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Modulation of neutrophil NADPH oxidase activity by alpha-1 antitrypsin

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A thesis presented to the Royal College of Surgeons in Ireland, Faculty of Medicine and Health Science, Royal College of Surgeons in Ireland.

Submitted for the Degree of Doctor of Philosophy

Supervisors:

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Candidate thesis declaration

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

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Table of Contents

Glossary of Abbreviations

AAT: Alpha-1 antitrypsin

AATD: Alpha-1 antitrypsin deficiency

AM: Alveolar macrophage

ANCA: Anti-neutrophil cytoplasmic antibodies

AMP: Adenosine monophosphate

ASL: Airway surface liquid

ATP: Adenosine triphosphate

BALF: Bronchoalveolar lavage fluid

BCA: Bicinchoninic acid assay

BLT1: LTB_4 receptor 1

BODE: Body-mass index, airflow Obstruction, Dyspnoea, and Exercise

BPI: Bacterial permeability-increasing protein

BSA: Bovine serum albumin

Ca2+: Calcium

cAMP: cyclic AMP

Cath: Cathepsin

C/EBPs: CCAAT/enhancer-binding proteins

CF: Cystic fibrosis

CGD: Chronic granulomatous disease

CLP: Common lymphoid progenitor

CMP: Common myeloid progenitor

COPD: Chronic obstructive pulmonary disease

CRP: C-reactive protein

CT: Computerised tomography

CXCR: G-coupled receptor C-X-C motif chemokine receptor

DAG: Diacylglycerol

DFP: Diisopropyl fluorophosphates

DNA: deoxyribonucleic acid

DMF: Dimethylformamide

dPBS: Dulbecco’s phosphate buffered saline

DTT: Dithiothreitol

EBC: Exhaled breath condensate

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

ELF: Epithelial lining fluid

ELISA: Enzyme linked immunosorbent assay

ER: Endoplasmic reticulum

ERK: Extracellular-signal regulated kinase

FACS: Fluorescence-activated cell sorting

FcγR: Glycosylphosphatidylinositol anchored FcγR receptor

FDA: U.S food and drug administration

FEV1: Forced expiratory volume in one second

FITC: Fluorescein isothiocyanate

fMLP: Formyl-Methionyl-Leucyl-Phenylalanine

FPR: Formyl peptide receptor

FRC: Functional residual capacity

FRET: Fluorescence resonance energy transfer

G-CSF: Granulocyte-colony stimulating factor

GDI: Guanine nucleotide dissociation inhibitors

GDP: Inactive-guanosine diphosphate

GFi-1: Growth factor independent -1

GM-CSF: Granulocyte-macrophage colony stimulating factor

GOLD: Global Initiative for Chronic Obstructive Lung Disease

GPCRs: G-protein coupled receptors

GSH: Glutathione

GTP: Active-guanosine triphosphate

H: Hours

hCAP-18: Human cathelicidin anti-microbial protein -18

HCT: Haematopoietic stem cell transplant

HIV: Human immunodeficiency virus

H_2O_2: Hydrogen peroxide

HOCl: Hypochlorous acid

HRP: Horseradish preoxidase

HSA: Human serum albumin

HSC: Haematopoietic stem cell

HVCN1: Hydrogen voltage gated channel-1

I-CAM1: Intercellular adhesion molecule 1

IEF: Isoelectric focusing

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IL-: Interleukin

IP3: Inositol triphosphate

iPSCs: Induced pluripotent stem cells

ITAM: Immunoreceptor tyrosine based activation motif domain

IV: Intravenous

JAK: Janus kinase

LBB: Lamberth break buffer

LFA-1: Lymphocyte function-associated antigen-1

LPS: Lipopolysaccharide

LTB_4: Leukotriene B4

MAC-1: Macrophage antigen 1

MAPK: Mitogen activated protein kinase

MCID: Minimally clinically important difference

MCP-1: Monocyte chemotactic protein-1

MDA: Malondialdehyde

MIG: Monokine induced by gamma interferon

Min: Minutes

MMP: Matrix metalloproteinase

MPO: Myeloperoxidase

mtDNA: mitochondrial DNA

NAC: N-acetylcysteine

NADPH: Nicotinamide adenine dinucleotide phosphate

NE: Neutrophil elastase

NETs: neutrophil extracellular traps

Nf-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NrF2: Nuclear factor erythroid 2–related factor 2

NTM: Non-tuberculous mycobacterial

O_2^- : Superoxide

OSM: Oncostatin M

PAD: Peptidyl arginine deiminases

PAF: Platelet activating factor

PAMPs: Pathogen-associated molecular patterns

PAS: Periodic acid Schiff

PBS: Phosphate Buffered Saline

PBSG: dPBS containing 5mM glucose

PBST: dPBS containing Tween 20

PGL-1: P-selectin glycoprotein ligand 1

PI: Protease inhibitor

PI3K: Phosphoinositide-3 kinase

PI*MM: Individual with MM AAT phenotype

PI*ZZ: Individual with ZZ AAT phenotype

pKa: Acid dissociation constant

PLCβ: Phospholipase C-β

PMA: Phorbol 12-myristate 13-acetate

PR3: Proteinase 3

PRR: Pattern-recognition receptors

PVDF: Polyvinylidene fluoride

qPCR: Quantitative polymerase chain reaction

rAAT: Recombinant alpha-1 antitrypsin

RAPID: Randomized, Placebo-controlled Trial of Augmentation Therapy in Alpha-1 Proteinase Inhibitor Deficiency

RAPID-OLE: RAPID-open-label extension

RCL: Reactive centre loop

RCT: Randomised controlled trial

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RT: Room temperature

SDS: Sodium dodecyl sulphate

SDS PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sec: Seconds

Serpin: Serine protease inhibitor

sIC: Soluble immune complex

SLPI: Secretory leukocyte protease inhibitor

SNAPS: Soluble N-ethymaleimide association protein

SNARE: Soluble N-ethymaleimide association protein receptors

SNARF: Seminaphthorhodafluor

SOD: Superoxide dismutase

STAT3: Signal transducer and activator of transcription 3

TACE: TNF-α converting enzyme

TBST: Tris-Buffered Saline with Tween 20

TGF-β: Transforming growth factor beta 1 TLR: Toll-like receptor TNF-α: Tumour necrosis factor alpha

TLC: Total lung capacity

TLR: Toll-like receptor

TMB: Tetramethylbenzidine

TNFα: Tumour necrosis factor α

TNFR: Tumour necrosis factor receptor

UPR: Unfolded protein response

UV: ultraviolet

VAMPs: Vesicle-associated membranes WBC: White blood cell count Y: Years

VCAM-1: Vascular cell adhesion molecule 1

WT: Wild-type

List of figures

List of Tables

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Abstract

Alpha-1 antitrypsin is a serine protease inhibitor that demonstrates an array of additional immunomodulatory functions. Individuals with the genetic condition, alpha-1 antitrypsin deficiency (AATD) are at increased risk of early onset emphysematous lung disease. This lung disease is partly driven by neutrophil mediated lung destruction in an environment of low AAT. The diverse functions of AAT appear to point to the pathogenesis of lung disease in AATD being more complex than a mere protease/anti-protease imbalance. There is growing evidence of the role of immune cell derived oxidative stress in the pathogenesis of non-genetic COPD. No such role has been firmly established in AATD related lung disease, while there is some evidence that AAT may affect immune cell O_2^- production, based on in vitro studies. The aim of this study was to examine the effects of AAT on neutrophil reactive oxygen species (ROS) production and to determine ROS production by circulating neutrophils in AATD individuals.

We investigated the effects of AAT on circulating neutrophils that had been exposed to fMLP and IL-8 using a cytochrome c reduction assay. We found that physiological concentrations of AAT were capable of significantly modulating neutrophil O_2^- production. We confirmed that the effects of AAT were directly related to NADPH oxidase activity by measuring O_2 consumption in these cells and by demonstrating the assembly of NADPH oxidase subunits at the neutrophil cell membrane. Further to this, we showed that circulating neutrophils from AATD individuals exhibit enhanced O_2^- production in response to soluble stimuli (fMLP). This enhanced activity did not affect the cytosolic pH of the neutrophil, despite our finding of a reduced abundance of HVCN1 in AATD neutrophils. The mechanism of enhanced responsiveness to fMLP by AATD neutrophils appears to be related to the ability of AAT to bind fMLP and prevent engagement with FPR1 on the cell membrane. AAT augmentation therapy was shown to reduce neutrophil O_2^- production. In summary, this study demonstrates that AAT can bind fMLP, preventing engagement with FPR1. This interaction prevents the assembly of NADPH oxidase at the cell membrane and therefore reduces ROS production. In AATD individuals this equates to a more robust ROS production in response to stimuli, an abnormality which is corrected by the administration of AAT augmentation therapy.

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Dedication

This thesis is dedicated to my parents, Seán and Bridget, for instilling in me a love of learning.

Chapter 1: Introduction

1.1 Neutrophils

Neutrophils are polymorphonuclear leucocytes that comprise the first line of host immune response to an invading pathogen. They are the most abundant white blood cell in humans and are considered a major effector cell of innate immunity. Their primary role is to clear infections, which can be accomplished via a number of different mechanisms including phagocytosis, production of reactive oxygen species (ROS), release of granular proteins and release of nuclear material in the form of neutrophil extracellular traps (NETs) (2-4). Neutrophils also play a role in the modulation of the adaptive immune response (5). These extraordinary defensive capacities carry with them the threat of significant tissue damage and destruction. I will review here the production, release, movement and bacterial killing properties of human neutrophils.

The major activity of the bone marrow is devoted to the production of granulocytes and monocytes. 60% of all leucocytes in the bone marrow consist of granulocyte precursors (6). The factors that determine whether a haematopoietic stem cell (HSC) will differentiate into a myeloid precursor or a lymphoid precursor remain, to this day, poorly understood. Two different models have been described (the classical and alternative pathways) by which HSCs in the bone marrow give rise to either common lymphoid progenitors (CLP) or a common myeloid progenitor (CMP), and there is not complete agreement on which model is correct. However, once destined to become a myeloid cell, a CMP will enter a well described process of differentiation (7).

The process of myeloid differentiation is a clearly regulated one. CCAAT/enhancer-binding proteins (C/EBPs), PU.1 and growth factor independent -1 (GFi-1), induce CMPs to differentiate into neutrophils and eosinophils. C/EBPα is an integral component in the initial stages of neutrophil development. The rate of production of neutrophils is modulated by external stimuli, which also influence the rate of neutrophil release from the bone marrow. >90% of mature neutrophils are stored within the bone marrow and their release is under the control of granulocyte-colony stimulating factor (G-CSF). In the steady-state, a normal adult human will produce $2x10^{11}$ neutrophils per day. In response to infections G-CSF

and other dendritic cell products (e.g. C-X-C motif ligand 1 (CXCL1) and CXCL10) are essential for increased neutrophil release from the bone marrow (7-9).

1.1.1 **Neutrophil granules**

Granulopoiesis is the term for the formation of granules within the developing neutrophil. Neutrophils are rich in cytosolic granules (3, 10-12). It is these granules that give them their characteristic appearance by light microscopy and led to them being termed granulocytes. Mature neutrophils contain primary, secondary and tertiary granules and secretory vesicles. Primary granules are formed first. They appear in the myeloblast to promyelocyte phase. This is followed by the formation of secondary granules in myelocytes and metamyelocytes. Tertiary granules form during the phase of transition from myelocyte to band neutrophil. Neutrophil granulopoeisis is complete with the formation of secretory vesicles in segmented neutrophils phase of neutrophil development. A fourth type of granule has been suggested to exist by Røving *et al*., which forms at the same time as secretory vesicles. This granule type was termed the ficolin-1-rich granule (9). It is not wholly accepted as a distinct granule subtype.

Once released into the circulation, neutrophils do not possess any proliferative capacity. Therefore, an important feature of their rapid response to injury or invading micro-organism is their ability to release pro-inflammatory mediators that have been pre-formed and stored in these so called intracellular granules. Granules were traditionally classified into two major groups: peroxidase-positive granules and peroxidase-negative granules. The now widely accepted classification of granule subtypes contains four members (Figure 1-1). All granules have distinct buoyant densities which means they can be separated by density gradient centrifugation. These granules are classified on a functional basis into: primary (azurophilic), secondary (specific), tertiary (gelatinase) and secretory vesicles. Primary granules contain high levels of myeloperoxidase (MPO) that account for 25% of the granule content (13). MPO mediates bacterial killing in the phagasome via the chlorination of reactive oxygen species (ROS) to form hypochlorous acid (HOCl). MPO is one of the targets of anti-neutrophil cytoplasmic antibodies (ANCA) that results in the clinical picture of vasculitis (e.g. microscopic polyangiitis). Primary granules also contain cathepsin G (cath G), neutrophil

elastase (NE), proteinase 3 (PR3), defensins, bacterial permeability-increasing protein (BPI) and lysozyme. NE directly damages membranes of micro-organisms, for example it can degrade the membrane protein A of *Eschericiae coli (12)*. It can also damage collagen, fibronectin and laminin which form an integral part of the extracellular matrix (ECM). NE is also important in regulating the function of other granule proteins, for example it cleaves matrixmetalloproteases (MMP) into their active forms. Other serine proteases include Cath G which can help to clear both gram negative and gram positive bacteria. PR3 is another granule protein that is a target of ANCA, in this case most often associated with granulomatosis with polyangiitis (13, 14).

Secondary granules contain lactoferrin and human cathelicidin anti-microbial protein -18 (hCAP-18). hCAP-18 is important in bacterial killing and the neutralisation of various endotoxins. hCAP-18 must undergo proteolytic cleavage by PR3 to become active. Lactoferrin, a further component of secondary granules, binds iron which reduces bacterial growth (15).

Tertiary granules release matrix metalloprotease 2 and 9 (MMP2 and MMP9). As mentioned these are released in a pro form and must undergo cleavage prior to their activation. MMP9 plays a role in neutrophil recruitment and tissue regeneration, it is a collagenase which can cleave a number of the structural components of ECM. It has been studied in chronic obstructive pulmonary disease (COPD) for its role in lung tissue destruction (16).

The release of granule contents involves the docking and fusion of granules to the outer plasma membrane or the phagocyte membrane. This is termed exocytosis or degranulation. It is important that the release of granule contents is regulated so that their toxic contents are released in response to a threat and not in an uncontrolled way that could lead to local tissue damage. The toxic mediators present in granules have the potential to cause significant destruction of human tissues. Exocytosis or degranulation is thought to take place in a number of discrete steps (13). The first step is the recruitment of the granule from the cytoplasm to the membrane (either the phagocytic vacuolar membrane or the outer plasma membrane). This step requires remodelling of the actin cytoskeleton. The functions of the actin cytoskeleton are many, including cell shape, polarity,

endocytosis, cell division, intracellular trafficking and cell motility. The next step in degranulation is tethering and docking of the granule, whereby the granules outer surface comes into contact with the target membrane. Granule fusion then occurs, which exposes the interior membrane surface of the granule to the exterior. It also means that the total surface area of the cell increases (14). The release of intracellular Ca^{2+} stores is essential for degranulation to occur, as is the hydrolysis of adenosine triphosphate (ATP) and guanine triphosphate (GTP). Annexins and GTP-binding proteins are the likely target of Ca^{2+}. The release of each type of granule appears to be regulated by different intracellular signalling pathways. Increased Ca^{2+} can be caused by a number of neutrophil receptors including fMLP and CXCR1 but the specific target of Ca^{2+} in causing degranulation has not been specifically identified (13).

GTPases are a group of GTP binding proteins, of which over 100 different types have been described. Ras-related GTPases are one of the most comprehensively studied of the GTPases. They can be found in the cytoplasm, actin cytoskeleton or the cell membrane. Three types of proteins have been described that can regulate the activity of Rho proteins. In general, the Ras-related GTPases are activated by binding to high-energy GTP, which is cleaved to form guanosine diphosphate (GDP) to activate the next effector molecule in the signalling pathway. Some Rho family proteins bind to guanine nucleotide dissociation inhibitors (GDI). These hold the Rho proteins in an inactive form in the cytoplasm and dissociation from the Rho protein needs to occur for initiation of downstream signalling. Secondly, Rho proteins may be activated by GDP–GTP exchange factors (GEFs), that is when bound to GDP they are inactive and requires conversion to GTP for the Rho to become active. Thirdly, Rho proteins can be downregulated by GTPase activating proteins (GAPs) (17). The Rho subfamily of GTPases serve a role in regulating the production of reactive oxygen species and in regulating actin cytoskeletal rearrangements. Rho, Rac and Cdc42 (cell division control protein 42) are the three predominant members of the Rho GTPases. Rac can be found as three distinct isoforms: Rac1, Rac2 and Rac3. Rac2 is predominantly found in haematopoietic cells and is expressed in human neutrophils. Rac 1 is found more ubiquitously. The role for Rac1 and Rac2 in superoxide (O_2^-) generation and chemotaxis are widely accepted (18). Neutrophils express both Rac1 and Rac2

but Rac2 is more abundant. Rac2 serves a selective role in neutrophil degranulation. Based on the use of *Rac2*$^{-/-}$ neutrophils it has been established that Rac2 is required for primary granule release (19). Degranulation of secondary and tertiary granules was not affected in the *Rac2*$^{-/-}$ neutrophils, however, the release of these latter granules is under the control of Rab27a another member of the Ras superfamily. Pohl *et al*. demonstrated that circulating CF neutrophils release less secondary and tertiary granules due to defective granule trafficking as a result of defective activation of Rab27a (20).

Adbel-Latif *et al.* isolated neutrophils from the bone marrow and peritoneal lavage of mice, wild type and *Rac2*$^{-/-}$. They measured the release of MPO and NE as markers of primary granule degranulation. MPO was measured using a tetramethylbenzidine (TMB) assay and NE using an elastase assay kit. Release of MPO and NE were measured over a time course in response to both fMLP and leukotriene B4 (LTB_4). Using flow cytometry to detect CD63 (a marker for primary granules), the authors established that primary granules in *Rac2*$^{-/-}$ neutrophils fail to translocate to the membrane is response to chemo-attractant stimuli (19).

The docking and fusion of granules to their target membrane is the final step in exocytosis. This step is likely guided by intracellular receptors and has been the subject of some interest. There have been numerous studies looking at a group of receptors known as SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), which interact with vesicle-associated membrane proteins (VAMP) (13, 21). It has been shown that degranulation is dependent on these proteins and that distinct SNARE isoforms may have specificity for the various granules. Indeed, VAMP-7 is essential for the SNARE-mediated release of primary, secondary and tertiary granule contents (22).

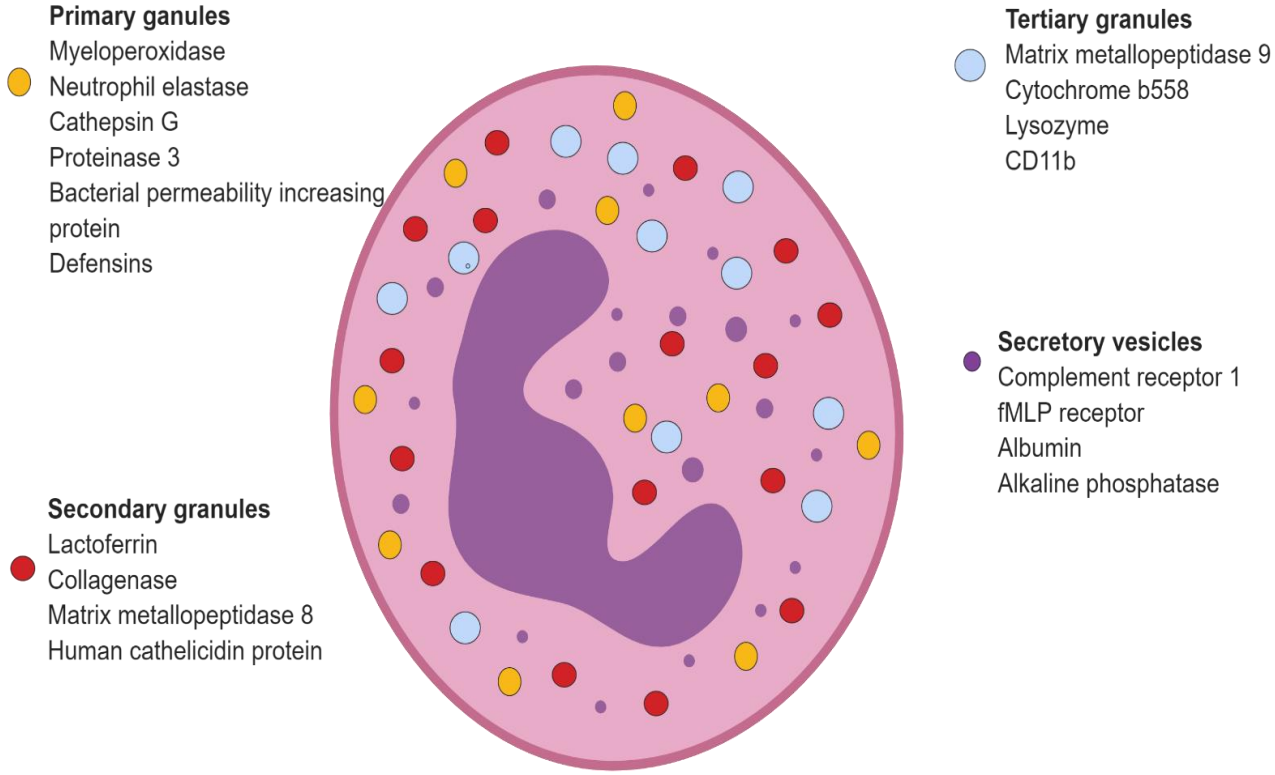


Figure 1-1: Neutrophil granule contents

Illustration depicting components of each of the four neutrophil granules; primary, secondary, tertiary and secretory. These granular contents are an array of antimicrobial agents and include MPO (primary), lactoferrin (secondary) and lysozyme (tertiary) which play a role in host defence during infection. Additionally, neutrophils contain cell receptors such as CD11b/CD18, in secretory granules, for adhesion and glycosylphosphatidylinositol anchored Fcγ receptor (FcγR) CD16b (secretory granules), also referred to as FcγRIIIb, for cell activation.

1.1.2 **Neutrophil recruitment**

Release of neutrophils from the bone marrow is orchestrated by the opposing effects of CXCR4 and CXCR2 (3, 7). Retention in the bone marrow requires active signalling through CXCR4 on the neutrophil surface and its ligand CXCL12. As a neutrophil matures in the bone marrow, it begins to lose CXCR4 and its CXCR2 activity increases. G-CSF stimulates GRO- β expression by bone marrow endothelium, which is a ligand for CXCR2, thus promoting neutrophil movement out of the bone marrow. In a similar way, aged neutrophils in the circulation begin to express more CXCR4, which is believed to direct them back to the bone marrow where they are phagocytosed by macrophages. Clearance of apoptotic neutrophils also occurs via Kupffer cells of the liver after P-selectin mediated hepatic sequestration (23). Circulating neutrophils survive for 8-24h in the circulation. The view that neutrophils have a short life span, are terminally differentiated in the circulation and that they are a homogenous cell in the circulation has been challenged in recent years (24, 25).

Neutrophils are found in three different reservoirs or pools in humans. That is the proliferative, circulating and marginating pools (6, 23). The proliferative pool represents those mature neutrophils in the bone marrow awaiting release. The circulating pool describes those neutrophils within the circulation (these are also the cells most easily accessed for experimental endeavour). The largest source of marginating neutrophils is the pulmonary vasculature and the lungs. The transit time of a neutrophil in the lung is longer than that for the liver or spleen. Some data suggests that a proportion of pulmonary neutrophils are crawling within the pulmonary vasculature during steady state conditions (26). Pulmonary vascular endothelium expresses CXCL12, which is a ligand for CXCR4 on neutrophils and may explain the retention of neutrophils in the lung. A CXCR4 antagonist in a mouse model resulted in neutrophil mobilisation from the lung, suggesting again the role of CXCR4:CXCL12 axis in pulmonary neutrophil sequestration (27).

The recruitment of neutrophils from the circulation to the site of infection involves the following steps: tethering, rolling, adhesion, crawling and transmigration (2). The presence of a large number of selectins, chemokine receptors and integrins allow neutrophils to be rapidly recruited to the site of infection. Neutrophil

recruitment is initiated by endothelial changes. The endothelium can be activated directly via pattern-recognition receptor mediated detection of molecules or indirectly by the release of leukotrienes, cytokines and histamine from tissue resident leucocytes that have come into contact with a pathogen. When activated, endothelial cells increase their surface expression of the adhesion molecules, P-selectin and E-selectin. P-selectin is released quickly, as it is found preformed in the endothelial cells whereas E-selectin must be synthesised de novo and is therefore expressed later (2). The circulating neutrophil has a selectin ligand (P-selectin glycoprotein ligand 1, PGL-1) which binds P and E-selectin leading to tethering of neutrophils to the surface of the vascular endothelium (28).

Once tethered, the neutrophil then rolls on the surface of the endothelium. This is not a passive process. It is achieved when the dissociation of a P-selectin-Ligand bond at the rear of the cell is balanced with the formation of another bond at the leading edge. These rolling neutrophils become activated due to exposure to endothelial cell cytokines that results in the expression of an integrin on the surface of the neutrophil, lymphocyte function associated antigen 1 (LFA-1). LFA-1 binds to intracellular adhesion molecule 1 (ICAM-1) on the endothelial surface. This allows for slow rolling and the eventual arrest of neutrophil rolling. Neutrophil crawling occurs in preparation for transmigration and involves the interaction with endothelial ICAM-1 and neutrophil expressed macrophage antigen 1 (MAC1). Transmigration (diapedesis) usually occurs at the cell-cell junction and requires movement across the endothelium and then the basement membrane. It involves a number of integrins including ICAM-1, ICAM-2 and vascular cell adhesion molecule 1 (VCAM1) (28). It takes up to 20min for a neutrophil to transmigrate from the circulation through the basement membrane. If transmigration occurs transcellularly, rather than via the cell-cell junction, the process takes up to 30min. Passage through the extracellular matrix may require the release of neutrophil proteases, specifically the matrix metalloproteases (MMPs). A fully transmigrated neutrophil will then follow a chemokine gradient to the site of infection (2).

1.1.3 **Neutrophil killing of microorganisms**

Neutrophils are the first immune cell to respond to an invading pathogen. We have already discussed how neutrophils are recruited to sites of infection. Once at the site of infection the neutrophil has an extensive antimicrobial arsenal at its disposal. I will next highlight the various microbial killing strategies of neutrophils including; ROS production, cytotoxic granule release, antimicrobial peptides and the production of NETs (29).

Neutrophils can perform both intracellular and extracellular killing of pathogens. Phagocytosis is the process by which a neutrophil engulfs a microbe, so that it is contained within the cell in a phagocytic vacuole, also called the vacuolar lumen (2). Phagocytosis of a microbe can be aided by opsonisation of that bacteria with opsonins that are recognised by specific neutrophil surface receptors. Neutrophils also express surface receptors that will recognise a microbe by its specific proteins (i.e. pathogen recognition). Complement and immunoglobulins (Ig) are the main factors in the circulation that enable efficient opsonisation. The human complement system is activated by one of three pathways; the classical pathway, the lectin pathway and the alternative pathway. The end product of these pathways are the release of potent chemoattractants (C5a/C5b) and opsonising proteins (30). Neutrophils express two major FcγRs that recognise Ig. FcγrII and FcγRIII. Ig are very abundant in the plasma. In particular, IgM and IgG play important roles in the opsonisation of bacteria and complement activation. Once opsonised, a pathogen can be recognised and phagocytosed by a neutrophil via receptor-mediated uptake. Neutrophils express large numbers of surface receptors for this purpose. Pattern-recognition receptors (PRRs) and certain G-protein coupled receptors (GPCRs) are relevant for the phagocytosis of microbes in this way. The receptor can recognise the host protein used to opsonise the microbe or they can recognise pathogen-associated molecular patterns (PAMPs). PRRs on the neutrophil surface can recognise PAMPs such as bacterial DNA and cell wall peptidoglycan. Toll-like receptors (TLRs) are an example of a PRR. TLRs can recognise lipids, carbohydrates and peptides relating to an invading organism and initiate phagocytosis. Formyl peptide receptor 1 (FPR1) is a GPCR expressed on the neutrophil that recognises bacterial products in the form of N-formyl peptides.

Activation of FPR1 can initiate phagocytosis, as well as chemotaxis and ROS production (31). The process of phagocytosis requires cytosolic actin filament rearrangement and the formation of pseudopodia. Phagocytosis itself does not result in pathogen death. The process by which a phagocytosed microbe is killed includes; the NADPH oxidase dependent production of ROS and the fusion and release of cytotoxic and antimicrobial proteins from neutrophil granules into the vacuolar lumen.

Neutrophils are also capable of extracellular pathogen killing and they exert this antimicrobial activity through the formation of neutrophil extracellular traps (NETs), a process also known as NETosis. NETosis was first described in 2004 (32). It is a process by which, on sensing the entry of a pathogen, neutrophils extrude a lattice-like structure composed of DNA, histones and high concentrations of granule derived antimicrobial molecules (32). This lattice of chromatin and histones can entrap bacteria and fungi while at the same time providing a scaffold to promote high concentrations of antimicrobial peptides and enzymes. Some of the granule contents that have been found associated with NETs include NE, PR3, CG, MPO, lactoferrin, LL-37 and Sp100A . NETosis may result in neutrophil cell death (suicidal NETosis) or with 'vital NETosis' neutrophils lose their nuclear material but retain the ability to phagocytose microorganisms. Chromatin decondensation during NETosis requires histone citrullination mediated by protein arginine deamminase 4 (PAD4) (33). Whether NETs represent a true host defence strategy is not accepted by all (34).

1.1.4 **NADPH oxidase and the production of superoxide**

The production of superoxide (O_2^-), via the neutrophil NADPH oxidase, is a further mechanism by which neutrophils can kill invading microbes. O_2^- is generated in response to various stimuli and is involved in both intracellular and extracellular killing. The importance of the NADPH oxidase of neutrophils for microbial killing is demonstrated by the genetic condition, chronic granulomatous disease. In this condition, neutrophils fail to produce ROS as a result of a genetic defect in one of the NADPH oxidase components. I will discuss this condition in greater detail in a later section of this introduction.

