimulates activity of p47\(^{\text{phox}}\) (Takeya et al, 2003). Therefore upon activation, the three phox proteins of the cytosolic complex (p40\(^{\text{phox}}\), p47\(^{\text{phox}}\) and p67\(^{\text{phox}}\)) translocate to the membrane, docking with gp91\(^{\text{phox}}\) and p22\(^{\text{phox}}\) (cytochrome b558) (Hordik et al, 2006). When assembled, NADPH oxidase generates the ROS, O\(_2^-\), by accepting electrons from cytoplasmic NADPH and donating them to molecular O\(_2\) (Bokoch et al, 2002, Quinn et al, 2004).

ROS play a crucial role in human physiological processes including cellular homeostasis, growth and apoptosis (D'Autréaux et al, 2007). In addition, ROS species, such as O\(_2^-\), dismutates to H\(_2\)O\(_2\), which is further converted to HOCl, facilitating the oxidative pathway of bacterial killing (Segal et al, 2005). Gene deletion of Rac2 in mice has been shown to lead to decreased O\(_2^-\) release from peripheral blood neutrophils in response to fMLP, TNF-\(\alpha\) and PMA (Roberts et al, 1999).

However, excessive ROS production can induce oxidative stress. Increased levels of ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors such as NF-\(\kappa\)B and activator protein-1 (AP-1), signal transduction, chromatin remodeling, and gene expression of pro-inflammatory mediators (Rahman and MacNee, 1998, Rahman et al, 2003).

1.4 Oxidative stress

1.4.1 Oxidative stress in airways disease

There is increasing evidence that oxidative stress is an important feature in chronic airways disease (Repine et al, 1997, MacNee et al, 2001). Oxidative stress causes oxidation of arachidonic acid and isoprostanes, which may
Neutrophilic MPO can play a crucial role in oxidative stress metabolising
H₂O₂ in the presence of Cl⁻ ions to the powerful oxidant HOCl. Oxidative
stress also activates haem oxygenase-1 (HO-1), which converts haem to
biliverdin, ferrous iron and carbon monoxide (CO) (Choi et al, 1996). HO-1 is
widely expressed in human airways (Lim et al, 2000) and CO production is
increased in COPD (Montuschi et al, 2001). In the airways, ROS can
indirectly activate signal transduction pathways and transcription factors, as
well as lead to the formation of oxidised mediators. ROS has been shown to
activate NFκβ in an epithelial cell line (Adcock et al, 1994) which can induce
neutrophilic inflammation via increased expression of IL-8, TNF-α and MMP-
9. Oxidative stress can also activate histone acetyltransferase activity in
chronic lung diseases associated with increased transcription of multiple
inflammatory genes (Rahman et al, 2002). Exogenous oxidants such as
cigarette smoke, ozone and nitrogen dioxide can all induce oxidative stress
in the airways (Devalia et al, 1997).
During exacerbations of COPD, an increased concentration of H₂O₂ in the
exhaled breath condensate has been detected (Dekhuijzen et al, 1996). In a
similar study, 8-isoprostane, produced from free radical-catalysed
peroxidation of essential fatty acids, was increased in the breath of normal
smokers, but to a much lesser extent than in COPD patients (Montuschi et
al, 2000).
Elevated systemic markers of oxidative stress, such as the specific lipid
peroxidation product, 4-hydroxy-2-nonenal, has previously been identified in
airway epithelial cells and neutrophils of patients with COPD (Rahman et al,
2002).
Oxidation of either methionine 351 or methionine 358 in AAT has been
shown to result in the loss of anti-NE activity, thereby accelerating the
breakdown of elastin in the lung parenchyma (Taggart et al, 2000).
Interestingly, alveolar macrophages from COPD patients show reduced
responsiveness to the anti-inflammatory effects of corticosteroids compared
to non-obstructed smokers (Culpitt et al, 2003). This may be explained by the
finding that oxidative stress impairs binding of glucocorticoid receptors to DNA and their translocation from the cytoplasm to the nucleus (Okamoto et al, 1999). Neutrophil-mediated oxidative stress in AATD may therefore have a significant impact on the efficacy of inhaled and systemic corticosteroid treatment routinely used in the management of AATD-associated airways disease.

1.4.2 Anti-oxidants in the lung

As oxidative stress is an important feature in the pathogenesis of COPD, targeting oxidative stress with antioxidants or boosting the endogenous levels of antioxidants is likely to be beneficial in the treatment of COPD. Dietary antioxidant supplementation with vitamin C, vitamin E and β-carotene has been attempted in cigarette smokers and patients with COPD and while reduced lipid peroxidation was detected in exhaled breath samples in a number of studies, no reduction in symptomatology of COPD patients was detected (Aghdassi et al, 1999, Lykkesfeldt et al, 2000).

In the human respiratory tract the normal production of oxidants is counteracted by several anti-oxidant mechanisms (Cantin et al, 1990). The predominant intracellular antioxidants in the airways are superoxide dismutase (SOD), catalase, glutathione and glutathione synthetase. SOD catalyses the dismutation of $O_2^-$ into $O_2$ or $H_2O_2$, which is further degraded to $H_2O$. Intracellular antioxidants in the lung are expressed at relatively low levels, whereas the major antioxidants are extracellular (Comhair et al, 2002). Cigarette smoke markedly upregulates the extracellular antioxidant glutathione peroxidase, which inactivates $H_2O_2$ and $O_2^-$ (Comhair et al, 2002). High concentrations of reduced glutathione have previously been detected in lung ELF (Cantin et al, 1990). The extracellular antioxidant superoxide dismutase3 (SOD3) is known to be highly expressed in the human lung, but its role in chronic airways disease is not yet clear (Bowler et al, 2002).
In addition to elucidating \( \text{O}_2^- \) production by neutrophils in response to both TNF-\( \alpha \)/fMLP and NE, this study will also investigate the potential protective anti-oxidant effect of SOD.

### 1.4.3 Activation of Protease Activated Receptor 2

While it has been shown that NE cleaves protease activated receptor 2 (PAR2) on nociceptive neurons directly causing release of ROS (Zhao et al, 2015), this has not been investigated in the neutrophil to date. Protease-activated receptors (PARs) act as sensors for active extracellular serine proteases and play an important role in cell proliferation, migration, matrix remodeling and inflammation. PAR2 is a seven trans-membrane domain G protein coupled receptor preferentially activated by trypsin and tryptase. It is expressed on neutrophils (Howells et al, 1997), airway epithelial cells (Cocks et al, 1999), lung macrophages (Steven et al, 2013) and vascular smooth muscle cells (D’Andrea et al, 1998). Lindner et al. (2000) identified that activation of PAR2 produced microvascular inflammation by rapid induction of P-selectin mediated-neutrophil rolling and that there was delayed onset of inflammation in PAR2- deficient mice. Zhao et al. (2015) reported that NE was shown to cleave human PAR2 in addition to stimulating PAR2-dependent cyclic adenosine monophosphate (cAMP) formation. This study also demonstrated that NE-activated PAR2 and transient receptor potential vaniloid-4 (TRPV-4) caused inflammation and pain, expanding the role of PAR2 as a mediator of protease-driven inflammation (Zhao et al, 2015). PAR2 is also known to be over-expressed in human airways of asthmatic and COPD patients (Cocks et al, 2001), as well as in bronchial vessels of patients with bronchitis (Miotto et al, 2002). In-vitro and in-vivo evidence supports up-regulation of PAR2 by inflammatory stimuli, such as TNF-\( \alpha \) and interleukin-1-alpha (IL-1-\( \alpha \)) (Nystedt et al, 1996). Thus, PAR2 may represent a novel pharmacological target in airways disease such as COPD (Cocks et al, 2001) and AATD and will be investigated in this study.
1.5 Autoantibodies in AATD

Autoimmune disease frequently involves elevated neutrophil activity in the absence of infectious agents. ANCA are autoantibodies directed against neutrophil antigens, predominantly PR3 and MPO, which have been implicated in the pathogenesis of small vessel vasculitis. AAT is known to be the main inhibitor of PR3. Several small studies have suggested that carriage of the Z allele in AATD is associated with the development of autoimmunity and ANCA (Audrain et al., 2001, Segelmark et al., 1995).

A study by Segelmark et al. (1995) suggested that the suboptimal response of plasma AAT to PR3 seen in PiZ-heterozygotes enhanced the risk of dissemination of the vasculitic process as reflected by the number of affected organs (p<0.01) and increased mortality rate (p=0.048). This study also concluded that AAT phenotyping was justified in all cases of PR3-ANCA-associated vasculitis. In addition, Morris et al. (2011) identified a link between ANCA-associated vasculitis and carriage of the Z-allele in AAT and its polymers and suggested this may promote inflammation by priming of neutrophils (Morris et al., 2011).

In 2001, Audrain et al. conducted a study to compare the incidence and specificity of ANCA among 273 ZZ-AATD vs. 272 MM sera samples using antigen-specific enzyme-linked immunosorbant assays (ELISA) for PR3, MPO, NE and lactoferrin. This study reported an increased incidence of anti-NE antibodies in the ZZ-AATD population compared to the MM group (p<0.05), but noted no difference in anti-MPO or anti-PR3 antibodies, suggesting that AATD was not sufficient to induce ANCA-positive vasculitis.

While circulating ANCA for MPO are strongly associated with the presence of vasculitis, they have been described in sera from patients with other conditions, including RA, systemic lupus erythematosis (SLE) and autoimmune thyroid disease, suggesting that a potentially pathogenic subset of anti-MPO autoantibodies exist. In 1998, Locke et al. compared the characteristics of anti-MPO antibodies in sera from 18 patients with active vasculitis with those present in remission (n=9) and in a disease control
group without clinical evidence of vasculitis (n=10). This study identified an over-expression of IgG4 subclass anti-MPO antibodies and a more frequent presence of IgM class anti-MPO antibodies in sera from patients with active vasculitis (Locke et al, 1999).

Having previously identified increased neutrophil degranulation of secondary and tertiary granules in AATD in response to TNF-α, Bergin et al. (2014) explored the potential for the development of autoantibodies in AATD. This study confirmed a significantly higher level of anti-lactoferrin IgG antibodies in a ZZ-AATD group compared with healthy control donors (p=0.001), but found no significant difference in the level of anti-hCAP-18 or anti-MMP-9 IgG autoantibodies (Bergin et al, 2014). Given that anti-lactoferrin IgG autoantibodies were the most prevalent in AATD, Bergin and colleagues (2014) also investigated the possibility of anti-lactoferrin antibody-mediated ROS production. MM and AATD neutrophils were exposed to lactoferrin antibodies (1 µg) for 30 min in the presence or absence of TNF-α (10 ng) and lactoferrin antibodies were found to induce significantly more O₂⁻ release from AATD neutrophils compared to MM cells (p=0.004). The authors therefore concluded that autoantibodies directed against lactoferrin circulating in the plasma of AATD patients could target antigen bound to the cell surface and trigger neutrophil ROS production. Importantly, this study also demonstrated that there was a 30% decrease in anti-lactoferrin antibody titres following long-term AAT augmentation therapy (p=0.04) (Bergin et al, 2014).

To date, the possibility of autoantibodies directed against the contents of neutrophil primary granules in AATD individuals compared with those receiving AAT augmentation therapy has not been explored and will be addressed in this study.
1.6 Aim

This translational research project aims to fully characterise neutrophil degranulation in AATD and to determine the effects of AAT augmentation therapy on the AATD neutrophil. This will add a new dimension to our current understanding of the crucial role of AAT in respiratory health.

To fulfil this aim, the following objectives were set:

1. To investigate neutrophil degranulation patterns in AATD

2. To determine the signaling mechanism underlying aberrant degranulation of neutrophil primary granules in AATD

3. To establish if there are increased autoantibodies directed against the contents of neutrophil primary granules in AATD

4. To explore the effect of AAT augmentation therapy on neutrophil degranulation and Rac2 activity in AATD
Chapter 2
Materials and Methods
2.1 Material suppliers

2.1.1 Chemicals and reagents
All chemicals and reagents were purchased from Sigma-Aldrich®, unless otherwise specified.

2.1.2 Antibodies
The primary antibodies employed for Western blot analysis are listed in Table 2.1 and secondary antibodies are listed in Table 2.2.
Table 2.1: Primary antibodies employed for Western blot analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Concentration</th>
<th>Molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit PAb anti-MPO</td>
<td>Novus Biological</td>
<td>1:1000 (WB)</td>
<td>53</td>
</tr>
<tr>
<td>Rabbit PAb anti-hCAP-18</td>
<td>Innovagen</td>
<td>1:2000 (WB)</td>
<td>16</td>
</tr>
<tr>
<td>Goat PAb anti-MMP-9</td>
<td>R&amp;D Systems</td>
<td>1:1000 (WB)</td>
<td>98</td>
</tr>
<tr>
<td>Rabbit PAb anti-Rac 1/2/3</td>
<td>Cell Signaling Technology</td>
<td>1:1000 (WB)</td>
<td>21</td>
</tr>
</tbody>
</table>

PAb= Polyclonal antibody; WB= Western blot analysis

Table 2.2: Secondary antibodies employed for Western blot analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit PAb HRP anti-goat</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000 (WB for Rac2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:2000 (WB for MPO)</td>
</tr>
<tr>
<td>Horse PAb HRP anti-mouse</td>
<td>Cell Signaling Technology</td>
<td>1:2000 (WB)</td>
</tr>
<tr>
<td>Goat PAb HRP anti-rabbit</td>
<td>Cell Signaling Technology</td>
<td>1:2000 (WB)</td>
</tr>
</tbody>
</table>

PAb= Polyclonal antibody; HRP= Horseradish Peroxidase; WB= Western blot analysis
2.2 Patient Samples

2.2.1 Patient Recruitment

Ethics approval was obtained from the Beaumont Hospital Ethics Committee, Dublin in accordance with the Declaration of Helsinki. Informed written consent was obtained from all participants. For the purpose of this research study, the term ‘AATD’ referred to study participants with AATD homozygous for the Z allele. Blood was collected from 6 clinically stable AATD patients attending the Alpha-1 clinic at Beaumont Hospital for analysis of neutrophil degranulation (Table 2.3). For our studies assessing the effect of AAT replacement therapy, 5 patients currently receiving AAT 60 mg/kg by once weekly infusion were recruited pre-infusion (day 0) and two days post-infusion (day 2) (Table 2.4). Demographics of healthy control (HC) and AATD individuals for the subsequent experiments are listed as follows; analysis of membrane-bound NE (Table 2.5), effect of exogenous AAT on primary granule release (Table 2.6 and 2.7), analysis of Rac2 activity (Table 2.8), analysis of the effect of AAT augmentation therapy on Rac2 activity (Table 2.9), ANCA quantification (Table 2.10) and O$_2^-$ release by neutrophils (Table 2.12). Of note, AATD individuals listed in Tables 2.3, 2.5, 2.7, 2.8 and 2.10 were on standard treatment for obstructive airways disease and were augmentation therapy-naïve. Control volunteers for all experiments did not have AATD, were non-smokers, showed no clinical evidence of any inflammation and were not taking any medications.