NADPH oxidase consists of a transmembrane flavocytochrome (b558) that interacts with a variety of cytosolic components to form an activated membrane complex. There are six subunits that must interact to form the active enzyme complex that is ultimately responsible for the generation of O_2^-, and subsequently other free radicals (35). A large, heterodimeric flavocytochrome b558 forms the membrane portion of the complex, it is often referred to as the 'catalytic core'. It consists of two subunits, $gp91^{PHOX}$ and $p22^{PHOX}$. Flavocytochrome b558 is located mostly on the membrane of specific granules, as well as on the plasma membrane and during phagocytosis it forms part of the vacuolar membrane. It is named flavocytochrome b558 because of the haem absorbance peak at 558nm in the reduced state (36). $gp91^{PHOX}$ is a large glycoprotein with a molecular mass of 91kDa, it is referred to as the β subunit and contains the apparatus that is required to transport electrons. $p22^{PHOX}$ is a much smaller protein and is referred to as the α subunit of the NADPH oxidase membrane components.

The *C*-terminal of $gp91^{PHOX}$ is hydrophilic and contains both NADPH and FAD binding sites. The *N*-terminal is hydrophobic and consists of six α-helices that span the membrane. Two of these helices contain two histidine residues each, which function to co-ordinate haem. These haem groups are essential for the transport of electrons (35). While flavocytochrome b558 is the 'work engine' of the NADPH oxidase it requires activation through interaction with a number of cytosolic proteins. When a phagocytic cell (e.g. a neutrophil) is in a quiescent state the cytosolic proteins $p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$ exist within the cytosol. Activation of this complex also requires the presence of two small guanine nucleotide-binding proteins, Rac2 and Rap1A.

We have already discussed the role of Rac2 in relation to neutrophil primary granule release. Rac2 is also required for the activation of the NADPH oxidase system. In the resting state, GDP-bound Rac2 is stabilised by GDP dissociation inhibitor (GDI). Upon activation, GTP-bound Rac2 translocates with $p67^{PHOX}$ to the plasma membrane (37).

There are various signalling pathways that regulate the activation of NADPH oxidase in neutrophils (38). The oxidase complex components are separated into distinct subcellular compartments of the cell to prevent inadvertent or spontaneous

activation of NADPH oxidase and potential damage to the resting neutrophil or its environmental surrounds. This spatial separation also allows for multiple points of regulation of ROS production.

$p47^{PHOX}$ becomes phosphorylated by ERK1/2 during neutrophil activation. Once activated via phosphorylation, $p47^{PHOX}$ functions to stabilise the interaction of the cytosolic components of NADPH oxidase with the flavocytochrome portion. It binds to the cytoplasmic portion of $gp91^{PHOX}$ while also forming an interaction with $p22^{PHOX}$ and $p67^{PHOX}$. $p67^{PHOX}$ itself binds to $p40^{PHOX}$ and also binds directly to the flavocytochrome. $p40^{PHOX}$ has been referred to as a shuttle partner and does not interact directly with the flavocytochrome (39).

Both ‘activation’ and ‘priming’ are terms used when discussing the signalling pathways involved in the functioning of NADPH oxidase. Activation can be considered to be a ligand-dependent response that results in the production of ROS (40). Priming is an intermediate step in activation. Following priming, a cell can be considered to be in a state of ‘enhanced responsiveness’. A primed neutrophil has been altered following exposure to a ligand (40). This ligand does not induce ROS production but it leaves the neutrophil more susceptible to activation of NADPH oxidase upon binding to a second ligand or stimulus. A neutrophil may be primed via G-protein coupled receptors (GPCRs), toll-like receptors and cytokine receptors. Direct activation of the NADPH oxidase of neutrophils can occur via activation of Fc and integrin. I will discuss each of these in some more detail below (36).

GPCRs possess seven membrane-spanning domains and are linked to a GTPase protein in the cytosol. Some of the agonists that can bind GPCRs include fMLP, IL-8, complement factor C5a, LTB_4 and platelet activating factor (PAF) (36). The binding of a ligand to a GPCR triggers an exchange of GDP for GTP which causes the release of the subunits Gβ and GβϒΥ from the cytoplasmic portion of the GPCR. The release of these G protein subunits causes activation of downstream pathways. GβϒΥ can activate phosphoinositide-3 kinase (PI3K) and phospholipase C-β (PLCβ), which leads to the breakdown of phosphatidylinositol biphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3), both of which influence Ca^{2+} flux. Ca^{2+} flux is essential for NADPH activation. PI3K also

mediates Rac2 activation. Binding of ligand to GPCRs may also stimulate Src family kinases which exerts downstream signalling through p38 MAPK (mitogen activated protein kinase) (41).

Tumour necrosis factor α (TNFα) is a cytokine that binds to TNF receptors (TNFR1 and TNFR2). TNFα has the ability to prime cells for ROS production through its effects on calcium flux and through the mobilisation of cytochrome b558 to the plasma membrane via the p38 MAPK-dependent pathway (36). TNFα also leads to the phosphorylation of $p47^{PHOX}$ which as previously mentioned leads to its translocation from the cytosol to the plasma membrane. Granulocyte macrophage colony stimulation factor also leads to the phosphorylation of $p47^{PHOX}$ via ERK 1/2 (extracellular-signal regulated kinase), which is a class of MAPK pathway also (42).

The formyl peptide receptor is a GPCR which recognises fMLP. These receptors can both activate and prime neutrophil NADPH oxidase (31). When fMLP binds its receptor in undergoes conformational change with the dissociation of its G protein cytosolic subunits. The subunits are then responsible for activating other signalling proteins that trigger secondary messengers like cAMP, Ca^{2+} and inositol phosphates, all of which can result in the production of ROS through NADPH oxidase (43).

Fc receptors are expressed on human neutrophils and have been shown to be linked to ROS production and involve FcγRIIIA and the FcγRIIIB receptors. They are transmembrane receptors which bind the Fc portion of IgG and signal via immunoreceptor tyrosine based activation motif domain (ITAM). Binding of IgG immune complexes to these receptors leads to the activation of SLP-76 signalling domain which results in the release of intracellular Ca^{2+} stores (36). Fc receptors also activate NADPH oxidase via phosphoinositide 3-kinase pathway.

The priming and activation of O_2^- production through the NAPDH oxidase can occur through a number of different signalling pathways. The consequences of NADPH oxidase activity include membrane depolarisation and an accumulation of H^+ ions liberated from NADPH. For sustained activity of the NADPH oxidase, it is essential that the cell must make allowances for these consequences of NADPH oxidase activity (44).

1.1.6 **Charge compensation and ion movement for neutrophil bacterial killing**

The electrogenic activity of NADPH oxidase affects both the membrane potential and pH of a cell and both of these can limit the function of this enzyme complex (45). It has been shown that pH affects electron current and O_2^- production and that NADPH activity is optimal closer to neutral pH (46). In the late 80's it was discovered that the electrogenic activity of NADPH oxidase is accompanied by an efflux of H^+. The proposal by Jones, Chappell and Henderson was that the efflux of H^+ ions acted to compensate for the charge generated by NADPH oxidase depositing protons in the cytoplasm (46). The idea that proton movement occurred as a result of a voltage-gated proton channel was first described by John Wood in the early 1970's. Wood was studying dinoflagellates and showed that a voltage-gated proton channel was responsible for H^+ movement that triggered a bioluminescent flash in this creature (47).

Hydrogen voltage gated channel-1 (HVCN1) is a proton-selective ion channel. It is found in many cell types, including neutrophils (48). I will discuss only its structure and function in human neutrophils. In neutrophils it can be found on the plasma membrane, the phagocyte vacuolar membrane and stored in secretory vesicles (48). The *hvcn1* gene was discovered in 2006. On the cell membrane HVCN1 is found as a dimer, with each monomer maintaining its ability to move protons. Its entire crystal structure has not as yet been described. It has four transmembrane domains (49) and consists of 273 amino acids. It has a voltage sensing domain but lacks a specific pore domain and HVCN1 is highly selective for protons.

HVCN1 channels become activated by a depolarising membrane and it's activity can be inhibited by Zn^{2+} (49). Unlike some ion channel blockers, Zn^{2+} does not block the channel but binds to the external surface of HVCN1 where it slows channel opening and shifts the voltage dependence positively (50). Its major role is to compensate the charge produced during the production of ROS mediated by NADPH oxidase. It extrudes protons from the cytosol, thus preventing excess cytosol acidification and preventing excess membrane depolarisation thereby enabling the continued activity of NADPH oxidase (51). In the phagocytic vacuole, HVCN1 transfers protons from the cytosol to the vacuolar lumen, which as well as maintaining activity of NADPH oxidase, by preventing excess membrane

depolarisation also lowers vacuolar pH. The lower pH in the neutrophil vacuole provides an ideal environment for MPO mediated production of HOCl (52) (Figure1-2).

Much has been learned about *hvcn-1* since the discovery of the gene in 2006. The use of a knock out mouse model has furthered our understanding of the function of this proton channel in neutrophils. Levine *et al.* used an *hvcn-1*$^{-/-}$ mouse neutrophil. The authors demonstrated that the pH of the phagocytic vacuole of the knock-out mouse neutrophils was significantly more alkaline than human and wild type (WT) neutrophils. *hvcn-1*$^{-/-}$ mouse neutrophils also swelled more after phagocytosis. Cytoplasmic pH was shown to fall in the *hvcn-1*$^{-/-}$ cell after phagocytosis in an exaggerated manner when compared to WT mouse or human neutrophils. In the *hvcn-1*$^{-/-}$ neutrophils, the minimum mean pH reached was 6.72, 2min after phagocytosis. In the WT mouse and human neutrophils the cytosolic pH went from a resting pH of 7.56 to a mean trough of 7.3, after phagocytosis. Levine *et al.* also examined the optimal pH for the activity of a number of essential granule constituents. They looked at cath G, MPO, and NE. MPO activity, in terms of both chlorination and peroxidation, was maximal at acid pH ~5 and was minimally active above pH 9. On the other hand, activity for cath G and NE were low at acidic pH ~5 and peaked for cath G at pH 7-9 and NE at pH 8-10.

Ramsey *et al.* also used *hvcn-1*$^{-/-}$ mouse neutrophils and showed that knock-out neutrophils produced significantly less O_2^-. O_2^- production was measured using a cytochrome C reduction assay (45). A reduction of ~75% in generation of O_2^- was seen, but despite this large difference the mice maintained the ability to clear various bacteria (e.g. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *and Burkholderia cepaciae*).

Okochi *et al.* also studied *hvcn-1*$^{-/-}$ neutrophils from mice (53). They showed that neutrophils from the *hvcn-1*$^{-/-}$ mice released significantly more primary granules after stimulation with PMA and IgG (they measured MPO and NE). Release of secondary and tertiary granules was not different in the *hvcn-1*$^{-/-}$ neutrophils compared to the WT. The authors also showed that the lungs of the mice had histologically more severe lung inflammation than WT mice when challenged with *Candida albicans*. Their conclusion was that HVCN1 inhibits the release of primary

granules in neutrophils, perhaps acting to protect against excessive tissue damage.

It is proposed by some authors that on their own NAPDH oxidase production of O_2^- into the phagosome or the release of granule contents cannot kill a microorganisms. These authors believe that the movement of ions into the phagocytic vacuole, which move across the vacuole membrane in order to compensate for NADPH oxidase activity, are essential to microbial killing in the phagosome (54). It is clear that the act of bacterial killing by neutrophils relies not on one mechanism but on the orchestration of a number of different aspects of neutrophil function. It is also evident that the environment within the neutrophil phagosome must be tightly regulated, in terms of the movement of ions and maintenance of pH, for effective bacterial killing to take place. Loss of control of any of these aspects could have the potential for impaired bacterial killing or lead to excessive inflammation.

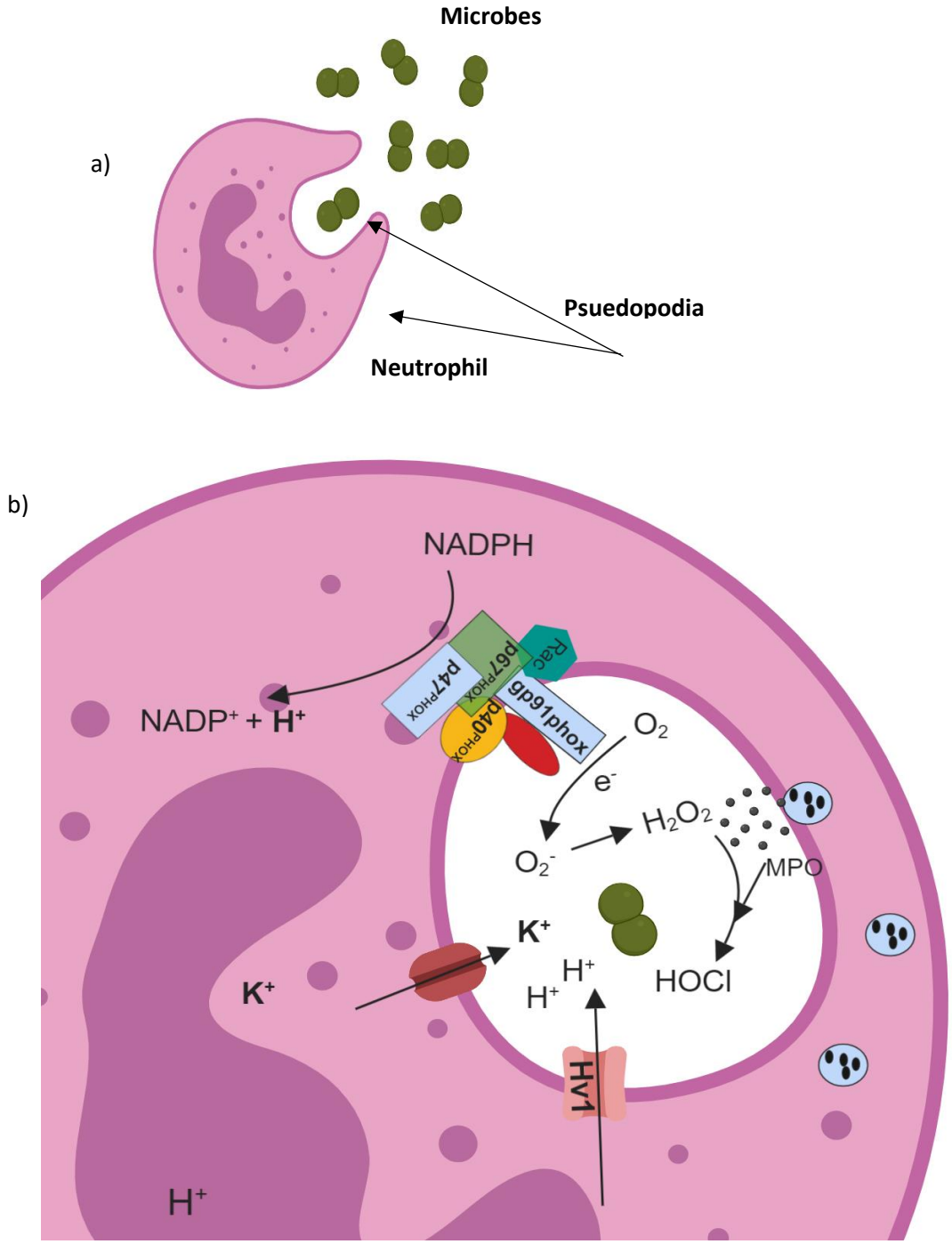


Figure 1-2: Phagocytosis and the intracellular killing of a microbe.

Diagrammatic illustration of how neutrophils kill pathogens within the phagosome. a) Neutrophil plasma membrane undergoes cytoskeletal rearrangement to form pseudopodia which encloses the bacteria into a phagosome. b) NADPH oxidase assembles on the phagosome membrane; electrons are transferred across for use in the production of ROS. O_2^- is converted into H_2O_2, by superoxide dismutase, which is then converted to HOCl in the presence of MPO. MPO and other proteases are released into the phagosome after fusion of granules with the phagosomal membrane. Killing of the microbe then occurs by the combined action of H_2O_2, HOCl and proteases. Ion channels compensate the membrane depolarisation and pH of the phagosome. HOCl; hypochlorous acid, Hv1;HVCN1, MPO; myeloperoxidase.

1.1.7 **Chronic granulomatous disease.**

The importance of NADPH oxidase to innate immunity is illustrated by the genetic condition chronic granulomatous disease (CGD). The discovery that this disease results from the disruption of the NADPH oxidase complex has led to further insights and understanding of the role of NADPH oxidase in health and disease (55). CGD is a rare, genetic disease with an incidence of approximately 1 in 250,000 (56). It is characterised clinically by recurrent and occasionally severe infections, most commonly of the skin and respiratory tract. The condition is also associated with dysregulated inflammation and autoimmunity. Characterised pathologically by granulomatous inflammation; skin abscesses, mouth ulcers, liver abscesses, and lymphadenitis are frequent clinical findings. While the disease was described first over 60 years ago it was only in the late 1960's that is was shown that neutrophils from CGD patients did not produce ROS after phagocytosis (57).

Initially believed to be X-linked there was subsequently shown to be autosomal inheritance of the condition also. The transmembrane glycoprotein, $gp91^{PHOX}$, is encoded by *CYBB* on the X chromosome and a mutation in this gene accounts for roughly 60% of cases of CGD. Autosomal recessive mutations can also occur. Mutations in *NCF1* ($p47^{PHOX}$) accounts for roughly 20% of cases and mutations in *CYBA* ($p22^{PHOX}$) and *NCF2* ($p67^{PHOX}$) each account for about 5% of cases. Abnormalities of $p40^{PHOX}$ have not been associated with CGD, perhaps not surprisingly given its limited role as a shuttle protein. In countries with high rates of consanguinity the rate of autosomal recessive CGD exceeds that of X-linked CGD. The severity of the disease and mortality is related to the amount of residual NAPDH oxidase activity and O_2^- production. In general X-linked CGD has a more severe course. Historically, most patients with CGD died by 10 years of age (55). Advances in both management and disease awareness have resulted in a markedly improved life expectancy, however, it is still reduced compared to the general population. The median age of death being 30-40 years of age (56, 58).

The most common sites of infection in CGD patients are the lung, skin, lymph nodes and liver. The most common pathogens *are Aspergillus spp*, *Staphylococcus aureus*, *Burkholderia cepacia*, *Serratia marcescens, Nocardia spp* and *Salmonella (56)*. Invasive fungal infection affect 20-40% of CGD patients.

Aspergillus fumigauts is the most commonly isolated fungal pathogen and it most commonly affects the lungs and chest wall. With the advent of azoles, death related to *Aspregillus fumigatus* is now uncommon (55).

In addition to recurrent and severe infections, evidence of dysregulated inflammation is seen commonly in CGD patients (approx. 70%).The mechanism of the dysregulated inflammation is under debate. The gastrointestinal (GI) tract is by far the most commonly affected site, in terms of inflammatory complications. GI involvement usually start as early as the first 10 years of life (59). Colitis with associated anal disease, such as fistulas and peri-rectal abscess, is the classical finding. Interstitial pulmonary fibrosis has also been described but is a much rare entity than the GI manifestations. Genitourinary and ocular involvement by this inflammatory complication have also been described.

The main treatment for patients with CGD is life-long antibiotic and antifungal prophylaxis. Trimethoprim-sulfamethoxazole and itraconazole have been shown to be effective in reducing the incidence of infections and are well tolerated. IFN-γ prophylaxis has demonstrated varying results in clinical trials and therefore it is generally used on a case by case basis only, reserved for those with ongoing infection despite antimicrobial prophylaxis. Allogenic haematopoietic stem cell transplant (HCT) is a curative treatment for CGD, both the infectious and inflammatory complications. Outcomes following HCT have improved greatly in recent years, in particular owing to advances in our understanding of graft versus host disease and better induction regimens (60, 61). There is still concern regarding late complication in CGD patients as they are often treated with HCT early in life. Gene therapy has been trialled with very little positive clinical results to date, however, trials are still ongoing (62).

1.2 Reactive oxygen species

1.2.1 **Reactive oxygen species: role in disease**

We have already discovered the important role of ROS in bacterial killing by neutrophils. However, oxidants in the form of ROS and RNS (reactive nitrogen species) have a dual function. In addition to their antimicrobial function, they act as signalling molecules that regulate a diverse range of physiological signalling

pathways (63). We will discuss this physiological role of ROS in brief here and subsequently we will focus our attention on the pathophysiological consequences of unregulated oxidants.

Qualities that make ROS good signalling molecules also make them difficult for us to measure in biological systems (64). These molecules are small and can be rapidly synthesised and destroyed. In general they can diffuse across cell membranes. ROS have a narrow concentration range in which they can function effectively as second messengers, above this and they exert their toxic effects. O_2^- is generated when O_2 receives a single electron through the activity of NADPH oxidase (or from mitochondrial respiration). O_2^- quickly dismutates to H_2O_2. The hydroxyl anion can then be formed in the Haber-Weiss reaction. When the Haber-Weiss reaction is catalysed by iron it is called the Fenton reaction. Hypochlorous acid (HOCl) can be formed from H_2O_2 in the presence of chloride, a reaction that is catalysed by MPO. The hydroxyl anion is the most reactive of the ROS, it rapidly combines with a target molecule (64). O_2^- is relatively less reactive and may diffuse a longer distance before combining with a target molecule. Their highly reactive nature can make it difficult, experimentally, to accurately characterise the exact reactive species that is responsible for a given reaction.

ROS can affect the redox state of a compound given their potent oxidising properties. ROS produced by NADPH oxidase are responsible for the induction of tyrosine phosphorylation and inhibition of tyrosine phosphatases (65). This tyrosine phosphorylation can positively or negatively regulate leucocyte tyrosine kinases. Tyrosine kinases are a group of close to 90 enzymes that are capable of phosphorylating tyrosine (an amino acid) on another protein, which leads to conformational changes that typically results in activation of that protein. There are many examples of tyrosine kinase activation through exposure to oxidants. For example, Ltk kinase is activated by an oxidising environment in lymphocytes (66). Exogenous oxidants induce tyrosine phosphorylation of the MAPK kinase family, which is a serine-threonine kinase (65). This can result in the activation of transcription factors and the production of pro-inflammatory cytokines. ROS through their action on protein tyrosine kinases and protein tyrosine phosphatases can participate in the regulation of apoptosis. A number of specific mechanisms for this have been suggested. ROS can activate the transcription of Nf-κB and

mediates the rapid induction of expression of genes involved in the acute inflammatory response. With regard to neutrophils, Nf-κB is involved in the regulation of production of IL-1, IL-6, IL-8 and MIP-2 (67).

ROS in health are limited by the quiescent nature of unstimulated leucocytes and the balancing effects of antioxidants such as, superoxide dismutase, glutathione peroxidase and catalase (68). Situations can arise, whereby the antioxidant capacity of the body are overwhelmed by oxidants. Oxidative stress is said to occur when exposure to oxidants or free radicals reaches sufficient levels to overwhelm antioxidant defences (68). Some have postulated that the lungs are vulnerable to the development of oxidative stress given; the high O_2 environment, its dual blood supply and the potential exposure to environmental pathogens and toxins via the air we breathe (69). Oxidative stress is recognised as a factor in the pathogenesis of COPD (63, 68-72).

ROS are capable of altering the structure of proteins, DNA and lipids. In relation to proteins, ROS can alter specific amino acid residues (e.g. cysteine, methionine, tyrosine) such that the structure, and therefore, function of protein is completely altered. For example, Taggart *et al*., in an in vitro model using plasma purified alpha-1 antitrypsin (AAT) and hydrogen peroxide (H_2O_2), demonstrated that methionine 351 and 358 in the active site of AAT can undergo oxidation. Strikingly, oxidation of either of these methionine residues in AATs active site resulted in a reduced ability of the protein to inhibit NE, therefore losing its primary function (73).

ROS effects on lipids have been predominantly demonstrated in vitro, with the possibility of chlorination of cholesterol and disruption of membrane integrity. Similarly, ROS may damage DNA, for example in the form of double stranded breaks (74). In the latter study, double stranded breaks in DNA caused by oxidative stress was thought to induce apoptosis, cell senescence and proinflammatory responses.

Cigarette smoke is a major source of oxidants. A cigarette contains between 4,000-6,000 compounds that burn at temperatures between 250-950^{o}C (69). This gives a single puff of a cigarette both a gas phase (higher temperature) and a tar phase, which together would contain 10^{17} free radicals. Smoking also decreases

levels of some antioxidants in smokers (vitamin C and E). Cigarette smoke increases neutrophil numbers in the lung and systemically even in the short term (75) and peripheral neutrophils of smokers produce more ROS. Phagocytes from the lungs of smokers release more ROS than those of non-smokers. In the subset of smokers who develop COPD there is a sustained recruitment of inflammatory cells into the airway, even following the cessation of cigarette smoking. Neutrophils are found with increased abundance in COPD airways and these represent an ongoing source of ROS.

There are a number of methods that have been used to demonstrate the presence of oxidative stress in COPD. Studies have directly measured ROS or antioxidants or products of oxidation reactions. These have been measured in various body compartments (e.g. plasma, sputum, BAL). H_2O_2 is increased in the exhaled breath condensate (EBC) of COPD patients (76). Isoprostanes are increased in the EBC of COPD patients also. Isoprostane is a product of the oxidation of arachidonic acid in vivo(76). Malondialdehyde (MDA) is another product of fatty acid peroxidation and thus considered to be a marker of oxidative stress. Zeng *et al.* have shown that COPD patients have increased expression of MDA in induced sputum, which further increased during exacerbation. This was associated with down regulation of glutathione (GSH) and superoxide dismutase (SOD) in induced sputum. The same findings were present in the plasma of these individuals in the same study of Zeng *et al.* (77). There is clearly robust evidence for the presence of an imbalance of oxidants and antioxidants in individuals with COPD. As with COPD itself, there is a heterogeneity to these findings. It is also unclear how big a role oxidants play in the propagation of lung disease in COPD. Endogenous oxidative stress is unlikely to be an initiating factor in the development of the disease but it is possible that it perpetuates the disease following an initial insult. This is relevant to individuals with alpha-1 antitrypsin deficiency (AATD), as they develop lung disease with many similarities to COPD.

Oxidants increase mucus production by epithelial cells in culture and impair cilia function (78). They also promote increased permeability of the bronchial epithelium (79). Some of what we understand of the pathophysiology of COPD is a result of the discovery of the genetic disease alpha-1 antitrypsin deficiency (AATD). These individuals have a severe deficiency of the serine protease inhibitor alpha-1

antitrypsin (AAT) and have an increased susceptibility to develop emphysema. This is largely due to the unopposed action of NE due to reduced release of polymerised AAT from hepatocytes. AAT is also susceptible to oxidation and inactivation (73). Therefore, in individuals with COPD with high levels of oxidants and lower levels of antioxidants the protease/antiprotease imbalance may be an aspect of the pathogenesis of the disease. There is also, as mentioned, an increased burden of neutrophils in the airway of COPD patients, further increasing the protease burden. CS can also oxidise and reduce the anti-elastase activity of another airway protease inhibitor, serine leucocyte protease inhibitor (SLPI) (80).

Carbonyl stress is the accumulation of reactive carbonyls and the subsequent protein carbonylation. Reactive carbonyl species are produced as a consequence of oxidation of carbohydrates, lipids and amino acids. Protein carbonylation is present in smokers and COPD patients (81). Reactive carbonyls are known to be involved in triggering various signalling pathways including apoptosis, protein function and in general disrupting normal cell function. Autoantibodies have also been found against carbonyl-modified self-proteins in COPD serum that increase with disease severity (82). It is not known if this autoantigen is destructive or pathogenic in COPD but there is growing evidence of the presence of autoantibodies in COPD.

CS upregulates MUC5AC expression by a well described mechanism. Oxidant generated hyaluronan fragments up regulate both MUC5AC and MUC5B in airway epithelium (83). MUC5AC and MUC5B are both gel-forming mucins responsible for forming the gel-like mucus layer that sits on top of the airway surface liquid (ASL), which coats the bronchial epithelium. Mucins are high molecular weight glycoproteins (84). MUC5B is the major mucins in the sputum of individuals with COPD (85)

Excessive oxidative stress may be deleterious to alveolar macrophage (AM) function. It has been shown that oxidation, by cigarette smoke, can lead to impaired phagocytosis of bacteria and impaired efferocytosis of apoptotic cells (86). Impaired AM mediated efferocytosis in COPD could be particularly damaging in COPD or AATD as neutrophils are persistently recruited into the airways in both of these conditions.

1.2.2 **Antioxidants: role in preventing disease.**

Asoutlined in section 1.2.1, ROS are balanced by the action of endogenous antioxidants. These are present in abundance in healthy individuals. They may be found intracellularly, circulating in the plasma or in the body fluids of various body compartments (e.g. epithelial lining fluid) (87). Antioxidants may be classified as either enzymatic or non-enzymatic oxidants. Non-enzymatic antioxidants include vitamin C, vitamin E, albumin, mucin, alpha-2 macroglobulin and glutathione. Enzymatic antioxidants include SOD, catalase and glutathione peroxidase. Albumin is the most abundant plasma protein. It is found in the plasma in great abundance (40g/L). Albumin may bind metal ions and scavenge free radicals through its thiol groups.

Given the links that have been demonstrated between oxidative stress and the development and progression of COPD, targeting this with antioxidant drug therapy has been the subject of a significant body of research (69). There are no therapies that have been shown to prevent the progression of COPD and such treatments are much needed. Some of the approaches that have been undertaken to target oxidants include; thiol compounds (including N-acetyl-cysteine), esters, carbocysteine, inducers of glutathione biosynthesis, Nrf2 activators, antioxidant vitamins (vitamin A,E,C and β-carotene), superoxide dismutase and glutathione peroxidase mimetics (89). A number of these products have undergone clinical trials. N-acetyl cysteine (NAC) is probably the most widely studied antioxidant in the treatment of COPD. In vivo NAC is rapidly metabolised to cysteine. This cysteine can scavenge oxidants via its thiol group. This cysteine is also available for GSH production. NAC can also reduce disulphide bonds which explains its mucolytic properties. NAC was studied in a Chinese COPD population in the PANTHEON trial (90). It was shown to significantly reduce exacerbation frequency when given in high doses in aerosol form via nebuliser. In stable COPD patients taking NAC 600mg daily they had reduced EBC levels of H_2O_2 suggesting an antioxidant effect in vivo (88).