2.2.2 Neutrophil Isolation

Human peripheral blood neutrophils were isolated from heparinised venous blood as described by Reeves et al. (2001). Neutrophils were isolated by mixing 5 mL Dulbecco’s phosphate buffered saline (DPBS) (Lonza, BE17-512F, BioWhittaker®) with 15 mL of blood and 2 mL of 10% (w/v) dextran in DPBS and allowed to settle for 15 min. The supernatant was collected and underlayed with 5 mL Lymphoprep™ (Axis-Shield Poc AS, Oslo, Norway) to separate neutrophils and erythrocytes from monocytes and macrophages by
Table 2.3: Demographics of HC and AATD individuals for analysis of neutrophil primary, secondary and tertiary granule release. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>AATD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>3/3</td>
<td>4/2</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>31.4 ± 4.1</td>
<td>53.4 ± 5.41</td>
</tr>
<tr>
<td>FEV₁ (% predicted ± SEM)</td>
<td>96.6 ± 1.34</td>
<td>31.8 ± 20.72</td>
</tr>
</tbody>
</table>

FEV₁ = Forced expiratory volume in one second

Table 2.4: Demographics of HC and AATD individuals receiving AAT augmentation therapy for analysis of neutrophil primary, secondary and tertiary granule release. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>AATD on AAT Tx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1/4</td>
<td>2/3</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>32.6 ± 7.64</td>
<td>62.4 ± 7.83</td>
</tr>
<tr>
<td>FEV₁ (% predicted ± SEM)</td>
<td>94.25 ± 2.65</td>
<td>44.8 ± 13.46</td>
</tr>
</tbody>
</table>
Table 2.5: Demographics of HC and AATD individuals for FRET analysis of membrane-bound NE. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>AATD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1/2</td>
<td>2/1</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>39.66 ± 3.66</td>
<td>52.5 ± 3.5</td>
</tr>
<tr>
<td>FEV₁ (% predicted ± SEM)</td>
<td>97.75 ± 0.66</td>
<td>27.5 ± 5.5</td>
</tr>
</tbody>
</table>

Table 2.6: Demographics of HC individuals for analysis of the effect of exogenous AAT on primary granule release. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>3</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1/2</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>40 ± 3.605</td>
</tr>
<tr>
<td>FEV₁ (% predicted ± SEM)</td>
<td>98 ± 0.57</td>
</tr>
</tbody>
</table>
Table 2.7: Demographics of HC and AATD individuals for analysis of the effect of exogenous AAT on primary granule release. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>AATD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1/2</td>
<td>3/0</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>32.33 ± 2.66</td>
<td>57 ± 4.358</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; (%)</td>
<td>96.66 ± 1.33</td>
<td>65 ± 24.11</td>
</tr>
</tbody>
</table>

Table 2.8: Demographics of HC and AATD individuals for analysis of Rac2 activity. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>AATD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
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<tr>
<td>Gender (male/female)</td>
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</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>31.5 ± 3.304</td>
<td>37.5 ± 8.003</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; (%)</td>
<td>98 ± 1.29</td>
<td>89.75 ± 16.8</td>
</tr>
</tbody>
</table>
Table 2.9: Demographics of HC and AATD individuals receiving AAT augmentation therapy for analysis of Rac2 activity. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>AATD on AAT Tx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1/2</td>
<td>2/1</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>25.33 ± 0.33</td>
<td>56.33 ± 2.18</td>
</tr>
<tr>
<td>FEV₁ (% predicted ± SEM)</td>
<td>96.33 ± 0.88</td>
<td>41 ± 2.645</td>
</tr>
</tbody>
</table>

Table 2.10: Demographics of individuals for quantification of ANCA. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>MM</th>
<th>AATD</th>
<th>AATD on AAT Tx</th>
</tr>
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<td>Number of subjects</td>
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<td>10</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>16/24</td>
<td>18/18</td>
<td>5/5</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>58.2 ± 1.92</td>
<td>54 ± 1.91</td>
<td>60.1 ± 7.2</td>
</tr>
<tr>
<td>FEV₁ (% predicted ± SEM)</td>
<td>97.3 ± 2.5</td>
<td>67 ± 6.41</td>
<td>48 ± 9.6</td>
</tr>
</tbody>
</table>
Table 2.11: Demographics of individuals for analysis of O$_2^-$ release by neutrophils. Values are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>3</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>1/2</td>
</tr>
<tr>
<td>Age (years ± SEM)</td>
<td>29.33 ± 2.403</td>
</tr>
<tr>
<td>FEV$_1$ (% predicted ± SEM)</td>
<td>98.66 ± 2.33</td>
</tr>
</tbody>
</table>
density gradient centrifugation (Megacentrifuge 1.0R, Heraeus Instruments) at 836 x g for 10 min at room temperature. Neutrophils were purified by re-suspending the obtained cell pellet in 20 mL high-grade water (H_2O) for 24 sec to achieve hypotonic lysis of erythrocytes. Tonicity was corrected by the addition of 20 mL 2X saline (1.8% (w/v) NaCl) and neutrophils were collected by centrifugation at 470 x g for 5 min at room temperature. The pellet containing neutrophils was re-suspended in DPBS pH 7.4 containing 5 mM glucose at room temperature and used immediately.

2.2.3 Trypan Blue Exclusion Test

The total number of neutrophils was counted by adding 10 µL of the neutrophil suspension to 90 µL of Trypan Blue and by use of a haemocytometer (Marienfeld Superior, Germany). Viable cells were unstained and non-viable cells stained blue. Cell viability was determined to be greater than 95% in all samples.
2.3 Preparation of protein samples

2.3.1 Preparation of whole cell lysates

For protein preparation of whole cell lysates, $1 \times 10^7$ cells were lysed in 75 µL of ice-cold radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 1% (w/v) Triton X-100, 1% (v/v) nonyl phenoxyethoxylethanol-40 (NP-40), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS) containing additional protease inhibitors (10 µg/mL N\textalpha-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 1 µg/mL phenylmethane-sulfonyl fluoride (PMSF), 10 µg/mL pepstatin A, 10 µg/mL leupeptin) and incubated on ice for 10 min. The suspension was spun at 20,817 x g for 10 min at 4°C to remove insoluble cell debris and the supernatant was collected. 10X sample buffer (SB) (2% (w/v) SDS, 1 mM 1,4 dithiothreitol (DTT), 1% (w/v) sucrose, 0.004% (w/v) bromphenol blue, 5 mM ethylenediaminetetraacetic acid (EDTA), 60 mM Tris HCl pH 6.7) was added to give a final concentration of 1 X. Samples were heated to 99°C for 3 min and stored at -20°C for subsequent visualisation of proteins by Coomassie blue staining of SDS-gels or Western blot analysis.
2.4 Protein quantification

2.4.1 Sodium dodecyl sulfate polyamide gel electrophoresis (SDS-PAGE)

Firstly two glass plates with a gasket between them were clamped together. Resolving gel (12.5% (w/v)) (Table 2.12) was prepared and placed between the plates to approximately 80% of the plate’s capacity and allowed to set with 200 µL isopropanol placed above the 12.5% (w/v) gel to even out the underlying layer of gel. The isopropanol was washed off with deionised H2O. Stacking gel (5% (w/v)) (Table 2.13) was placed above the resolving gel and a loading comb positioned and stacking gel was allowed to set. The plates were placed in a tank containing 1X Running buffer (6 g Tris, 2.88 g Glycine and 2 g sodium dodecyl sulfate (SDS)). Prepared protein containing samples were mixed with 10X SB. Samples were boiled at 99°C for 3 min and then centrifuged at 4°C for 1 min. Samples (15 µL) with 10X sample buffer (1.6 µL) were run alongside 4 µL of molecular weight ladder, SeeBlue® Plus 2 Prestained Standard (Invitrogen, Bio Sciences Ltd, Ireland), for approximately 2 h at 130V. The gel was further analysed by Western blotting to determine protein content and profile.
Table 2.12: Components of 12.5% (w/v) resolving gel.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 mL</td>
<td>1.5 M Trizma base pH 8.9</td>
</tr>
<tr>
<td>7.6 mL</td>
<td>Deionised H₂O</td>
</tr>
<tr>
<td>10 mL</td>
<td>30% (w/v) Protogel (acrylamide) (National Diagnostics, Atlanta, USA)</td>
</tr>
<tr>
<td>240 µL</td>
<td>10% (w/v) SDS</td>
</tr>
<tr>
<td>150 µL</td>
<td>10% (w/v) Ammonium persulfate (APS) (0.1 g APS in 1 ml H₂O)</td>
</tr>
<tr>
<td>6 µL</td>
<td>Tetramethylethylenenediamine (TEMED)</td>
</tr>
</tbody>
</table>

Table 2.13: Components of 5% (w/v) stacking gel.

<table>
<thead>
<tr>
<th>Volume</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>630 µL</td>
<td>0.5 M Trizma base pH 6.8</td>
</tr>
<tr>
<td>3.4 mL</td>
<td>Deionised H₂O</td>
</tr>
<tr>
<td>830 µL</td>
<td>30% (w/v) Protogel (acrylamide) (National Diagnostics, Atlanta, USA)</td>
</tr>
<tr>
<td>50 µL</td>
<td>10% (w/v) SDS</td>
</tr>
<tr>
<td>50 µL</td>
<td>10% (w/v) APS</td>
</tr>
<tr>
<td>5 µL</td>
<td>TEMED</td>
</tr>
</tbody>
</table>
2.4.2 Coomassie blue staining

Gels containing protein bands were visualised using Coomassie blue staining (R250). Gels were placed in Coomassie blue stain, the components of which were 10% (v/v) acetic acid, 45% (v/v) methanol, 45% (v/v) deionised H$_2$O and 0.2% (w/v) Coomassie, for 1 h. Gels were then destained for 1 h or until the bands were visible using 10% (v/v) acetic acid, 25% (v/v) methanol and 65% (v/v) deionised H$_2$O. Images were taken using the G:Box SynGene machine (Synoptics, U.K.).

2.4.3 Western blot analysis

Western blotting was performed with 1X Transfer buffer (3.03 g Trizma base, 14.4 g Glycine, 800 mL deionised H$_2$O and 200 mL methanol) using the XCell II SureLock Mini-Cell wet Western blotter™ blot module (Invitrogen, Bio Sciences Ltd., Ireland). Methanol was used to activate polyvinylidene fluoride (PVDF) membranes (Roche) and the sponges and Whatman™ filter paper were pre-soaked in 1X Transfer buffer. Following electrophoresis, gels were sandwiched between activated PVDF, presoaked Whatman™ filter paper and sponges to allow for the transfer of proteins by wet transfer. Transfer was carried out at 30 V for 120 min for two blots. Following transfer, PVDF membranes containing transferred proteins were placed in Blocking buffer containing 3% (w/v) dried skimmed milk (Marvel, Chivers Ireland Ltd, Ireland) and 1% (w/v) bovine serum albumin (BSA) in DPBS solution containing 0.1% (v/v) Tween® for 1 h at room temperature. The blots were incubated overnight at 4°C with various antibodies and individual concentrations as specified in Table 2.1. The blots were then washed in DPBS-Tween® (0.01M DPBS, 0.5% Tween®) every 10 min for 40 min and then probed with HRP-linked antibodies against the primary antibody for 1 h at room temperature (Table 2.2). Blots were washed again every 10 min for 40 min in DPBS-Tween®. The blots were incubated with Immobilon Western Chemiluminescent HRP-substrate solution (Millipore, Billerica, MA, USA) and the bands were visualised with the G:BOX SynGene machine (Syngene,
GeneTools® software was employed to quantify the bands.

2.5 Neutrophil degranulation assay

Neutrophils (1 x 10^7) from AATD patients and HC were either unstimulated (time point 0) or stimulated with either 1 µg/mL phorbol 12-myristate 13-acetate (PMA) (Huber-Lang et al, 2002) or 1 ng/mL tumour necrosis factor-alpha (TNF-α) (Cross et al, 2008) with 100 ng/mL formyl-methionyl-leucyl-phenylalanine (fMLP) (Palmer et al, 1983) at 37°C. Cross et al. (2008) have previously shown that a TNF-α concentration less than 10 ng/mL does not cause apoptosis. Samples (100 µL) aliquots were removed after each time point (including 0, 5, 10 or 20 min) and added to ice-cold protease inhibitor cocktail in DPBS to prevent cleavage of released proteins (Table 2.14). After centrifugation at 425 x g x 5 min at 4°C, 20 µL of 10X sample buffer was added to 100 µL of supernatant containing degranulated proteins. Samples were boiled at 99°C for 3 min and stored at -80°C. Remaining cells were subjected to whole cell lysis as previously described for loading controls.
Table 2.14: Constituents of Protease Inhibitor Cocktail.

<table>
<thead>
<tr>
<th></th>
<th>Inhibition target</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mL DPBS</td>
<td></td>
</tr>
<tr>
<td>10 µL TLCK</td>
<td>Trypsin inhibitor</td>
</tr>
<tr>
<td>10 µL Leupeptin</td>
<td>Serine and cysteine protease inhibitor</td>
</tr>
<tr>
<td>20 µL Pepstatin A</td>
<td>Aspartic acid protease inhibitor</td>
</tr>
<tr>
<td>25 µL PMSF</td>
<td>Serine protease inhibitor</td>
</tr>
</tbody>
</table>
2.6 Fluorescence resonance energy transfer (FRET) analysis

The following protocol was adapted from Korkmaz et al. (2008), Nature Protocols 3.6.

Neutrophils (1x10^7) from HC and AATD individuals (Table 2.5) were isolated and either unstimulated or stimulated with TNF-α (1 ng/10^7 cells) and fMLP (100 ng/10^7 cells) at 37°C for 0, 5, 10 and 20 min. Extracellular supernatant obtained at each time-point was removed after centrifugation at 425 x g x 5 min at 4°C. The neutrophil pellet was re-suspended in 100 µL DPBS and 50 µL of this suspension was loaded in duplicate onto wells on a white polypropylene plate (Nunc® Microwell™ 96 well polypropylene plates, P6866, Sigma Aldrich). The standard curve for NE was derived from NE (TS563, Elastin Products Company, Inc) standards of increasing concentrations (0 nanomolar (nM), 5 nM, 10 nM, 20 nM, 30 nM, 40 nM, 50 nM and 60 nM in 50 µL) which were added in duplicate to the first two columns of the 96-well plate. The slope of the standard curve was used to extrapolate unknown concentrations of membrane-bound NE. FRET substrate for human NE was formed by adding 152 µL of 30% (w/v) dimethylformamide (DMF) into a vial of Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp (3230-v, Peptide Institute, Inc), and adding a further 608 µL DPBS for a final concentration of 1 mM. NE FRET (50 µL) substrate was then added to each well immediately prior to plate reading at a wavelength 320-420 nanometres (nm) set at 28°C for 1 h at 20 sec intervals using a spectrofluorometer (SpectraMax® M3 multi-mode microplate reader, Molecular Devices). Samples were analysed in duplicate and membrane-bound NE concentration was expressed in nM.
2.7 Rac2 activation assay

Neutrophils (1 x 10^7) from HC and AATD individuals (Table 2.8 and Table 2.9) were isolated and either unstimulated or stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C for 2.5 min. Of note, Benard et al. (1999) previously demonstrated peaks in Rac2 activity between 30 sec and 5 min following stimulation. Aliquots (100 µL) were removed at 0 and 1.25 min/2.5 min and added to 100 µL of protease inhibitor cocktail (2mL 5X lysis buffer (Abcam, catalogue # ab139586), 8 mL deionised H2O, 5 µL PMSF, 2 µL pepstatin A, 1 µL leupeptin and 1 µL TLCK). Cell-free supernatants were harvested by centrifugation at 425 x g x 5 min at 4°C and the remaining neutrophil pellets retained. Diisopropylfluorophosphatate (DFP) (1 µL) and 1X lysis buffer (Table 2.15) (200 µL) were added to each neutrophil pellet which were lysed by passing through a 25 gauge needle x 3. Neutrophil lysates were cleared by centrifugation at 12,000 x g x 10 min at 4°C. Lysis solution (Table 2.16) (1 mL) was added to the neutrophil lysates from each time point and lysates were again cleared by centrifugation at 12,000 x g x 15 min at 4°C. Neutrophil lysate (40 µL) from each time point was removed for total Rac2 analysis. The Abcam Rac activation kit (catalogue # ab139586) was used to pull down active GTP-bound Rac from the neutrophil lysates. This kit exploits the selective interaction of the Cdc42/Rac interactive binding domain (CRIB) of the effector p21 activated kinase-1 (PAK-1) with the active Rac-GTP conformation. 200 µL of the remaining neutrophil lysate from each time point and 40 µL glutathione sepharose slurry was incubated on a rotating wheel for 30 min at 4°C. Glutathione sepharose slurry binds to the glutathione sepharose-tagged-p21 activated kinase-Cdc42/Rac interactive binding domain (GST-PAK-CRIB) complex containing active GTP-bound Rac. The beads are washed with 1 mL ice-cold 1X Lysis buffer x 3. SDS-PAGE SB (2X) was added to each total and active GTP-bound Rac sample and boiled at 99°C for 3 min followed by centrifugation at 425 x g x 2 min at 4°C for subsequent PAGE and Western blot analysis. Anti-Rac2 antibody (Cell Signalling Technology Rac 1/2/3 antibody, 1:1000) was used to detect total and active GTP-bound Rac2 in neutrophil lysates. Of note, human neutrophils primarily express Rac2 rather than Rac1 (Heyworth et al, 1994).
and while Rac2 is limited to cells of the haematopoietic system (Didsbury et al, 1989), human neutrophils do not express Rac3 (proteinatlas.org).

The bands were visualised with the Molecular Imager® Gel Doc™ XR System (BioRad Laboratories, Inc.). Quantification of immunoband intensity was carried out employing ImageLab® 5.2.1 software (BioRad Laboratories, Inc.).
Table 2.15: Constituents of 1X Lysis Buffer.

<table>
<thead>
<tr>
<th>Volume (mL)</th>
<th>Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Deionised H₂O</td>
</tr>
<tr>
<td>2</td>
<td>5X lysis buffer stock (Rac activation kit (Abcam, catalogue # ab139586), containing Tris buffer pH 7.5, NaCl, MgCl₂, Nonyl phenoxypolyethoxyethanol (NP-40), PMSF, Pefabloc, Aprotinin, Pepstatin A). Note individual concentrations not released by company.</td>
</tr>
</tbody>
</table>

Table 2.16: Constituents of Lysis Solution.