Carbocysteine is a more novel thiol than NAC. It also has mucolytic properties. When taken orally it reaches the lung tissue effectively and has demonstrated oxidant scavenging capacity. Carbocysteine is used as a mucolytic in clinical

practice, is undergoing further trial for its use as an antioxidant in chronic bronchitic phenotype of COPD and in idiopathic pulmonary fibrosis patients. Both carbocysteine and NAC currently have U.S food and drug administration (FDA) approval for their use as antioxidants. Nrf2 activators, vitamin C and vitamin E supplementation and glutathione replacement has been studied also in COPD with disappointing results (70).

So there is clear evidence that in COPD there is an increase in oxidative stress, which appears to play an important role in the pathogenesis of the disease. There are some small molecule targets that have been developed to target oxidants and some antioxidants have even shown clinical efficacy in randomised controlled trials (RCTs). The vast heterogeneity of COPD, including its clinical presentation, clinical course and the variation in proteins, enzymes, molecules and cells involved in COPD are a challenge when searching for potential therapies. There has been very little study on the role of oxidative stress in AATD individuals. The study of oxidative stress in AATD may shed more light on mechanisms underlying the pathogenesis of both diseases.

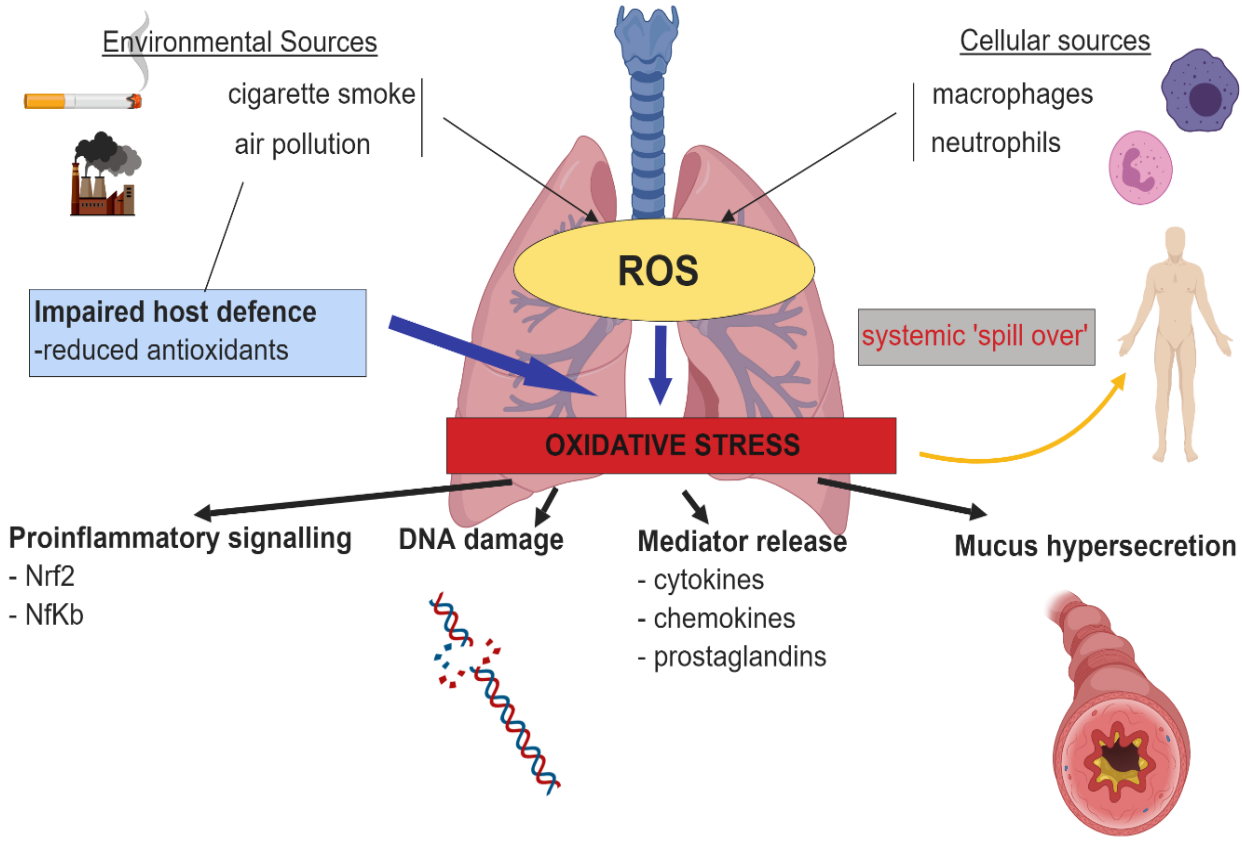


Figure 1-3: The contribution of oxidative stress to the development of COPD.

Cigarette smoke is a source of oxidants and can attract inflammatory cells into the lungs. The NADPH oxidase is also a source of ROS. Inhaled oxidants can act on cell surface pattern recognition receptors to induce the production of pro-inflammatory cytokines and chemokines. In smokers, the antioxidant defence of the lung is impaired which leads to a state of oxidative stress. There is 'spill over' of markers of oxidative stress into the circulation of smokers and patients with COPD. The consequences of unopposed oxidants are many fold, they include; DNA damage, the activation of pro-inflammatory signalling pathways with the release of inflammatory mediators; mucus hypersecretion can occur with production of MUC5AC and MUC5B. DNA: deoxyribonucleic acid, Nfkb: nuclear factor kappa-light-chain-enhancer of activated B cells, NrF2: Nuclear factor erythroid 2–related factor 2, ROS: reactive oxygen species.

1.3 Alpha-1 antitrypsin

1.3.1 **Alpha-1 antitrypsin: The protein**

Alpha-1 antitrypsin (AAT) is a member of the serine protease inhibitor (serpin) superfamily. AAT is the archetypal member of this family of protease inhibitors that includes alpha-1 antichymotrypsin, antithrombin, C1 inhibitor and others (89, 90). AAT was named in 1955 by Schultze and colleagues, who discovered its presence in the alpha globulin fraction of plasma purified proteins, and also noted its ability to inhibit trypsin (91). Much has been learned about AAT in the proceeding 60 years since its discovery. Its ability to inhibit the serine protease, NE, is unrivalled and AAT is considered the major NE neutralising protein of the lower respiratory tract. AAT also has other biological functions that have been extensively researched since its discovery (92).

About 80% of AAT that is found in plasma is produced by hepatocytes. AAT is also synthesised by neutrophils, macrophages and epithelial cells of the lung, kidney, cornea, intestine, pancreas, ovaries and testis (93-96). The normal plasma concentration of AAT is 0.9-1.75g/L and it has a half-life of approximately 5 days once in the circulation. The rate of synthesis is 34mg/kg/day, with about a third of circulating AAT being degraded daily (97). From the circulation, AAT passes into many body compartments, likely via transcytosis across the endothelial cell layer (98). AAT is found in most body fluids but its distribution is not uniform. AAT is found in urine, tears, saliva, semen and breast milk (96, 99-101). In epithelial lining fluid from BAL samples concentrations are about 10% of that of the circulation (102).

During inflammation, circulating levels of AAT can increase 3-4 fold. For this reason, AAT is referred to as an acute phase protein. C-reactive protein, complement and ferritin are other acute phase proteins that are up-regulated during times of inflammation or infection. Transferrin and albumin are examples of negative acute phase proteins, their level in plasma falls with the presence of inflammation (103). The liver is not the only source of increased AAT production during inflammation. It is reported that AAT expression by lung tissue, induced by cytokines, can increase by up to 30-fold (104). The differences in tissue expression are due to tissue-specific AAT promoters. In hepatocytes, under basal

conditions, AAT is regulated by the actions of hepatocyte nuclear factor 1a and 4 (HNF1a, HNF4; tissue-specific transcription factors) on the hepatocyte promoter (105). Acute phase AAT expression by hepatocytes is regulated by the interaction of IL-6 and oncostatin M with an enhancer sequence. In the latter, oncostatin M stimulation of hepatocyte AAT production occurs following an interaction between the hepatocyte promoter and an element at the 3′-end of the AAT gene, which is oncostatin M responsive. This is a signal transducer and activator of transcription 3 (STAT3) mediated process (106). IL-1, TNFα and lipopolysaccharide (LPS) can also stimulate AAT synthesis by hepatocytes. The signalling pathways activated by these cytokine receptors on hepatocytes are either JAK/STAT or MAPK pathways (107).

The AAT gene (SEPRINA1) is 12.2kb long, consists of seven exons and six introns. SERPINA1 is located on the long arm of chromosome 14q31-32.2 (97, 108). The exons are designated; Ia, Ib, Ic, II, III, IV and V, with the four coding exons being II, III, IV and V. Exon V contains the so called ‘active site’ (109). AAT undergoes posttranslational glycosylation, whereby, three oligosaccharide side chains are added to the surface of the protein at three distinct asparginine residues (position 46, 83 and 247). The glycans are important initially for recruitment of the chaperone molecules that regulate protein folding and secretion. Glycans also increase the stability and solubility of AAT, thus giving it a longer half-life in the circulation (110). The carbohydrate side chains of AAT account for 15% of the molecules weight. Variations in the oligosaccharide side chains give rise to different glycoforms of AAT. These different glycoforms have different charges and effect the migration of the protein on an isoelectric focusing (IEF) gel (111). N-glycans of AAT are composed of sialic acid, mannose, galactase, fucose and N-acetyl glucosamine, which exist in bi-, tri- or tetra-antennary structure. Alterations in the glycan structures of AAT in individuals with pneumonia have been shown to alter the immune regulatory function of AAT (112).

The mature AAT protein secreted into the circulation is a 394-peptide, 52kDa protein with three carbohydrate side chains (90). By crystallography, AAT is a globular structure 6.7nmx3.2nm in dimensions. The AAT polypeptide chain consists of nine α-helices (A-I) and three β-pleated sheets (A-C). The molecule contains three internal salt bridges. The structure of all serpin superfamily

members bare many similarities. The reactive centre loop (RCL) of AAT contains a methionine at position 358 and a serine at position 359, that are held together under tension (113). Thus, AAT is said to be in a metastable state and cleavage of the RCL is energetically favourable to the protein. The predominant function of AAT is believed to be the inhibition of proteases. This process is particularly important in the lung. AAT is capable of inhibiting the neutrophil proteases NE, PR3 and Cath G (114). The RCL is recognised as a substrate by the active site of the target enzyme (e.g. NE). This results in the formation of a covalently bound serpin:enzyme complex. Once bound, NE cleaves AAT's RCL (at Met 358), which releases stored potential energy, some of which is used to disable the catalytic machinery of the protease. Cleavage of the RCL results in a conformational change, likened to a mousetrap action, which flips NE to the opposite end of the AAT molecule, rendering the protease inactive and AAT unable to bind further NE. This complex is then recognised by serpin-enzyme complex receptors found on hepatocytes, due to the exposure of new binding sites revealed by cleavage of the RCL, and the complex is removed from the circulation. NE and AAT bind in a 1:1 (i.e. equimolar) ratio. The proteolytic activity of AAT can be reduced by a number of proteases and also by oxidation of residues in the RCL. Oxidation of both met 358 and met 351 have been shown to reduce the NE inhibiting capacity of AAT dramatically (73). MMP-9 can also proteolytically inactivate AAT (115).

It is now well accepted that AAT does not simply function as a serine protease inhibitor (116). It also functions to inhibit some cysteine proteases and metalloprotease (107). In addition to this, a large body of work has revealed the role of AAT as an effector of immune cell function, in the regulation of apoptosis and as an antimicrobial. The immune-modulatory functions of AAT have been studied, in vitro and in vivo, in a range of diseases including, alpha-1 antitrypsin deficiency (AATD). The study of individuals with AATD has perhaps been most instrumental in delineating the many functions of AAT in both health and disease.

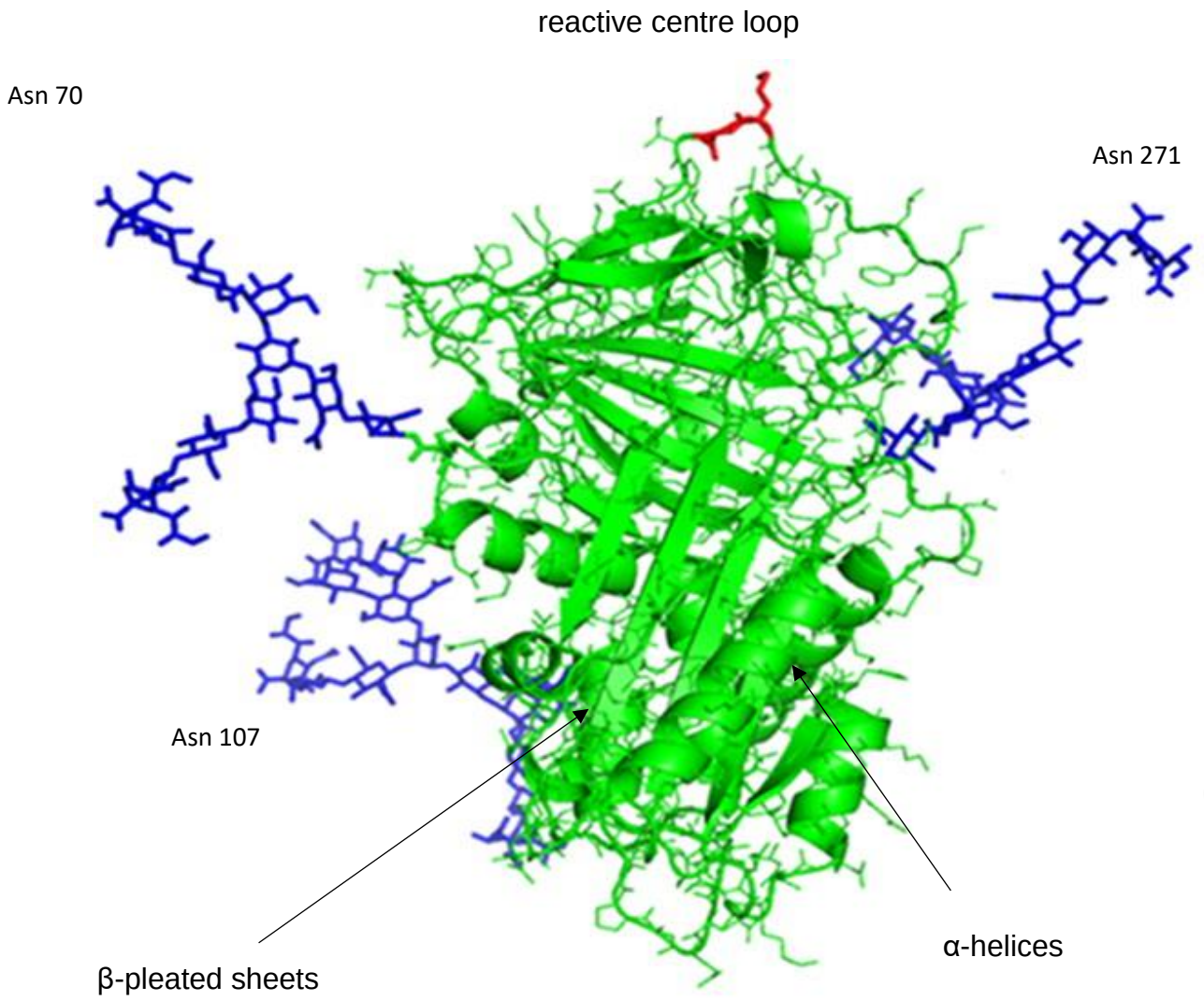


Figure 1-4: The 3D structure of AAT.

Diagrammatic representation of the 3D ribbon structure of AAT. The protein backbone is shown in green, with the β-pleated sheets represented as arrows and α-helices shown as coils. The reactive centre loop (RCL) is shown in red and the three glycan side chains are in blue. (McCarthy *et al.,* 2014 (1))

1.4 Alpha-1 antitrypsin deficiency

1.4.1 **History of alpha-one antitrypsin deficiency**

AATD was discovered in Malmo, Sweden by Carl-Bertil Laurell and Sten Eriksson (117). Laurell had spent the preceding years refining a paper electrophoretic technique for the separation of plasma proteins. He observed a missing α1 band on a number of these strips. At that time globulins could be separated into α1, α2, β1, β2 and γ- globulins. He thought initially that this was due to bacterial contamination of the samples and neuraminidase activity. However, no other glycoprotein was affected in its ability to migrate towards the anode on his electrophoretic paper in these samples. So it seemed that it was a real finding. The missing fraction corresponded with AAT. AAT had been isolated from human serum in 1962 by Schultze *et al.* (91). The protein was named AAT due to its ability to inhibit trypsin. Laurell and Eriksson could deduce from work by Jacobson *et al.*, that the missing fraction in their samples corresponded to AAT (118). They coined the term alpha-1 antitrypsin deficiency (AATD) in their paper of 1963, in which they described the clinical details of five individuals who were missing this α1 band. Three of these individuals had obstructive lung disease, in the form of; chronic bronchitis, bronchiectasis and emphysema. One young woman had no lung disease and an elderly woman had no lung disease but had severe rheumatoid arthritis. The authors suggested a link between AATD and 'degenerative pulmonary disease'.

Two years after this 1963 paper, Eriksson published a case series of 33 deficient homozygous probands (119). The type of inheritance was apparent from this study (Mendelian inheritance, autosomal recessive) and intermediate AAT levels (measured as total trypsin inhibitory capacity) were felt to represent heterozygotes. It was clear from the series of 33 probands that there was a high degree of variability in the clinical manifestations of AATD. Some patients had no apparent lung disease whilst others had obstructive lung disease of early onset. There was significant ascertainment bias in this study population. Eriksson noted a basal predominance to the disease in the lungs of affected patients and a pattern of panacinar emphysema (based on post-mortem and radiological findings). Further

clinical features noted in this group were, significant weight loss and a lack of hypercapnia. Patients with bronchiectasis were also observed.

Interestingly, in their seminal paper, Laurell and Eriksson demonstrated that AAT in deficient serum had decreased immunoelectrophoretic mobility, suggesting a structural abnormality of the protein. The reason for this slowing in the electric field was not known at the time. We now know that it is related to a less negative net charge of the mutant protein, as discussed later in this introduction.

The inhibiting capacity of AAT on trypsin was not considered to be of significance in the pathogenesis of emphysema in AATD. Trypsin did not induce emphysema in animal models. In the early 1970's Gross *et al*., demonstrated the presence of emphysema in mice after the direct intra-tracheal installation of papain (120). Papain is a protease derived from papaya fruit. The findings in mice was subsequently repeated in guinea pigs (121). It was later discovered that the ability of papain to induce emphysema was related to its ability to degrade elastin; a lung connective tissue protein (122). Further work demonstrated the ability of other elastases to produce histologic and physiologic findings of pulmonary emphysema in various animal models. None of these elastases, however, were found in humans. Janoff *et al*. were the first to describe human NE (123). They discovered this in the primary granules of neutrophils and found it to be a potent elastolytic protease. Soon after they discovered NE they demonstrated, in vitro, the ability of NE to digest lung elastin (124). In dogs, NE was shown to induce emphysema (125). Over the following years it was revealed that AAT was a potent inhibitor of NE, with a high association rate constant and inhibitory constant (126). There was a 1,000-fold more rapid rate of association between NE and AAT than between AAT and trypsin. The key role of unopposed NE activity in the development of emphysema in AATD became unequivocal (127).

In the years following the discovery of AATD, Laurell and Eriksson did not link the risk of emphysema in AATD with cigarette smoking. This was described in 1978, when smoking was demonstrated to be an important prognostic factor in AATD individuals (128). The discovery that NE could produce emphysema-like changes in animal models and that NE was inhibited by AAT was a major advance in our understanding of emphysema in AATD. However, the importance of this finding for

emphysema in those replete in AAT was less clear. That was until the discovery in 1977 by Janoff *et al*. that cigarette smoke condensate had the ability to disrupt the elastase inhibiting capacity of AAT (129). Further studies revealed that oxidation of specific residues of the active site of AAT (methionine 358 and 351) could reduce AATs ability to inhibit elastase (73). So these findings strengthened the concept of a protease:anti-protease imbalance in the development of emphysema in those deficient and replete in AAT. The latter group having a functional deficiency of AAT due to oxidation of methionine residues by cigarette smoke.

In 1969, Sharp *et al*. described a series of children with AATD who developed fulminant liver failure and death. At autopsy, the livers contained inclusion bodies that almost certainly represented misfolded AAT protein, polymerised within the ER of hepatocytes (130). These can be identified (although not specifically) on biopsy by periodic acid Schiff (PAS) staining followed by diastase treatment. The liver disease in AATD was found not only to affect children but could affect a small proportion of adults with the condition also. There was also an increased risk of hepatocellular carcinoma in AATD individuals (131).

As electrophoretic techniques advanced, with the introduction of potato starch gel electrophoresis and as sampling increased, additional abnormal AAT proteins were discovered. A naming system was developed for the AAT proteins based on their migration within a starch gel (132). This was called the protease inhibitor (Pi) system. It was set up in such a way that the normal AAT protein migrated to the middle of the gel and was named Pi*M. The originally identified abnormal protein barely migrated at all in the starch gel (due to a change in charge, as mentioned previously) and is designated PI*Z. The autosomal co-dominant mode of inheritance of AATD was discovered by analysis of AAT protein migration on a starch gel. In heterozygotes both proteins are expressed and found in the serum, and therefore both an M and a Z band will be seen on the starch gel (132).

1.4.2 **Alpha one-antitrypsin deficiency: Epidemiology**

AATD has been described in all populations, races and in many countries throughout the world. It is generally accepted that prevalence is highest in Scandinavia, Portugal and Spain (133). Information on prevalence is generally derived from population-based screening studies or targeted detection programmes. A 2013 U.S. review outlined the fact that AATD is under-recognised with only a small minority of expected cases being diagnosed clinically (134). In a study by Silverman *et al*. in St Louis, they randomly tested 20,000 blood specimens from the St Louis blood bank (blood donations) and detected 7 individuals who were PI*ZZ (135). Given the population of St Louis, this would mean an estimated prevalence of 700 PI*ZZ individuals. From surveys of local doctors they identified only 28 recognised PI*ZZ cases. Another study gave estimates of the prevalence of PI*ZZ in the US and 21 other countries to be 100,000 based on epidemiologic surveys, while the international and US alpha one registry had identified only 2.4% of these individuals (133).

It is understandable that heterozygotes may not develop symptoms and therefore may not come to attention for diagnostic testing. This likely accounts for much of the under-diagnosis (134). However, there is also evidence that in symptomatic individuals there is a long delay to diagnosis. Stoller *et al*. showed that the mean delay to diagnosis was 7.2 years and that 50% of patients reported seeing at least three physicians before a diagnosis of AATD was confirmed (136). Campos *et al.* in 2005 measured a mean delay to diagnosis of 8.3 year (137).

A number of population based screening studies have been undertaken to study the prevalence of AATD. The largest of these were performed in Sweden (n=200,000 new-borns) and Oregon, USA (n=107,000 new-borns). In the Swedish study, 95% of all infants born between 1972 and 1974 were measured (138). The estimated prevalence of PI*ZZ was 1 per 1,639 individuals. In the Oregon based study, in which 107,038 infants were screened with heel stick blood specimens, it was 1 per 5,097 (139).

Targeted detection programmes have also been undertaken. In 2010 in Poland an AATD screening programme was established targeting patients with respiratory

disease. Following four years of data collection 2,500 patients with COPD had been screened. 13% of this population had at least on AATD mutant allele. The frequency of PI*ZZ in the COPD population was 1.6% (more than 15 times than estimated in the general population in Poland). Interestingly, of all the patients diagnosed with PI*ZZ through this targeted detection programme, only 1/3 had COPD as their clinical presentation, while 11% were referred for testing due to asthma and 13% for bronchiectasis (140).

The Alpha One Foundation of Ireland launched the National AATD targeted detection programme at Beaumont Hospital, Dublin in 2004. In 2011, Carroll *et al*. published data from their first 3,000 individuals screened for AATD, they compared this to 1,100 samples from the Trinity Biobank, Dublin that represented a random sampling of the general population. The main reason for referral for testing was a diagnosis of COPD (50%), while family history accounted for 14% of those tested and cryptogenic liver disease 9%. The authors found that the Z allele was more commonly found than the S allele in the targeted detection programme suggesting the more prominent role of the Z allele in disease development. The estimated prevalence for PI*ZZ in the general population was 1 per 2,104 of population (based on the biobank samples randomly collected). This suggested that for a population of 4.24 million there would be expected to be 2,015 ZZ individuals in Ireland. This would give Ireland one of the highest prevalence's of the disease in the world (141).

The American Thoracic Society/European Respiratory Society recommend testing for AATD in the following populations: all COPD patients, all non-responsive asthmatics, cryptogenic liver disease and first degree relatives of AATD individuals (142).

1.4.3 **The Z allele.**

The Z allele is the most common mutation resulting in severely deficient levels of AAT (90). It is caused by a single point mutation in SERPINA1 that results in the substitution of glutamic acid for lysine at position 342 (Glu342Lys) (143). The other common deficiency variant is the S allele. This results from a single point mutation also, with a resultant substitution of glutamic acid for valine at position 246

(Glu246Val). Null mutations also occur, they are rare and their diagnosis can be more difficult due to the interpretation of isoelectric focusing. Null mutations are important because they may result in more severe emphysema (144). Null mutations result from frameshift or nonsense mutations that results in a premature stop codon in the coding region of the mRNA. This results in the production of a truncated protein which in some instances results in removal of the aberrant protein at the cell level (111). Individuals with null mutations do not develop liver disease as there is a complete lack of the AAT protein and so no accumulation of AAT in hepatocytes.

A characteristic feature of the AAT protein produced by a Z allele (Z-AAT) is its ability to misfold and to form polymers (17). Z-AAT polymers form when the reactive centre loop (RCL) links to the b-sheet A of an adjacent Z-AAT. This is referred to as loop-sheet polymerization. Due to accumulation of polymerized Z-AAT in the endoplasmic reticulum of hepatocytes in PI*ZZ individuals, serum AAT levels are very low (113). Hepatocytes of PI*ZZ individuals secrete only about 15% of Z protein into the plasma. As well as resulting in reduced serum levels of AAT, Z-AAT polymers in the liver can cause unfolded protein response (UPR) and impaired autophagy in the liver, which can lead to cirrhosis (145). The Z polymers in hepatocytes are visible by electron microscopy and stain PAS positive.

Z polymers can also be found in the airways (epithelial lining fluid, ELF), as well as within monocytes, and neutrophils (143). They can cause endoplasmic reticulum (ER) stress in monocytes via UPR, as well as in neutrophils leading to apoptosis (146). In the airways they can act as a chemoattractant for neutrophils. Z-AAT polymers have been visualized at skin biopsy of AATD individuals with panniculitis (147).

1.4.4 **The effects of AAT on neutrophil function in health and disease.**

It is well recognised that AAT impacts on immune cell function. This includes neutrophils, macrophages, lymphocytes and eosinophils. There has been a large body of pioneering work in this field in recent years (148-153). However, as this thesis is focussed on neutrophil function the following text will discuss the impact of AAT on neutrophil activity predominantly (Figure 1-5).

AAT is first and foremost an inhibitor of neutrophil derived serine proteases. These include NE, PR3 and Cath G, with AAT having the highest affinity for NE, then PR3 and finally Cath G. Some serpins function primarily as protein carriers (e.g. thyroglobulin, corticotrophin binding globulin) and the similar conformational arrangement of AAT makes it a suitable binding partner for a range of plasma constituents. Another characteristic of AAT that lends it immunomodulatory capabilities are its glycosylation profile which can alter its electrostatic binding capacity. AAT is thought to be produced in small amounts by neutrophils. It was felt that perhaps it was then stored in the primary granules of these cells. It is more likely that it is localised to the plasma membrane where it co-localises with FcγRIIIb (154).

There is an accumulation of neutrophils into the lungs of individuals with AATD and this has been characterised to be directly related to AAT in a number of ways. Hubbard *et al.* (155), demonstrated that AATD alveolar macrophages released significant quantities of LTB_4 (nearly three times more than controls) and that this acted as a neutrophil chemoattractant. It was shown that macrophages could be stimulated to release more LTB_4 by exposure to NE and this effect could be reversed by purified human AAT. Further evidence of the interaction of AAT with LTB_4 came from a study by O'Dwyer *et al.* that demonstrated that AATD individuals have increased neutrophil adhesion and degranulation in response to LTB_4. The authors showed that AAT can bind LTB_4 and modulate it's interaction with its receptor, BLT1. Circulating levels of LTB_4 were found to be reduced in AATD individuals receiving intravenous AAT augmentation therapy (156).

Bergin *et al.* (2010) (154) gave us some insights into the mechanism by which the AAT protein regulates neutrophil chemotaxis. The authors also demonstrated how neutrophil chemotaxis is augmented in AATD and corrected by AAT replacement

therapy in AATD individuals. In this study, it was demonstrated that the AAT protein binds to IL-8 and prevents binding to its cognate receptor CXCR1, resulting in reduced neutrophil migration. IL-8 is well known to be a neutrophil chemoattractant through its interaction with the CXCR1 receptor on the neutrophil surface (157). Bergin *et al.* found that in patients with AATD there is increased neutrophil migration, as IL-8 is not bound to AAT, due to serum deficiency of the latter. In the same study, the authors showed that treatment with AAT augmentation therapy reduced neutrophil migration by the above mechanism.

A further mechanism of neutrophil recruitment involving AAT was elucidated by Bergin *et al*. This mechanism involved the inhibition of ADAM-17 (a membrane-associated metalloprotease) activity by AAT in response to soluble immune complexes and inhibiting the release of the FcγRIIIb receptor from the cell membrane. The interaction of IL-8 and AAT was likely related to its electrostatic activity as recombinant AAT (non-glycosylated) did not bind to IL-8 with the same affinity as glycosylated AAT (154). AAT has also been shown to inactivate calpain-1, thereby inhibiting neutrophil directional migration (158). Haemin is not found in plasma in large quantities in health but is found in the circulation in certain disease states. Haemin is the oxidised form of haem which is liberated from haemoglobin (i.e. haemolysis). Haemin has been shown to induce neutrophil migration and ROS production. In vitro, AAT has been shown to bind free haemin. It does not do so with as high an affinity as other plasma proteins, such as haemopexin. Janciauskiene *et al.* showed that AAT can ‘scavenge’ free haemin, and thus reduce haemin induced ROS production, IL-8 release and neutrophil migration (159).