<table>
<thead>
<tr>
<th>Volume (mL/µL)</th>
<th>Constituent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 mL</td>
<td>5X lysis buffer (Rac activation kit, Abcam, catalogue # ab139586)</td>
</tr>
<tr>
<td>4.8 mL</td>
<td>Deionised H₂O</td>
</tr>
<tr>
<td>60 µL</td>
<td>100X protease inhibitor mix (Rac activation kit, Abcam, catalogue # ab139586)</td>
</tr>
<tr>
<td>6 µL</td>
<td>GDP 100 mM (Rac activation kit, Abcam, catalogue # ab139586)</td>
</tr>
<tr>
<td>150 µg</td>
<td>GST-PAK-CRIB (Rac activation kit, Abcam, catalogue # ab139586)</td>
</tr>
</tbody>
</table>
2.8 Determination of the effect of exogenous AAT on neutrophil degranulation

Neutrophils (1 x 10^7) from HC and AATD individuals were isolated and stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C in the presence or absence of increasing concentrations of AAT; 2, 6.875, 13.75 or the physiological concentration, 27.5 µM. Aliquots (500 µL) were removed at 0 and 5 min and added to 100 µL ice-cold protease inhibitor cocktail in DPBS to prevent cleavage of released proteins (Table 2.13). Cell-free supernatants were harvested by centrifugation at 425 x g x 5 min at 4°C. 10X SB (20 µL) was added to 100 µL of supernatant containing degranulated proteins. Samples were boiled at 99°C for 3 min and stored at -80°C for subsequent SDS-PAGE and Western blot analysis. Rabbit PAb anti-MPO antibody (Novus Biological, 1:1000) was employed as a marker of neutrophil primary granule degranulation.
2.9 Autoantibody quantification

2.9.1 Anti-MPO and anti-PR3 autoantibody quantification

An ELISA-based method was used for evaluation of autoantibodies against neutrophil primary granule proteins, MPO and PR3, as per the ‘Routine use of the Phadia® 250 for anti-MPO and anti-PR3 antibody testing’ protocol set out by the Clinical Directorate of Laboratory Medicine, Beaumont Hospital, Dublin (document no. LP-IMM-ICAP0004, revision 3.6, active date 09/09/14).

The PHADIA® 250 EliA™ system (Phadia GmbH, Freiburg, Germany) is a fully automated test that measures the concentrations of antigen specific IgG antibodies in human plasma or serum. The EliA™ MPO wells (article no. 14-5537-01) were coated with human MPO protein, while the EliA™ PR3 (article no. 14-5536-01) wells were coated with human PR3 protein. The plates were incubated with plasma samples from HC (MM) (n=40), AATD (n=36) and AATD individuals receiving augmentation therapy (n=10) (Table 2.10).

If present in the patient’s plasma sample, antibodies to MPO or PR3 bound to their specific antigen. After washing away non-bound antibodies, enzyme-labelled antibodies against human IgG antibodies, EliA™ IgG conjugate (article no. 83-1017-01), were added to form an antibody-conjugate complex. Following incubation, non-bound conjugate was washed away with washing solution (article no. 10-9202-01) and the bound complex was incubated with a development solution (article no. 10-9441-01). The reaction was then stopped with stop solution (article no. 10-9442-01) and the fluorescence of the eluate was measured. The test results were calculated from a calibration curve specific for the anti-MPO and anti-PR3 antibodies being measured.

The intensity of the fluorescence was directly proportional to the concentration of relevant antibody in the plasma sample. Both, EliA™ MPO and EliA™ PR3 were calibrated against the new Centre for Disease Control (CDC) ANCA references: CDC PR3-ANCA Human Reference Serum #16 and CDC MPO-ANCA Human Reference Serum #15 (CDC 2008). Results were given in international units per millilitre (IU/mL). The references ranges are listed in Table 2.17.
Table 2.17: Reference ranges for EliA™ MPO and EliA™ PR3 autoantibody quantification.

<table>
<thead>
<tr>
<th></th>
<th>Negative (IU/mL)</th>
<th>Equivocal (IU/mL)</th>
<th>Positive (IU/mL)</th>
<th>Measuring range (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EliA™ MPO</td>
<td>&lt; 3.5</td>
<td>3.5-5</td>
<td>&gt;5</td>
<td>0.2-134</td>
</tr>
<tr>
<td>EliA™ PR3</td>
<td>&lt; 2</td>
<td>2-3</td>
<td>&gt;3</td>
<td>0.2-177</td>
</tr>
</tbody>
</table>

Table 2.18: Reference ranges for Alegria® anti-NE autoantibody quantification.

<table>
<thead>
<tr>
<th></th>
<th>Normal (U/mL)</th>
<th>Elevated (U/mL)</th>
<th>Measuring range (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alegria® anti-NE assay</td>
<td>&lt; 10</td>
<td>≥ 10</td>
<td>0-100</td>
</tr>
</tbody>
</table>
2.9.2 Anti-NE autoantibody quantification

Anti-NE IgG autoantibodies were detected in plasma samples using the anti-
elastase Alegria® ELISA-based assay (ORG 224 ORGENTEC Diagnostika GmbH Mainz, Germany). The Alegria® assay features barcoded 8-well-
microstrips, called Alegria® test strips. Each strip was designed for a single
determination of one patient sample and holds a complete set of reagents,
including enzyme conjugate, enzyme substrate, sample buffer and a test
specific control. Furthermore each strip has two antigen-coated wells which
serve as reaction wells for one control and one patient sample. The
determination was based on an indirect enzyme linked immune reaction with
the following steps: antibodies present in positive samples bind to the
antigen coated on the surface of the two reaction wells forming an antibody
antigen complex. After incubation, a first washing step removed unbound
molecules. Subsequently, added enzyme conjugate binds to the immobilised
antibody-antigen complex. After incubation, a second washing step removed
unbound enzyme conjugate. Addition of enzyme substrate solution resulted
in hydrolysis and colour development during incubation. The intensity of
the blue colour correlated with the concentration of the antibody-antigen-
complex and was measured photometrically at 650 nm. The Alegria® test
strip was used with the diagnostic instrument Alegria® - a fully automated
random access analyser. By means of SMC®-Technology data encoded on
the barcode were transferred from the Alegria® test strip to the instrument
and the assay was automatically processed and evaluated. The reference
ranges are listed in Table 2.18.
2.10 Cytochrome c reduction assay

A cytochrome c reduction assay was used to measure the production of $O_2^-$ by neutrophils. This protocol is adapted from Babior et al. (1973). Neutrophils from HC were isolated as per section 2.2.2. At the final step, neutrophils were suspended in 500 µL cytochrome c buffer (100 µM cytochrome c (C2506 Sigma Aldrich), 0.5 mM MgCl$_2$, 0.5 mM CaCl$_2$, 5 mM glucose in DPBS) and a neutrophil count performed. Neutrophils ($1 \times 10^6$) were then added to wells of a 96 well UV-transparent polystyrene plate (TC plate 96 well standard F, Sarstedt). Pre-heated cytochrome c buffer at 37°C was added to each well to make up a final volume of 200 µL. Neutrophils ($1 \times 10^6$) in cytochrome c buffer were then either untreated (control) or stimulated with TNF-α (100 pg/10$^6$ cells)/fMLP (10 ng/10$^6$ cells) or NE (25 µM) or NE (25 µM)/AAT (27.5 µM) or NE (25 µM)/AZD9668 (27.5 µM) (Polyphor Ltd., Switzerland) or NE (25 µM)/SOD (12.5 µg/mL) (S2515 Sigma Aldrich) or NE (25 µM)/protease-activator receptor-2 (PAR2) blocking antibody (10 µg/mL) (C62022 R&D systems). The reduction of cytochrome c was recorded at a wavelength of 550 nm over 30 min at 10 min intervals at 37°C on a spectrophotometer, SpectraMax® M3 multi-mode microplate reader, Molecular Devices. $O_2^-$ production (nmoles/1 x 10$^6$ cells) was determined by correcting for absolute absorbance at 30 min (t=30 min absorbance - t=0 min absorbance), volume per reaction (200 µL) and the extinction coefficient of cytochrome c (0.021) (Babior et al, 1973, Guengerich et al, 2009).
2.11 Statistical Analysis

All data investigated in this report was analysed using GraphPad Prism 4.00 (GraphPad Software, San Diego, CA, USA). Results are expressed as mean ± SEM. Statistical significance was calculated by student t-test, where a p value ≤ 0.05 was deemed statistically significant.
Chapter 3
Impaired neutrophil degranulation in AATD
3.1 Introduction

AATD is characterised by a protease: anti-protease imbalance due to low circulating levels of AAT and the development of neutrophil-driven lung destruction resulting in premature emphysema (McElvaney et al, 1997, Tobin et al, 1983).

Dysregulated neutrophil degranulation is recognised as an important event in inflammatory lung conditions such as asthma and acute lung injury (Skubitz et al, 1999). Taggart et al. (2000) previously demonstrated increased NE release by CF neutrophils mediated by TNF-α and IL-8, while Pohl et al. (2014) demonstrated decreased neutrophil degranulation of secondary and tertiary granules in CF compared with healthy control cells in response to TNF-α. In 2014, Bergin et al. identified increased plasma concentrations of neutrophil-released secondary and tertiary granule proteins from AATD neutrophils in response to TNF-α. However, degranulation patterns of neutrophil granules in AATD, in particular primary granules, in response to a combination of the physiological stimuli TNF-α and fMLP, have not been characterised to date.

Neutrophils are known to contain a potent antimicrobial arsenal within primary, secondary and tertiary intracellular granules. This chapter focuses on MPO, hCAP-18 and MMP-9 as markers of primary, secondary and tertiary granule release in AATD, respectively.

MPO is the most abundant peroxidase in neutrophil primary granules and excessive levels of MPO are associated with high oxidative stress (Shindhelm et al, 2009).

Enhanced levels of the C-terminal peptide of hCAP-18, LL-37, released from neutrophil secondary granules, inhibit neutrophil apoptosis contributing to enhanced neutrophil accumulation and inflammation in primary airway epithelial cells. (Barlow et al, 2006).

Elevated levels of the neutrophil tertiary granule metalloproteinase, MMP-9, have been identified in plasma and BAL fluid of patients with acute
respiratory distress syndrome (ARDS) and correlates with clinical severity (Fligiel et al, 2006).

In addition to MPO, experiments in this chapter will also examine the serine protease, NE, as an alternative marker of neutrophil primary granule behaviour in AATD. NE causes proteolysis of collagen IV and elastin on the extracellular matrix and therefore plays a key role in the development of emphysema in AATD (Kawabata et al, 2002, Janoff et al, 1977). Owen et al. (1995) demonstrated that human neutrophils express cell surface-bound NE that can be strikingly upregulated by pro-inflammatory mediators and substantially resistant to inhibition by naturally occurring protease inhibitors. The authors suggested that given the strong positive charge of serine proteases at physiological pH, the mechanism of neutrophil membrane binding was predominantly electrostatic. This study also detected an inverse relationship between the molecular weight of AAT and its capacity to inhibit cell surface-bound NE, suggesting that steric hindrance was the major mechanism by which membrane-bound serine proteases could evade protease inhibitors such as AAT (Owen et al, 1995). Long-lived catalytically active neutrophil-surface proteases such as NE could contribute to the pathogenesis of tissue destruction by facilitating neutrophil egress from the vasculature and penetration of tissue barriers allowing local degradation of extracellular matrix proteins (Owen et al, 1995).

Neutrophil degranulation is under the tight control of small GTPases of the Ras superfamily that cycle between an inactive GDP-bound state and an active GTP-bound state. Rac2 has been shown to serve a selective role in neutrophil degranulation of primary granules and primary granule release of NE and MPO has previously been demonstrated to be absent in Rac2 knockout mice (Abdel-Latif et al, 2004). The signaling mechanisms underlying dysregulated neutrophil degranulation, particularly of primary granules, in AATD have not yet been established and this study aims to resolve this.
**Aims of this chapter**

In this chapter therefore we hypothesised that neutrophils of AATD individuals illustrate dysregulated degranulation processes leading to enhanced extracellular release of granule components.

To test this hypothesis, the following objectives were set:

1. To explore neutrophil degranulation activity patterns of AATD patients compared to healthy control cells
2. To determine whether AATD neutrophils exhibit increased NE membrane activity
3. To investigate if the molecular basis for the enhanced degranulation of primary granules in AATD involved increased Rac2 activation.
3.2 Impaired neutrophil degranulation in AATD

3.2.1 Impaired degranulation of neutrophil primary granules analysed by Western blot

To assess degranulation of primary granules, healthy control (HC) and AATD circulating neutrophils were isolated and then stimulated with PMA (1 µg/10⁷ cells) or TNF-α (1 ng/10⁷ cells)/fMLP (100 ng/10⁷ cells) at 37°C. Extracellular supernatants were analysed for degranulated proteins after 0 and 20 min. MPO, a marker of primary granule release, was detected by Western blot analysis employing a rabbit polyclonal anti-MPO antibody.

Figure 3.1 (A) displays a representative Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates post 20 min demonstrating equal protein loading indicative of equal cell numbers used per reaction. Figure 3.1 (B) displays a representative Western blot showing increased release of MPO from AATD primary granules at 20 min post PMA stimulation. Densitometry values of immunoblots of 6 separate experiments (Figure 3.1 (C), n=6 individuals per group) revealed increased degranulation of primary granules by AATD neutrophils compared with healthy controls with the difference being significant after 20 min stimulation (p= 0.04).

As PMA is a non-physiological stimulus, the ensuing experiment investigated degranulation in response to TNF-α/fMLP. Bergin et al. (2014) previously demonstrated increased membrane levels of TNF-α and increased plasma concentrations of TNFR-1 in AATD patients homozygous for the Z allele and therefore this is a relevant stimulus to use. In support of the use of fMLP, this bacterium-derived peptide is a potent neutrophil chemoattractant coordinating neutrophils into alveolar spaces during acute infective exacerbations (Gauthier et al, 2007). Figure 3.2 (B) displays a representative Western blot showing increased release of MPO from AATD primary granules evident at 5 min after TNF-α/fMLP stimulation.
Densitometry values of immunoblots of 6 separate experiments (Figure 3.2 (C), n=6 individuals per group) revealed a 369% increase in degranulation of primary granules of AATD neutrophils compared with healthy control cells with the difference being significant after 5 min stimulation (p= 0.023).

In conclusion, there is increased degranulation of primary granules by AATD neutrophils compared with healthy control neutrophils in response to both PMA and a combination of TNF-α/fMLP. This result is concerning for our AATD patients as MPO has a negative correlation with FEV₁ in patients with COPD (Park et al, 2013), suggesting that neutrophil MPO-mediated oxidative stress plays a role in the pathogenesis of airways obstruction in AATD-associated emphysema. Following on from this, our next set of experiments sought to determine neutrophil degranulation patterns of secondary granules in AATD.
Figure 3.1: MPO release determined by Western blot analysis is increased in AATD neutrophils in response to PMA. Healthy control (HC) and AATD circulating neutrophils were either unstimulated (time 0) or stimulated with PMA (1 µg/10^7 cells) at 37°C and extracellular supernatants were obtained at 0 and 20 min. (A) Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates post 20 min demonstrating equal protein loading indicative of equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for MPO using a rabbit polyclonal anti-MPO antibody (Novus Biological, 1:1000 dilution). Note supernatants were loaded on a contiguous gel. The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was carried out employing the GeneTools software® (HC t=0 min set as 1 and other time point data normalised to HC t=0 min). Statistical significance was calculated by student t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ± SEM, (n=6 individuals per group).
Figure 3.2: MPO release determined by Western blot analysis is increased in AATD neutrophils in response to TNF-α/fMLP. Healthy control (HC) and AATD circulating neutrophils were either unstimulated or stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C and extracellular supernatants were obtained at 0 and 5 min. (A) Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates post 5 min demonstrating equal protein loading indicative of equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for MPO using a rabbit polyclonal anti-MPO antibody (Novus Biological, 1:1000 dilution). Note supernatants were loaded on a contiguous gel. The molecular weight (kDa) is indicated on the left. (C) Immunoband quantification is expressed as densitometry arbitrary units. (HC t= 0 min set as 1 and remaining time point data is normalised to HC t=0 min). Statistical significance was calculated by student t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ± SEM, (n=6 individuals per group).
3.2.2 Impaired degranulation of neutrophil secondary granules analysed by Western blot

Bergin et al. (2004) reported increased neutrophil degranulation of secondary and tertiary granules in response to TNF-α alone. To expand on this, we chose to look at neutrophil degranulation of secondary granules in response to three additional stimuli. In this experiment, HC and AATD circulating neutrophils were isolated and then stimulated with PMA (1 µg/10^7 cells) or a combination of TNF-α (1 ng/10^7 cells)/ fMLP (100 ng/10^7 cells) at 37°C. Extracellular supernatants were analysed for degranulated proteins after 0, 5, 10 or 20 min.

hCAP-18, a marker of secondary granule release, was detected by Western blot analysis employing a rabbit polyclonal anti-hCAP-18 antibody. Of note, the protein detected on immunoblotting was 16 kDa and not the 8 kDa active form, LL-37. hCAP-18 is activated by PR3 and NE. However it is likely that the presence of protease inhibitors in the stop buffer prevented PR3- and NE-induced hCAP-18 activation from occurring.