AAT has been shown to modulate the production of ROS by neutrophils in an in vitro model (160). This may be by a mechanism of oxidation of methionine residues as shown by Taggart *et al* (73). The mechanism by which AAT may modulate the production of ROS by neutrophils has not as yet been elucidated. AAT has also been shown to interrupt ligand receptor interaction between TNFα and TNFR1/TNFR2 and modulates neutrophil degranulation by this pathway (161). Hurley *et al.* showed, in vitro, that plasma purified AAT can reduce the percentage of TNFα-induced neutrophil apoptosis. In this study, it was shown that AATD neutrophils have exaggerated apoptosis and that AAT therapy in these individuals

diminishes neutrophil apoptosis by reducingADAM-17 activity, suggesting a role for endogenous AAT in the regulation of neutrophil apoptosis (146).

Some of the anti-inflammatory effects of AAT appear to be independent of its antiprotease activity. Oxidised AAT, can still inhibit the LPS-induced production of IL-8 and TNFα in human monocytes and in freshly isolated human neutrophils and LPS challenged mice (162). As mentioned previously, the glycosylation status of AAT is of relevance to some of its anti-inflammatory effects. AAT has some antimicrobial activity also. An example of this is the ability of AAT to reduce human immunodeficiency virus-1 (HIV-1) infectivity and block HIV-1 production. VIRIP (a 20 residue fragment of AAT) binds to gp41, a fusion peptide of HIV-1 (163).

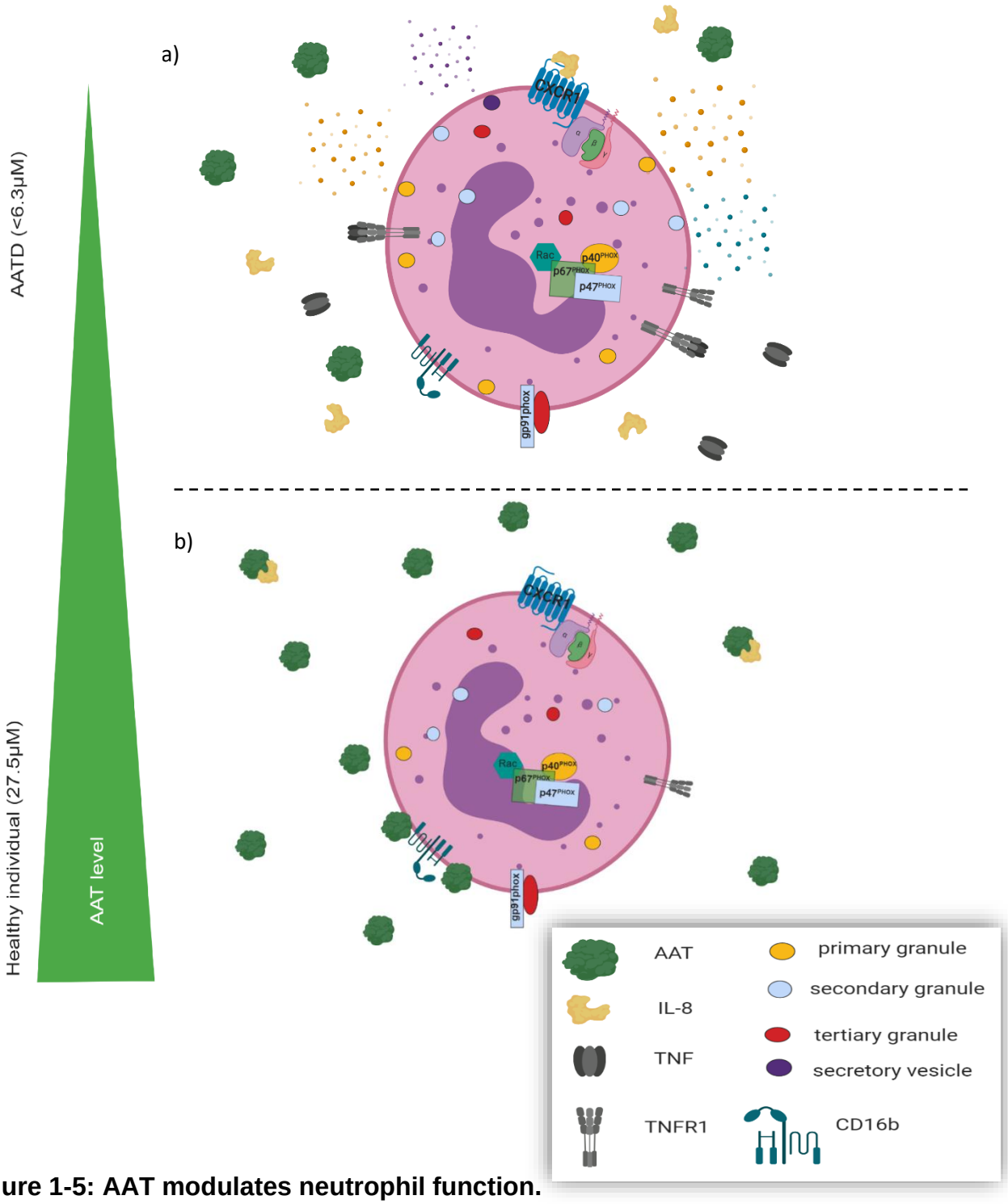


Figure 1-5: AAT modulates neutrophil function.

a) In a low AAT environment IL-8 will engage with its receptor to promote neutrophil chemotaxis. TNFα will also engage with TNFR1 and cause secondary and tertiary granule release. ADAM-17 is able to cleave CD16b in response to soluble immune complexes inducing chemotaxis. Uninhibited NE is available and can signal to macrophages to release LTB_4 resulting in further recruitment of neutrophils. b) In a high AAT environment the glycoprotein moieties of AAT bind IL-8 preventing engagement with CXR1. AAT also inhibits ADAM-17 thus preventing cleavage of CD16b and activation of neutrophil chemotaxis. AAT reduces available soluble TNFα for engagement with TNFR1.

1.4.5 **Diagnostic testing for AATD**

In general, the diagnosis of AATD is made through one of three diagnostic pathways; 1) testing of individuals with clinical signs or symptoms consistent with a diagnosis of AATD (e.g. early onset emphysema), 2) predispositional testing (e.g. family history) and 3) targeted detection (i.e. COPD). Initial diagnostic testing involves the quantification of plasma AAT levels using nephelometry. Further testing is carried out if the plasma level of AAT is found to be low. The next test to be performed is either genotyping or isoelectric focusing (protease inhibitor typing) and different centres will have different testing pathways. Genotyping would initially be performed looking for the two most common disease associated alleles (S and Z) through the use of quantitative polymerase chain reaction (qPCR) and allele specific amplification. In some countries, such as the USA and Germany, genotyping is performed from DNA extracted from dried blood spots. In a patient with low circulating levels of AAT and genotyping negative for S and Z allele the next diagnostic test would be isoelectric focusing of serum. Isoelectric focusing (IEF) is a technique of separating proteins based on their charge. In some centres, isoelectric focusing is performed as the initial test, following the finding of a low AAT level. Isoelectric focusing requires significant skill and expertise in interpretation and will not identify null mutations, thus results must be interpreted with the clinical details of the patient taken into account. Each of the phenotypes of AATD exhibits a characteristic pattern (that can be compared to a reference), on an IEF gel, due to the charge of the protein. Occasionally, sequencing of SERPINA1 is required if the above testing proves inconclusive (107).

1.4.6 **Clinical manifestations of AATD**

The predominant clinical manifestations of AATD are lung disease, liver disease and rarely panniculitis (90). Granulomatosis with polyangiitis is also a rare association (164). The risk of lung disease in AATD is dependent of the mutation in SERPINA1, environmental exposures and probably other genetic factors. Pi*ZZ is the most common deficiency phenotype that is associated with lung disease. Even those with Pi*ZZ may lead their entire lives without any clinical evidence of

AATD related disease. Pi*ZZ never smokers who were identified by screening had a similar mortality rate to control never smokers in a Swedish group (165).

When lung disease is present, COPD is the most common manifestation in individuals with AATD. Smoking is the major factor that accelerates the development of COPD in AATD individuals. COPD is defined as a ‘common, preventable, treatable disease that is characterised by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by exposure to noxious particles or gases’ (166). Although widely varied, the onset of respiratory symptoms in an individual PI*ZZ is between age 40 and 50 years (167). The lung disease seen in AATD is classically emphysema, which is a histological description for destruction and dilatation of the airways distal to the terminal bronchioles. Emphysema results in reduced surface area for gas exchange to occur and the loss of elastic recoil in the distal airways that causes airway collapse and subsequent dynamic hyperinflation. It is these two mechanisms that cause dyspnoea and impaired exercise tolerance, in patients with emphysema.

The emphysema of individuals with AATD has some characteristic differences to the emphysema seen in smokers replete with AAT. In AATD individuals the pattern may be panacinar and it classically affects the lung bases (Figure 1-6). There is, however, heterogeneity amongst these changes and upper lobe predominant and centrilobular emphysema may be seen (168). When lung disease is present spirometry typically shows an obstructive defect. In non-smokers, the likelihood of having obstructive spirometry increased after the age of 50, but was found to be normal up to that age. In the Swedish registry only 28% of never smokers fulfilled spirometric criteria for COPD diagnosis while in ever-smokers this was 72%, in PI*ZZ individuals (169). In the NHLBI registry, 61% of individuals with AATD demonstrated reversibility and 35 % reported having been diagnosed with asthma (168).

Estimates of the prevalence of bronchiectasis have varied significantly. The NHLBI registry reported 2% of patients having bronchiectasis (168) while another study reported that amongst symptomatic PI*ZZ individuals the incidence of radiologically diagnosed bronchiectasis was 95% (170). The Bronchiectasis

Research Registry has published some findings on the characteristics of bronchiectasis in AATD and in idiopathic bronchiectasis. This is a US based registry covering 13 different sites. 58 adults with severe AATD were included (64% SZ, 35% ZZ). The most striking finding of this study was that 62% of AATD individuals had cultures that were positive for non-tuberculous mycobacterial (NTM) disease, compared to 45% of idiopathic bronchiectasis (171). This was in accordance with findings of Kim *et al.* who found in their cohort of 85 patients with NTM disease that 15% had an abnormal AAT phenotype (172).

Smoking status of PI*ZZ individuals affects the time of onset, severity, rate of decline of lung disease and mortality rate (167). The most common cause of death in PI*ZZ individuals is respiratory failure, followed by cirrhosis. PI*ZZ never smokers show fewer deaths from emphysema but relatively more deaths from cirrhosis (139). In a 2016 study from the Swedish national AAT deficiency registry PI*ZZ individuals had excess mortality compared with the Swedish general population. PI*ZZ individuals had increased mortality from the following diseases: respiratory disease, primary liver cancer, complicated colon diverticulitis and pulmonary embolism. Mean age at death was lower in ever smokers. Smoking status of the general population in this study was not available to draw those comparisons, unfortunately (173). Molloy *et al.* showed that PI*MZ was associated with an odds ratio for COPD of 5.18 in non-smokers and 10.8 in ever-smokers (174).

Panniculitis refers to inflammation of the subcutaneous fat. The panniculitis associated with AATD is rare, with an estimated prevalence of 1 in 1000 PI*ZZ individuals (175). Since this original case there have been over fifty cases reported in the literature (175). It usually occurs in individuals with a PI*ZZ phenotype but it has been described in PI*SZ, PI*MZ, PI*MS and PI*SS phenotypes (168). It is more common in women and tends to occur in patients aged 30-60 years old. Approximately 35% of cases are preceded by an episode of trauma. Ulceration and oily discharge appear to be distinctive clinical features of the AATD panniculitis (175).

The treatment of AATD associated panniculitis has not been fully established and has been hampered by the small number. For this reason there is a lack of

evidence for specific therapies. Decisions regarding treatment are often made based on expert opinion and clinical experience. The immune modulating effects of AAT augmentation therapy make it an obvious choice in the management of the neutrophil driven inflammation of AATD associated panniculitis. Smith *et al.* were the first to treat successfully two PI*ZZ patients with severe panniculitis with intravenous AAT augmentation therapy in 1987 (the same year this therapy was approved by the FDA to prevent progression of emphysema in AATD). There are an accumulating number of cases in the literature of panniculitis associated with AATD that have shown a rapid and dramatic response to AAT augmentation therapy (176). Blanco *et al.* in their comprehensive review of AATD associated panniculitis give details of thirteen such cases (175). Alternative therapies that have been used in the past include: prednisolone, colchicine, dapsone, antibiotics, antimalarials and azathioprine. None of these has a good evidence base and remain experimental therapies. Dapsone is considered to be first line therapy by some as it is widely available and cheap. Unfortunately, up to 15% of patients will develop methaemoglobinaemia on long term dapsone therapy (177).

The curative effect of liver transplant on cases of AATD associated panniculitis would also support the role of intravenous augmentation therapy (8, 9). Augmentation therapy is also a very well tolerated treatment.

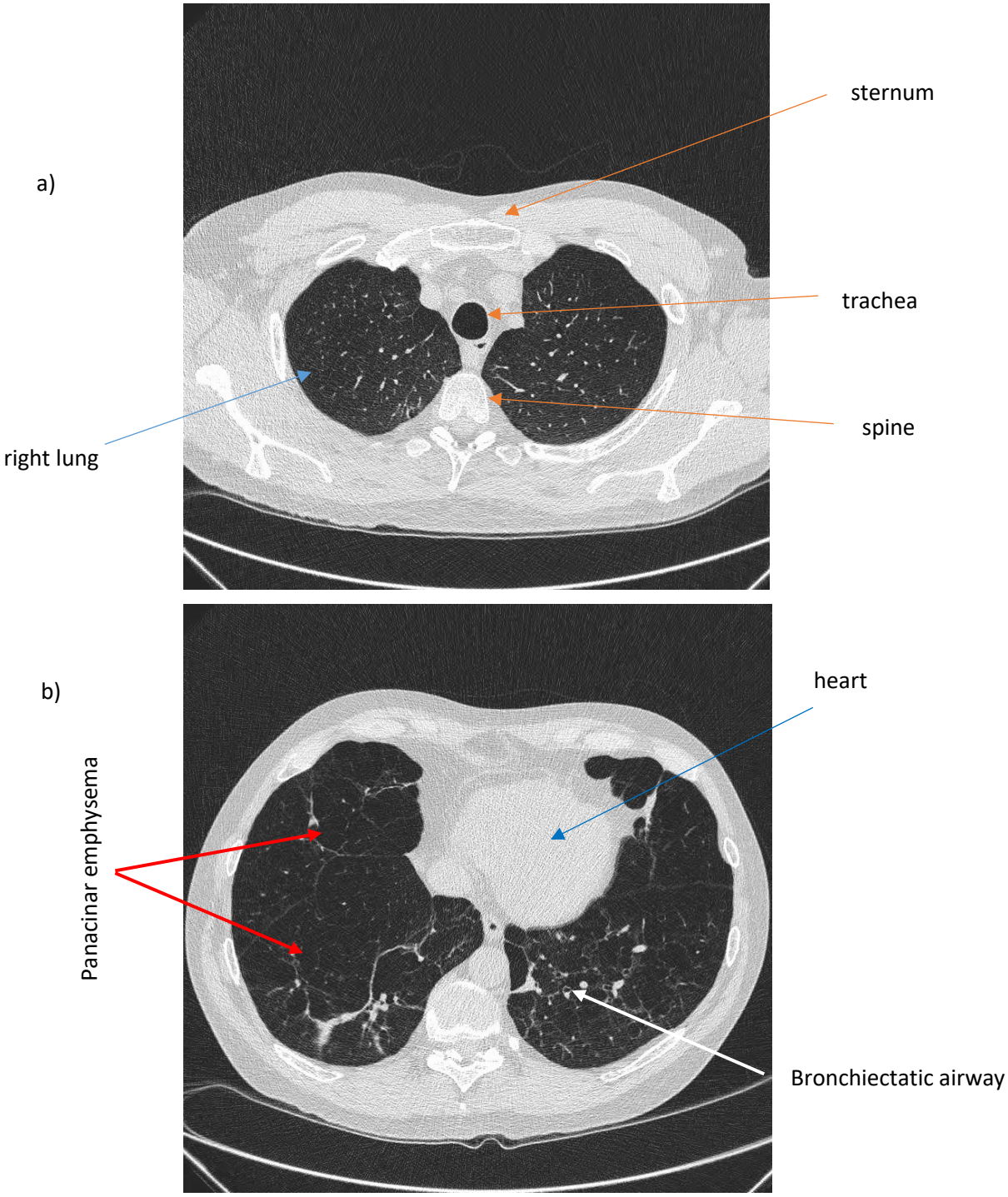


Figure 1-6: Emphysema in a patient with AATD.

High resolution CT thorax, cross sectional images, from a patient with AATD. a) images of upper lobes, showing mild panacinar emphysema. b) lower lung zones reveal moderate panacinar emphysema (red arrow) with mild bronchiectatic changes present in both lower lobes also (white arrow) (reproduced with permission form patient).

1.4.7 **Treatment of AATD: lung and liver disease.**

Avoidance of cigarette smoke and other environmental exposures is of paramount importance in the treatment of individuals with AATD (90). While the development of lung disease and spirometric abnormalities occurs in non-smoker PI*ZZ individuals, as evidenced by data from the Swedish registry, non-smoker PI*ZZ individuals have a mortality rate similar to non-smoker controls. The only specific therapy approved by the FDA for the treatment of AATD related lung disease is intravenous (IV) plasma purified AAT, often called AAT augmentation therapy. General treatment of COPD secondary to AATD should follow international guidelines as laid out by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (2019 update) (178). This includes education, exercise, pulmonary rehabilitation, vaccination to influenza and pneumococcus as well as inhaled therapy with corticosteroids, b_2 agonists and anti-muscarinics (166). Below I will focus on specific, novel and experimental therapies for the treatment of individuals with AATD and I will outline the evidence base for AAT augmentation therapy in AATD. Treatments that have been studied in human patients with AATD are summarised in Table 1-1 and Table 1-2, for lung and liver disease respectively.

Plasma purified AAT augmentation therapy is the only specific therapy that is approved by the FDA for the treatment of AATD. It was first approved in 1987 for AATD associated emphysema. Data showing an increase in serum levels of AAT, increase in BAL levels of AAT and increased anti-NE capacity in ELF was used in the initial study that led to approval of the drug for treatment of the disease (179). Augmentation therapy addresses, to some extent, the low circulating levels of AAT in severe deficient states. Clearly, it is of no benefit in liver disease. Although long approved by the FDA its use is not universally accepted and a Cochrane review did not recommend its use (180). The small population being studied in AATD makes it difficult to power an RCT for mortality and forced expiratory volume in 1 second (FEV_1). FEV_1 is perhaps not a robust clinical outcome measure in a rare disease. Computerised tomography (CT) densitometry is a more recent marker of disease progression that has been used in place of FEV_1, as a primary outcome measure in AATD trials (181). Although a relatively new technology, CT

densitometry has undergone some validation in COPD populations (182, 183). More longitudinal studies are certainly needed and a threshold of minimal clinically important difference (MCID) needs to be confirmed as has been done for outcomes such as FEV_1 (184).

The Randomized placebo controlled trial of augmentation therapy in Alpha-1 Proteinase Inhibitor Deficiency (RAPID) study is the most recent trial of AAT augmentation therapy. The multicentre study took place over 13 countries in patients with severe AATD defined by a serum concentration of AAT < 11mM, with an FEV_1 of 35-70% of predicted (185). Patients were randomly assigned to receive human plasma purified AAT intravenously at 60mg/kg per week or placebo for 24 months. 93 patients were randomised to augmentation therapy and 87 to placebo. The primary end points were CT lung density at total lung capacity (TLC) or functional residual capacity (FRC) and the two combined. Lung CT scans were performed at 0, 3, 12, 21 and 24 months. While the annual rate of loss of lung density at TLC and FRC combined didn't differ between the two study arms, the annual rate of loss of lung density at TLC was significantly less in patients receiving IV AAT augmentation therapy. The placebo group had a loss of 2.19g/L per year while the treatment arm had a loss of 1.45g/L per year. There was no difference in severe adverse events. In the RAPID trial there was no slowing in decline of FEV1 and no difference in exacerbation frequency. The study was not powered to meet these outcomes specifically. RAPID-OLE was the open label extension of the RAPID trial. The study was extended for a further 24 months and all patients were given IV AAT augmentation therapy. This study supported the findings of the initial RAPID trial (186). Augmentation therapy is expensive. In Europe it costs approx. €70,000 euro and in the USA $100,000. It is also a limited resource as it relies on blood donors.

As with all plasma purified proteins used therapeutically there is some concern about the potential for transmissible disease and it is also a limit resource that relies on blood donation. For that reason, there have been attempts to produce recombinant forms of AAT (rAAT). This has been done successfully in bacteria and yeast (187, 188). Unfortunately, from a therapeutic stand point, rAAT is non-glycosylated and is removed from plasma rapidly. Scientist next looked at the production of AAT by transgenic animals. AAT was produced in the milk of sheep,

unfortunately even small concentrations were found to trigger an inflammatory antibody-mediated response (189). Recombinant human AAT has been produced via transgenic brown rice seed and up to a third of this is glycosylated (190). Plants, such as *Nicotiana bethaminana,* have also been engineered to produce native AAT. This relative of the nicotine plant can produce large quantities of AAT (6mg/g of leaf tissue) but the AAT released from the ER was enzymatically inactive and was not glycosylated. The authors found that the plant was however highly amenable to glycan engineering and they could produce a disialyated AAT (191).

The main rationale of specific treatment in AATD is to restore the protease/antiprotease balance. This is either through increasing circulating AAT or reducing the NE burden. While most studied therapies to date have focused on the former, there has been some investigation into antagonists or inhibitors of NE. Sivelestat, is a selective NE inhibitor that has been studied in many lung diseases including ARDS, severe pneumonia, and cystic fibrosis. It has not demonstrated any favourable effects on clinical outcome (192). There are concerns, from a mechanistic view point, that inhibiting NE may decrease the antimicrobial defences within the lung. In a trial that has just begun recruiting in April 2019, the authors will assess the safety, tolerability and mechanistic effect of alvelestat (MPH996; oral NE inhibitor; NCT03679598) in subjects with emphysema related to severe AATD (i.e. ZZ, SZ or Pi*Null).

Increasing the availability of circulating AAT is another obvious approach to treating AATD. This can be done by administering exogenous AAT. IV AAT can be given in a human plasma purified form, as discussed, or it may be given as a recombinant preparation. Current practice is to give patients 60mg/kg of AAT augmentation therapy intravenously every week. This is based on the results of the 1987 paper, on which the decision was made to approve augmentation therapy for the treatment of AATD lung disease (179). Campos *et al.* studied the effects of double dose (DD) (120mg/kg) augmentation therapy in a group of patients already on long term standard dose (SD) (60mg/kg) therapy. This was a pilot study (193). The cohort were studied over a 12 week period. During the first 4 weeks the patients were receiving SD, this was followed by 4 weeks of DD and a final 4 weeks on SD once again. DD therapy was found to increase trough levels of AAT to normal levels (>25μM), which is not achieved by SD therapy (which improves

AAT trough levels to roughly 50% of normal). DD therapy also reduced serine protease activity (cath G and NE) in BAL fluid. Isodesmosine and desmosine, markers of elastin degradation, were also reduced in the DD group. It was an important study, as it showed that DD therapy could reduce inflammation in those already receiving SD augmentation therapy. A limiting factor of the study was that it included only 10 patients (7 ZZ, 1 SZ). Some improvements that were seen at the end of the 4 weeks of DD therapy (e.g. cytokine profile, immune cell activation) persisted to the end of the trial (i.e. after 4 weeks back on SD). This study was funded by CSL Behring.

Inhaled AAT as a form of therapy has been shown to have biochemical efficacy and its safety profile is well established (194). It has in fact been studied in neutrophilic lung diseases other than AATD (e.g. cystic fibrosis) (195). Large phase III trials will be needed to determine its clinical utility. An advantage of inhaled AAT is that there would be no requirement for weekly intravenous access for drug administration. This would significantly reduce health care utilisation costs. Inhaled AAT can be detected in ELF and serum of individual's receiving the treatment. Inhaled AAT has also been shown to increase the NE inhibitory capacity of ELF (194, 196). In a study performed with funding from Bayer pharmaceuticals, following 52 patients with cystic fibrosis who received inhaled AAT for 4 weeks, it was found that treated patients had a reduction in neutrophil numbers, elastase activity, pro-inflammatory cytokines and *Pseudomonas aeruginosa* in their BAL samples. In this study, there was no effect on lung function (197). A phase III study of inhaled AAT therapy in AATD is complete and the results of this are awaited (Kamada Pharmaceuticals) (198).

There has been some research focusing on increasing endogenous AAT production by hepatocytes as a treatment for AATD liver disease (199). Danazol and tamoxifen, which stimulate oestrogen and progesterone receptors on the liver, failed to augment AAT levels significantly (200, 201). Short interfering RNA (siRNA) have been studied to stop aberrant AAT production. Two different Phase I/II human trials of siRNA inhibition of mutant Z protein synthesis as liver disease therapy have been performed to date. The first, by Alnylam pharmaceuticals, in healthy control and Pi*ZZ individuals with liver disease (NCT02503683). Preliminary reports suggest successful reduction in circulating AAT, with monthly

subcutaneous injections of the drug. The trial was stopped as 3 individuals experienced a rise in liver function tests on the highest dose of the compound. Arrowhead pharmaceuticals also performed a phase I study of their own siRNA AAT compound, delivered in multiple centres in healthy control and AATD patients. They found the compound to be well tolerated and produced a significant reduction in circulating AAT levels (NCT02363946) (202). The Arrowhead study was terminated early because of toxicity findings related to the delivery vehicle (ARC-EX1) that were seen in a non-human primate study. Carbamazpeine and sirolimus have been studied in mouse models and shown to increase autophagic degradation of the Z protein (203-205). A trial of carbamazepine in AATD patients with cirrhosis in underway (NCT01379469). These treatments will clearly not treat lung disease related to AAT as they will further reduce circulating AAT levels.

Inhibiting polymerisation of the Z protein may represent a target for both liver and lung disease, by reducing the burden of polymers in hepatocytes and by increasing the secretion of Z protein into the circulation, which although less effective at NE inhibition than the M protein does retain some of this capability (206). A number of therapeutic strategies have been studied aiming to stabilise the AAT protein against pathological conformational changes. The polymerisation mechanism in AATD involves the filling of the s4A site by residues of the reactive centre loop of another molecule. Analog peptides to the s4A site have been developed that block polymerization in vitro. A tetrapeptide (TTAI) has shown effective blockade of polymerisation. It is hoped that the identification of an optimal small peptide will aid in the development of a mimetic small molecule that could be given orally rather than intravenously (207-209). A monoclonal antibody, 4B12 mAb, blocks in vitro polymerisation of Z AAT but retains anti-NE activity (210).

Gene therapy has undergone extensive study in AATD. Given that PI*ZZ results from a single point mutation, correction of this via gene therapy would appear to be an attractive treatment target. However, despite the use of varied vectors (plasmid, adenovirus) and routes of administration (systemic, muscle, intrapleural) there has been failure to produce sustained increase in AAT levels in these, mostly, mouse models (33). Human trials of gene therapy in AATD began in the early 2000’s and the first results were published in 2006 (211). Because AAT is a secreted protein, gene therapy delivery options for AATD are broadened, such that AAT could be

produced from sites other than the liver or lungs. The most successful form of AATD gene therapy to date has been delivered via intramuscular (IM) injection, as the first trial in 2006 was. With the intramuscular route there was increased AAT production but to very low levels, despite changing the vector type and dose escalation (212). This dose escalation was impractical as 100 IM injections were required which was invariably associated with some local injection site reactions (213). The intrapleural route of administration has been and is still undergoing investigation as a potential for gene therapy in AATD. Much of this work has been pioneered by the Crystal lab in Weill Cornell University in New York, USA. The major advantage of intrapleural administration is that both local and systemic delivery are targeted by the gene therapy vector, thus, increasing the possibility of producing adequate quantities of AAT to provide a therapeutic effect. In a mouse model, AAT levels were increased to >11μM up to 40 weeks post administration of the intrapleural gene therapy (214). A phase I/II clinical trial is underway to investigate safety and to determine preliminary efficacy in humans (NCT02168686).

Another target of gene therapy for AATD liver disease is the transcription factor TFEB. In a PI*Z mouse model, delivery of TFEB induces autophagy in the liver and reduces liver fibrosis and inflammation. Autophagy is a homeostatic process, in which, subcellular structures (e.g. Z-AAT) are enveloped and degraded in double-membraned vacuoles, known as autophagosomes. These autophagosomes fuse with lysosomes where their contents can be repurposed by the cell. In PI*ZZ individuals, the formation of autophagosomes is increased in hepatocytes.

Induced pluripotent stem cells (iPSCs) are a growing area of interest and research in many fields of medicine, including AATD. The development of lung organoids that are patient specific show promise in studying drug response in cystic fibrosis (215). There has been real-world application of iPSCs in the treatment of macular degeneration (216). AATD being a monogenic disease makes it an attractive option to study with the use of iPSCs. Wilson *et al*. have produced AATD patient-derived iPSC-hepatocytes (217). These cells have been shown to have many of the features of hepatocellular toxicity that are seen in AATD livers. Globular inclusions of Z-AAT are seen to accumulate in the ER of iPSC-hepatocytes and

similar to findings in vivo, these stain PAS positive and are resistant to digestion with diastase. Wilson *et al.* were also able to demonstrate aberrant protein glycosylation and secretion using the iPSC model. They showed increased autophagic flux in PI*ZZ iPSC-hepatocytes relative to PI*MM controls. They also showed that the increased autophagic flux could be further augmented with carbamazepine (217). The variability of the liver disease that is seen at liver biopsy in PI*ZZ individuals is also true of iPSC-hepatocytes from PI*ZZ individuals (e.g. only 25% of iPSC hepatocytes demonstrated ER inclusions) (218). Z-AAT expression in iPSC hepatocytes showed UPR activation in early stage liver progenitor cells and ER stress response in mature hepatocytes (219).

iPSCs clearly offer a role in gaining further understanding of the pathogenesis of liver disease in AATD. They may also serve as an adjunct to clinical trials, as shown with the studies of carbamazepine mentioned above. These are autologous cells that do not carry with them the many risk of organ transplantation. There are clearly many obstacles in this regard and the field remains in its infancy. Patient derived iPSCs may also in the future have the potential to restore function to tissue (218). Genetic manipulation of Z-AAT hepatocytes to form M-AAT hepatocytes using Zinc finger nucleases or CRISPR-cas9 technology could offer an attractive potential treatment strategy for AATD and has been shown to be possible in a mouse model by adeno-associated virus (AAV) delivery (220, 221).

COPD (including AATD) is the most common indication for lung transplantation worldwide, accounting for up to 40% of lung transplants worldwide (222). AATD accounts for approximately 6% of all lung transplants and at least 10% of those performed for COPD (223). Referral for lung transplant is generally made based on a prognostic score known as the BODE index (Body-mass index, airflow Obstruction, Dyspnoea, and Exercise). Predicting survival without transplant is an important factor in referring a patient for this procedure. The median five year survival post transplant for COPD is approximately 60% (222), which will likely not differ to the life expectancy of patients who do not undergo lung transplant. Similarly, lung transplantation in AATD improves quality of life but does not improve survival (224). For this reason, patient selection is of the utmost importance. Given the heterogeneity of COPD, including in AATD individuals, the BODE index is a poor prognostic tool. Survival post lung transplantation, in the

modern era, appears to be equivalent between matched ‘normal’ COPD and AATD COPD patients (225).

Some studies have suggested that survival for patients with AATD may be somewhat improved post lung transplantation (167). Others have suggested that with double lung transplant in AATD there may be a slightly faster rate of decline of FEV_1 (226). 25 year data from the Swedish lung transplant programme, up to 2014, showed no significant difference in survival between those transplanted for ‘normal’ COPD versus AATD (227).

Table 1-1: Treatments for lung disease associated with AATD. studied in humans.

Treatment	Goal	Outcome	References
Human plasma purified IV AAT (60mg/kg)	Augment plasma/body fluid levels of AAT	Slows decline in lung CT density measurements at TLC	Dirksen *et al.* Dirksen *et al.* Chapman *et al.* McElvaney *et al.*
Human plasma purified IV AAT (120mg/kg)	Augment plasma/body fluid levels of AAT	Decreased NE, Cath G activity. Decreased markers of elastin degradation	Campos *et al.*
Inhaled AAT	Augment ELF and interstitial levels of AAT	Safety established Increased anti-NE capacity of lung ELF Phase II/III (complete;results pendning)	NCT01217671
NE inhibitor (oral)	Correct protease-antiprotease	Safety in bronchiectasis, COPD, CF Phase II (recruiting)	NCT03679598
Gene therapy (intramuscular, intrapleural)	Increase endogenous AAT production by correcting Z mutation	IM (AAV): Phase 2, safety and feasibility without therapeutic AAT levels Intrapleural: Phase I/II results awaited	Hubbard *et al.* NCT02168686
Lung volume reduction surgery		Effective improvement in physiological parameters and symptoms (less than 'normal' COPD) Improved FEV_1 at 2 years (vlaves)	Cassine *et al.* Dauriat *et al.* Stoller *et al.* Hillerdal *et al.*
Lung transplant		No difference in long term survival in COPD vs AATD Possibly more post-operative complications	Tanash *et al.* Banga *et al.* Cassivi *et al.* Kleinerova *et al.*

Table 1-2: Treatments for liver disease associated with AATD. studied in humans.

Treatment		Outcome	References
Carbamazepine	Enhancement of autophagic degradation of Z-AAT		NCT01379469
siRNA	Reduce Z-AAT production in hepatocytes		NCT02363946
4-Phenylbutyrat	Chaperone	Did not increase serum AAT levels	Teckman *et al.*
Liver Transplant	End stage liver disease	80% 5 yr survival in adults	Townsend et al.

1.5 Aims and study objectives

There has been some preliminary work demonstrating that AAT modulates the detection of O_2^- from stimulated healthy control neutrophils. There is an established role for oxidative stress in the pathogenesis of COPD, but this has not been studied in great detail in AATD. We hypothesise that AAT modulates neutrophil ROS production by directly affecting the assembly of the cytosolic subunits of NADPH oxidase at the plasma membrane and therefore its activity. We set out to also demonstrate the mechanism of this modulating effect of AAT. In this study we will also explore O_2^- release by neutrophils from individuals with AATD. In order to fully investigate this aim, the following objectives were set out:

1. To investigate the role, if any, that AAT plays in the assembly of the NADPH oxidase subunits.
2. To determine the mechanism by which AAT modulates O2- production by healthy control neutrophils
3. To examine the effects of AAT augmentation therapy on neutrophil NADPH oxidase activity in individuals with AATD

Chapter 2: Materials and Methods

2.1 Chemicals and Reagents

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

2.2 Study design

Volunteers recruited to this study are outlined in Table 2-1. Healthy non-smoking control subjects were recruited and were defined as having an MM phenotype by isoelectric focusing with serum AAT concentrations within the normal range (25–50 μM). Non-smoking PI*ZZ patients were recruited from the Irish Alpha-1 Antitrypsin Deficiency outpatient clinic in Beaumont Hospital.

Study groups: non-obstructed PI*ZZ, PI*ZZ with COPD, PI*ZZ receiving augmentation therapy on day 0 and day 2 of treatment.

All subjects were free from pulmonary exacerbation in the eight weeks prior to enrolment to the study. All participants provided written informed consent, which was approved by the Beaumont Hospital Institutional Review Board (reference 13/92).

2.3 Neutrophil isolation

Blood was collected in Sarstedt Monovette® tubes containing lithium-heparin. Human peripheral blood neutrophils were isolated using the method previously described by Reeves et al 2002 (54). All steps were performed at room temperature (RT). Blood was added to a 50ml tube containing 5ml of 1X saline (0.45% (w/v) NaCl) and 10% (w/v) dextran. The solutions were gently mixed and then left to stand for 15min, until the erythrocytes had sedimented. The supernatant was carefully pipetted into a separate 50ml falcon, underlayed with lymphoprep (5ml) (Axis-Shield POC, Oslo, Norway) and centrifuged at 836 X g for 10min. The supernatant was discarded, the red blood cells were quickly (10sec) lysed by hypotonic shock in molecular grade water and 2X saline (1.8% (w/v) NaCl) was quickly added to prevent neutrophil lyses. The sample was then centrifuged at 470 x g for 5min. The supernatant was removed and the neutrophil

pellet was resuspended in Dulbecco's phosphate buffered saline (dPBS) (pH 7.4) containing 5mM glucose.

2.3.1 **Cell viability and purity**

Trypan blue exclusion test was used to determine cell viability. The neutrophil cell count was obtained by adding 10 µl cell suspension to 90 µl trypan blue on a haemocytometer (Superior, Marienfeld, Germany). The proportion of viable (unstained) cells in the final cell number was calculated with the resultant cell viability being greater than 95% in all samples used. Neutrophil purity was determined at >98% by assessment for cd16b by flow cytometry analysis (161).

Table 2-1: Study subjects

	PI*ZZ	PI*MM
No. of Subjects	40	16
Gender, male/female	25/15	10/6
Age, years (Range)	18-86	26-45
FEV1 (% predicted) (Mean ±SEM)	60.52 ± 3.86	N/A

2.4 SDS-polyacrylamide gel electrophoresis

In order to carry out SDS-PAGE both separating and stacking gels were cast as per Table 2-2. Two glass plates were assembled and the separating gel (12.5% v/v) was filled to approximately 80% capacity. A layer of isopropanol was overlaid and the gels were left for 20 min to solidify. Upon solidification the isopropanol layer was discarded, the stacking gel (5% v/v) was added on top of the set separating gel and a plastic comb was inserted. After setting, the comb was gently removed. Gels were transferred to the ATTO AE6500 module (Medical Supply Company, Ireland) which was then filled with 1X Running Buffer made from a 10X stock (Tris 30g, glycine 144g, SDS 10g). The wells were flushed with Running Buffer using a Hamilton syringe. Proteins that were intended for analyses were suspended in 2X Sample Buffer (3M Tris-HCL (pH6.8), 0.2% bromophenol blue, 50% (w/v) sucrose, 1% (w/v) SDS, 200mM dithiothreitol (DTT) and 200mM ethylenediaminetetraacetic acid (EDTA). Samples were loaded into the wells alongside a pre-stained protein ladder Seeblue Plus 2® (Fisher Scientific, Ireland). Samples were then separated by applying 90 volts for 15 min and 120 volts for the remainder of the separation.

Table 2-2: SDS-PAGE gel components

12.5% (w/v) Separating Gel	5% (w/v) Stacking gel
3ml - 1.5 M Tris pH 9.8	315µl - 0.5 M Tris pH 6.8
3.8ml - Deionized water (dH2O)	1.7ml - dH_2O
5ml – Protogel* (30% v/v)	415µl - Protogel (30% v/v)
120µl - SDS (10% w/v)	25µl - SDS (10% w/v)
75µl - Ammonium persulfate (10% v/v)	25µl - Ammonium persulfate (10% v/v)
6µl - Tetramethylethylenediamine	5µl –Tetramethylethylenediamine

*Fisher Scientific

2.4.1 **Coomassie blue stain**

For protein visualisation of SDS-PAGE, Coomassie blue stain was employed. SDS gels were stained with filtered Coomassie blue stain (10% (v/v) acetic acid, 45% (v/v) methanol, 45% dH_2O (v/v) and 0.2% (w/v) Coomassie Blue R250) for 1 h with gentle rocking at RT. After this time, the Coomassie stain was removed and the destain solution (10% (v/v) acetic acid, 25% (v/v) methanol and 65% dH2O) was added with gentle rocking for 15 min. After 15 min, fresh destain solution was added and this process was repeated four times. Protein bands were visualised using a QUP/A4-SL Lightbox (Scientific Laboratory Supplies, United Kingdom).

2.4.2 **Western blot analysis**

Proteins to be analysed by Western blotting were transferred from SDS gels onto polyvinylidene fluoride (PVDF) membranes (Roche, Ireland) that had been preactivated in methanol (100% v/v). Transfer Buffer (0.3% (w/v) Tris base, 1.4% (w/v) glycine and 20% (v/v) ethanol) was pre-prepared and stored at 4ºC. Sponges and Whatman filter paper were pre-soaked in Transfer Buffer prior to transfer. A XCell 11 Blot Module (Bio-Sciences, Ireland) was prepared by layering 2 pre-soaked sponges, filter paper, SDS gel, membrane, filter paper and sponges. This module was then placed in a Novex Mini-Cell module (BioSciences, Ireland) for wet transfer. Transfer was carried out at 30 volts for 90 min for 1 gel or 120 min for 2 gels. Upon transfer completion, membranes were stained with Ponceau S for confirmation of protein transfer. Membranes were then washed in deionized water and blocked in Blocking Buffer containing 5% (w/v) milk marvel in Tris-Buffered Saline with Tween 20 (TBST) (24g Tris pH (7.6), 88g NaCl, 0.05%, (v/v) tween 20,) for 1 h at RT. Membranes were then incubated in the appropriate primary antibody in Blocking Buffer for either 1 h at RT (AAT) or overnight at 4ºC (See Table 2-3 for complete list of antibodies and concentrations). Membranes were washed in TBST 10min at which time the old TBST was discarded and new TBST was added. The washing step was repeated three times. Membranes were then incubated with appropriate HRP-linked secondary antibody (Table 2-3) in Blocking Buffer for 1 h at RT. Membranes were then washed in TBST for 30 min with wash

changes every 10 min. Immuno-reactive protein bands were detected by incubating the membrane in Immobilon Western Chemiluminescent HRP substrate solution (Merck Millipore, Ireland) using a Chemi Doc MP System (Fannin, Ireland). A ladder of known molecular weight was used to determine correct protein size. Densitometry was carried out using Image Lab (Fannin, Ireland) and results displayed as arbitrary units. Control experiments were performed with and without primary and secondary antibody, to assess for non-specific binding of these antibodies and to ensure that false positive results were not recorded.

Table 2-3: Antibodies used in Western blot experiments

Primary antibody	Supplier	Working conc.	Secondary antibody	Supplier	Working conc.
HVCN-1 Rabbit polycloncal IgG	Santa Cruz (SAB3500536)	1µg/ml	Anti-rabbit	7074S Brennan & Co	0.5µg/ml
$p67^{PHOX}$ (Goat)	Segal lab, UCL.	0.5µg/ml	Rabbit anti-goat	Sigma (sc-2768)	1µg/ml
$p47^{PHOX}$ (Goat)	Segal lab, UCL	0.5µg/ml	Rabbit anti-goat	Sigma (sc-2768)	1µg/ml
β-Actin (mouse)	Santa Cruz, Sc-47778	1µg/ml	Anti-mouse	7076S Brennan & Co	0.5µg/ml

2.5 Whole cell lysate (neutrophil) preparation for HVCN1 expression

After neutrophil isolation, as above, $1x10^7$ neutrophils (in PBS-glucose solution) were placed in a 1.5ml Eppendorf. This was then centrifuged at 4ºC at 470 x g for 5 min. The supernatant was discarded. The neutrophil pellet was then incubated in 2µl of diisopropyl fluorophosphates (DFP) on ice for 10 min. The neutrophil pellet was then re-suspended in 300µl of K-lysis buffer (150mM NaCl, 50mM TrisHCl, 1% (v/v) Triton X-100, 0.2mM $NaVO_4$, 1mM DTT, 10mM NaF, and 1mM EDTA). The following protease inhibitors were added fresh to the K-lysis buffer on the day of the experiment TLCK, Pepstatin A, Leupeptin, Phenylmethanesulfonyl fluoride (PMSF) and Pefabloc (Table 2-4). This was then centrifuged at 4ºC at 470 x g for 5 min. The supernatant was removed and stored at -80ºC.

2.6 Subcellular fractionation: isolation of neutrophil membranes for $p67^{PHOX}$ and $p47^{PHOX}$ expression

Cell fractionation was carried out as previously described (228). Isolated and counted neutrophils were lysed by sonication. A post-nuclear supernatant was generated by centrifugation at 500 x g for 5 min. A sucrose gradient was generated by carefully pipetting 7 ml of 60% (w/w) sucrose in Lamberth break buffer (LBB) (Table 2-5) and protease and phosphatase inhibitors (PI) into the bottom of a 12.5 ml ultracentrifuge tube. On top of this, 2 ml of 35% (w/w) sucrose was carefully added and then 2 ml of 17.5% (w/w) sucrose was added to complete the gradient. The above generated post-nuclear supernatant was then pipetted on top of the 17.5% (w/w) sucrose layer. All steps after neutrophil isolation were carried out at 4ºC. Fractionation of subcellular components was achieved by ultracentrifugation at 100,000g for 1 h using a swing-out rotor in Beckman Coulter Optima Ultracentrifuge at 4ºC. Following centrifugation, the cytosolic fraction was removed from above the 17.5% (w/w) sucrose layer and immediately stored at -80 °C. The membrane layer was found at the interface between the 17.5% and 35% (w/w) layers. Membranes were removed by pipetting and were then diluted in 3-4 fold volume of ice-cold LBB + PI. This solution was then centrifuged at 20,000g in a benchtop centrifuge at 4 °C in order to pellet the membranes. Following this centrifugation step, the supernatant was removed and the membranes were re-suspended in 50 µl of LBB + PI before storage at -80 °C.

Table 2-4: Protease and Phosphatase inhibitors

Protease Inhibitor	Stock concentration	Working concentration	Class/Target
TLCK	10mg/ml in H_2O	10µg/ml	Trypsin and trypsin-like serine proteases
Leupeptin	10mg/ml in H_2O	10 µg/ml	Serine and cysteine proteases
Pepstatin A	5mg/ml in methanol	10 µg/ml	Aspartic proteases
Phenylmethane sulfonyl fluoride (PMSF)	200nM in ethanol	500µM	Serine, acetlycholineesterases and thiol proteases
Pefabloc	417mM in H_2O	1mM	Serine proteases
Diisopropyl fluorophosphates (DFP)	liquid	2.5 mM	Broad spectrum serine protease, actelycholinesterases
Ethylenediamine tetraacetic acid (EDTA)	200mM	1mM	metallopeptidases
NaF	in H_2O	10mM	Phosphatase inhibitor
$NaVO_4$	in H_2O	0.2mM	Phosphatase inhibitor

Table 2-5: Lamberth break buffer

Reagents	Protease and phosphatase inhibitors
0.746 g KCl	Pefabloc (1 mM)
0.175 g NaCl	PMSF (500 µM)
0.381 g $MgCl_2$	Leupeptin (10 µg/ml)
3.024 g PIPES(1,4-Piperazinediethanesulfonic acid)	TLCK (10 µg/ml)
800 ml H_2O	Pepstatin A (10 µg/ml)
Adjust pH to 7.0 using NaOH	NaF (10 mM)
Fill up to 1 litre	$NaVO_4$ (0.2 mM)

2.7 Flow cytometry analysis

Neutrophils were isolated, as above, counted and divided into 15ml tubes each containing $2x10^6$ neutrophils. Paraformaldehyde (4% w/v, 500µl) was added to each cell pellet for 10min. The cells were centrifuged at 470 x g for 5min, the supernatant was discarded and the cells were washed with 500µl of dPBS. This wash step was repeated three times. The cells were then blocked with 300µl of 1% (w/v) bovine serum albumin (BSA) in dPBS for 30min. Primary antibody was added to the relevant samples (anti-HVCN1 rabbit polyclonal, Sigma-Aldrich). An isotype control was also used (control rabbit IgG, Santa Cruz) (see Table 2-6). These were left to incubate while rolling for 1h. Three washing steps as above were then undertaken. Following this, the cells were resuspended in 1% (w/v) BSA and secondary antibody was added (FITC-labelled goat anti-rabbit Abcam) in the dark for 30min. Three further washing steps were then completed. Samples were analysed for fluorescence with FACSCalibur BD, to 10,000 events (156). FlowJo® software was used to analyse the results.

Table 2-6: Antibodies employed for flow cytometry.

Name	Manufacturer	Concentration
Anti-HVCN-1 (SAB3500536) Rabbit polycloncal IgG	Santa Cruz Biotechnology, Germany	1µg/ml
Normal rabbit IgG (sc 2027)	Santa Cruz Biotechnology, Germany	1µg/ml
Goat anti-Rabbit IgG FITC (ab6717)	Abcam, Cambridge, UK	1µg/ml
Mouse MAb CD16b	Santa Cruz Biotechnology	1µg/1x10^6 cells

2.8 Superoxide and Oxygen consumption quantification

2.8.1 **Cytochrome c reduction assay**

A cytochrome c reduction assay was used to measure the production of O_2^- by circulating neutrophils. The protocol was adapted from Babior *et al.* (229). 1x 10^7 neutrophils were isolated, as above, then re-suspended in 1ml of DPBS-glucose. Cytochrome C buffer (5mM glucose, 0.5mM $MgCl_2$, 0.5mM $CaCl_2$ and 100µM cytochrome c in DPBS) had been prepared in aliquots and stored in the freezer at -20^{0}C. Prior to neutrophil isolation an aliquot of cytochrome c buffer was placed in the water bath at 37^{o}C. The neutrophils were centrifuged at 4^{0}C at 470 x g for 5 min. The neutrophil pellet was then resuspended in 1ml of cytochrome C, to give 1x10^6 neutrophils per 100µl. Cells were added to a 96 well plate. Various stimulants were used including TNFα (0.5ng per 1x10^6 cells), IL-8 (0.5ng/1x10^6 cells) (161), fMLP (1µM) and PMA (200nM). These were added immediately prior to the reaction being recorded spectrophotometrically at a wavelength of 550nm at 37^{o}C (SpectraMax® M3 multimode microplate reader, Molecular Devices). In a subset of experiments $ZnCl_2$ was included. Various concentrations of $ZnCl_2$ were used (500µM, 100µM, 50µM, 10µmM). SOD was employed as a negative control (12.5µg/ml, S2515 Sigma Aldrich). O_2^- quantity was determined by correcting for absolute absorbance and the extinction coefficient of cytochrome c (0.021) and results were expressed in nmoles/1x10^6 neutrophils.

2.8.2 **Oxygen consumption**

A Clarke-type oxygen electrode was used. All experiments carried out using 2x10^7cells per treatment. O_2 consumption was recorded over 4min at 1mm/sec recording speed. The inner chamber was bathed in 20µl of 3M KCl, while the outer electrode was slightly moistened with 3M KCl. The O-ring was placed over the Teflon membrane and a stirring bar placed within the chamber to oxygenate the reaction. Cells were pre-treated with AAT (27.5µM) for 5min following which cells were exposed to IL-8 (10ng/2x10^7cells) for 5min. fMLP (10µM) was employed immediately prior to commencing O_2 consumption readings. Sodium dithionate was used as a negative control buffer in the calibration of the electrode.

2.9 Cytosolic pH measurement using the ratiometric pH indicator carboxy-SNARF-1 (seminaphthorhodafluor)

Neutrophils were isolated as already described. $1x10^7$ cells were made up to 1ml with dPBS and 50µl of SNARF-1 (12.5mg/ml). This was left on the bench for 10min at RT. The cells were then centrifuged at 470 x g for 5min at RT and resuspended in 500µl of fresh dPBS. pH standards, ranging from 5.8 to 8.6, were placed in a labelled 96 well plate. Cells ($1x10^5$ per well) to be used to form the pH standard were exposed to nigericin (10µl, 6.7nM) and then added to the various pH standards in the 96 well plate. The plate reader was then set to read at a single time point, monitoring emission at 580nm and 640nm at RT. pH was calculated based on the ratio of fluorescence intensities measured at two different wave lengths and interpolated from a standard curve.

Cells ($1x10^5$) were added to the wells. $ZnCl_2$ (100µM) was added to a number of pre-determined samples at RT for 5 min. Various stimulants were employed, including PMA (200nM), fMLP (1µM), and TNF-α (0.5ng/$1x10^7$cells).The stimulants were added immediately prior to recording at RT at 580nm and 640nm. A standard curve was created for each experiment. This resulted in a sigmoidal dose-response curve, from which results were interpolated.

2.10 Fluorescence Resonance Energy Transfer (FRET) assay for neutrophil elastase activity

The protocol was adapted from Korkmaz *et al.* 2008, Nature protocols (230). Neutrophils were isolated as described above. Exogenous NE (TS563, Elastin Products Company, Inc) was used to make standards of various concentrations (5nM to 60nM) in order to create a standard curve. An NE specific FRET substrate was used (Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp, 3230-v, Peptide Institute, Inc). The FRET substrate was reconstituted in 30% (w/v) dimethylformamide (DMF) to give a final concentration of 1mM. It was stored in the dark as it is ultraviolet (UV) sensitive. FRET substrate was added to the samples, which were loaded in a 96 well plate (Nunc Microwell™ polypropylene plates, P6866, Sigma Aldrich), immediately prior to being placed in the plate reader at 320-420nm (excitation-emission wavelengths) at 28ºC (SpectraMax® M3

multimode microplate reader, Molecular Devices). 2µl of FRET substrate was added per well followed by $1x10^6$ neutrophils. Samples were analysed in duplicate.

2.11 AAT/fMLP binding experiments.

2.11.1 **Slot blot analysis**

Slot blot analysis was used to assess protein binding, with all steps occurring at RT. A Minifold Slot Blot System (GE Healthcare, Ireland) was set up as in Figure 2-1. Two sheets of Whatman® filter paper and PVDF membrane (Roche, Ireland) were pre-soaked in dPBS. dPBS (500µl) was flushed through each well and filtered through the membrane under a vacuum set at a flow rate of 1ml/min. fMLP (10µM) was loaded into the wells and filtered through the membrane under vacuum and following this the vacuum was switched off. Next, 500µl of AAT (27.5µM) or dPBS was loaded into the wells and left to incubate for 1 h. The unbound sample was discarded and the wells were washed gently with dPBS (500µl) x2. Wells were blocked with 5% (w/v) milk marvel in Tris-Buffered Saline with Tween 20 (TBST) (24g TRIS, 88g NaCl, 0.05%, (v/v) tween 20, pH 7.6) for 1h. A goat anti-AAT HRP-conjugated antibody (Abcam, ab191350) was then added and left to incubate for 1 h (anti-AAT HRP linked). Immuno-reactive protein bands were detected by covering the membrane with Immobilon Western Chemiluminescent HRP substrate solution and visualised using a Chemi Doc MP System (Fannin, Ireland). Densitometry was carried out using Image Lab (Fannin, Ireland).

2.11.2 **Polystyrene beads**

For experiments investigating binding of fMLP to AAT, 10 µm polystyrene beads (Polysciences Europe, Eppelheim, Germany) were coated with AAT in saline solution (0.9% NaCl [w/v]) as previously described (231). In brief, the beads were suspended in saline with added AAT (100µg in 100µl) for 24 h in the dark at 4°C with gentle rotation. The beads were centrifuged (15,000 × g for 4 min at 4°C) and the amount of AAT binding to the beads was indirectly determined by measuring the unbound levels by UV spectrometry. After washing with saline, the beads were

blocked with 2% (w/v) BSA. AAT-loaded beads were washed and resuspended in solutions of a FITC-labelled fMLP for 30 min at room temperature. Uncoated beads (no added AAT) were also suspended in solutions with the FITC-labelled fMLP. Fluorescence was counted using a BD FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) with a total of 10,000 events acquired. The data were analysed and the mean fluorescence units (MFU) for each experiment were determined using FlowJo software.

2.12 Nephelometry to determine serum AAT levels

Blood was collected in 5ml serm blood tubes. Nephelometry was performed by the biochemistry department of Beaumont Hospital using Beckman Coulter IMMAGE® Immunochemistry systems. This AAT test measures the rate of increase in light scattered from particles suspended in solution as a direct result of complexes that are formed during an antigen-antibody reaction (i.e. antibody directed against AAT and compared to a standard).

2.13 Statistical analysis

All data sets were analysed for statistical significance using GraphPad Prism 5.0 software package (GraphPad Software, United States of America) with significance determined at $p< 0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Where no significance was achieved ‘ns’ is displayed. Data were tested for normality using the D’Agostino & Pearson omnibus normality test. For parametric analysis comparing two data sets a Student’s two-tailed t-test was performed. For parametric data comparing three or more groups an ANOVA was employed followed by post hoc Bonferroni multiple comparisons test. Bonferroni multiple comparisons test was selected as it enables comparison between selected columns. Dunn’s multiple comparion test was also used post hoc ANOVA of non-parametric data sets. All data sets are presented as mean values (+/- s.e.m.).

Chapter 3: AAT modulates the assembly and activity of NADPH oxidase

3.1 Introduction

The phagocytic respiratory burst of neutrophils was first observed in the 1930's when it was thought to be as a result of increased mitochondrial activity (232). It was initially assumed that this consumption of oxygen was as a result of an increase in mitochondrial demands of the cell as it engulfed a bacteria. Some twenty years later it was shown that inhibiting mitochondrial function with cyanide failed to inhibit the respiratory burst thus debunking the theory that the increased oxygen consumption was related to mitochondrial function (233). It was subsequently shown that a complex of proteins on the cell membrane was responsible for transferring an electron to O_2 (234) and was indeed responsible for the dramatic increase in oxygen consumption seen when a neutrophil phagocytosed a bacteria. This complex of proteins is known as NADPH oxidase. The discovery of chronic granulomatous disease (CGD) in the late 1960's helped in the further description of the NADPH oxidase complex of phagocytic cells. The respiratory burst of phagocytic cells is absent in patients with CGD and they are unable to kill bacteria and fungi in an efficient manner. Patients with CGD develop acute and chronic infections predominantly of their lungs and skin (57). The various genetic defects of CGD were described in the 1980's following the discovery and description of the membrane and cytosolic components of neutrophil NAPDH oxidase (235, 236). NADPH oxidase can be found in numerous phagocytic cells of the innate immune system including, neutrophils, macrophages, monocytes and eosinophils (237).

The NADPH oxidase complex of neutrophils is composed of five subunits (see Figure 3-0). $gp91^{PHOX}$ and $p22^{PHOX}$ form the catalytic core of the NADPH oxidase that is responsible for transferring electrons across the cell membrane to reduce O_2 to O_2^- (237). These two subunits, in resting neutrophils, reside predominantly at the plasma membrane. They are also found in primary granules and secretory vesicles (36). Even at rest $gp91^{PHOX}$ and $p22^{PHOX}$ are found together as a heterodimer, known as cytochrome b558. $p22^{PHOX}$ also acts as a docking station for the cytosolic components of NADPH oxidase. The cytosolic components of NADPH oxidase are $p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$. These exist as a complex in the cytosol of resting neutrophils. Rac2 is another cytosolic component that is essential for NADPH oxidase activity. The spatial separation of the cytosolic

components of NADPH oxidase is important. It allows a graded production of ROS and prevents the occurrence of spontaneous NADPH oxidase activation with the subsequent release of cytotoxic mediators.

The products of NAPDH oxidase activity or the respiratory burst warrant further consideration and description. ROS is a collective term for a group of oxygen-derived molecules that are formed often as a product of normal cellular metabolism and have important roles in healthy cell homeostasis. Of most interest in this group of molecules are the oxygen radicals, namely superoxide (O_2^-) and hydroxide (OH^-). These free radicals possess an unpaired electron that make them likely to react with and damage a myriad of molecules through oxidation reactions. Hypochlorous acid (HOCl), ozone (O_3) and hydrogen peroxide (H_2O_2) are reactive oxygen species that are non-radicals as they do not possess an unpaired electron (35). The generation of ROS is a cascade of reactions. This cascade begins with the generation of O_2^- from O_2. The main fate of O_2^- is dismutation to H_2O_2, which can then be converted to HOCl by MPO in the presence of Cl^-. O_2^- may also react with nitric oxide to form peroxynitrite or it may react with iron leading to the generation of the hydroxyl radical ($^{\cdot}OH$), the latter being known as the Fenton reaction (36). The primary producers of ROS are the NOX family of NAPDH oxidases.

The activity of the NADPH oxidase of circulating neutrophils will be the focus of this chapter. Neutrophils are the most abundant white blood cell in humans and they are the first line of defence in terms of innate immunity. ROS from neutrophils can be released into the vacuolar lumen of the phagosome and it can also be released extracellularly, typically at the site of an invading organism. Neutrophils express a large number of cell surface receptors, particularly when in an inflammatory environment (2). There are many signalling pathways by which NADPH oxidase can be primed or activated. For example, TNFα binding to its receptor leads to phosphorylation of $p47^{PHOX}$ that is mediated by p38MAPK phosphorylation of Ser345 (42). This results in priming, but not activation, of NADPH oxidase. IL-8 binding to CXCR1 induces the activation of phospholipase A_2, Ca^{2+} release and up regulation of the surface expression of N-formyl peptide receptors (36). Collectively, this primes the neutrophil for enhanced NADPH oxidase activity pending the addition of a second stimulus. Formyl receptors, such

as FPR1, which recognises fMLP are unlike other GPCRs as they can either prime or directly activate neutrophil NADPH oxidase activity (31). The priming of neutrophil NADPH oxidase is an important concept for experiments performed in this chapter. Priming may involve phosphorylation of cytosolic components of NADPH oxidase, membrane translocation of the cytosolic components or the translocation of cytochrome b558 from secretory vesicles to the plasma membrane.