A representative Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates indicates equal cell numbers used per reaction (Figure 3.3 (A)). Figure 3.3 (B) displays a representative Western blot showing increased release of hCAP-18 from AATD secondary granules particularly evident at 5 and 20 min after PMA stimulation.

Densitometry values of immunoblots of 3 separate experiments (Figure 3.3 (C), n=3) revealed increased degranulation of secondary granules by AATD neutrophils compared with HC, with the difference being significant after 5 min (p= 0.05) and 20 min stimulation (p= 0.03).

Ensuing experiments investigated degranulation of secondary granules in response to TNF-α/fMLP. Figure 3.4(B) displays a representative Western blot showing increased release of hCAP-18 from AATD neutrophils particularly evident at 10 min post TNF-α/fMLP stimulation.

Densitometry values of immunoblots of 3 separate experiments (Figure 3.4 (C), n=3) revealed a 290% increase in degranulation of secondary granules
by AATD neutrophils compared with healthy control cells at 10 min stimulation (p=0.038).
Collectively these experiments demonstrate that there is increased degranulation of secondary granules by AATD neutrophils compared to healthy control cells in response to both PMA and a combination of TNF-\(\alpha\)/fMLP. This aberrant process bodes poorly for AATD patients, as excessive levels of the hCAP-18 are known to inhibit neutrophil apoptosis and promote neutrophil-mediated inflammation in airway epithelial cells (Barlow et al, 2006).
Our third set of experiments built on these results and explored neutrophil degranulation of tertiary granules in AATD.
Figure 3.3: hCAP-18 release determined by Western blot analysis is increased in AATD neutrophils in response to PMA. Healthy control (HC) and AATD circulating neutrophils were either unstimulated or stimulated with PMA (1 µg/10^7 cells) at 37°C and extracellular supernatants were obtained after 0, 5, 10 and 20 min. (A) Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates demonstrating equal protein loading supporting equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for hCAP-18 using a rabbit polyclonal anti-hCAP-18 antibody (Innovagen, 1:2000 dilution). The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity (HC t= 0 min set as 1 and remaining time point data is normalised to HC t=0 min). Statistical significance was calculated by student t-test, n=3 individuals per group.
Figure 3.4: hCAP-18 release determined by Western blot analysis is increased in AATD neutrophils in response to TNF-α/fMLP. Healthy control (HC) and AATD circulating neutrophils were either unstimulated or stimulated with TNF-α (1 ng/10^7 cells)/ fMLP (100 ng/10^7 cells) at 37°C and extracellular supernatants were obtained after 0, 5 and 10 min. (A) Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates demonstrating equal protein loading supporting equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for hCAP-18 using a rabbit polyclonal anti-hCAP-18 antibody (Innovagen, 1:2000 dilution). The molecular weight (kDa) is indicated on the left. (C) Immunoband quantification is expressed as densitometry arbitrary units. (HC t= 0 min set as 1 and remaining time point data is normalised to HC t=0 min). Statistical significance was calculated by student t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ± SEM, (n=3 individuals per group).
3.2.3 Impaired degranulation of tertiary granules by AATD neutrophils analysed by Western blot

To assess degranulation of tertiary granules, healthy control and AATD circulating neutrophils were isolated and then either unstimulated or stimulated with PMA (1 µg/10⁷ cells) or TNF-α (1 ng/10⁷ cells)/fMLP (100 ng/10⁷ cells) at 37°C. Extracellular supernatants were analysed for degranulated proteins after 0, 5, 10 and 20 min. MMP-9, a marker of tertiary granule release, was detected by Western blot analysis employing a goat polyclonal anti-MMP-9 antibody.

A representative Coomassie blue-stained gel is seen in Figure 3.5 (A), while Figure 3.5 (B) displays a representative Western blot showing increased release of MMP-9 from AATD primary granules evident at 5 min after PMA stimulation. Densitometry values of immunoblots of 6 separate experiments (Figure 3.5 (C), n=6) revealed increased degranulation of tertiary granules by AATD neutrophils compared with healthy control cells with the difference being significant after 5 min stimulation (p= 0.028).

The next experiment assessed degranulation of tertiary granules in response to TNF-α/fMLP. Figure 3.6 (B) displays a representative Western blot showing increased release of MMP-9 from AATD tertiary granules particularly evident at 20 min after TNF-α/fMLP stimulation. Densitometry values of immunoblots of 6 separate experiments (Figure 3.6 (C), n=6) revealed a 293% increase in degranulation of tertiary granules by AATD neutrophils compared with healthy control cells with the difference being significant after 20 min stimulation (p= 0.042).

In conclusion, there is increased degranulation of tertiary granules by AATD neutrophils compared with healthy control neutrophils in response to both PMA and a combination of TNF-α/fMLP. This neutrophil degranulation pattern negatively impacts on AATD individuals, as increased plasma levels of MMP-9 have previously been shown to predict pulmonary status decline, including worsening FEV₁ and lung density as well as greater COPD exacerbations in AATD-associated emphysema (Omachi et al, 2011).
Figure 3.5: MMP-9 release determined by Western blot analysis is increased in AATD neutrophils in response to PMA. Neutrophils isolated from HC and AATD individuals were either unstimulated or stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C and cell-free supernatants were collected after 0 and 5 min. (A) Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates demonstrating equal protein loading indicative of equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for MMP-9 using a goat polyclonal anti-MMP-9 antibody (R&D Systems 1:1000 dilution). Note supernatants were loaded on a contiguous gel and blot has been divided to reflect a representative image. (C) Immunoband quantification is expressed as densitometry arbitrary units. Statistical significance was calculated by student t-test, (n=6 individuals per group).
Figure 3.6: MMP-9 release determined by Western blot analysis is increased in AATD neutrophils in response to TNF-α/fMLP. Neutrophils isolated from HC and AATD individuals were either unstimulated or stimulated with TNF-α (1 ng/10^7 cells)/ fMLP (100 ng/10^7 cells) at 37°C and cell-free supernatants were collected after 0, 5, 10 and 20 min. (A) Coomassie blue stained gel of unstimulated and TNF-α/fMLP-stimulated neutrophil whole cell lysates demonstrating equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for MMP-9 using a goat polyclonal anti-MMP-9 antibody (Innovagen, 1:1000 dilution). The molecular weight (kDa) is indicated on the left. (C) Immunoband quantification is expressed as densitometry arbitrary units. Statistical significance was calculated by student t-test, (n=6 individuals per group).
3.3 Excessive membrane-bound NE in AATD

3.3.1 Excessive membrane-bound NE in AATD neutrophils analysed by FRET

Thus far we have demonstrated excessive release of MPO, as our marker of primary granules, by AATD neutrophils. As an alternative method of exploring degranulation patterns of primary granules, we sought to measure membrane-bound NE. As NE is a highly positively charged serine protease (Navia et al, 1989; Pham et al, 2006), it has the ability to bind to the negatively charged neutrophil membrane at physiological pH.

To determine membrane-bound NE, healthy control and AATD neutrophils were isolated and either unstimulated or stimulated with TNF-α (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) for 0, 5, 10 and 20 min, n=3 individuals per group. Extracellular supernatant obtained at each time-point was removed after centrifugation and the neutrophil pellet was re-suspended in DPBS and loaded onto a 96-well plate. Fluorescence substrate for human NE (Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp, 3230-v, Peptide Institute, Inc) was added to each well immediately prior to plate reading at 320-420 nm set at 28°C for 1 h. The standard curve was derived from NE standards of increasing concentrations (0 nM, 5 nM, 10 nM, 20 nM, 30 nM, 40 nM, 50 nM and 60 nM) and the slope of the graph was used to extrapolate the unknown concentrations. Membrane-bound NE activity was detected by FRET analysis as previously described (Korkmaz et al, 2008). Figure 3.7 displays a graph of membrane-bound NE activity measured by FRET after 0, 5, 10 and 20 min stimulation with TNF-α/fMLP revealing significantly increased membrane-bound NE on AATD neutrophils compared with healthy control cells with the difference being significant across all stimulation time-points (p=0.001, p=0.043 and p=0.034, respectively). In addition, membrane-bound NE on AATD neutrophils increased by 131% following 20 min stimulation with TNF-α/fMLP (p=0.029).
In conclusion, there is increased membrane-bound NE in AATD neutrophils compared with healthy control neutrophils in response to TNF-α/fMLP. This is of particular concern for AATD individuals as excess membrane-bound NE facilitates neutrophil egress from the vasculature promoting neutrophil-driven lung destruction and development of premature emphysema (Owen et al., 1995).

The signaling mechanism underlying dysregulated neutrophil degranulation of primary granules has not been elucidated to date and the next experiment was designed to explore this.
Figure 3.7: Membrane-bound NE activity determined by FRET is increased in AATD neutrophils in response to TNF-α/FMLP. Healthy control (HC) and AATD circulating neutrophils were stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C. Extracellular supernatants obtained after 0, 5, 10 and 20 min were removed after centrifugation and the neutrophil pellet was re-suspended in DPBS. Quantification of NE activity was employed using the plate reader (SpectraMax® M3 multi-mode microplate reader, Molecular Devices) at a wavelength of 320-420 nm and at 28°C. Statistical significance was calculated by student t-test, where a p value ≤ 0.05 was deemed statistically significant and shown as mean ± SEM, (n=3 individuals per group).
3.4 Excessive Rac2 activity in AATD

3.4.1 Excessive Rac2 activity of AATD neutrophils analysed by Western blot

Thus far, we have identified excessive release of MPO as well as increased membrane-bound NE activity in AATD neutrophils. However, to date, the molecular basis for this enhanced degranulation of neutrophil primary granules has not been explored. To assess Rac2 activity of neutrophils following activation, healthy control and AATD circulating neutrophils were isolated and then either unstimulated (0 min) or stimulated with TNF-α (1 ng/10^7 cells) and fMLP (100 ng/10^7 cells) for 2.5 min, n=4 individuals per group. Neutrophil lysates were obtained for each time-point. The Abcam Rac activation kit (catalogue # ab139586) was used to pull down active GTP-bound Rac. This kit exploits the selective interaction of PAK-CRIB with the active Rac-GTP conformation.

Total and active GTP-bound Rac2 content in neutrophil lysates was then detected by Western blot analysis using an anti-Rac2 antibody (Cell Signalling Technology Rac 1/2/3 antibody, 1:1000). Heyworth et al. (1994) previously identified that neutrophils primarily express Rac2 rather than Rac1, while Rac3 is not expressed by neutrophils (proteinatlas.org).

Figure 3.8 (A) displays a representative Western blot showing equal total Rac2 in HC and AATD neutrophil lysates. Figure 3.8 (B) demonstrates increased active Rac2 in HC and AATD neutrophils following stimulation with TNF-α/fMLP.

Densitometry values of immunoblots of 4 separate experiments (Figure 3.8 (C), n=4) revealed a 239% increase in active Rac2 content in AATD neutrophil lysates compared with HC neutrophil lysates after 2.5 min stimulation (p=0.016). Of note, Benard et al. (1999) previously demonstrated peaks in Rac2 activity between 30 sec and 5 min following stimulation.
In conclusion, there is increased active Rac2 content in AATD neutrophils compared with HC cells in response to a combination of TNF-α/fMLP. This new finding is of particular interest in understanding the pathogenesis of AATD-associated emphysema, as active Rac2 is also known to interact with nitric oxide synthase 2A stimulating NO production (Kone, 2000) known to be increased in the exhaled breath of patients with inflammatory airways disease (Hatzigorou et al, 2007), as well as stimulating activity of NADPH oxidase subunits (Kim et al, 2001) that can mediate ROS generation in neutrophils (Nathan, 2006).
Figure 3.8: Active Rac 2 as a percentage of total Rac2 determined by Western blot analysis is increased in AATD neutrophils in response to TNF-α/fMLP. Healthy control (HC) and AATD circulating neutrophils were either unstimulated (control) or stimulated (stim) with TNF-α (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) at 37°C for 2.5 min and neutrophil lysates were obtained. (A) and (B) Representative Western blots of neutrophil lysates probed for anti-Rac2 antibody (Cell Signalling Technology, 1:1000 dilution) displaying total Rac (A) and active GTP-bound Rac (B). The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was carried out using the ImageLab software® (Densitometry values for active Rac2 were divided by total Rac2 for each time-point. HC t= baseline set as 1 and other time-course data is normalised to HC t=0). Statistical significance was calculated by student t-test, (n=4 individuals per group).
3.5 Discussion

The aims of chapter 3 were to explore neutrophil degranulation patterns and membrane activity of NE in AATD and also to determine the signaling mechanisms involved in aberrant degranulation of primary granules in AATD.

Neutrophil degranulation plays an important role in innate defense by releasing the antimicrobial contents of its primary, secondary and tertiary granules (Faurshou et al, 2003). However, excessive release of neutrophil granule proteins can damage a broad range of extracellular matrix proteins including elastin (Shapiro et al, 1991), collagen (Borregaard et al, 1997), fibronectin (Vissers et al, 1991) and laminin (Heck et al, 1990). This process is seen in AATD, which is characterised by a protease: anti-protease imbalance, leading to alveolar matrix damage of the lung resulting in premature emphysema (Guenter et al, 1981).

In this chapter, we have demonstrated that AATD neutrophils release enhanced levels of MPO, hCAP-18 and MMP-9, markers of primary, secondary and tertiary granule components, respectively, when compared with healthy control neutrophils following stimulation with PMA and a combination of the physiological stimuli TNF-α/fMLP (p< 0.05). These results are supported by previous work by Bergin et al. (2014) who identified increased plasma concentrations of neutrophil-released secondary and tertiary granule proteins from AATD neutrophils in response to TNF-α alone.

Excessive MPO levels have previously been identified in the BALF of patients with ARDS (Weiland et al, 1986) and are known to have a negative correlation with FEV₁ (Park et al, 2013). In addition, excessive MPO activity is associated with high levels of oxidative stress (Shindhelm et al, 2009).

The antimicrobial polypeptide LL-37 released from neutrophil secondary granules in its inactive pro-form hCAP-18 can promote inflammatory responses by activation of neutrophils (Kai-Larsen et al, 2007). LL-37 also inhibits neutrophil apoptosis thereby contributing to enhanced neutrophil accumulation at the site of tissue injury (Barlow et al, 2006).
MMP-9 released from neutrophil tertiary granules is known to activate pro-inflammatory cytokines such as TNF-α. Elevated levels of MMP-9 have been demonstrated in both BALF and plasma of patients with ARDS which correlated with clinical severity (Fligiel et al, 2006).

The next experiment used FRET analysis to confirm that there was increased membrane-bound NE in AATD neutrophils compared with healthy control neutrophils in response to TNF-α/fMLP. This finding is of particular concern for AATD individuals as membrane-bound NE has been shown to be strikingly resistant to inhibition by anti-proteases such as AAT due to steric hindrance (Owen et al, 1995).

In addition, NE has been shown to up-regulate expression of the potent chemokine, IL-8, via TLR-4 in bronchial epithelium of CF airways (Devaney et al, 2003). Hubbard et al. (1991) demonstrated that NE could induce secretion of the neutrophil chemoattractant LTB4 in airway epithelial cells and macrophages in AATD. Excess NE has also been shown to cleave and inactivate the C5a receptor in BALF of CF patients, contributing to suboptimal local neutrophil priming and bacterial clearance (van den Berg et al, 2014).

Given our findings of enhanced MPO release and increased membrane-bound NE in AATD neutrophils, the final experiment in this chapter sought to explore if excessive Rac2 activity could be the underlying signaling mechanism for dysregulated degranulation of primary granules in AATD. Rac2 is known to be critical for neutrophil primary granule exocytosis (Abdel-Latif et al, 2004). While very little is currently known about Rac2 and inflammatory airways disease, haplotypes of Rac2 have been shown to be associated with increased susceptibility to multiple sclerosis (MS) and Crohn’s disease, supporting a causal role for Rac2 variants in the pathogenesis of autoimmune disease (Sironi et al, 2011). Furthermore, sustained activation of Rac2 has previously been associated with increased concentration of NADPH-oxidase-mediated ROS in lipase-deficient murine macrophages (Aflaki et al, 2011), raising the possibility of increased NADPH-oxidase-driven O2− release by AATD neutrophils with excessive active Rac2 content.
For the final experiment, a recombinant GST-tagged PAK-CRIB was used to pull down active Rac-GTP, and total and active Rac2 content was detected by Western blot analysis. This confirmed that there was a 239% increase in active Rac2 content in AATD neutrophils compared with HC neutrophils in response to TNF-α/fMLP (p=0.016).