The mechanism by which AAT may influence oxidative stress has been studied with some interesting results. Bucurenci *et al.* demonstrated the inhibition of neutrophil O_2^- production by human AAT (160). Experiments in this study had been designed to show the mechanism by which AAT was inactivated by human neutrophils. During their experiments the authors discovered that the amount of detectable O_2^- from cells stimulated with concavalin A and cytochalasin E was lowered in the presence of AAT. A dose response was also shown whereby 3.8µM AAT significantly inhibited O_2^- and with 11.5µM AAT O_2^- release was almost completely inhibited. This is, to our knowledge, the only study to demonstrate the effects of exogenous AAT on neutrophil O_2^- production. A mechanism for the findings by Bucurenci and colleagues was not elucidated and we feel that the role of AAT in the modulation of neutrophil ROS production warrants further study.

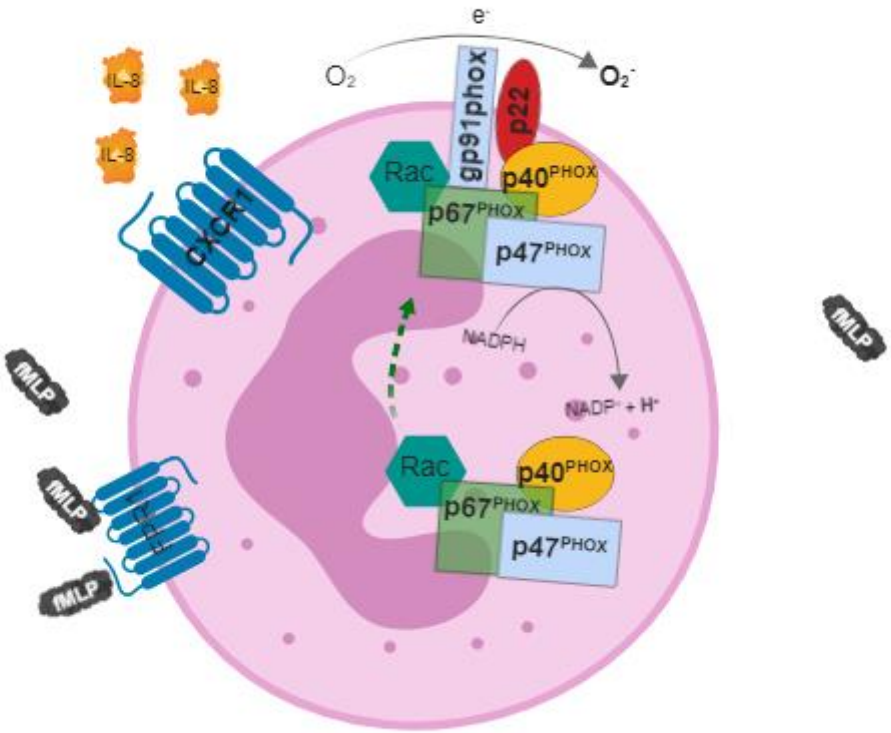


Figure 3-0: The assembly of neutrophil NADPH oxidase subunits.

The cytosolic components of NADPH oxidase consists of $p67^{PHOX}$, $p47^{PHOX}$, $p40^{PHOX}$ and Rac2. Upon exposure to a soluble stimulus (e.g. IL-8, TNFα, fMLP) the cytosolic components undergo phosphorylation and translocate to the plasma membrane. In the resting state, Rac2 resides bound to GDI. Upon activation, Rac2 dissociates from GDI and is converted to GTP:bound Rac, which translocates to the plasma membrane where it binds $p67^{PHOX}$ and is required for activation of NADPH oxidase. An electron is transferred across the plasma membrane which consumes O_2 to form O_2^-. At the same time NADPH is reduced to $NADP^+$ and H^+, both of which are liberated to the cytosol.

3.2 Aims of this chapter

The role of ROS in lung diseases such as COPD is well established. It has been shown that AAT can modulate the production of O_2^- by neutrophils (160). The mechanism by which this occurs is not known. We hypothesised that AAT modulates neutrophil ROS production by effecting the assembly of the cytosolic subunits of NADPH oxidase at the plasma membrane, and that individuals with AATD will display increased O_2^- release. In order to fully investigate this aim, the following objectives were set out:

4. To determine the mechanism by which AAT modulates O_2^- production by healthy control neutrophils
5. To investigate the role, if any, that AAT plays in the assembly of the NADPH oxidase subunits.
6. To explore the production of O_2^- by neutrophils of individuals with AATD.

3.3 NADPH oxidase activity and its modulation by AAT

In resting neutrophils, the components of NADPH oxidase are dormant and become activated in response to a variety of pro-inflammatory stimuli. This stimulus might be the presence of a microbe, the formation of a phagocytic vacuole or the binding of a ligand to its cognate cell surface receptor (237). Neutrophils express a large number of surface receptors that are increased greatly at sites of infection or sterile inflammation (10). Activation of these receptors triggers a variety of intracellular signalling cascades that can prime or activate NADPH oxidase. There are many signalling pathways by which NADPH oxidase can be primed or activated, as previously described.

The aim of our initial experiments were to quantify the extracellular generation of O_2^- by neutrophils in response to various exogenous stimuli. We wanted to determine that our experimental technique was adequate to successfully repeat the published results of others (Bucurenci *et al.* (160)). PMA (200nM) was used as a positive control. PMA is known to produce robust O_2^- generation, it is an un-physiological stimulus that does not act directly on a surface receptor but rather permeates the membrane and activates PKC (238). SOD was used as a negative control as it catalyses the dismutation of O_2^- to H_2O_2. fMLP is known to prime NADPH oxidase and to cause low levels of activation. TNFα and IL-8 cause priming of NADPH oxidase when used individually, but do not cause activation.

A cytochrome c reduction assay was used to quantify the abundance of extracellular O_2^-. Isolated neutrophils were treated with PMA (200nM), fMLP (10μM), TNFα (10ng/2x10^7 cells) and fMLP, IL-8 (10ng/2x10^7 cells) and fMLP or in the presence of SOD. Cells were incubated with either TNFα or IL-8 for 5min. All other stimulants and SOD were added immediately prior to addition of the cytochrome c buffer. The cytochrome c buffer (100nM cytochrome c, 2mM MgCl2, 2 mM CaCl2, and 5mM glucose in PBS) was kept at 37ºC in a water bath and added to the 96 well plate immediately prior to the samples being read in the spectrophotometer. The reduction of cytochrome c was recorded at 550nm over a 30min time course at 37ºC.

Our results show, O_2^- production at 5min in response to the various stimuli (Figure 3-1). Stimulation with PMA resulted in a 10-fold increase in O_2^- production

compared to control cells, from 0.5nM to 5nmol/1x10^6 cells (n=3 biological repeats, $p<0.001$, ANOVA). Exposure to fMLP alone resulted in a 5-fold increase in O_2^- release from neutrophils (2.9nmol/1x10^6 cells) ($p<0.05$). When TNFα or IL-8 were combined with fMLP there was a 7-fold increase in O_2^- production compared to resting levels (3.7nmol and 3.8nmol/1x10^6 cells, respectively) ($p<0.01$).

These experiments confirm previous findings of a graded activation of NADPH oxidase in response to various exogenous stimuli. PMA was the most robust activator of NADPH oxidase activity. IL-8 and TNFα primed the cell for a more robust activation of NADPH oxidase when subsequently exposed to fMLP. The action of TNFα and IL-8 were found to be similar in regard to O_2^- generation. Both of these cytokines are relevant in the pathogenesis of AATD (154, 161). In future experiments we would use IL-8 as our priming agent given its well described role in AATD (154).

We next set out to investigate the effect of AAT on O_2^- production, in vitro, once again using the cytochrome c reduction assay.

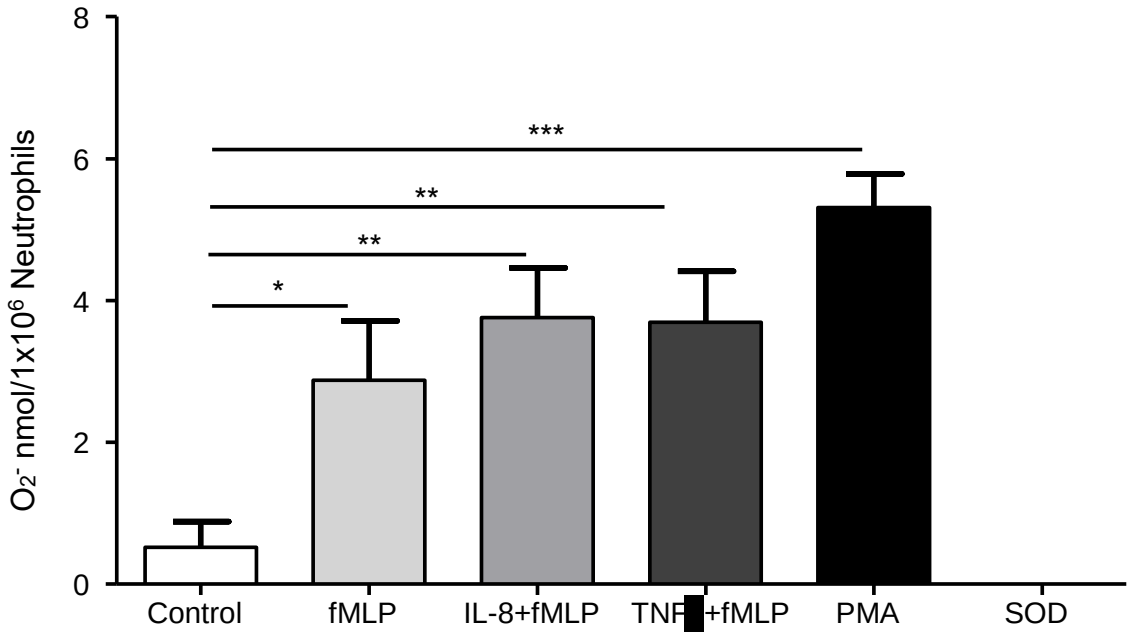


Figure 3-1: O_2^- production by neutrophils in response to exogenous stimuli.

Graph illustrating O_2^- production by purified neutrophils using a cytochrome c reduction assay. Control represents resting cells. SOD was used as a negative control. IL-8 (10ng/1x10^7 cells), fMLP (10µM), TNFα (10ng/2x10^7 cells), PMA (200nM) were used as soluble stimuli (n=3 biological repeats, *p<0.05, **p<0.001, ***p<0.0001, ANOVA post hoc Bonferroni test).

3.4 AAT modulates the production of O_2^- by stimulated neutrophils.

Previous research has shown that O_2^- production by neutrophils in response to certain stimuli can be modulated by AAT (160). Ensuing experiments of this study were designed to confirm this finding. IL-8 and fMLP were used as the chosen stimuli of NADPH oxidase activity.IL-8 is a known neutrophil chemoattractant (239) and it also acts on the CXCR1 receptor on the neutrophil surface to prime NADPH oxidase (240). IL-8 is of particular relevance in AATD. The chemotactic response of neutrophils in response to IL-8 is exaggerated in AATD individuals. Bergin *et al.*, have also shown that AAT, in its glycosylated form, can bind to IL-8 and that this complex prevents the interaction of IL-8 with CXCR1 (154). fMLP is a potent neutrophil chemotactic factor known to prime and activate NADPH oxidase. fMLP and IL-8 in combination, or fMLP, alone would be used for all future experiments.

Circulating neutrophils were isolated from healthy control individuals as previously described. Cells were incubated with varying concentrations of exogenous AAT (1.7µM to 27.5µM) for 5min. These concentrations of AAT were chosen based on the varying concentration of AAT in different body compartments, in health and disease. 27.5µM represents the concentration of AAT in plasma of PI*MM individuals. PI*ZZ individuals have plasma concentrations below 11µM. The concentration of AAT of various body fluids varies as AAT diffuses to various body compartments. For example, AAT levels of the interstitial fluid are 10-40µM and in ELF are 2-5µM (241). Two aliquots of cells were not pre-incubated with AAT. Cells were then exposed to IL-8 (10ng/1x10^7 cells) for 5min. Following this fMLP (10µM) was added to cells. This last step was performed immediately prior to addition of the cytochrome c buffer and reading the plate with the spectrophotometer over a 5 min time course.

Our results, show a time course of O_2^- production over 5 min (Figure 3-2a). This is a representative time course of five separate experiments. This demonstrates that the extracellular production of O_2^- in response to IL-8 and fMLP was modulated in a dose-dependent manner by pre-incubation with AAT. In IL-8+fMLP stimulated cells, extracellular O_2^- production was significantly reduced by pre-incubation with 3.4µM AAT (Fig 3-2b) ($n=5$, $p<0.05$, ANOVA, post Bonferroni test). At concentrations equivalent to physiological plasma concentration (27.5µM) O_2^-

detection was almost completely inhibited (0.05nmol/1x10^6 neutrophils). Cells that were treated with IL-8+fMLP without pre-incubation with AAT produced 8nmol/1x10^6 neutrophils, 160-fold greater than those cells that were pre-incubated with 27.5µM AAT.

These experiments confirm the findings of Bucerenci *et al.* that, in vitro, AAT at and below physiological plasma concentrations modulates neutrophil O_2^- production. We next set out to examine where along the pathway of NADPH oxidase activation that AAT was exerting this effect.

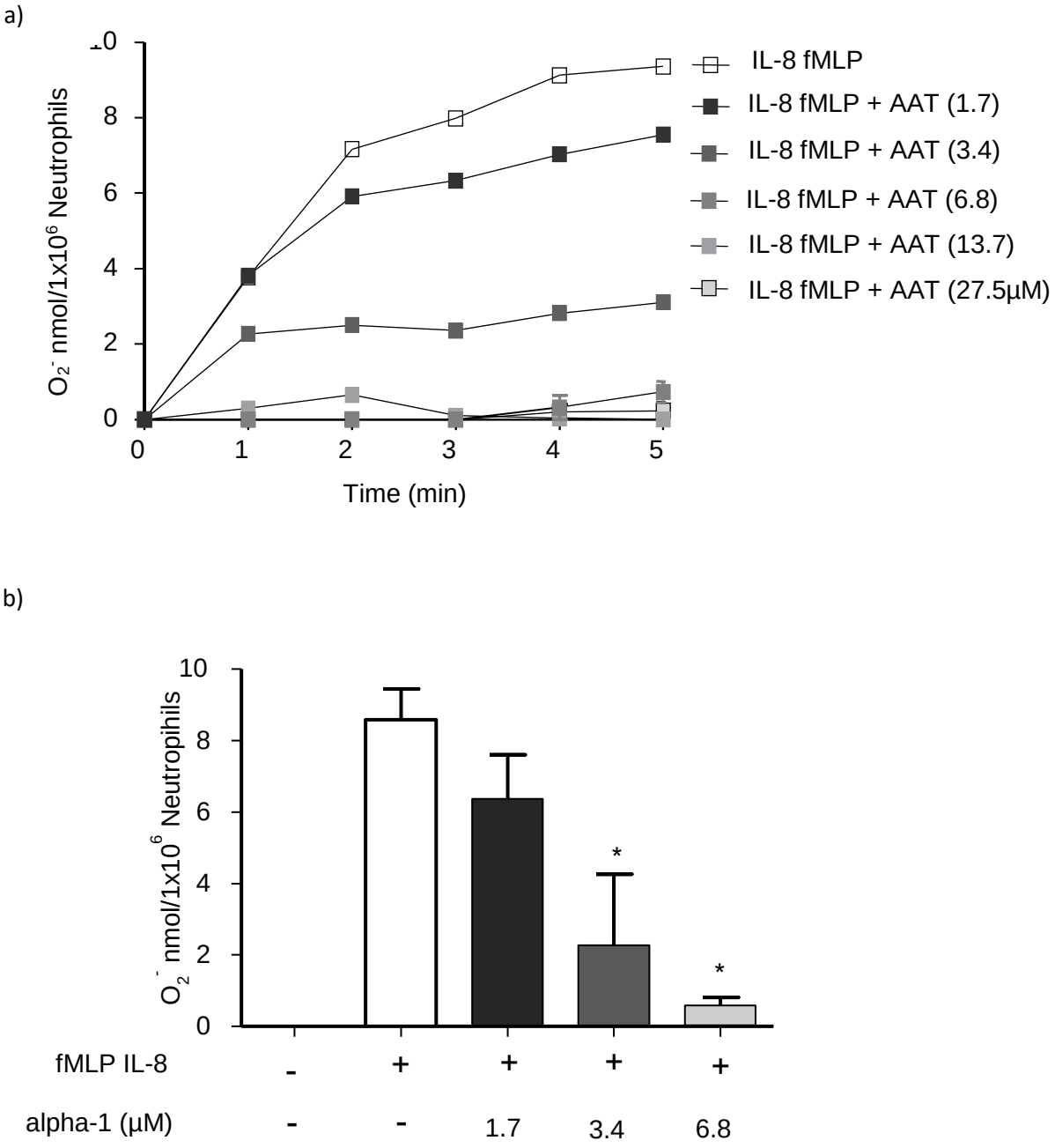


Figure 3-2: The effect of exogenous AAT on O_2^- production by stimulated neutrophils.

a) Representative time course of O_2^- production by circulating neutrophils, stimulated with IL-8 (10ng/2x10^{7}cells) and fMLP (10µM) in the presence of various concentrations of AAT (1.7µM-27.7µM). Measured using a cytochrome c reduction assay. b) A graph illustrating the significant inhibition of O_2^- detection, at 5 min, with 3.4µM and 6.8µM AAT (n=5 biological repeats, *p<0.05, ANOVA, post hoc Bonferroni test,).

3.5 The assembly of NADPH oxidase cytosolic subunits at the plasma membrane is influenced by AAT.

As previously discussed, IL-8 exposure results in priming of neutrophil NAPDH oxidase (240). fMLP, on the other hand, can induce low levels of NAPDH oxidase activity. The combination of IL-8 and fMLP will result in higher levels of O_2^- production than would be expected for either agent alone (240). We demonstrated, that IL-8 and fMLP combined resulted in a 7-fold increase in O_2^- from baseline (Fig 3-1). Activation of NAPDH oxidase requires the phosphorylation of $p47^{PHOX}$ and the translocation of $p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$ (i.e. cytosolic components) to the plasma membrane for electron transport across the membrane to occur (237). We have already shown that AAT can modulate the amount of detectable O_2^- produced by NADPH oxidase in response to IL-8 and fMLP (Figure 3.2). It is well documented that AAT is sensitive to oxidation (242) and in this manner the findings of Figure 3-2 may be explained by exogenous AAT scavenging O_2^- during its own oxidation. However, AAT may also modulate NADPH oxidase activation by impacting on signalling events up-stream from this. For this reason, the ensuing experiments were designed with the aim of assessing the effects of AAT on the membrane translocation of $p67^{PHOX}$ and $p47^{PHOX}$ in neutrophils, in response to various stimuli.

Isolated neutrophils were stimulated with IL-8 and fMLP in the presence or absence of exogenous AAT. Aliquots of cells were taken at 0, 30 and 60 seconds post stimulation. Following cell lysis, in the presence of protease inhibitors, neutrophil membranes were isolated by subcellular fractionation, as described previously (243). These isolated neutrophil membranes were electrophoresed and Western blotted for the presence of $p67^{PHOX}$ and $p47^{PHOX}$ (antibodies for $p67^{PHOX}$ and $p47^{PHOX}$ courtesy of Segal laboratory, University College London).

Western blot analysis revealed that stimulating cells with IL-8 and fMLP resulted in protein expression of both $p67^{PHOX}$ and $p47^{PHOX}$ at the plasma membrane (Figure 3-3a). When neutrophils were pre-incubated with AAT (27.5µM) membrane expression of both $p67^{PHOX}$ and $p47^{PHOX}$ decreased. Membrane expression of $p47^{PHOX}$ decreased by 45% in those neutrophils pre- incubated with AAT (n=3 biological repeats, $p<0.01$, ANOVA). Similarly, $p67^{PHOX}$ was decreased by 75% in

those neutrophils pre-incubated with AAT ($p<0.01$), compared to cells not incubated with AAT.

In conclusion, these results suggest that AAT is modulating NADPH oxidase assembly at the plasma membrane. The reduced detection of O_2^- cannot be fully explained by the oxidation of AAT by O_2^- (i.e. O_2^- scavenging), although this may still be a factor. To further elucidate this, we next set out to examine the effect of AAT on the O_2 consumption of neutrophils in response to the same stimuli.

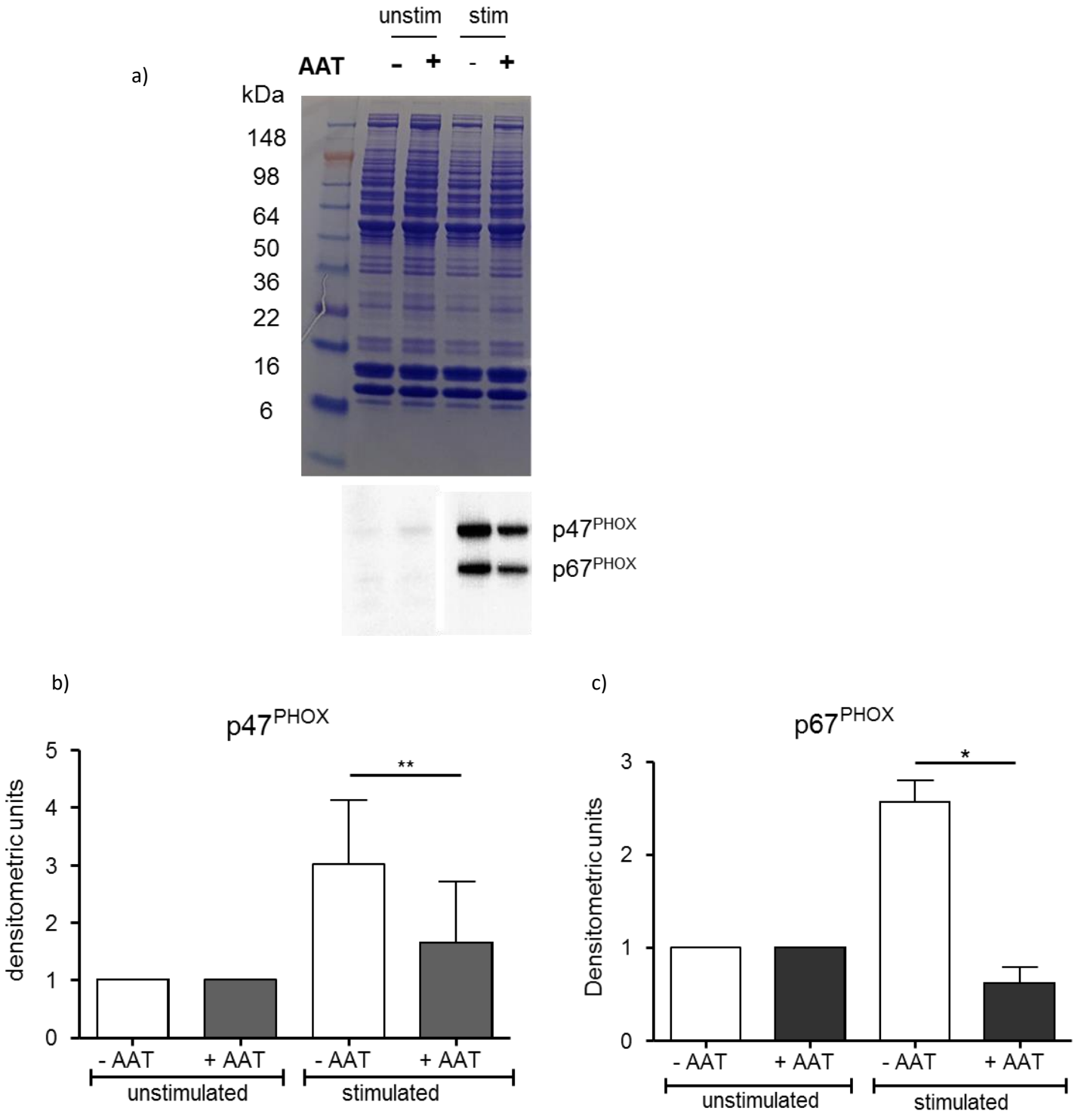


Figure 3-3: AAT reduces translocation of the cytosolic subunits of NADPH oxidase to the plasma membrane.

a) A Coomasie gel is shown representing isolated, circulating neutrophil membranes post stimulation with IL-8 (10ng/1x10^7cells) and fMLP (10µM), or unstimulated, in the presence or absence of AAT (27.5µM). Also shown is a representative Western blot depicting membrane protein expression of $p67^{PHOX}$ and $p47^{PHOX}$ (white line indicates point of splicing between two separate blots) (b) Graph depicting the densitometry of immunobands for $p47^{PHOX}$ and (c) $p67^{PHOX}$ (n=3 biological repeats, *p<0.05, **p<0.01, Student's t test).

3.6 O_2 consumption and its modulation by AAT.

We have demonstrated that AAT reduces detection of O_2^- using a cytochrome c reduction assay. This experiment does not confirm that AAT is directly influencing NADPH oxidase activity. AAT is known to act as a scavenger of O_2^-. The methionine residues of AAT can undergo oxidation to form oxidized AAT. In this way, AAT could lead to a reduction in detection of O_2^-, as we saw in the first experiments of this chapter (Figure 3-1). NADPH oxidase activation has many steps and a number of different pathways, however, the ultimate step is the consumption of O_2 (see Figure 3-0) which is converted to O_2^- with the transfer of an electron via NADPH oxidase. In our next set of experiments, we set out to demonstrate the effects of AAT on neutrophil O_2 consumption in response to NADPH oxidase activation.

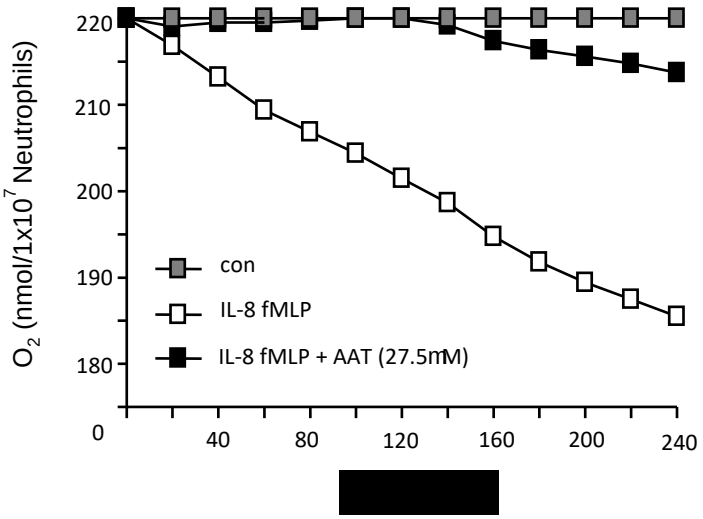
For this experiment we used a Clark-type oxygen electrode to measure O_2 content of cell suspensions. All experiments were carried out in 500µl of dPBS containing glucose (5mM), using $2x10^7$cells per treatment. O_2 consumption was recorded over 4 min. Cells were pre-treated with AAT (27.5µM) for 5min. Subsequently, they were treated with IL-8 for 5min (10ng/$2x10^7$cells) and fMLP (10µM) was added immediately prior to commencing O_2 consumption readings.

A 4min time course of O_2 content of neutrophil suspensions following various treatments is shown (Fig 3-4a). The O_2 content of cell suspensions treated with IL-8/fMLP drops dramatically over the 4min time course. This represents O_2 consumption related to the activity of NADPH oxidase. There is little change in the O_2 content of cell suspensions with control cells or cells that were pre-incubated with AAT and then stimulated with IL-8/fMLP. At 4min the O_2 content of the neutrophil suspensions, in those cells treated with IL8/fMLP, decreased by approximately 15%, from 220nmol/$1x10^7$ neutrophils to 185nmol/$1x10^7$ neutrophils (Fig 3-4b) ($n=3$, $p<0.001$, ANOVA, post Bonferroni test). There was no significant change in O_2 content at 4min in those stimulated neutrophils that were pre-incubated with AAT.

This result confirms that AAT is directly modulating NADPH oxidase activity and not merely acting as a scavenger of O_2^-, as we have shown that AAT modulates neutrophil O_2 consumption induced by IL-8/fMLP. Given the role of AAT in the

activity of NADPH oxidase activation we next set out to examine the assembly and activity of NADPH oxidase in individuals with AATD, in whom plasma levels of AAT are chronically low.

a)



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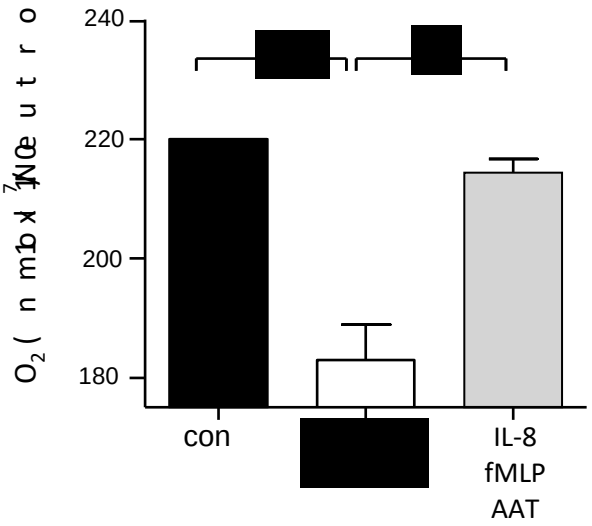


Figure 3-4: O_2 consumption by neutrophils is modulated by AAT.

a) A time course of the O_2 content of neutrophil suspension in response to stimuli and the effect of AAT on stimulated cells, measured using a cytochrome c reduction assay. b) Neutrophils exposed to IL-8 (10ng/2x107cells) and fMLP (10µM) had significantly increased O_2 consumption and this was inhibited by pre-incubation with exogenous AAT (27.5µM) (n=3 biological repeats, **p<0.01, ***p<0.001 one-way ANOVA, post Bonferroni test).