In summary, the experiments performed in this chapter confirm that there is dysregulated degranulation in neutrophil primary, secondary and tertiary granules in AATD. Additionally, NE has been shown to re-bind to the membrane of AATD neutrophils and, importantly, excessive Rac2 activity has been identified as a possible molecular basis for the enhanced degranulation of primary granules in AATD. These aberrant processes may cause extravagant release of proteolytic enzymes and up-regulation of pro-inflammatory mediators leading to the sustained alveolar matrix damage and premature emphysema seen in AATD. Zhao et al. (2015) has demonstrated that NE cleaves PAR2 on nociceptor neurons causing direct release of reactive oxygen species (ROS) and the possibility of activation of PAR2 by NE is explored later in chapter 5.
Chapter 4

The effect of AAT on neutrophil degranulation
4.1 Introduction

In chapter 3, we confirmed that AATD neutrophils exhibited dysregulated degranulation processes of neutrophil primary, secondary and tertiary granules, in addition to re-binding of NE to the neutrophil membrane and excessive Rac2 activation, all of which may contribute to sustained alveolar matrix damage in the lung.

Treatment of AATD includes smoking cessation (O’Brien et al, 2015), long-acting beta agonists and anti-cholinergic inhalers (Campos et al, 2009), inhaled and systemic corticosteroids (Corda et al, 2008) and pulmonary rehabilitation (Sahin et al, 2016). Selected patients with ZZ-AATD and moderate to severe airflow obstruction on spirometry may be considered for AAT augmentation therapy. This consists of intravenous infusion of plasma purified human AAT at a dose of 60 mg/kg/week with the aim of raising serum AAT levels above the protective threshold ≥ 11 µmol/L in both plasma and respiratory ELF (Juvelekian et al, 2004). AAT augmentation therapy is regarded as a safe and well-tolerated treatment with Stoller et al. (2003) reporting a very low absolute frequency of adverse events following AAT augmentation therapy (0.019 to 0.023 events per patient-month, 95% confidence interval) without transmission of viral hepatitis, HIV infection or prion disease. A meta-analysis conducted by Chapman et al. (2009) reported a 23% slower rate of FEV₁ decline among all patients receiving AAT augmentation therapy across five clinical trials, while augmentation therapy has also been shown to reduce hospitalisation costs by decreasing the incidence and severity of pulmonary exacerbations (Barros-Tizón et al, 2012). Last year, the RAPID study was the first randomised placebo-controlled trial of AAT augmentation therapy to confirm a significantly lower annual rate of lung density loss on CT in those patients receiving AAT augmentation therapy (Chapman et al, 2015).

Despite the observed clinical effects of AAT therapy further evidence of the biochemical effectiveness of augmentation therapy is required (Russi et al, 2008). Moreover, the mechanism by which AAT augmentation therapy
modulates the immune system of individuals with lung disease is poorly understood. An important question is whether establishment of protective threshold serum levels of AAT in patients receiving AAT augmentation therapy influences circulating neutrophils known to be essential in the development of AATD-associated lung disease.

Intravenous AAT augmentation therapy has been shown to decrease neutrophil membrane expression of TNF-α (Bergin et al., 2014), as well as preventing neutrophil chemotaxis induced by IL-8, by direct binding to IL-8 preventing its interaction with its own receptor, CXCR1 (Bergin et al., 2010). Hurley et al. (2014) demonstrated that AAT augmentation therapy decreased ADAM-17/TACE activity in AATD individuals. AAT augmentation therapy has also been shown to reduce the plasma levels of IgG-class autoantibodies (Bergin et al., 2014).

Bergin et al. (2014) previously demonstrated that exogenous AAT at physiological concentrations of 27.5 µM significantly reduced degranulation of neutrophil secondary and tertiary granules of MM neutrophils following stimulation with TNF-α. However, the effect of exogenous AAT on neutrophil primary granules in HC or AATD individuals has not been explored to date.

As Rac2 is critical for neutrophil primary granule exocytosis (Abdel-Latif et al., 2004), our finding of excessive active Rac2 content in AATD neutrophils supports our hypothesis that Rac2 is the underlying signaling mechanism for aberrant neutrophil primary granule release in AATD. In this chapter the effect of AAT augmentation therapy on Rac2 activity is elucidated for the first time.

**Aims of this chapter**

In this chapter we hypothesised that AAT normalises aberrant neutrophil degranulation patterns and excessive Rac2 activity in AATD.
To test this hypothesis, the following objectives were set:

1. To determine whether exogenous AAT modulates primary granule release in HC and AATD neutrophils *in-vitro*
2. To investigate if AAT augmentation normalises aberrant degranulation of neutrophil primary, secondary and tertiary granules in AATD *ex-vivo*
3. To explore the effect of AAT augmentation therapy on excessive Rac2 activity in AATD *ex-vivo*
4.2 The effect of AAT on neutrophil degranulation in AATD

4.2.1 The effect of exogenous AAT on MPO release from neutrophil primary granules of HC and AATD cells

To assess the effect of exogenous AAT on neutrophil primary granule release, healthy control neutrophils were isolated and stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C in the presence or absence of increasing concentrations of AAT; 2 µM, 6.875 µM, 13.75 µM and 27.5 µM. Extracellular supernatants were analysed for degranulated proteins at 5 min. MPO, a marker of primary granule release, was detected by Western blot analysis employing a rabbit polyclonal anti-MPO Ab.

Figure 4.1 (A) displays a representative Western blot showing decreased release of MPO from HC primary granules as the AAT concentration increased. Densitometry values of immunoblots (Figure 4.1 (B), n=3) revealed decreased degranulation of primary granules by HC neutrophils following stimulation with TNF-α/fMLP as the AAT concentration increases to 13.75 µM and 27.5 µM (71.3% reduction, p=0.021 and 80.8% reduction, p=0.002, respectively).

The next experiment investigated degranulation of primary granules of HC neutrophils compared with AATD neutrophils following stimulation with TNF-α/fMLP for 5 min in the presence of AAT, used at two different concentrations; 2 µM and 27.5 µM. Figure 4.2 (A) displays a representative Western blot showing decreased release of MPO from primary granules of both HC and AATD neutrophils as the AAT concentration increased. Densitometry values of immunoblots (Figure 4.2 (B), n=3) revealed an 88.1% decrease in degranulation of primary granules by AATD neutrophils following stimulation with TNF-α/fMLP at an AAT concentration of 27.5 µM (p=0.04).

Moreover, degranulation of primary granules by AATD neutrophils was normalised to that of HC levels at an AAT concentration of 27.5 µM (p=0.34).
In conclusion, physiological levels of exogenous AAT significantly reduces excessive degranulation of primary granules by AATD neutrophils, in addition to normalising degranulation to HC levels following stimulation with TNF-α/fMLP in-vitro. This is a very encouraging result for AATD individuals, as it highlights the ability of exogenous AAT to correct aberrant degranulation patterns of primary granules in AATD which may in turn reduce excessive MPO-driven oxidative stress and DNA damage seen in lung epithelial cells (Haegens et al, 2008). Therefore, the aim of our next set of experiments was to investigate if AAT augmentation normalises aberrant degranulation of neutrophil primary, secondary and tertiary granules in AATD ex-vivo.
Figure 4.1: MPO release determined by Western blot analysis is decreased in HC neutrophils in response to TNF-α/fMLP as the AAT concentration increases. Purified healthy control (HC) circulating neutrophils were stimulated with TNF-α (1 ng/10^7 cells) and fMLP (100 ng/10^7 cells) at 37°C in the presence or absence of increasing concentrations of AAT and supernatants were obtained after 5 min. (A) Representative Western blot of supernatants probed for MPO using a rabbit polyclonal anti-MPO Ab (Novus Biological, 1:1000 dilution). The molecular weight (kDa) is indicated on the left. (B) Quantification of immunoband intensity was carried out employing the GeneTools software® (No AAT control set as 1 and other AAT concentration data was normalised to this value). Statistical significance was calculated by student t-test, (n=3 individuals per group).
Figure 4.2: MPO release determined by Western blot analysis is decreased in HC and AATD neutrophils in response to TNF-α/fMLP as the AAT concentration increases. Isolated healthy control (HC) and AATD circulating neutrophils were stimulated with TNF-α (1 ng/10^7 cells) and fMLP (100 ng/10^7 cells) at 37°C in the presence or absence of increasing concentrations of AAT and supernatants were obtained after 5 min. (A) Representative Western blot of supernatants probed for MPO using a rabbit polyclonal anti-MPO Ab (Novus Biological, 1:1000 dilution). The molecular weight (kDa) is indicated on the left. (B) Quantification of immunoband intensity (No AAT control set as 1 and other data normalised to this value), n=3 individuals per group. Of note, there was no difference between MPO release by AATD neutrophils when exposed to a concentration of 2 µM exogenous AAT vs no exogenous AAT (p=0.08).
4.2.2 The ex-vivo effect of AAT augmentation therapy on neutrophil degranulation in AATD patients

Thus far we have demonstrated that neutrophils of AATD patients degranulate increased levels of primary, secondary and tertiary granule proteins compared with healthy controls. This excessive neutrophil degranulation in AATD is known to perpetuate airway inflammation and cause premature emphysema in the AAT lung (Hoenderdos et al, 2013). Current specialised treatment consists of weekly intravenous infusion of purified human plasma AAT known as augmentation therapy. Numerous studies have investigated the effect of augmentation therapy on patient outcomes and to date illustrate a trend towards a slower rate of FEV₁ decline (Vreim et al, 1998, Chapman et al, 2009), a reduction in the incidence of respiratory infections (Lieberman et al, 2000) and a lower risk ratio for death (p< 0.001) (Russi et al, 2008). Despite these observed effects, further evidence of the efficacy of this treatment is required. We sought to explore ex-vivo neutrophil degranulation activity patterns of AATD patients pre and post AAT augmentation therapy.

In order to understand the effect of AAT augmentation therapy on neutrophil degranulation, neutrophils were isolated from AATD patients pre-AAT augmentation therapy (day 0/morning before AAT infusion) and post-AAT augmentation therapy (day 2 post-infusion, paired samples n=5). Isolated neutrophils were stimulated with TNF-α (1 ng/10⁷ cells)/ fMLP (100 ng/10⁷ cells) at 37°C and extracellular supernatants were obtained after 10 min stimulation. MPO, a marker of primary granule release, hCAP-18, a marker of secondary granule release and MMP-9, a marker of tertiary granule release were detected by Western blot analysis as already described. AAT augmentation therapy was found to decrease the levels of neutrophil degranulation of primary (Figure 4.3), secondary (Figure 4.4) and tertiary granules (Figure 4.5) (98% reduction, p= 0.003, 91% reduction, p= 0.048 and 89.7% reduction, p= 0.06, respectively). Moreover, there was no significant difference between primary, secondary and tertiary granule release from AATD neutrophils post-infusion of AAT compared to healthy control cells,
demonstrating that neutrophil granule release from AATD neutrophils normalised to healthy control levels just two days following augmentation therapy. Collectively, these results illustrate the ability of AAT augmentation therapy to dramatically reduce aberrant neutrophil degranulation of AATD patients to healthy control levels ex-vivo.
Figure 4.3: MPO release from primary granules determined by Western blot analysis is significantly reduced in AATD neutrophils post-augmentation therapy. Isolated healthy control (HC) and AATD circulating neutrophils were stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C on day 0 and day 2 post augmentation therapy. Extracellular supernatants were obtained after 10 min on both days. (A) Coomassie blue stained gel of TNF-α/fMLP stimulated neutrophil whole cell lysates demonstrating equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for MPO using a rabbit polyclonal anti-MPO antibody (Novus Biological, 1:1000 dilution). The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was carried out employing the GeneTools® software. Statistical significance was calculated by student t-test (n=5 individuals per group).
Figure 4.4: AAT augmentation therapy significantly reduces hCAP-18 release from secondary granules determined by Western blot analysis. Neutrophils isolated from HC and AATD individuals on day 0 and day 2 augmentation therapy were stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C and cell-free supernatants were collected after 10 min on both days. (A) Coomassie blue stained gel of TNF-α/fMLP stimulated neutrophil whole cell lysates demonstrating equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for hCAP-18 using a rabbit polyclonal anti-hCAP-18 antibody (Innovagen, 1:2000 dilution). The molecular weight (kDa) is indicated on the left. (C) Immunoband quantification is expressed as densitometry arbitrary units, n=5 individuals per group.
Figure 4.5: MMP-9 release from tertiary granules determined by Western blot analysis is reduced in AATD neutrophils post-augmentation therapy. Isolated healthy control (HC) and AATD circulating neutrophils were stimulated with TNF-α (1 ng/10^7 cells)/fMLP (100 ng/10^7 cells) at 37°C on day 0 and day 2 augmentation therapy. Extracellular supernatants were obtained after 10 min on both days. (A) Coomassie blue stained gel of TNF-α/fMLP stimulated neutrophil whole cell lysates demonstrating equal cell numbers used per reaction. (B) Representative Western blot of supernatants probed for MMP-9 using a goat polyclonal anti-MMP-9 antibody (R&D Systems, 1:1000 dilution). The molecular weight (kDa) is indicated on the left. (C) Immunoband quantification is expressed as arbitrary densitometry units, n=5 individuals per group.
4.2.3 The ex-vivo effect of AAT augmentation therapy on excessive Rac2 activity in AATD patients

To assess the effect of AAT augmentation on excessive Rac2 activity, circulating neutrophils were isolated from AATD patients pre-AAT augmentation therapy (Day 0) and post-AAT augmentation therapy (Day 2). Neutrophils (1 x 10^7) were stimulated with TNF-α (1 ng/10^7 cells) and fMLP (100 ng/10^7 cells) at 37°C for 1.25 min and neutrophil lysates obtained, n=3 individuals per group. Total and active GTP-bound Rac2 content in neutrophil lysates was then detected by Western blot analysis using an anti-Rac2 antibody (Cell Signalling Technology Rac 1/2/3 antibody, 1:1000). Figure 4.6 (A) displays a representative Western blot showing equal total Rac2 in AATD neutrophils pre- and post-AAT augmentation therapy, while Figure 4.6 (B) highlights a representative Western blot showing decreased active GTP-bound Rac2 in neutrophil lysates two days post-AAT augmentation therapy. Densitometry values of immunoblots of 3 separate experiments (Figure 4.6 (C), n=3) revealed a 75% decrease in active Rac2 content by AATD neutrophil lysates day 2 post-AAT augmentation therapy compared with neutrophil lysates pre-AAT augmentation therapy after 1.25 min stimulation with TNF-α/fMLP (p=0.03).

In conclusion, AAT augmentation therapy significantly reduces excess Rac2 activity in AATD neutrophils in response to a combination of TNF-α/fMLP.
Figure 4.6: Active Rac2 as a percentage of total Rac2 determined by Western blot analysis is decreased in AATD neutrophils in response to TNF-α/fMLP following AAT augmentation therapy. AATD circulating neutrophils were stimulated with TNF-α (1 ng/10⁷ cells) and fMLP (100 ng/10⁷ cells) at 37°C for 1.25 min and neutrophil lysates were obtained. (A) and (B) Representative Western blots of neutrophil lysates probed for anti-Rac2 antibody (Cell Signalling Technology, 1:1000 dilution) displaying total Rac (A) and active GTP-bound Rac (B). The molecular weight (kDa) is indicated on the left. (C) Quantification of immunoband intensity was carried out using the ImageLab software®. Densitometry values for active Rac2 were divided by total Rac2 for each time-point. Statistical significance was calculated by student t-test and n=3 individuals per group.
4.3 Discussion

The aim of chapter 4 was to explore whether AAT normalised aberrant neutrophil degranulation patterns in AATD *in-vitro and ex-vivo* and to determine if AAT augmentation therapy reduced excessive Rac2 activity in AATD neutrophils.

As discussed, specialised treatment for AATD is available in the form of weekly intravenous infusions of plasma purified human AAT referred to as augmentation therapy. AAT augmentation therapy restores the concentration of AAT both in the blood and in bronchoalveolar lavage fluid delaying the progression of AATD-associated lung disease (Wewers et al., 1987).

To determine the effect of exogenous AAT on neutrophil primary granule release, HC and AATD neutrophils were stimulated with TNF-α/fMLP in the presence and absence of AAT at concentrations of 2 µM and 27.5 µM and Western blot was used to detect MPO in the extracellular supernatants. This experiment confirmed that physiological levels of exogenous AAT (27.5 µM) significantly reduced excessive degranulation of primary granules by AATD neutrophils by 88.1% (p=0.04), in addition to normalising degranulation to HC levels following stimulation with TNF-α/fMLP *in-vitro*.