3.7 The activity of NADPH oxidase in circulating neutrophils of individuals with AATD.

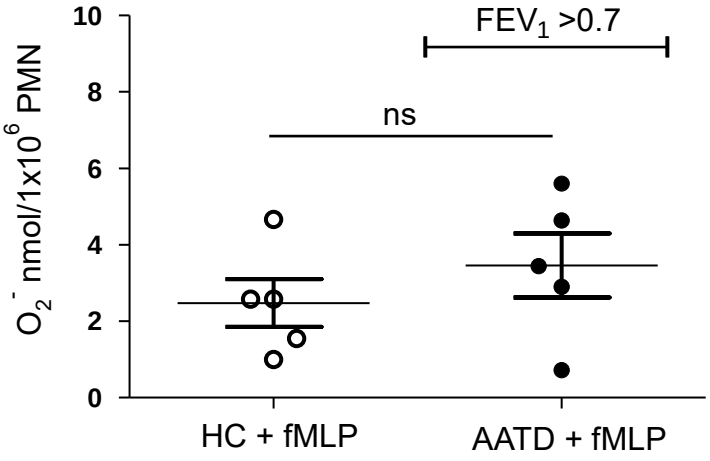
Preceding results have demonstrated that, in healthy control neutrophils, IL-8 and fMLP result in a robust activation of the neutrophil NADPH oxidase and fMLP alone results in lower levels of O_2^- production. Further to this, we have shown that this effect can be modulated, in vitro, by AAT and that this modulation is intrinsically related to NADPH oxidase activity. In order to further evaluate the role of AAT in NADPH oxidase activity we next set out to explore O_2^- production by circulating neutrophils in individuals with AATD. AAT is an acute phase reactant, therefore, our inclusion criteria for patient recruitment stipulated that they were free of pulmonary exacerbations in the past eight weeks. We hypothesised that, as a consequence of low levels of AAT in AATD individuals, extracellular O_2^- would be increased relative to control cells.

Circulating neutrophils were isolated from healthy control and AATD individuals. These cells were treated with fMLP (10µM) immediately prior to addition of the cytochrome c buffer and reading the plate with the spectrophotometer over a 5min time course.

Our results illustrate that when neutrophils of AATD individuals with obstructive spirometry were treated with fMLP alone they produced greater amounts of O_2^- then healthy control neutrophils treated in the same way (Figure 3-5b) (n=11 subjects, p=0.002, student's t test). Healthy control neutrophils stimulated with fMLP produced O_2^- at a rate of 3.9nmol/1x10^6 cells. Neutrophils from AATD individuals with FEV_1 <0.7 showed a 60% greater rate of O_2^- production (6.4nmol/1x10^6 cells) than healthy control cells. There is a suggestion from this result that circulating neutrophils of AATD individuals are in a 'primed state' whereby exposure to fMLP results in exaggerated NADPH oxidase activity and ROS production. In AATD individuals with normal spirometry there was no difference in O_2^- production compared to healthy controls.

We next set out to examine the assembly of the NADPH oxidase subunits on the neutrophil membrane in response to fMLP in AATD individuals to further elucidate the mechanism by which AATD individuals have greater neutrophil O_2^- production in response to this soluble stimulus.

a)



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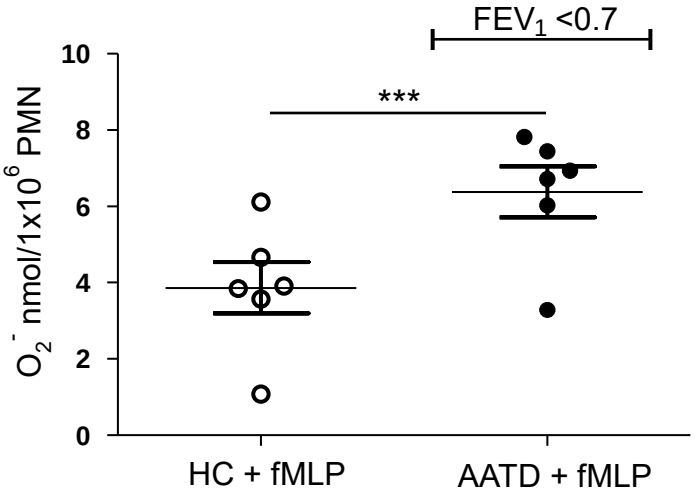


Figure 3-5: O_2^- production in response to fMLP is exaggerated in circulating neutrophils of individuals with AATD.

a) O_2^- production of circulating AATD neutrophils (FEV_1>0.7) was not different compared to healthy control cells (n=5, p=0.2, Student's t test). b) O_2^- production isolated from AATD individuals with FEV_1<0.7 was increased compared to healthy control neutrophils (n=6, p=0.0004, Student's t test, two-tailed). Healthy control (HC) or AATD neutrophils were treated with fMLP (10µM).

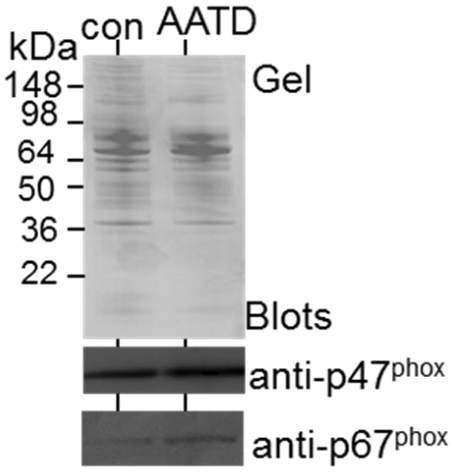
3.8 The assembly of NADPH oxidase cytosolic subunits at the plasma membrane in AATD neutrophils.

Our previous experiments demonstrated exaggerated O_2^- production by AATD neutrophils in response to fMLP. In our previous in vitro studies (Figure 3-3) we have shown that exogenous AAT directly modulates the activity of NADPH oxidase by modulating the translocation of the NADPH oxidase cytosolic subunits $p67^{PHOX}$ and $p47^{PHOX}$ to the plasma membrane in healthy control cells. For this reason, we hypothesised that AATD neutrophils would display increased expression of the cytosolic components of NADPH oxidase at the plasma membrane. Ensuing experiments were designed to examine the membrane expression of $p67^{PHOX}$ and $p47^{PHOX}$ in AATD neutrophils stimulated with fMLP.

Isolated neutrophils from healthy control and AATD individuals were stimulated with fMLP (10µM) for 5min. These cells were then lysed in a lysis buffer containing protease inhibitors. Neutrophil plasma membranes were isolated by sucrose gradient subcellular fractionation, as described previously (54). These neutrophil membranes were electrophoresed and Western blotted for the presence of $p67^{PHOX}$ and $p47^{PHOX}$.

Our results revealed increased expression of $p67^{PHOX}$ and $p47^{PHOX}$ on the plasma membrane of circulating AATD neutrophils (n=3 subjects per group, **p<0.01, Student's t test). There was approximately a 50% increase in the expression of both $p67^{PHOX}$ and $p47^{PHOX}$ in AATD neutrophils treated with fMLP compared to healthy control cells treated similarly. These results support our previous findings that circulating AATD neutrophils produce approx. 35% more O_2^- in response to fMLP. Therefore, these results further suggest that circulating neutrophils from individuals with AATD are primed. In our next experiments, we wanted to examine the response of AATD neutrophils to activation of NADPH oxidase with a more robust stimulus that is known to be of relevance in AATD (154).

a)



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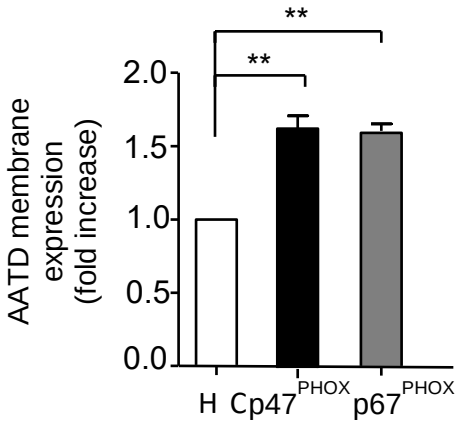


Figure 3-6: Neutrophil membrane expression of p67^{PHOX} and p47^{PHOX} is increased in AATD neutrophils in response to fMLP.

a) A Coomasie gel is shown of neutrophil membrane fractions from healthy control (HC) individuals and those with AATD, both treated with fMLP (10µM). Also shown are representative Western blots depicting protein expression of p47^{PHOX} (middle panel) and p67^{PHOX} (lower panel). b) Graph depicting the densitometry of immunobands for p67^{PHOX} and p47^{PHOX} shown as the fold increase in p67^{PHOX} and p47^{PHOX} in AATD relative to healthy control. Expression of both p67^{PHOX} and p47^{PHOX} were increased by 50% in AATD membrane fractions compared to healthy controls (n=3 subjects per group, **p<0.01, ANOVA post hoc Bonferroni test).

3.9 The activity of NADPH oxidase in individuals with AATD in response to IL-8 and fMLP.

We have shown that AATD neutrophils exhibit an exaggerated response to fMLP. They exhibit increased O_2^- production associated with increased assembly of NADPH oxidase complex at the plasma membrane in response to this stimulus. We wanted to examine the production of O_2^- by AATD neutrophils in response to a more robust activation of NADPH oxidase by inclusion of IL-8 in combination with fMLP. The activity of IL-8 is known to be modulated by AAT, whereby, AAT binds IL-8 to prevent interaction with its receptor CXCR1. Therefore, in AATD, neutrophils are more susceptible to the chemotactic effects of IL-8 (154). The aim of the current experiment was to compare the O_2^- production of AATD neutrophils and healthy control neutrophils in response to IL-8 and fMLP.

Similar to previous experiments, we used IL-8 and fMLP as exogenous stimuli. Circulating neutrophils were isolated from healthy control or AATD individuals. These cells were treated with IL-8 (10ng/1x10^7cells) and fMLP (10μM) immediately prior to addition of the cytochrome C buffer and reading of the plate at 550nm over a 5min time course.

AATD neutrophils produced 25% more O_2^- in response to the combination of IL-8 and fMLP than did healthy control cells treated similarly (Fig 3-7) (n=6 subjects per group, p=0.04, Student's t test). The rate of O_2^- production at 5min was 3.7nmol/1x10^6 by healthy controls neutrophils while in AATD the rate was 5nmol/1x10^6 cells.

We can conclude, that circulating AATD neutrophils are primed for a more robust activation of NADPH oxidase, as demonstrated by their response to fMLP alone and to IL-8 in combination with fMLP.

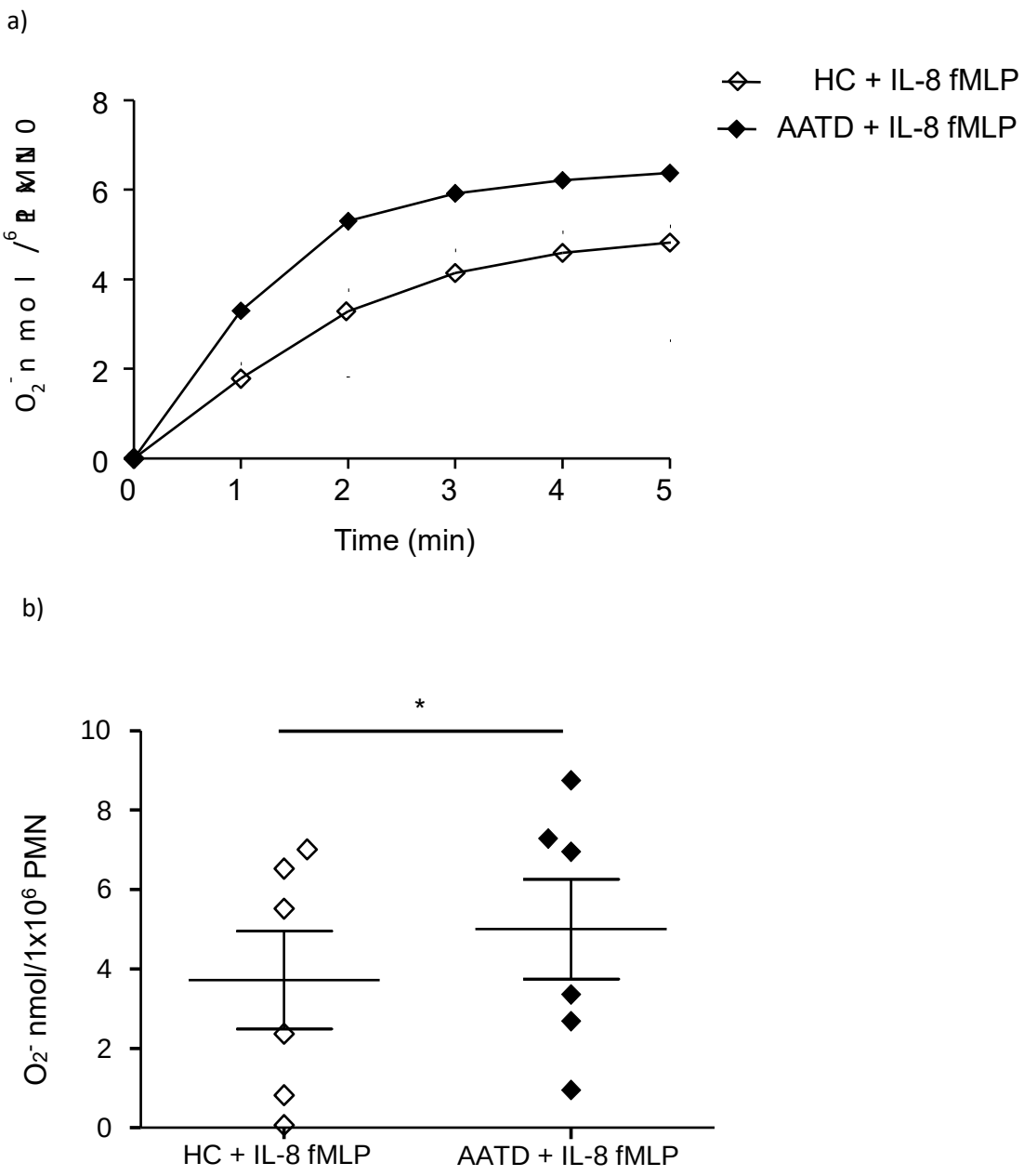


Figure 3-7: Exaggerated O_2^- production by neutrophils of individuals with AATD in response to IL-8/fMLP.

a) 5 min time course of O_2^- production (representative graph of a single individual), as measured by a cytochrome c reduction assay. Healthy control (HC) or AATD neutrophils were treated with IL-8 (10ng/1x10^7cells) and fMLP (10µM). b) The amount of detectable O_2^- in AATD cells treated with IL-8 and fMLP was significantly greater than that from healthy control cells treated similarly (n=6, p=0.04, Student's t test, two-tailed). Data normally distributed, as assessed by the D'Agostino & Pearson omnibus normality test.

3.10 Discussion

The first aim of this chapter was to measure the production of O_2^- by neutrophils in response to various stimuli. A cytochrome c reduction assay was used. This has the benefit of being able to record the extracellular production of O_2^- and it is considered by many to be the 'gold standard' of cell based O_2^- quantification (244). Using this assay, we have demonstrated that PMA is a robust activator of NAPDH oxidase, as would be expected given its un-physiological membrane-permeating action. We showed that fMLP alone stimulated low levels of O_2^- production and that this was augmented by first priming neutrophils with either IL-8 or TNFα. These results confirm the findings of others (44).

The second aim of this chapter was to study, in vitro, the effects of exogenous AAT on neutrophil O_2^- detection. Bucurenci *et al.* in 1992 reported the effects of human plasma AAT on neutrophil O_2^- production (160). While the authors of this study initially set out to study the mechanism of inactivation of AAT by human neutrophils they came upon the finding that AAT modulates O_2^- detection from stimulated neutrophils. We aimed to replicate the experiments of Bucerenci using more physiological stimuli (i.e. IL-8 and fMLP) with more relevance to AATD. IL-8 is a well-studied chemokine (in AATD and other pulmonary diseases (154, 245)) that primes the NAPDH oxidase of neutrophils via CXCR1 and CXCR2. fMLP is a synthetic analogue of the chemotactic bacterial peptide that can prime and activate NADPH oxidase via FPR1 and FPR2 receptors (240).

We demonstrated that incubating neutrophils with physiological concentrations of AAT prior to stimulation with IL-8 and fMLP resulted in reduced production of extracellular O_2^-. When we used physiological concentrations of AAT (i.e. plasma levels) detection of O_2^- was almost completely abolished. The response to AAT was found to be dose dependent and was still significant at 3.4μM. This is consistent with the findings of Bucurenci *et al*. The dose dependent response is important to consider. In normal individuals, plasma AAT levels are 20-53μM and it is said that the putative protective threshold level is 11μM (179). While AAT is found in high abundance in the plasma of replete individuals its concentration falls as it moves to other body compartments. AAT levels of the interstitial fluid for example are 10-40μM and in ELF are 2-5μM (179). These compartments are

clearly relevant to lung pathologies such as COPD and emphysema. Therefore, our finding that 3.4μM of AAT significantly modulates the production and detection of O_2^- is an important one. This level of AAT in the interstitium and ELF is sufficient to significantly reduce O_2^- production by NAPDH oxidase. In AATD, the concentration of AAT in ELF is obviously significantly reduced compared to AAT replete individuals. The target for successful protection of the alveolar structures from neutrophil proteolytic activity is thought to be 1.2μM in alveolar ELF (246). Our results suggest that, perhaps, the protective target for O_2^- activity is greater than this.

These results confirmed that, in vitro, AAT is modulating O_2^- production. However, we are not directly measuring NADPH oxidase activity with the cytochrome c reduction assay, rather we are determining the amount of O_2^- available to reduce cytochrome c to ferrocytochrome (by the resultant colour change) . There are alternate ways in which AAT may be altering the detection of O_2^-. Foremost in this consideration is the oxidation of AAT methionine residues by O_2^- resulting in less available O_2^- for the reduction of cytochrome c in our assay. Further experiments set out to clarify this. We demonstrated, in vitro, that neutrophils stimulated with fMLP and IL-8 had detectable levels of the cytosolic components of NAPDH oxidase, $p67^{PHOX}$ and $p47^{PHOX}$, present at the plasma membrane. When these cells were exposed to exogenous AAT prior to the addition of the stimuli, the detection of $p67^{PHOX}$ and $p47^{PHOX}$ at the plasma membrane was reduced by 40% and 20% respectively. Moreover, use of a Clark-type oxygen electrode we measured O_2 consumption of neutrophils in response to IL-8 and fMLP and the effect of AAT on O_2 consumption. We found that AAT modulates the O_2 consumption induced by IL-8 and fMLP. Therefore, these experiments together provide evidence that AAT is directly modulating the assembly and activation of neutrophil NADPH oxidase.

With the role of AAT in NADPH oxidase activity slightly better defined by these experiments, it provoked the question as to the assembly and activity of NADPH oxidase in AATD individuals. A total of 11 patients with AATD were recruited to the next part of the study and the same number of healthy control volunteers. All AATD individuals were PI*ZZ and were free from exacerbations in the past 8

weeks. None were current smokers. The healthy control group were all non-smokers with no history of lung disease.

Our first experiments in this patient cohort measured O_2^- production by circulating neutrophils in response to various stimuli. We found that, in response to fMLP, AATD neutrophils had significantly more O_2^- production. There was a 1.5 fold increase in detectable O_2^- in the AATD group stimulated with fMLP alone. This would suggest that circulating AATD neutrophils are in a primed state, thus having a more robust response to fMLP.

To clarify that this effect was related to NADPH oxidase activity we measured the assembly of NADPH oxidase components on the plasma membrane of both AATD and healthy control neutrophils in response to fMLP. We demonstrated that AATD neutrophils had a 50% increase in membrane expression of both $p67^{PHOX}$ and $p47^{PHOX}$ in response to fMLP compared to healthy control neutrophils. This confirmed that AATD individuals have exaggerated NAPDH oxidase activity may be related to their chronic deficiency of plasma AAT.

Overall, from the results of this chapter we can conclude that, AAT modulates the production of O_2^- in circulating neutrophils through effects on the assembly of NADPH oxidase subunits at the plasma membrane. This effect is seen in individuals with AATD, who demonstrate augmented O_2^- production and augmented assembly of NAPDH oxidase in circulating neutrophils. The mechanism by which AAT modulates NADPH oxidase activity is not known and will be investigated in the coming chapters. Firstly, in the next chapter we will investigate the physiological consequences to the neutrophil of enhanced NADPH oxidase activity.

Chapter 4: Evaluation of the consequences of enhanced NADPH oxidase activity on neutrophil cytosolic pH in AATD.

4.1 Introduction

In the previous chapter we have shown that AAT modulates the activity of NADPH oxidase. We also demonstrated that circulating neutrophils from individuals with AATD exhibit enhanced ROS production via increased NADPH oxidase assembly at the cell membrane. The activity of NADPH oxidase has a number of consequences for the neutrophil. NADPH oxidase liberates two electrons from NADPH which cross the membrane through the cytochrome b558 complex and reduce two molecules of O_2 to two molecules of O_2^- (35). Two H^+ are generated intracellularly for each NADPH that is consumed (see Figure 4-1). One proton comes directly from oxidized NADPH and another is generated when $NADP^+$ enters the hexose monophosphate shunt. The compartment into which the electrons are transported from cytochrome b558 depends on the location of the assembled NADPH oxidase complex. It can be assembled on the plasma membrane, in which case, movement of electrons is extracellular (and H^+ accumulates in the cytosol) or NADPH oxidase may assemble on the vacuolar membrane (e.g. during phagocytosis). In the latter case, electrons are transferred to the vacuolar lumen and H^+ to the neutrophil cytosol (237).

In 1988 it was discovered that the activity of neutrophil NADPH oxidase is electrogenic and results in membrane depolarisation (247). Its activity also results in the accumulation of cytosolic H^+ and a transient acidification of the cytosol (248). Both of these consequences of NADPH oxidase activity can terminate its own activity if the membrane depolarisation and cytosolic acidification are not compensated for. In neutrophils, HVCN1, a voltage-gated proton channel is activated by both membrane depolarisation and changes in cytosolic pH and acts to counteract both of these effects (248). Its action is intrinsically linked to the activity of NADPH oxidase and it can move large quantities of H^+ when required. Detailed studies by Pick *et al*., and other groups, have shown that NADPH oxidase transports electrons at a rate of 300 electrons/sec and that subsequent activation of a single HVCN1 channel results in the movement of 34,000 protons/sec. Thus giving HVCN1 the capacity to compensate for the electrogenic activity of over 100 NADPH oxidase molecules (249).

HVCN1 clearly possesses large compensating capabilities in terms of H^+ movement. This is important, as under physiological conditions the concentration of H^+ ions in intracellular and extracellular fluids is low, 40nM and 100nM respectively. Therefore, flux of relatively small quantities of protons through HVCN1 can cause rapid variations in pH within the cell, as well as extracellularly. The regulation of intracellular pH by HVCN-1 is important for a multitude of reasons. Paramount to this is that the function of NADPH oxidase itself is dependent on intracellular pH. Its function is optimal at pH 7.5 and drastically decreases at either lower or higher pH (51). In addition to this, it has been shown that a number of phagosomal granule contents show activity that is dependent on pH. Within the phagosome, MPO has predominantly peroxidase activity at low pH and catalase activity at high pH, while NE has greater activity at high pH (250).

Neutrophils have a high abundance of NADPH oxidase relative to other cells and also have the highest abundance of proton channels (51). The ratio of NADPH oxidase to HVCN1 channels is approximately 10:1, as per DeCoursey and colleagues (249). The Na^+/H^+ antiport and the vacuolar-type ATPase are other ion transporters that can buffer pH changes within the neutrophil. Morgan et al studied pH changes in human neutrophils following phagocytosis of zymosan, they used the pH indicator SNARF. They studied the role of not only HVCN-1, but also Na+/H+ antiport and V-type H+ ATPase in pH regulation following phagocytosis. The authors demonstrated significant cytosolic acidification after zymosan phagocytosis. By sequentially inhibiting each of these channels the authors showed that HVCN-1 is the first channel to respond to this acidification but that both Na+/H+ antiport and V-type H+ ATPase are also required (251).

The consequence of absent HVCN1 has been studied in mouse and human neutrophils with effects on both ROS production and cytosolic pH. A number of knock out studies of *hvcn-1* mouse neutrophils have been performed which show that HVCN-1 is essential for O_2^- production (45). These studies showed that mouse neutrophils lacking HVCN-1 demonstrated impaired killing of *Staphylococcus aureus* and increased lung inflammation (53). From the results of our first chapter we have shown exaggerated NADPH oxidase activity and assembly in circulating neutrophils from individuals with AATD. The physiological

consequences of this in relation to both cytosolic pH and proton channel activity have not been studied in this population.

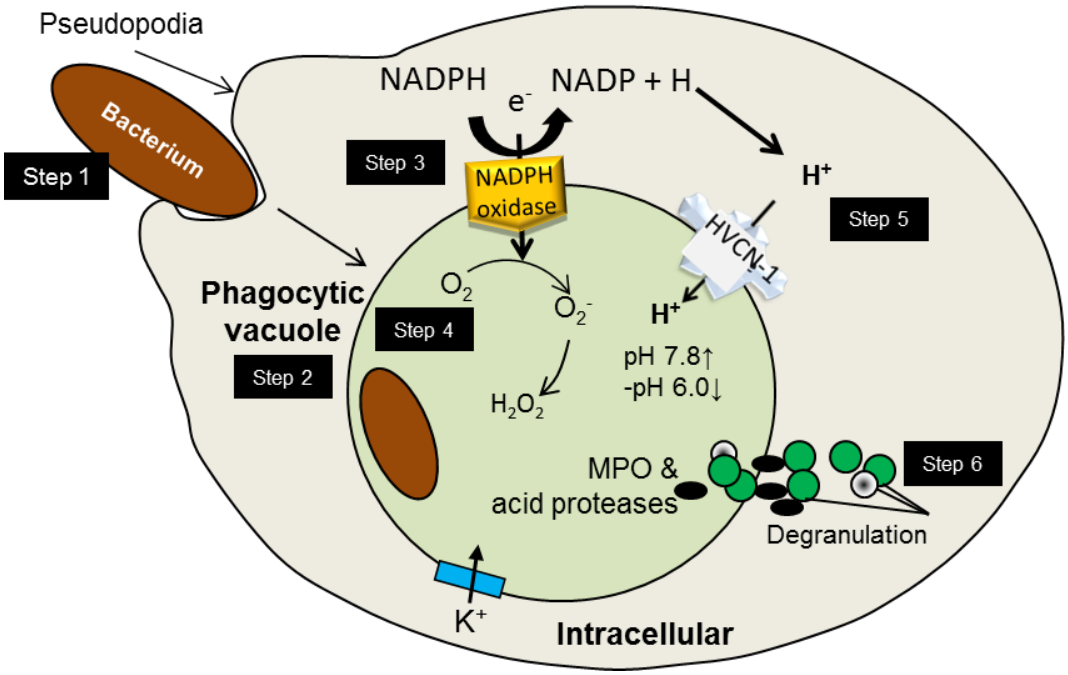


Figure 4-1: Diagrammatic representation of activation of the NADPH oxidase of neutrophils upon engulfment of a bacterium

Step 1: A bacterium is recognised by receptors on the plasma membrane of the neutrophil. The interaction triggers the formation of pseudopodia around the bacterium. Step 2: The bacterium becomes fully enclosed within a phagocytic vacuole. Step 3: The NADPH oxidase is selectively activated in the membrane of the phagocytic vacuole and donates electrons across the membrane to generate O_2^- in the vacuolar lumen (Step 4). Step 5: Activity of the NADPH oxidase depolarises the membrane and the nature of the compensating charge involves influx of potassium (K^+) and protons (H^+). HVCN1 pumps H^+ across the vacuolar membrane and the initial rise in pH to ~7.8 is followed by a decrease to pH ~6.5, which is facilitated by HVNC-1 activity and H^+ flux. Step 6: Antimicrobial peptides and proteins including acid proteases and MPO are released into the phagocytic vacuole by a process called degranulation.

4.2 Aims of this chapter

We have shown that circulating neutrophils from individuals with AATD have enhanced production of O_2^- due to NADPH oxidase activity. The aim of this chapter is to understand the consequences for the neutrophil of increased NADPH oxidase activity, investigating cytosol pH and proton channel activity. Our hypothesis was that circulating neutrophils of individuals with AATD would show excess cytosolic acidification in response to exogenous stimuli. In response to the cytosolic acidification, our aim was to investigate whether there would be a compensatory increase in the abundance of HVCN1. To fulfil this aim, the following objectives were set:

1. To determine the abundance of HVCN1 in circulating AATD neutrophils
2. To investigate the cytosolic pH of neutrophils from AATD individuals
3. To explore the consequences of any differences found in pH or HVCN1 abundance

4.3 The abundance of HVCN1 in neutrophils of individuals with AATD.

HVCN1 is a highly selective proton channel found on epithelial cells, macrophages, lymphocytes and granulocytes. In neutrophils, HVCN1 is essential for balancing and compensating the membrane depolarisation and cytosolic H^+ accumulation. HVCN1 resides on the plasma membrane and in intracellular stores. It is predominantly found in secondary and tertiary granules and secretory vesicles in resting cells (48). Phosphorylation of Thr29 in the N-terminus results in enhanced HVCN1 activity (51). The movement of protons upon activation of HVCN1 from the cytosol is either to the extracellular space or intra-phagosomal (252).

We demonstrated in the previous chapter that the assembly and activity of NADPH oxidase was increased in neutrophils from individuals with AATD. This increase in NADPH oxidase activity must be associated with an increased extrusion of protons from the neutrophil in order that activity of the oxidase continues uninterrupted. We hypothesised that HVCN1 would be expressed with increased abundance on plasma membranes of circulating neutrophils of individuals with AATD. In the next set of experiments, we aimed to measure the abundance of HVCN1 in circulating neutrophils of AATD individuals and healthy control resting cells.