This result echoes previous work by Bergin et al. (2014) who demonstrated that exogenous AAT (27.5 µM) significantly reduced degranulation of neutrophil secondary and tertiary granules of MM neutrophils following stimulation with TNF-α alone. In addition, AAT has been shown to decrease plasma LTB₄ concentrations and reduce levels of membrane-bound NE in AATD neutrophils (O'Dwyer et al., 2015).

In this chapter we have further illustrated that degranulation activity of primary, secondary and tertiary granules in AATD neutrophils following stimulation with TNF-α/fMLP is reduced two days following AAT augmentation therapy (98% reduction, p= 0.003, 91% reduction, p= 0.048 and 89.7% reduction, p= 0.06, respectively). Moreover, there was no significant difference between primary, secondary and tertiary granule release from AATD neutrophils post-infusion of AAT, demonstrating that
neutrophil granule release from AATD neutrophils was normalised to healthy control levels just two days following augmentation therapy.

This result is complimented by previous work by Bergin et al. (2014) who identified that AAT augmentation therapy decreased neutrophil membrane expression of TNF-α. AATD patients homozygous for the Z allele were characterised by increased activation of the TNF-α system as demonstrated by increased membrane TNF-α levels and increased plasma concentrations of TNFR1 and neutrophil-released secondary and tertiary granule proteins. Treatment of AATD patients with AAT augmentation therapy resulted in decreased membrane TNF-α expression. His study demonstrated that serum AAT coordinates TNF-α intracellular signaling and neutrophil degranulation of secondary and tertiary granules via modulation of ligand-receptor interactions. These results therefore provide a potential mechanism by which AAT augmentation therapy reduces excessive neutrophil degranulation in our study by AAT binding to TNFR1 thereby preventing TNF-α engagement with its receptor. AAT has also been shown to inhibit the interaction of fMLP with its receptors, formyl peptide receptor 1 (FPR1) and FPR2, on the neutrophil membrane (Alfawaz et al, 2013), thereby reducing fMLP-induced neutrophil migration in vitro (Stockley et al, 1990).

In addition to the ability of AAT augmentation therapy to decrease the activity of the TACE/ADAM-17 and correct accelerated neutrophil apoptosis in AATD (Hurley et al, 2014), our confirmatory results of the normalisation of aberrant neutrophil degranulation of primary, secondary and tertiary granules lends further support to the use of AAT augmentation therapy for AATD.

In 2004, Abdel-Latif et al. confirmed that Rac2 is critical for neutrophil primary granule exocytosis. In chapter 3 we postulated that the molecular basis for the enhanced degranulation of primary granules in AATD was increased Rac2 activation and indeed excessive Rac2 activity was detected in AATD neutrophils. In this chapter we have confirmed that just two days following AAT augmentation therapy when plasma AAT levels are high, Rac2 activity in AATD neutrophils was significantly reduced by 75% following stimulation with TNF-α/fMLP (p=0.03). This mirrors the normalisation of primary granule MPO release post-AAT augmentation therapy. While little is
known about the role of Rac2 in chronic airways disease, previous studies have linked Rac2 with autoimmune demyelinating disease (Sironi et al, 2011). Bergin et al. (2014) previously demonstrated that AAT augmentation therapy reduced plasma levels of IgG class autoantibodies in AATD individuals. Our findings of the normalising effect of AAT augmentation therapy on Rac2 activity therefore provide further support for the use of augmentation therapy not only for AATD, but also as a potential therapeutic avenue for other autoimmune diseases.

In summary, the experiments performed in this chapter confirm that AAT not only reduces excessive degranulation by AATD neutrophils post-AAT augmentation therapy, but also normalises excessive neutrophil degranulation patterns in AATD in-vitro and ex-vivo. These findings support our hypothesis of the ability of AAT to restore protease: anti-protease harmony by inhibiting the excessive release of these neutrophil proteases as well as enhancing our understanding of the mechanism by which augmentation therapy reduces lung tissue damage.
Chapter 5

Consequence of excessive primary granule release by AATD neutrophils
5.1 Introduction

ANCA are autoantibodies implicated in the pathogenesis of autoimmune necrotising vasculitis affecting small vessels seen in GPA (van der Woude et al., 1985) and microscopic polyangiitis (Savage et al., 1987). The main antigenic targets for ANCA are MPO (Falk et al., 1988), PR3 (Lüdemann et al., 1990) and more infrequently, lactoferrin (Esnault et al., 1994) and NE (Tervaert et al., 1993).

A few studies have implied that ANCA are associated with heterozygosity of the Z allele in AATD (Audrain et al., 2001, Segelmark et al., 1995, Morris et al., 2011), while a small number of case reports have described systemic necrotising vasculitis in patients with ZZ-AATD (Fortin et al., 1991, Fregonese et al., 2008).

In 2014, Bergin et al. demonstrated increased membrane surface expression of the secondary granule protein, lactoferrin, on AATD neutrophils compared with HC cells (p<0.0001), as well as a higher incidence of anti-lactoferrin IgG autoantibodies in the plasma of AATD individuals (p=0.001). In chapter 3, we illustrated increased membrane-bound NE on AATD neutrophils compared with HC cells (p<0.05). To date, the presence of autoantibodies directed against the contents of neutrophil primary granules in AATD compared with AATD individuals receiving AAT augmentation therapy has not been fully explored and will be the initial focus of this chapter.

Furthermore, increased release of O$_2^-$ by AATD neutrophils exposed to lactoferrin antibodies for 30 min in the presence of TNF-α (10 ng) compared with HC cells (p=0.004) was detected, thereby concluding that anti-lactoferrin autoantibodies led to an enhanced rate of neutrophil ROS production in AATD (Bergin et al., 2004). Significant interest is growing in the role of oxidative stress as a critical driver of chronic airways disease (Repine et al., 1997, MacNee et al., 2001, Holguin et al., 2013). Rahman et al. (2002) previously demonstrated elevated levels of systemic markers of oxidative stress, such as 4-hydroxy-2-nonenal, in neutrophils and airway epithelial cells of patients with COPD, while Dekhuijzen et al. (1996) detected
increased concentrations of the oxidant $H_2O_2$ in exhaled breath condensate of COPD patients with intercurrent exacerbations.

In chapter 3, we illustrated increased release of MPO from primary granules, increased membrane-bound NE and increased Rac 2 activity in AATD neutrophils compared with HC cells. Excess levels of MPO may play a significant role in oxidative stress by metabolising $H_2O_2$ to the powerful oxidant HOCl in the presence of Cl$^-$ ions (Harrison et al., 1976), while Rac2 is an essential component of one of the principal cellular sources of ROS, the NADPH oxidase complex (Dorseuil et al., 1996). In addition, it has been demonstrated that NE cleaves the G-protein coupled receptor, PAR2, on nociceptor neurons causing direct release of ROS (Zhao et al., 2015). Furthermore, Taggart et al. (2000) demonstrated that oxidation of methionine residues in AAT resulted in loss of its anti-NE activity, thereby expediting elastin breakdown in the lung parenchyma of AATD individuals. Therefore, our second set of experiments will concentrate on ROS production by the neutrophil in response to NE as well as the role of PAR2 in $O_2^-$ release.

Importantly, in chapter 4, we demonstrated the ability of AAT augmentation therapy to normalise aberrant neutrophil degranulation patterns and reduce excessive Rac2 activity by AATD neutrophils. While increased release of ROS by AATD neutrophils exposed to lactoferrin antibodies has previously been described, a significant decrease in anti-lactoferrin antibody titres following long-term AAT augmentation therapy ($p=0.04$) has also been detected (Bergin et al., 2014). Our final set of experiments will therefore explore the ability of anti-NE agents to reduce ROS production by the neutrophil following NE stimulation.

**Aims of this chapter**

In this chapter we took a dual approach to understanding the consequence of enhanced primary granule release by AATD neutrophils. Firstly, we hypothesised that AATD neutrophils exhibit enhanced levels of anti-NE, anti-
MPO and anti-PR3 autoantibodies, and secondly, that NE drives excessive $\mathrm{O}_2^-$ release via PAR2 activation on neutrophils that can be corrected by anti-protease treatments.

To test this hypothesis, the following objectives were set:

1. To establish if there are increased autoantibodies directed against the contents of neutrophil primary granules in AATD
2. To determine if NE induces ROS production by the neutrophil
3. To identify if NE acts via PAR2 on the neutrophil membrane to trigger release of ROS
4. To explore the effect of exogenous AAT on ROS production by the neutrophil
5.2 Investigation of ANCA in AATD

5.2.1 Assessment of ANCA directed against neutrophil primary granule proteins in AATD

Thus far, we have identified increased degranulation of neutrophil primary granules in AATD compared with HC, as well as confirming excessive membrane-bound NE in AATD neutrophils. Elevated anti-lactoferrin IgG autoantibodies have previously been described in AATD individuals (p=0.001), in addition to a reduction in anti-lactoferrin autoantibody titres post long-term AAT augmentation therapy (p=0.04) (Bergin et al, 2014). To assess the presence of autoantibodies directed against NE, MPO and PR3, plasma samples were obtained from non-smoking HC (MM) individuals (n=40), AATD individuals (n=36) and AATD individuals on AAT augmentation therapy (AAT Tx) (n=10). An ELISA-based method for evaluation of autoantibodies was carried out by the Department of Immunology, Beaumont Hospital, Ireland. Anti-NE autoantibody quantification was performed on plasma samples using the anti-elastase Alegria® ELISA-based assay (ORG 224 ORGENTEC Diagnostika GmbH Mainz, Germany) with anti-NE autoantibody pre-defined positivity set by the manufacturer at 10 IU/mL, as described in section 2.9.2. Assessment of anti-MPO and anti-PR3 autoantibodies was performed as per the ‘Routine use of the Phadia® 250 for anti-MPO and anti-PR3 antibody testing’ protocol set out by the Clinical Directorate of Laboratory Medicine, Beaumont Hospital, Ireland (document no. LP-IMM-ICAP0004, revision 3.6) as previously detailed in section 2.9.1. Positivity was set by the manufacturer as 3 SDs above the mean of healthy control individuals and was defined as > 5 IU/mL and > 3 IU/mL for anti-MPO and anti-PR3 autoantibodies, respectively.

Figure 5.1 displays the graphical representation of anti-NE autoantibodies detected in MM, AATD and AATD individuals receiving AAT augmentation therapy, revealing that there was no increase in anti-NE autoantibodies in AATD individuals compared with the other groups. While none of the AATD
individuals or those receiving AAT augmentation therapy were positive for anti-NE autoantibodies, two of the MM individuals were. Of these, one MM individual had recently been diagnosed with RA, while the other individual had no clinical manifestations of autoimmune disease.

In addition to anti-NE autoantibodies, the ensuing experiments investigated the presence of autoantibodies against two other neutrophil primary granule proteins, MPO and anti-PR3. Anti-MPO autoantibody distribution in MM, AATD and AATD individuals receiving AAT augmentation therapy is represented in Figure 5.2. Analysis confirmed that none of the individuals were positive for ANCA against MPO and that there was no significant difference in titres between the three groups. Figure 5.3 displays the distribution of anti-PR3 autoantibodies in MM, AATD and AATD individuals receiving AAT augmentation therapy and revealed that there was no increase in anti-PR3 autoantibodies in AATD individuals compared with the other groups.

For all autoantibody analyses, statistical significance was calculated by student’s t-test, where a p value of ≤ 0.05 was deemed statistically significant. However, the absence of autoantibody concentrations above the designated positive cut-off values between groups negated the relevance of these results. Collectively, while our experiments did not identify any increase in ANCA directed against neutrophil primary granule proteins in AATD, the next set of experiments were designed to explore if primary granule-derived NE could induce ROS production in the neutrophil.
Figure 5.1: No increase in anti-NE autoantibodies in AATD individuals determined by ELISA. Plasma samples from HC (MM) individuals (n=40), AATD individuals (n=36) and AATD individuals on AAT augmentation therapy (n=10) were analysed for anti-NE autoantibodies using the Alegria® ELISA-based assay (ORG 224 ORGENTEC Diagnostika GmbH Mainz, Germany). The dashed line represents the pre-defined positive cut-off for anti-NE autoantibody set by the manufacturer at 10 IU/mL. Two of the MM individuals were positive for anti-NE autoantibodies, while none of the AATD individuals or those receiving AAT augmentation therapy were positive.
Figure 5.2: No increase in anti-MPO autoantibodies in AATD individuals determined by ELISA. Plasma samples from MM individuals (n=40), AATD individuals (n=36) and AATD individuals on AAT augmentation therapy (n=10) were analysed for anti-MPO autoantibodies using the Phadia® 250 system. The dashed line represents the cut-off for anti-MPO autoantibody positivity set by the manufacturer at 5 IU/mL (3SDs above the mean for healthy control individuals). None of the individuals in the MM, AATD or AAT augmentation therapy group were positive for anti-MPO autoantibodies and there was no significant difference in anti-MPO autoantibody titres detected between the three groups.
Figure 5.3: Anti-PR3 autoantibodies determined by ELISA are not increased in AATD individuals. Plasma samples from MM individuals (n=40), AATD individuals (n=36) and AATD individuals on AAT augmentation therapy (n=10) were analysed for anti-PR3 autoantibodies using the Phadia® 250 system. The dashed line represents the cut-off for anti-PR3 autoantibody positivity set by the manufacturer at 3 IU/mL (3SDs above the mean for healthy control individuals). Again, none of the individuals were positive for anti-PR3 autoantibodies and there was no significant difference in PR3 ANCA titres detected between the three groups.
5.3 ROS production by neutrophils

5.3.1 Effect of NE, AAT and PAR2 on ROS production by neutrophils

Having confirmed increased membrane-bound NE in AATD neutrophils in chapter 3, the focus of this set of experiments was to determine the potential for NE to drive ROS production in neutrophils. Zhao et al. (2015) recently highlighted a role for PAR2 as a mediator for protease-driven inflammation after demonstrating that NE cleaves PAR2 on nociceptor neurons triggering inflammation and pain. However, to date, ROS production mediated by activation of neutrophilic PAR2 by NE and the effect of exogenous AAT on ROS production by neutrophils has not been elucidated (Figure 5.4).

To address this, a cytochrome c reduction assay was performed to assess production of O$_2^-$ by neutrophils using a protocol adapted from Babior et al. (1973) as per section 2.10. Firstly, isolated neutrophils (1 x 10$^6$) from HC were added to wells on a 96-well plate with cytochrome c buffer pre-heated to 37°C to make up a final volume of 200 µL, n=3 individuals per treatment. Neutrophils were either unstimulated or stimulated with TNF-α (100 pg/10$^6$ cells)/fMLP (10 ng/10$^6$ cells), NE (25 µM), NE (25 µM)/AAT (27.5 µM) or NE (25 µM)/PAR2 blocking Ab (10 µg/mL) as per section 2.10. The reduction of cytochrome c was recorded at a wavelength of 550 nm over 30 min at 37°C on a spectrophotometer.

Figure 5.5 displays a graph of O$_2^-$ production (nmoles/L x 10$^6$ cells) by HC neutrophils which were either unstimulated or treated with TNF-α/fMLP, NE, NE/AAT and NE/PAR2 blocking Ab as above (n=3 individuals per treatment). Both TNF-α/fMLP and NE significantly increased O$_2^-$ production by 235% and 107%, respectively (p=0.017 and p=0.011, respectively), while addition of the physiological concentration of AAT (27.5 µM) to NE not only significantly reduced O$_2^-$ production by neutrophils by 41.8% (p=0.005), but normalised O$_2^-$ production to unstimulated neutrophil levels. Furthermore, addition of a blocking concentration of PAR2 Ab (10 µg/mL) to NE also significantly decreased O$_2^-$ production by neutrophils by 66.5% (p=0.039), normalising O$_2^-$ production to HC levels.
Figure 5.4: Schematic representation of the proposed mechanism of ROS release from neutrophil via PAR2. NE released from neutrophil primary granules activates the seven-transmembrane G-protein coupled PAR2 causing release of ROS.
SOD (12.5 µg/mL) was added to NE-stimulated neutrophils (25 µM) as a positive control and highlighted the anti-oxidant effect of SOD, n=1 (Figure 5.5).

AAT obtained from purified human plasma for the treatment of AATD is a precious resource and it is important to explore the efficacy of alternative anti-NE agents. The ensuing experiment sought to determine the ability of an alternative anti-protease agent to reduce ROS production by neutrophils.
Figure 5.5: Excessive ROS production by HC neutrophils in response to NE, the normalising effect of AAT and the impact of PAR2 blocking Ab.

Cytochrome c buffer at 37°C was added to healthy control (HC) neutrophils (1 x 10^6) to a total volume of 200 µL/well and neutrophils were either unstimulated (unstim) or treated with TNF-α (100 pg/10^6 cells)/fMLP (10 ng/10^6 cells), NE (25 µM), NE (25 µM)/AAT (27.5 µM) or NE (25 µM)/PAR2 blocking Ab (PAR2 blocking Ab) (10 µg/mL). Reduction of cytochrome c was recorded on a spectrophotometer (SpectraMax® M3 multi-mode microplate reader, Molecular Devices) at a wavelength of 550 nm over 30 min at 37°C. Statistical significance was calculated by student’s t-test, where a p value of ≤ 0.05 was deemed statistically significant and shown as mean ± SEM (n=3 individuals per treatment). In addition, SOD (12.5 µg/mL) was added to NE-stimulated neutrophils (25 µM) to demonstrate the trend in reduction of O_2^- by SOD (n=1).
5.3.2 Effect of alternative anti-NE agents on ROS production by neutrophils

Thus far, we have established that NE stimulates significant release of $O_2^-$ from neutrophils ($p=0.011$) compared to unstimulated cells and that AAT (27.5 $\mu$M) not only reduces ROS production by NE-stimulated neutrophils ($p=0.005$) but also normalises $O_2^-$ production to healthy control levels. This experiment investigated the comparative ability of an alternative commercially-available anti-NE agent to reduce ROS production by neutrophils. A cytochrome c reduction assay to compare neutrophilic production of $O_2^-$ was performed as previously described in section 2.10.

Neutrophils ($1 \times 10^6$) were either unstimulated or treated with NE (25 $\mu$M), NE (25 $\mu$M)/AAT (27.5 $\mu$M) or NE (25 $\mu$M)/AZD9668 (27.5 $\mu$M) (Sigma Aldrich®), $n=3$ individuals per treatment. The reduction of cytochrome c was recorded at a wavelength of 550 nm over 30 min.

Figure 5.6 displays a graph of $O_2^-$ production (nmoles/1 x 10$^6$ cells) by NE-stimulated HC neutrophils in response to two anti-NE agents, AAT and AZD9668. This showed that there was a slight trend but no significant reduction in $O_2^-$ release by NE-stimulated neutrophils treated with AZD9668. However, treatment with AAT (27.5 $\mu$M) dramatically decreased excessive ROS production by NE-stimulated neutrophils compared with the same physiological concentration of AZD9668 (63.3% vs. 12.6%, $p=0.002$), reassuringly confirming an important additional benefit of AAT therapy for our AATD patients.
Figure 5.6: Effect of alternative anti-NE agents on ROS production by neutrophils. Cytochrome c buffer at 37°C was added to healthy control (HC) neutrophils (1 x 10^6) to a total volume of 200 µL/well and neutrophils were either unstimulated or treated with NE (25 µM), NE (25 µM)/AAT (27.5 µM) and NE (25 µM)/AZD9668 (AZD) (27.5 µM). Reduction of cytochrome c was recorded at a wavelength of 550 nm over 30 min at 37°C, (n=3 individuals per group). AAT significantly reduced O$_2^-$ production by NE-stimulated neutrophils (p=0.008), while AZD had minimal effect. In addition, AAT (27.5 µM) dramatically decreased excessive ROS production by NE-stimulated neutrophils compared with the same concentration of AZD (p=0.002). Statistical significance was calculated by student’s t-test, where a p value of ≤ 0.05 was deemed statistically significant and shown as mean ± SEM (n=3 individuals per treatment).
5.4 Discussion

The aim of chapter 5 was to explore if there were increased autoantibodies directed against neutrophil primary granule proteins in AATD, as well as determining ROS production by the neutrophil in response to NE, the role of PAR2 in O$_2^-$ release and finally the effect of physiological concentrations of anti-NE therapies on neutrophil ROS production.

The anti-NE Alegria® ELISA-based assay and the Phadia® 250 system for anti-MPO and anti-PR3 antibody testing were employed to determine the presence of IgG autoantibodies directed against neutrophil primary granule proteins in plasma samples of MM, AATD and AATD individuals on AAT augmentation therapy as described in sections 2.91 and 2.92. These assays identified that there was no increase in anti-MPO or anti-PR3 autoantibodies directed against neutrophil granule proteins in AATD individuals compared with MM individuals, suggesting that AATD alone was insufficient to induce ANCA-positive vasculitis. These results are supported by previous work by Audrain et al. (2001). However, while Audrain and colleagues reported an increased incidence of anti-NE IgG antibodies in the serum of AATD individuals compared to the MM group (p< 0.05), our study found no increase in the presence of anti-NE IgG autoantibodies in AATD plasma samples. This may be explained by differences in ELISA techniques and interpretation of results, as Audrain et al.(2001) used an alkaline phosphatase-conjugated goat polyclonal anti-human IgG reagent (Sigma Aldrich-1350, St. Louis, MO, USA) to detect serum anti-NE autoantibodies and defined a positivity level as a value higher than 2 SD over the mean binding of normal human sera. Nevertheless, this result was surprising given our previous finding of increased membrane-bound NE on AATD neutrophils compared with HC cells.

Our focus therefore shifted to the impact of NE on ROS production by the neutrophil and the potential of anti-NE therapies to ameliorate this.

To determine O$_2^-$ production by neutrophils, a cytochrome c reduction assay was performed on unstimulated neutrophils and neutrophils that were treated
with TNF-α/fMLP, NE, NE/AAT or NE/PAR2 blocking Ab. This experiment confirmed a significant increase in \( \text{O}_2^- \) release by neutrophils in response to TNF-α/fMLP and NE (235% increase, \( p=0.017 \) and 107% increase, \( p=0.011 \), respectively). Importantly however, addition of AAT (27.5 µM) to NE-stimulated neutrophils normalised \( \text{O}_2^- \) production to control levels, adding a further dimension to the benefits of AAT augmentation therapy for our AATD patients. Furthermore, addition of a blocking concentration of PAR2 Ab (10 µg/mL) to NE significantly decreased \( \text{O}_2^- \) production by 66.5% and normalised it to HC levels. This is an interesting finding as it not only confirms that NE activates PAR2 on neutrophil membranes to produce ROS, but also suggests that blocking of PAR2 may represent a novel therapeutic avenue in the management of the ROS-mediated pathway of AATD. In addition to elucidating \( \text{O}_2^- \) release by NE-stimulated neutrophils, this study also identified a protective anti-oxidant role for SOD (Figure 5.5). SOD is one of the predominant intracellular antioxidants in the airways (Cantin \textit{et al}, 1990), though its role in chronic airways disease has not yet been clearly established (Bowler \textit{et al}, 2002). Similar to the positive effects of anti-oxidant use with aerosolised glutathione in CF (Snyder \textit{et al}, 2002, Calabrese \textit{et al}, 2015); targeting oxidative stress with antioxidants is likely to be beneficial in the management of COPD and AATD.

AAT obtained from purified human plasma for the treatment of AATD is a precious resource and interest is growing in alternative anti-NE agents such as AZD9668. AZD9668 is a selective oral inhibitor of NE. In 2006, Stockley \textit{et al.} demonstrated a significant improvement of 100 mL in FEV\textsubscript{1} in the AZD9668 group compared with the placebo group (\( p=0.006 \)) in a phase 2 study of bronchiectasis patients receiving 60 mg twice daily over four weeks. However, Kuna \textit{et al.} (2011) did not detect any improvement in lung function or respiratory signs and symptoms in a phase IIb trial of over 600 symptomatic COPD patients treated with AZD9668 60 mg twice daily for three months in combination with their maintenance budesonide/formoterol inhaler, suggesting that further research is needed to determine the optimal duration of studies to evaluate NE inhibitors as disease-modifying agents.
In the present study, the final experiment sought to determine the efficacy of the alternative commercially-available NE inhibitor, AZD9668, in reducing neutrophil ROS production. A cytochrome c reduction assay was again performed to compare O$_2^-$ release by neutrophils stimulated with NE in the presence of AAT (27.5 µM) or AZD9668 (27.5 µM). This experiment confirmed that AAT significantly decreased O$_2^-$ production by NE-stimulated neutrophils compared with the same concentration of AZD (63.3% reduction vs. 12.6% reduction, p=0.002).

AAT has been previously shown to reduce neutrophil adhesion and chemotaxis, independent of its anti-elastase activity (Jonigk et al, 2013). This may therefore explain the superior performance of AAT over a commercially-available NE inhibitor in reducing neutrophilic ROS production.

While this result is certainly very reassuring for our AATD patients, it also highlights the ongoing importance of developing alternative anti-NE agents in the fight against AATD-driven airways disease.
Chapter 6
General Discussion
6.1 Discussion

This study demonstrates that there is aberrant degranulation of primary, secondary and tertiary granules, increased membrane-bound NE and excessive Rac2 activity in neutrophils of individuals with AATD (Figure 6.1). Importantly, this study shows for the first time that AAT augmentation therapy not only normalises excessive degranulation patterns but also decreases elevated Rac2 activity by AATD neutrophils. We have confirmed an additional pathway of neutrophil ROS production via activation of transmembrane PAR2 by NE and identified that blocking of PAR2 normalises excessive $O_2^-$ release by NE-stimulated neutrophils. Furthermore, exogenous AAT at physiological concentrations (27.5 µM) normalises increased $O_2^-$ production by NE-stimulated neutrophils to healthy control levels, adding a further dimension to the benefits of AAT augmentation therapy for our AATD patients (Figure 6.2).

These observations support our hypotheses that neutrophils of AATD individuals illustrate dysregulated degranulation processes leading to enhanced extracellular release of granule proteins and that AAT normalises aberrant neutrophil degranulation patterns and excessive Rac2 activity in AATD, as well as normalising ROS production by NE-stimulated neutrophils.

While neutrophil degranulation plays an essential role in innate immunity (Faurshou et al, 2003), dysregulated neutrophil degranulation plays a critical role in the pathogenesis of AATD-associated airways disease, as well as other respiratory conditions such as asthma (Carlson et al, 1991) and CF (Pohl et al, 2014). Significant neutrophil influx (Bergin et al, 2010) and increased degranulation of secondary and tertiary granules (Bergin et al, 2014) lead to a protease: anti-protease imbalance that favours development of premature emphysema (Guenter et al, 1981). Significantly increased levels of MPO and NE have also been detected in the sputum of AATD patients compared with AAT-sufficient COPD individuals (Hill et al, 2000), while excessive levels of LL-37 and MMP-9 have been identified in induced sputum of COPD individuals (Golec et al, 2009, Lowrey et al, 2008).
Figure 6.1: Neutrophils in AATD. Schematic illustration demonstrating that there is aberrant degranulation of primary, secondary and tertiary granules by AATD neutrophils, in response to inflammatory mediators, leading to excessive release of NE and MPO, hCAP-18 and MMP-9, respectively. In addition, excessive activity of the GTP-binding protein Rac2 occurs, driving increased degranulation of primary granules. Furthermore, there is enhanced re-binding of the NE to the neutrophil membrane in AATD, activating PAR2, which in turn causes release of ROS.
Figure 6.2: Effect of AAT on the AATD neutrophil. Schematic illustration demonstrating that AAT augmentation therapy reduces excessive degranulation of primary, secondary and tertiary granules by AATD neutrophils, in response to inflammatory mediators, resulting in decreased release of NE and MPO, hCAP-18 and MMP-9, respectively. AAT augmentation therapy also reduces exaggerated activity of Rac2, thereby normalising primary granule exocytosis by AATD neutrophils. In addition to potentially ameliorating excessive NADPH oxidase-mediated ROS production by its dampening effects on increased activity of Rac2 by AATD neutrophils, AAT also decreases O$_2^-$ release by NE-stimulated neutrophils normalising it to HC levels.
In 2013, Ma et al. identified that AAT augmentation therapy significantly reduced levels of desmosine and isodesmosine, biomarkers of elastin degradation, in both plasma and BALF of treated AATD patients.

The results of our study reveal that there are 3-fold increased levels of hCAP-18 and MMP-9, released from secondary and tertiary granules of AATD neutrophils, respectively, in response to TNF-α/fMLP (p=0.038, p=0.042, respectively) supporting previous work by Bergin et al. (2014). This is an important finding as enhanced levels of hCAP-18 and its active peptide, LL-37, have been shown to inhibit neutrophil apoptosis leading to sustained inflammation in airway epithelial cells (Barlow et al, 2006). Excess levels of LL-37 and MMP-9 have also been shown to correlate negatively with FEV₁ in CF patients (Chen et al, 2004) and COPD patients, respectively (Linder et al, 2015).

This study adds to the field and has demonstrated for the first time the presence of aberrant degranulation of neutrophil primary granules in AATD as demonstrated by a 3.7-fold increased in MPO release by AATD neutrophils compared with HC cells in response to TNF-α/fMLP (p=0.023). This is a concerning finding as MPO activity of acid extracts of sputum is directly correlated with airflow obstruction in homozygotic delF508 patients with CF (Garner et al, 2004), while Weiland et al. (1986) demonstrated increased levels of neutrophilic MPO in the BALF of ARDS patients. Elevated levels of MPO have been detected in serum and BALF of patients with lung cancer compared with HC (p<0.05), positively correlating with increased intracellular ROS in peripheral blood neutrophils of lung cancer patients (Vaguliene et al, 2013). In addition to respiratory disease, increased leucocyte and plasma levels of MPO have been shown to promote oxidation of lipoproteins in atheroma in individuals with coronary artery disease (Zhang et al, 2001). Interestingly, Churg et al. (2012) reported that AZ1, an MPO inhibitor, reduced progression of emphysema and small airway remodelling while partially protecting against pulmonary hypertension in a cigarette smoke exposure model in guinea pigs, suggesting a potential therapeutic role for MPO inhibitors in the management of obstructive Airways disease.
In addition to MPO as a marker of neutrophil primary granule release, this study also focused on NE. We used FRET analysis as an alternative technique to demonstrate a 61.9% increase in membrane-bound NE in AATD neutrophils in response to 20 min stimulation with TNF-α/fMLP compared with HC (p=0.034). In AATD, NE has previously been shown to induce secretion of LTB₄ in airway epithelial cells and macrophages (Hubbard et al, 1991), while Devaney et al, (2003) demonstrated the ability of NE to up-regulate the potent pro-inflammatory chemokine, IL-8, via TLR-4. NE has also been demonstrated to cleave important cell membrane receptors. Ordinarily following a bacterial infection, galectin-9 binds to T cell Ig and mucin-domain-containing molecule-3 (TIM-3) on neutrophils, inducing phosphorylation and production of IL-8, subsequent neutrophil recruitment and resolution of infection. However, Vega-Carrascal et al. (2014) identified that TIM-3 and galectin-9 underwent rapid proteolytic degradation by NE, contributing to increased pseudomonas infection and suggested a potential benefit to aerosolised AAT augmentation therapy for CF patients. In addition, excess NE in BALF of CF patients has the ability to inactivate the complement 5a (C5a) receptor, further contributing to suboptimal neutrophil priming and bacterial clearance (van den Berg et al, 2014).

Given our findings of enhanced MPO release and increased membrane-bound NE in AATD neutrophils, we hypothesised that increased activity of the GTP-binding protein, Rac2, was the underlying molecular mechanism for excessive degranulation of primary granules in AATD. Rac2 is known to be essential for degranulation of neutrophil primary granules (Abdel-Latif et al, 2004), as well as being required for F-actin formation in neutrophils which is crucial for chemotaxis and O₂⁻ release (Filippi et al, 2004). In a mouse model of IgG immune-complex mediated ALI, Dooley et al. (2009) identified that ALI was attenuated in Rac2 knockout mice compared with wild-type mice, suggesting that lung injury in response to immune complex deposition was dependent on Rac2 in alveolar macrophages and neutrophils. However, very little is currently known about the role of Rac2 in obstructive airways disease. Using recombinant GST-PAK-CRIB to pull down active Rac-GTP, our study identified a 239% increase in active Rac 2 content in AATD neutrophils.
compared with HC neutrophils in response to TNF-α/fMLP (p=0.016). As Rac2 is also an intrinsic component of the NADPH oxidase complex that mediates ROS production by neutrophils (Nathan et al, 2006), increased Rac2 activity in AATD neutrophils may drive neutrophil-mediated excess $O_2^-$ production in AATD.

Having identified dysregulated degranulation of neutrophil primary granules, increased membrane-bound NE and excessive Rac2 activity in neutrophils of AATD individuals, the essential question remained as to whether AAT augmentation therapy ameliorated these aberrant processes exhibited by the AATD neutrophil.

AAT augmentation therapy consisting of weekly infusions of plasma purified human AAT (60 mg/kg) in order to maintain the plasma AAT above the protective threshold remains the mainstay of treatment for ZZ-AATD individuals with a plasma AAT level < 11 μmol/L and moderate to severe airflow obstruction (ATS/ERS 2003). Most of the original efficacy studies of AAT augmentation therapy relied on non-randomised observational studies (Petrache et al, 2009) and included findings by Seersholm et al. (1997), the National Heart Lung and Blood Institute (1998) and Chapman et al. (2009) of a significantly slower rate of FEV₁ decline in patients with moderate airflow obstruction at baseline treated with AAT augmentation therapy. Clinical studies have also reported a reduction in the frequency of severe infective exacerbations (Dirksen et al, 2009, Barros-Tizón et al, 2012), as well as improved quality of life for patients receiving home-based weekly AAT augmentation therapy (Wilke et al, 2013). The RAPID trial was the first prospective randomised placebo-controlled trial that confirmed the annual rate of lung density loss was significantly less in patients receiving AAT augmentation therapy than in the placebo group (p=0.03) (Chapman et al, 2015). AAT augmentation therapy has a relative paucity of side effects (Wencker et al, 1998, Stoller et al, 2003), but remains an expensive therapy (Stoller et al, 2004), with the direct medical cost of augmentation therapy averaging $40,000 per individual patient (Mullins et al, 2001). As AATD individuals often experience frequent pulmonary exacerbations and a progressive decline in lung function, the clinical and economic burden of
illness can be high. Hay and Robin (1991) estimated that at 30% efficacy, the cost of augmentation therapy ranged from $50,000 to $128,000 per life-year saved and that cost-effectiveness at 30% efficacy or higher would be comparable to other widely used medical interventions. More recently, Alkins and O’Malley (2000) showed a lower incremental cost-effectiveness ratio of $13,971 for individuals with severe AATD on the National Institutes of Health Registry. While cost-effectiveness of augmentation therapy remains controversial (McCarthy and Dimitrov, 2010), recent results from the RAPID trial are strongly supportive of continued use for individuals with severe AAT deficiency.

Despite the reported clinical effects of AAT augmentation therapy, the mechanisms by which AAT modulates the immune system of HC and AATD individuals are not fully delineated and further evidence of the biochemical efficacy of AAT augmentation therapy is required (Russi et al, 2008).

Importantly, we have illustrated a significant reduction of > 89% in degranulation of neutrophil granules by AATD neutrophils following stimulation with TNF-α/fMLP two days after receiving AAT augmentation therapy. Of even greater significance for our AATD patients, neutrophil primary, secondary and tertiary granule release by AATD neutrophils ex-vivo, was strikingly normalised to HC levels just two days post therapy. These results compliment previous findings of reduced neutrophil membrane expression of TNF-α (Bergin et al, 2014), decreased ADAM-17 activity and correction of aberrant neutrophil apoptosis (Hurley et al, 2014) and reduced plasma LTB₄ concentrations (O’Dwyer et al, 2015) in response to AAT augmentation therapy, further strengthening our knowledge of the biochemical benefits of this treatment and enhancing our understanding of the mechanism by which AAT augmentation therapy reduces alveolar damage.

In addition to the crucial role of AAT as an anti-protease in the lung, emerging evidence suggests that AAT has unique anti-inflammatory properties, leading to its potential therapeutic use for a variety of other important inflammatory diseases. In CF patients, aerosolised AAT has already been shown to reduce excessive NE activity in respiratory ELF
(McElvaney et al, 1991, Griese et al, 2007), as well as reduce time to first acute exacerbation (Martin et al, 2006). While development of the CFTR potentiator, ivacaftor (kalydeco®), and combination therapy with ivacaftor/lumacaftor (orkambi®) mark significant milestones in treatment to improve CFTR protein function in patients with G551D mutations and delF508 mutations, respectively, (Ramsey et al, 2011, Wainwright et al, 2015), consideration should be given to the potential supportive role of therapeutic intravenous AAT in addressing inflammation in CF patients.

In addition to inflammatory airways disease, AAT has been investigated as a potential therapy for RA. Progression of RA involves a sustained inflammatory process by immune cells (McInnes et al, 2010) and treatment of moderate to severe active RA often involves the use of biologics such as TNF-α inhibitors (Navarro-Sarabia et al, 2006). It has previously been shown that AAT significantly reduced TNF-α binding to TNFR1 on neutrophils (Bergin et al, 2014). In addition, Yoshida et al. (2005) reported that AAT can inhibit a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4) known to be involved in the pathogenesis of RA. AAT has also been shown to delay development of RA in a mouse model (Grimstein et al, 2011), suggesting that AAT may limit the positive inflammatory feedback loop that perpetuates RA. Again, the safety profile and ability of AAT therapy to positively impact neutrophil-mediated killing of P. aeruginosa in CF patients (McElvaney et al, 1991) should be acknowledged when considering the association of TNF-α inhibitors with opportunistic infections (Bergstrom et al, 2004, Wallis et al, 2004) and reactivation of latent Mycobacterium tuberculosis (M. tuberculosis) (Keane 2005).

The role of AAT as a potent anti-inflammatory has also been investigated in Type 1 diabetes mellitus resulting from autoimmune destruction of insulin-producing pancreatic beta cells. Hashemi et al. (2007) identified impaired activity of circulating AAT in individuals with diabetes mellitus, while a study by Koulmanda et al. (2008) demonstrated curative and beta cell regenerative effects of AAT treatment in autoimmune non-obese diabetic mice. Preliminary data from a number of clinical trials addressing the safety and efficacy of intravenous AAT in type 1 diabetes mellitus suggest improved
pancreatic islet cell function in up to half of the participants (National Institutes of Health clinical trial registry NCT01304537, NCT01319331).

In addition to the beneficial role of AAT in ameliorating excessive neutrophil degranulation in AATD, we have also determined for the first time, that AAT augmentation therapy decreased active Rac2 content in AATD neutrophil lysates two days following treatment (p=0.03). This mirrored the timeline in the reduction of aberrant degranulation of primary granules by AATD neutrophils following AAT augmentation therapy and supports our hypothesis that AAT augmentation therapy decreases excessive Rac2 activity in AATD. While further research is required into the relationship between Rac2 and chronic lung disease, a causal role for Rac2 has been hypothesised in the pathogenesis of autoimmune diseases such as Crohn's disease and multiple sclerosis (Sironi et al., 2011). This, along with findings of reduced plasma levels of IgG autoantibodies following AAT treatment (Bergin et al., 2014), suggests that AAT augmentation therapy may be considered as a therapeutic avenue for autoimmune disorders.

Our findings of increased membrane-bound NE and excessive degranulation of primary granules by AATD neutrophils prompted us to consider the potential for the development of ANCA in AATD. The role of autoimmune pathology in the development and progression of COPD is becoming increasingly appreciated. Packard et al. (2013) identified that COPD patients produce autoantibodies reactive to a broad range of self-antigens, including anti-cardiolipin and anti-histone antibodies and anti-La/ Sjögren syndrome antigen B (anti-La/SS-B) antibodies associated with SLE and Sjögren's syndrome, respectively. In addition, combined pulmonary fibrosis and emphysema (CPFE) is a frequent radiological finding in patients with ANCA-associated microscopic polyangiitis (Homma et al., 2013). A number of studies have indicated that the Z-allele is increased in ANCA-associated vasculitis (Borgmann et al., 2001, Elzouki et al., 1994, Esnault et al, 1994). Previous research has identified a higher incidence of anti-lactoferrin IgG autoantibodies in the plasma of AATD individuals compared with HC, while no significant difference was detected in the level of anti-hCAP-18 or anti-MMP-9 IgG autoantibodies (Bergin et al, 2014). In addition, increased
incidence of anti-NE IgG antibodies in the serum of ZZ-AATD individuals has previously been reported by Audrain et al. (2001). However, our study did not identify any increase in the presence of anti-NE, anti-MPO or anti-PR3 IgG autoantibodies in AATD plasma samples compared with MM individuals. While variation in ELISA technique and interpretation of positive cut-off values may have contributed to the conflicting findings, further investigation of ANCA directed against neutrophil primary granule proteins in AATD is warranted to ensure an additional therapeutic benefit of AAT in AATD is not overlooked.

The complexities of oxidative stress are particularly evident in the respiratory tract which is continuously exposed to external environmental oxidants as well as recruited activated inflammatory cells internally (Holguin et al, 2013). Airway oxidative stress has been associated with air trapping, bronchitic symptoms and loss of lung function (Drost et al, 2005) as well as diminished response to corticosteroids by alveolar macrophages in COPD (Culpitt et al, 2003). This study has confirmed excessive MPO release as well as increased membrane-bound NE and Rac2 activity by AATD neutrophils. While MPO metabolises H$_2$O$_2$ to the powerful oxidant HOCl (Harrison et al, 1976), Rac2 is the main p67$^{phox}$-interacting GTPase in the NADPH oxidase complex of human cells (Dorseuil et al, 1996).

Therefore, our interest was sparked in the potential impact of excessive NE on ROS production as a mechanism of oxidative stress in the neutrophil. A cytochrome c reduction assay confirmed a significant 107% increase in O$_2^-$ production by neutrophils in response to NE (25 µM), (p=0.011).

Based on previous findings of ROS production following binding of NE to PAR2 on nociceptive neurons (Zhao et al. 2015), we postulated that NE may activate PAR2 on the neutrophil membrane to induce release of O$_2^-$. Using a blocking concentration of PAR2 Ab (10 µg/mL) (Julovi et al, 2011), we confirmed that O$_2^-$ production by NE-stimulated neutrophils was significantly inhibited by 66.5% (p=0.039) and normalised to unstimulated neutrophil levels. To our knowledge, this is the first confirmation that NE activates PAR2 on neutrophil membranes to produce ROS and implies that PAR2 antagonists may be used as a novel anti-oxidant therapy in obstructive
airways disease where PAR2 has been shown to be over-expressed (Cocks et al., 2001). In addition, it has been demonstrated that PAR2 expression in endothelial cells causes vascular smooth muscle relaxation and hypotension leading to organ hypoperfusion, suggesting that PAR2 antagonists may also form the basis of a new class of therapeutic agents for the treatment of endothelium-based vascular diseases such as peripheral vascular disease, stroke and heart disease (McGuire et al., 2004).

Most importantly, this experiment also demonstrated normalisation of $O_2^-$ release by NE-stimulated neutrophils in the presence of AAT (27.5 µM). As an oxidant: anti-oxidant imbalance is a pathogenic mechanism recognised in interstitial lung diseases secondary to systemic sclerosis, sarcoidosis and pneumoconiosis (Rottoli et al., 2005), our findings also expand the potential therapeutic benefits of AAT therapy beyond AATD to the spectrum of diffuse lung disease (DLD).

When considering the impact of oxidative stress in AATD, it is important to be cognisant of the extreme relevance of smoking cessation for our AATD patients. Cigarette smoke contains a staggering $1 \times 10^{15}$ free oxygen radicals per puff (Pryor et al., 1993), while $H_2O_2$ in tobacco smoke is known to be essential for loss of anti-NE activity of AAT via oxidation of its methionine residues 351 and 358 (Taggart et al., 2000). O’Brien et al. (2015) identified that cigarette smoking was the greatest predictor of impairment in FEV$_1$ and DLCO in AATD individuals, and that the extent of radiological emphysema correlated most significantly with DLCO. These results highlight the importance of close liaison with smoking cessation services for our AATD patients.

Our final experiment demonstrated that AZD9668, an alternative anti-NE treatment, made no significant difference to ROS production by NE-stimulated neutrophils, while AAT significantly reduced $O_2^-$ release compared with AZD9668 (63.3% vs. 12.6%, p=0.002). Accumulating evidence suggests that, in addition to its classic serpin activity, AAT exerts anti-inflammatory and cytoprotective effects (Ehlers et al., 2014). Interestingly in 2013, Jonigk et al. demonstrated reduced neutrophil adhesion and chemotaxis using a recombinant form of AAT without anti-elastase activity in a
lipopolysaccharide-stimulated mouse model, confirming that the anti-inflammatory and immunomodulatory properties of AAT could be independent of NE inhibition. The presence of biological effects of AAT other than the NE-inhibitory effect may therefore explain why a commercially-available NE inhibitor did not have the same effect on ROS production as AAT. In addition, the 27.5 μM concentration of AZD9668 used in this experiment was based on the physiological concentration of AAT and further investigation is therefore warranted to establish if the AZD9668 compound is active against NE at this concentration.

While significant strides have been made toward our understanding of the cellular and biochemical events associated with AATD-associated airways disease, there remains a need for ongoing research and development of alternative and supportive therapies capable of limiting progression of this condition. AAT augmentation therapy is a precious commodity derived from blood plasma of healthy human donors and as such is a lifelong weekly intravenous therapy sometimes necessitating the insertion of an implanted vascular access device. Low-molecular weight NE inhibitors may be important alternatives in the defence against AATD-driven airways disease.

The design of NE inhibitors has primarily focused on the attachment of a serine trap to a peptidyl recognition element that facilitates binding of the inhibitor to the active site of NE (Groutas et al., 2011). Additional NE inhibitors include plant-derived mini-protein cyclotides (Craik et al., 2006) and alternate substrate inhibitors that acylate the active serine site and undergo slow deacylation (Zhong et al., 2004). The NE acylating agent, sivelestat (elaspol®) is marketed in Japan and Korea for the treatment of ALI associated with systemic inflammatory response syndrome (Hyashida et al., 2011, Miyoshi et al., 2013).

Experimental therapies for AATD include aerosolised AAT (Brand et al., 2009), intravenous recombinant AAT (Alkins et al., 2000) complicated by high rates of allergic sensitisation and intramuscular AAT gene therapy which has shown promise (Loring and Flotte, 2015). Interestingly, Castilho et al. (2014) recently pursued recombinant expression of human AAT in Nicotiana benthamiana, a close relative of the tobacco plant (Nicotiana tabacum).
Unusually high levels of two forms of recombinant AAT were expressed in the leaves; a full-length AAT in the ER displaying inhibitory activity and a secreted AAT processed in the RCL rendering it unable to interact with target proteases. However, subsequent intensive glycoengineering led to development of secreted AAT carrying sialated N-glycan structures largely resembling serum-derived AAT (Castilho et al, 2014).

Future studies anticipate maximising delivery of recombinant AAT via intrapleural and airway routes (Loring et al, 2015). Nonetheless, biochemical and clinical efficacy of these experimental treatments compared with traditional AAT augmentation therapy remains to be seen.

Collectively, the findings of this study deepen our understanding of the role of aberrant neutrophil degranulation in the pathogenesis of AATD as well as determining Rac2 as the underlying molecular mechanism of excessive degranulation of neutrophil primary granules in AATD. Most importantly, this study sheds further light on additional biochemical benefits of AAT through its ability to normalise aberrant degranulation, extravagant Rac2 activity and excessive ROS production by AATD neutrophils, strongly advocating the sustained use of AAT augmentation therapy for our AATD patients.
6.2 Future direction

As the AATD neutrophil may shape the inflammatory response and influence patient outcomes, further research investigating the AATD neutrophil is required.

The findings of this study have confirmed the ability of AAT augmentation therapy to restore excessive degranulation of primary, secondary and tertiary granules to normal levels and decrease elevated active Rac2 content of AATD neutrophils within two days of treatment. In addition, NE has been shown to mediate ROS production via PAR2 activation on the neutrophil, while AAT has been shown to normalise excessive $O_2^-$ release by NE-stimulated neutrophils.

While defective Rac2 activity has been implicated in ARDS (Dooley et al., 2009) and autoimmune neurological disease (Sironi et al., 2011), very little is currently understood about the role of Rac2 in chronic lung disease. Furthermore, Rac2 is a critical component of the NADPH oxidase complex responsible for ROS production. While this study has demonstrated the ability of AAT to correct excessive Rac2 activity in AATD neutrophils and normalise $O_2^-$ production by NE-stimulated neutrophils, the nature of the interaction between AAT and Rac2 has not yet been elucidated and requires further investigation, especially in the context of its potential use in the treatment of other respiratory and autoimmune conditions.

While Rac2 is associated with primary granule release, Rab27a has been implicated in the regulation of secondary and tertiary granule trafficking. Pohl et al. (2014) previously demonstrated defective activation of Rab27a associated with less secondary and tertiary granule component release by CF neutrophils. Therefore, future exploration of the role of Rab27a in excessive degranulation of secondary and tertiary granules by the AATD neutrophil is important.

In contrast to an earlier study by Audrain et al. (2001), this study did not identify increased anti-NE IgG autoantibodies in AATD individuals. As discussed, while ELISA technique variation and positive value interpretation
may have contributed to this difference, further investigation of ANCA in AATD is essential.

While this study focused on neutrophils of AATD individuals homozygous for the Z allele, it is anticipated that similar studies will investigate these neutrophil patterns in AATD individuals with other SERPINA 1 variants, as well as AATD carriers.

Collectively, the findings of this translational research study add a new dimension to our understanding of the biochemical benefits of AAT augmentation therapy in ameliorating aberrant neutrophil degranulation processes in AATD. This understanding supports not only the exciting development of aerosolised AAT delivery systems and AAT gene therapies, but also has wider therapeutic implications for other neutrophil-associated respiratory and autoimmune disorders.
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