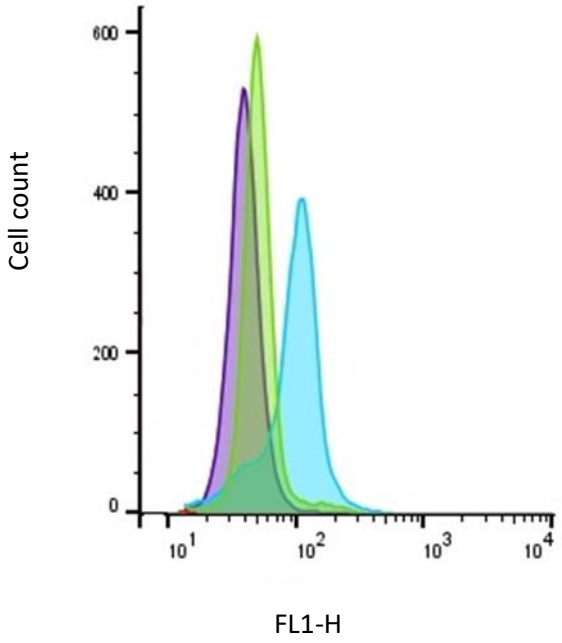
Firstly, we measured the abundance of HVCN1 on the surface of circulating neutrophils by flow cytometry. Isolated neutrophils were fixed in 4% (w/v) paraformaldehyde and probed with a rabbit anti-HVCN1 antibody (SAB3500536, Sigma). A rabbit isotype control antibody was used as a control with a goat anti-rabbit IgG FITC-labelled secondary antibody. Results revealed a greater than 20% reduction in the abundance of HVCN1 on the neutrophil cell surface in AATD neutrophils (Figure 4-2) (n=9 subjects per group, p=0.049, Student's t test).

Secondly, we quantified the abundance of HVCN1 in circulating neutrophils by Western blot analysis. Circulating neutrophils were isolated and whole cell lysates prepared using a lysis buffer containing protease inhibitors. Following this, samples were electrophoresed and Western blotted and probed for the presence of HVCN1 using a rabbit, anti-HVCN1 antibody (SAB3500536, Sigma). Abundance of HVCN1 was quantified by densitometric analysis of immunobands. Results of this experiment demonstrated that the abundance of HVCN1 in AATD neutrophil

whole cell lysates was reduced by approximately 15% compared to healthy controls (Figure 4-3) (n=7 subjects per group, p=0.025, Student's t test).

These results demonstrate a reduced abundance of HVCN1 in circulating AATD neutrophils, as measured by flow cytometry and Western blot analysis. The reduction was of the magnitude of 15-20%. This is contrary to our hypothesis. We next set about determining the mechanism by which AATD neutrophils have a lower abundance of HVCN1.

a)



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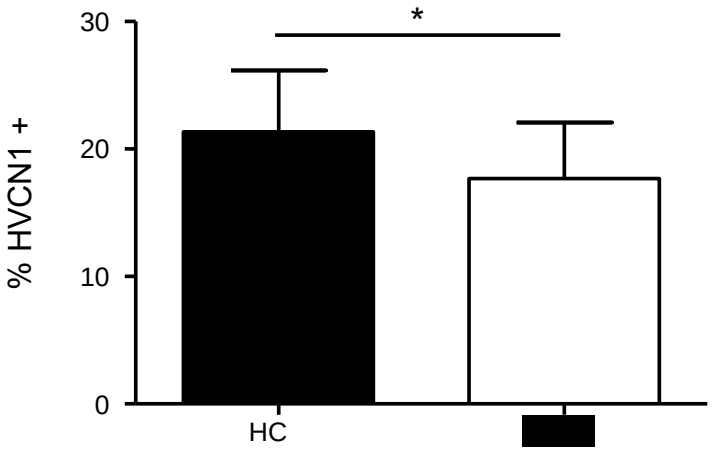


Figure 4-2: Cell surface expression of HVCN1 in AATD neutrophils.

a) Representative FACS analysis histogram showing abundance of cell surface HVCN1 in healthy control (blue) and AATD (green) neutrophils compared to the isotype control (purple). The x-axis (FL1-H) represents the mean fluorescent intensity (MFI) as detected in the FITC channel. Isolated circulating neutrophils were fixed with paraformaldehyde and analysed by flow cytometry using an anti-HVCN1 antibody. b) Graph illustrating a significantly lower abundance of HVCN1 in AATD neutrophils, as measured by flow cytometry (n=9 subjects per group, p=0.049, Student's t test). (%HVCN1 + represents the percentage of cells which stained positively for the presence of HVCN1 relative to the isotype control, set to read 10,000 events)

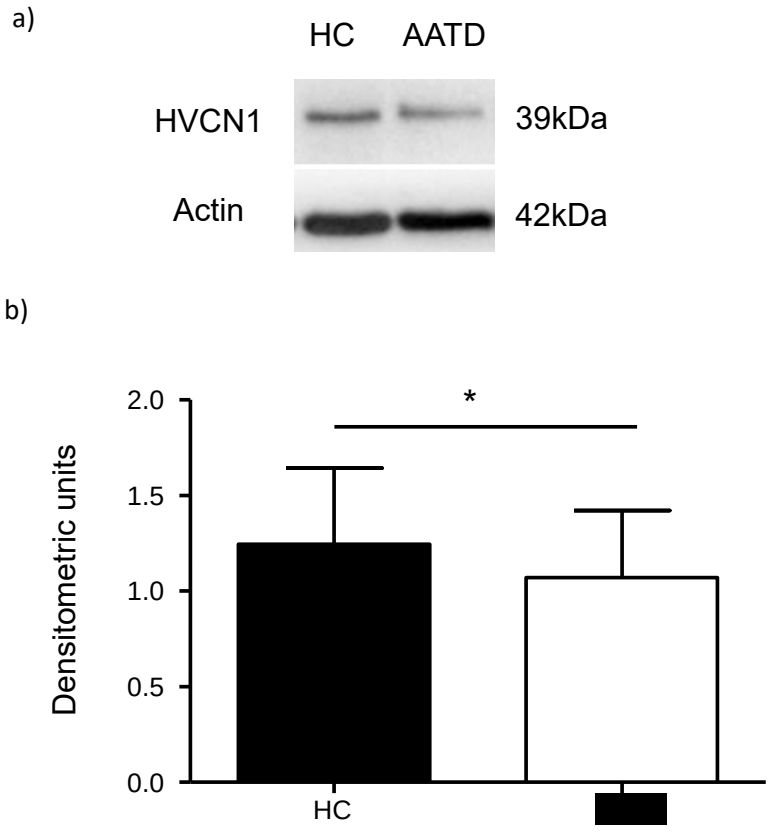


Figure 4-3: Abundance of HVCN1 in neutrophil whole cell lysates by Western blot analysis.

a) Representative Western blot depicting HVCN1 expression in neutrophil whole cell lysates. Actin was used as a loading control. b) Graph depicting densitometry for HVCN1 immunobands, showing significantly decreased protein level of HVCN1 in neutrophil whole cell lysates of individuals with AATD versus healthy controls (HC) (n=7 subjects per group, p=0.025, Student's t test).

4.3.1 **Surface expression of HVCN1 in response to exogenous stimuli.**

Our results show that the abundance of HVCN1 is reduced in neutrophils of individuals with AATD. These experiments were performed on resting neutrophils. The majority of HVCN1 in resting neutrophils is stored in intracellular granules. The aim of our next set of experiments, was to examine the expression of plasma membrane HVCN1 in response to soluble stimuli that would mobilise the intracellular stores of HVCN1 to the outer plasma membrane.

PMA is known to stimulate the release of primary, secondary, tertiary granules and secretory vesicles and was used as a positive control. TNFα and fMLP are responsible for a similar but less robust degranulation in neutrophils and they represent a more physiological stimulus as they both act on neutrophil surface receptors. TNFα had been studied in many neutrophil driven diseases and its relevance in AATD has been well described (161).

Isolated healthy control neutrophils were incubated with PMA (200nM) alone, or a combination of TNFα (10ng/2x10^7cells) and fMLP (10μM). At the pre-determined time points, aliquots of 2x10^6 cells were fixed with 4% (w/v) paraformaldehyde. The fixed cells were then probed with anti-HVCN1 antibody. The abundance of HVCN1 was determined after probing with a secondary FITC labelled antibody and analysis by flow cytometry.

Our results show, that following stimulation with PMA there was a greater than 2-fold increase in the surface expression of HVCN1 (Figure 4-4a) (n=6, *$p<0.05$, ANOVA, post Bonferroni test). After stimulation with TNFα and fMLP the surface expression of HVCN1 increased by 1.5 fold (Figure 4-4b) (n=6, *$p<0.05$, ANOVA, post Bonferroni test). In the next set of experiments, we evaluated the plasma membrane expression of HVCN1 in response to TNFα and fMLP over a longer time course. Our results show, while there is an initial increase in HVCN1 expression on healthy control cells, at 60min post exposure to TNFα and fMLP there was a greater than 30% decrease in the cell surface abundance of HVCN1 (n=4, $p=0.0154$, ANOVA, post Bonferroni test) .

These results show that HVCN1 is mobilised from internal stores to the plasma membrane after exposure to various exogenous stimuli. Furthermore, the latter

result demonstrates that after prolonged exposure (60 min) to TNFα and fMLP, the surface expression of HVCN1 falls below resting levels. TNFα and fMLP are responsible for degranulation of primary, secondary and tertiary granules and secretory vesicles. In subsequent experiments we would endeavour to uncover the mechanism by which HVCN1 is being depleted from the outer plasma membrane of neutrophils, as seen in this set of experiments.

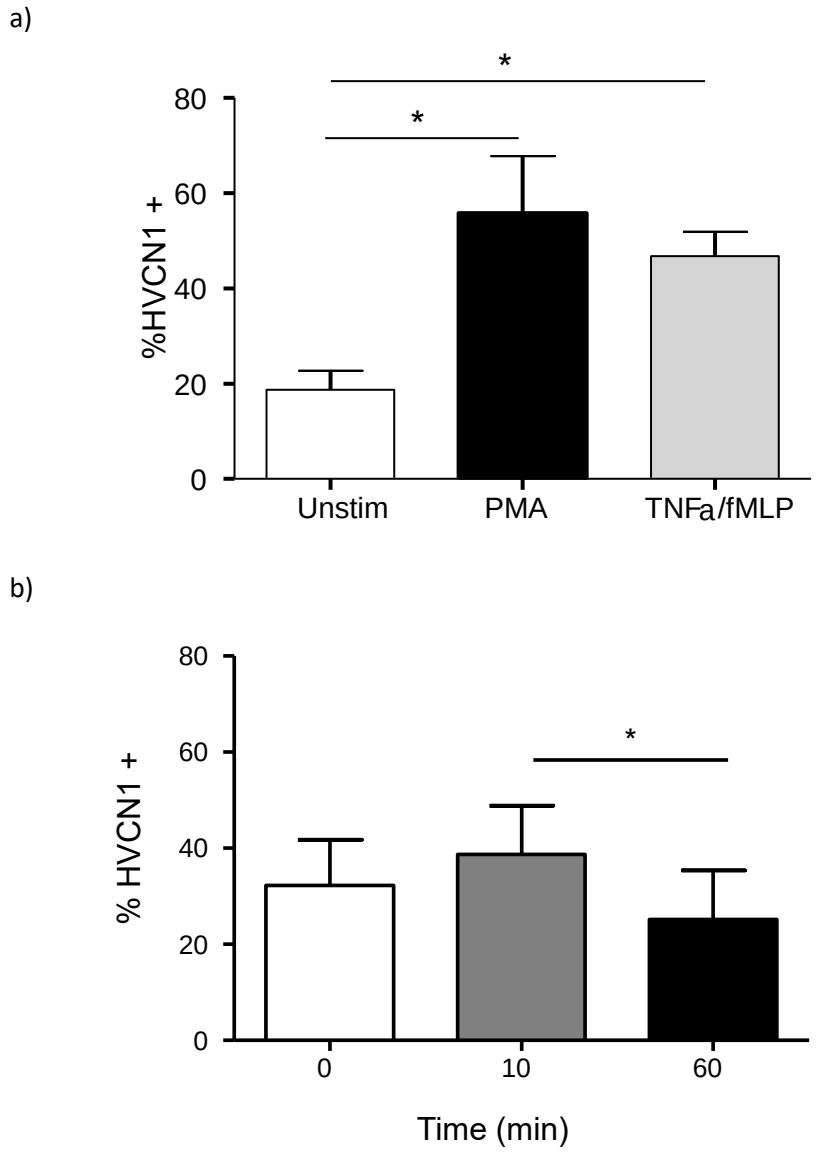


Figure 4-4: Abundance of HVCN1 on the neutrophil plasma membrane in response to exogenous stimuli over a time course, in healthy controls.

a) The surface abundance of neutrophil HVCN1 as measured by flow cytometry in response to PMA (200nM) or TNFα (10ng/2x10^7 cells) and fMLP (10µM) is shown at 10min post stimulation. There is a significant increase in membrane HVCN1 in response to PMA or TNFα/fMLP compared to unstimulated cells (unstim) (n=6 biological repeats, *p<0.05, ANOVA, post Bonferroni test). (b) Shown is a graph depicting the surface abundance of HVCN1 on the healthy control neutrophil plasma membrane after exposure to TNFα (10ng/2x10^7 cells) and fMLP (10µM) at various time points. At 10 min there was an increase in the abundance of HVCN1 followed by a significant reduction in the abundance of HVCN1 by 60min (n=4 biological repeats, p=0.015, ANOVA, post hoc Bonferroni test). Both results represent healthy control circulating neutrophils.

4.3.2 **A serine protease inhibitor maintains surface expression of HVCN1.**

We have demonstrated in the previous experiments reduced expression of HVCN1 on neutrophils from AATD individuals. Bergin *et al.*, showed that AATD neutrophils have increased degranulation of secondary and tertiary granules (161). Unpublished data from the laboratory of Professor McElvaney has also shown that AATD neutrophils have increased degranulation of primary granules also (253). As discussed, neutrophil granules contain a plethora of enzymes that serve many functions. Proteases are found in abundance in neutrophil granules. Primary granules contain MPO (peroxidase and catalase), defensins and the related serine proteases: NE, cath G and PR3 (10). These serine proteases all have proteolytic capacity and must be considered as possibly being responsible for the cleavage of HVCN-1 in AATD neutrophils. Secondary granules contain lactoferrin, lysozyme and hCAP-18. Tertiary granules contain MMPs, such as MMP-9. MMPs are also known to cause protein degradation and should also be considered as a possible cause of HVCN-1 cleavage. All these granules are released to a greater extent by neutrophils of AATD individuals, leading to an increased abundance of all these proteases into an environment low in circulating AAT (161).

Taking account of this previously published and unpublished data, we hypothesised that HVCN1 was being cleaved from the surface of neutrophils by protease activity. Our aim was to examine the effects of a broad range of protease inhibitors on HVCN1 abundance following exposure to exogenous stimuli of neutrophil degranulation.

In our next set of experiments we incubated isolated neutrophils with an array of protease inhibitors prior to stimulation with TNFα (10ng/2x10^7cells) and fMLP (10µM). This included the serine protease inhibitor, pefabloc (1mM), E-64 (10µM), a cysteine protease inhibitor, and EDTA (5mM), a metalloprotease inhibitor. After 60min, 2x10^6 neutrophils from each treatment group were fixed with 4% (w/v) paraformaldehyde. These cells were then probed with anti-HVCN1 primary antibody and a secondary, FITC labelled complementary antibody. HVCN1 abundance was then determined by flow cytometry and expressed as the percentage of cells demonstrating fluorescence above that of the isotype control (%HVCN1+).

Our results revealed, that when cells were incubated first with the serine protease inhibitor pefabloc the abundance of HVCN1 was greatly increased compared to cells not treated with this protease inhibitor (Figure 4-5) (n=9 biological repeats, p=0.007, ANOVA, post hoc Bonferroni test). More importantly, while pefabloc appeared to maintain the expression of HVCN1, E-64 (cysteine protease inhibitor) and EDTA (metalloprotease inhibitor) had no protective effect on the surface expression of HVCN1. These results suggest that it is likely a serine protease that is responsible for the loss of HVCN1 from the neutrophil membrane that we have shown. We next set out to determine if circulating AATD neutrophils have altered abundance of proteolytically active serine proteases on their plasma membranes that could be responsible for the altered abundance of HVCN1 that we have demonstrated.

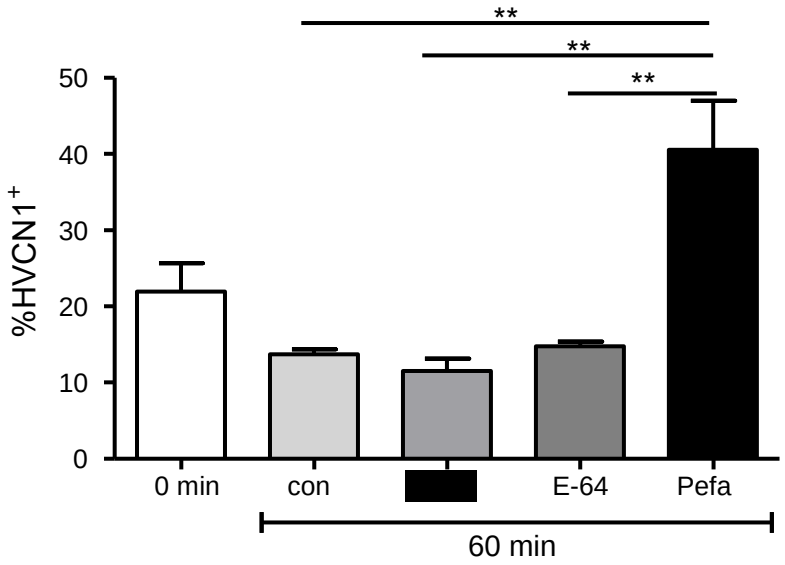


Figure 4-5: The effect of various protease inhibitors on the HVCN1 expression on the surface of stimulated neutrophils.

A graph depicting the abundance of HVCN1 on neutrophil cell surface by flow cytometry. Cells were treated with protease inhibitors EDTA (5mM), E-64 (10µM) or pefabloc (1mM)) or not (control, (con)) prior to the addition of TNFα (10ng/2x10^7 cells) and fMLP (10µM).The abundance of HVCN1 was measured at 0 and 60 min for control cells and 60 min following the addition of the protease inhibitor treatments. In the presence of pefabloc (Pefa) (serine protesase inhibitor), stimulating cells with TNFα and fMLP resulted in significantly increased abundance of HVCN1 on the plasma membrane (n=9 biological repeats, p=0.007, ANOVA, post hoc Bonferroni test). Pre-treating cells with metalloprotease inhibitor (EDTA) or the cysteine protease inhibitor (E-64) had not effect on the abundance of HVCN1.

4.3.3 **TNFα and fMLP stimulate the release of NE at the membrane of circulating neutrophils.**

We have shown that exposing neutrophils to TNFα and fMLP results in an initial increase in the surface expression of HVCN1 (Figure 4-4b). However, 60min post exposure of neutrophils to this stimuli the abundance of HVCN1 was found to be reduced. We hypothesised that this was the result of TNFα and fMLP mediated release of proteases from neutrophil granules that cleave HVCN1 on the plasma membrane. Our aim in the following set of experiments was to measure the abundance of NE (a constituent of primary granules) on the neutrophil membrane following exposure to TNFα and fMLP.

Isolated neutrophils were incubated with TNFα (10ng/2x10^7 cells) and fMLP (10µM) for 60 min. Aliquots were removed at time 0, 10 and 60 min post stimulation. Cells were centrifuged and resuspended in dPBS with 5mM glucose. A FRET assay specific for NE was performed to measure the membrane activity of NE (Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp, 3230-v, Peptide Institute, Inc) (230).

Our results show that after stimulation with TNFα and fMLP, there was a greater than 2.5 fold increase in membrane bound NE activity at 60min (Figure 4-6) (n=5, $p<0.01$, ANOVA, post hoc Bonferroni). We did not measure the activity of secondary and tertiary granule contents or secretory vesicle contents in response to TNFα and fMLP. Nor did we measure the abundance or activity of other primary granule contents such as PR3 or CG. We know that NE activity is increased in AATD individuals with and without obstructive lung disease (156) and that NE binds to the plasma membrane (154). This is another reason why we have focussed solely on measuring NE activity in the following experiments. It is clear from this set of experiments, that in response to TNFα and fMLP, membrane bound NE activity is dramatically increased. Our next experiments would specifically examine whether NE can cleave HVCN1 from the neutrophil membrane.

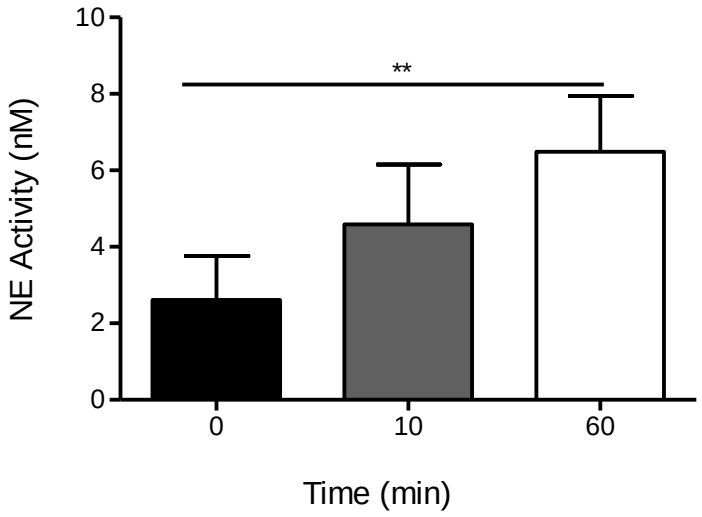


Figure 4-6: Membrane NE activity of healthy control neutrophils in response to TNFα and fMLP.

A graph depicting the activity of membrane bound NE of circulating healthy control neutrophils after stimulation with TNFα (10ng/2x10^7cells) and fMLP (10^{-6}M). NE activity was measured using a NE specific FRET assay. Membrane bound NE activity was increased 60 min post stimulation (n=5 biological repeats, **p<0.01, one-way ANOVA, post hoc Bonferroni test). All samples were analysed to 10,000 events.

4.3.4 **NE cleaves HVCN1 from the neutrophil membrane, in vitro.**

We have demonstrated that exposure of neutrophils to TNFα and fMLP results in reduced detection of HVCN1 on the plasma membrane, while at the same time increasing the release and activity of primary granule contents, specifically NE, on the cell membrane. The loss of HVCN1 from the plasma membrane is inhibited by a serine protease inhibitor but not cysteine or metalloprotease inhibitors. This suggests that a serine protease released in response to TNFα and fMLP is responsible for degradation of HVCN1 and therefore reduced detection of same by flow cytometry. We hypothesised that NE was the responsible protease. In our next experiments, we set out to determine the surface expression of HVCN1 following exposure of neutrophils to exogenous NE.

Isolated, circulating healthy control neutrophils were incubated at 37^{o}C in the presence or absence of 20nM of exogenous NE (Elastin products, derived from purulent human sputum). Aliquots of 2x10^{6} cells were removed at doubling time points (0, 15, 30 and 60min) and fixed with 4% (w/v) paraformaldehyde. As previously described, these cells were then stained with a polyclonal anti-HVCN1 antibody and a complementary FITC-labelled secondary antibody. The abundance of HVCN1 was then determined by flow cytometry.

Our results show that exposing neutrophils to 20nM NE resulted in a 40% reduction in membrane expression levels of HVCN1 compared to untreated cells as determined by flow cytometry analysis (Figure 4-7) (n=5 biological repeats, p=0.005, ANOVA, post Bonferroni). HVCN1 abundance was at its lowest 30 min post exposure to NE. In those cells not treated with NE there was an 8% increase in surface expression of HVCN1, likely due to mobilisation of internal stores after prolonged incubation of the cells.

These results confirm our hypothesis that NE is capable of reducing HVCN1 from the neutrophil membrane. We next wanted to examine the membrane activity of NE in neutrophils from AATD individuals.

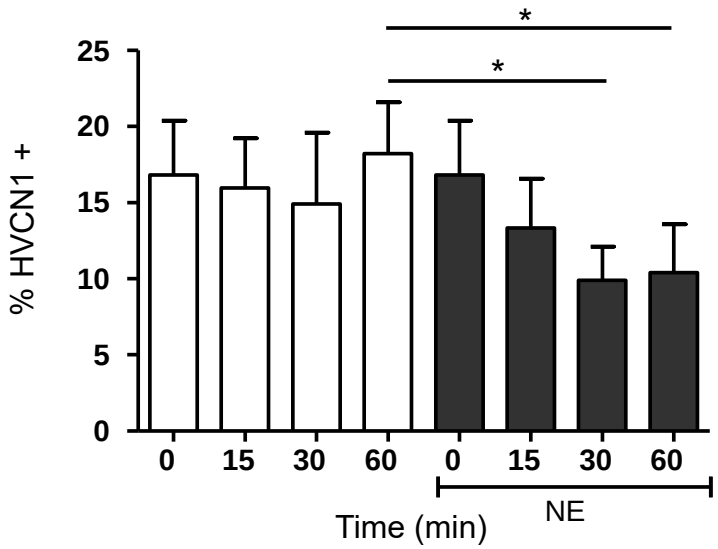


Figure 4-7: The effect of exogenous NE on neutrophil membrane expression of HVCN1.

A graph illustrating neutrophil membrane expression of HVCN1 as measured by flow cytometry analysis using a rabbit isotype control and a rabbit anti-HVCN1 primary antibody. Isolated neutrophils ($2x10^6$), were untreated (white bars) or exposed to NE (20nM) (black bars) over a 60 min time course. At 30 and 60 min following the addition of exogenous NE (20nM) there was a reduction in the abundance of HVCN1 compared to untreated cell at 30min and 60 min (n=5 biological repeats, p=0.005, ANOVA, post hoc Bonferroni).

4.4 Increased membrane bound NE activity on circulating AATD neutrophils.

We have shown that NE is capable of cleaving HVCN1 from the surface of neutrophils in vitro. Individuals with AATD have low circulating levels of AAT with the main substrate target of AAT being NE. We hypothesised that NE is responsible for the reduced abundance of HVCN1 seen in our previous experiments on AATD neutrophils. The aim of our next experiment was to determine the activity of NE on the neutrophil membrane of AATD individuals.

As previously described the activity of NE was measured using a NE specific FRET substrate (Abz-Ala-Pro-Glu-Glu-Ile-Met-Arg-Arg-Gln-EDDnp, 3230-v, Peptide Institute, Inc). Circulating neutrophils were isolated as previously described. A healthy control sample was randomly matched to each AATD sample.

We found that the activity of membrane bound NE on AATD neutrophils was more than double that of healthy controls (n=3, p=0.03, Student's t-test). This result suggests excessive primary granules release by AATD neutrophils and sustained activity of NE on the neutrophil membrane. In our next set of experiments, we would examine the effects of intravenous AAT replacement therapy on both the abundance of HVCN1 and NE activity.

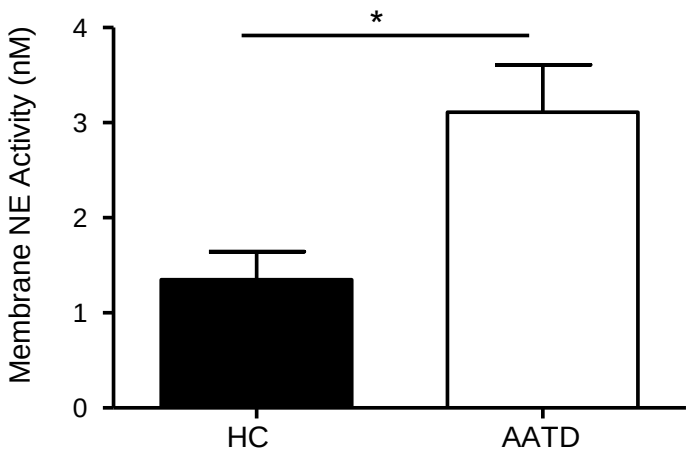


Figure 4-8: Membrane bound NE activity on neutrophils of individuals with AATD.

A graph illustrating the membrane activity of NE on healthy control (HC) or AATD circulating neutrophil plasma membranes, as measured by FRET assay. AATD neutrophils exhibited twice the activity of NE, as compared to healthy controls (n=3 subjects per group, p=0.03, Student's t test).

4.5 The effects of intravenous AAT augmentation therapy on membrane bound NE activity and surface expression of HVCN1 in AATD neutrophils.

We have demonstrated that AATD neutrophils have a lower abundance of HVCN1 while at the same time having increased plasma membrane NE activity. We have also shown, in vitro, that NE can cleave HVCN1 from the neutrophil surface. It would appear, to us, that the reduced abundance of HVCN1 in AATD neutrophils is due to the unopposed action of NE. To further examine this hypothesis our next experiments involved patients receiving intravenous AAT replacement therapy. Our aim was to measure HVCN1 abundance before and after treatment and at the same time to quantify neutrophil membrane bound NE activity.

Patients with AATD receiving IV AAT replacement therapy were recruited. These AATD patients had blood samples drawn on day 0 (on the day of their infusion, immediately prior to administration) and on day 2, post infusion. This is similar timing of sample collection as performed by Bergin *et al.* (154, 161). A matched healthy control was used for day 0 and day 2, with a different healthy control being used for each individual AATD patient. Isolated neutrophils were fixed with 4% (w/v) paraformaldehyde and as previously described the abundance of HVCN1 was determined by flow cytometry.

Our results show that membrane HVCN1 on AATD neutrophils increases by 40% on day 2 compared to day 0 (Figure 4-9a) (n=3, p=0.01, Student's t test). We also measured NE activity on circulating neutrophils using a NE specific FRET assay, as previously described. Again samples were taken on Day 0 and Day 2. We show here (Figure 4-9b) that there was no difference in NE activity on Day 0 (2.1nM) compared to Day 2 (1.3nM) (n=3, p=0.6, Student's t test).

These results show the IV AAT replacement therapy was associated with significantly increased abundance of HVCN1 on neutrophil plasma membrane. Possibly owing to the small patient numbers we were unable to show a difference in neutrophil membrane NE activity between day 0 and day 2. We have demonstrated that AATD neutrophils have greater NADPH oxidase activity as demonstrated by increased extracellular O_2^- production (chapter 3) and that HVCN1 is reduced in AATD neutrophils likely due to cleavage by high levels of membrane bound NE. The increased activity of NADPH oxidase in the setting of

reduced abundance of HVCN1 may lead to an accumulation of H^+ in the cytoplasm as it is the role of ion channels such as HVCN1 to compensate for an increased H^+ load. We hypothesised that the cytosolic pH of AATD neutrophils would be lower than healthy controls due to the increased activity of NADPH oxidase in these neutrophils and their reduced abundance of HVCN1. This was explored in the experiments that follow.

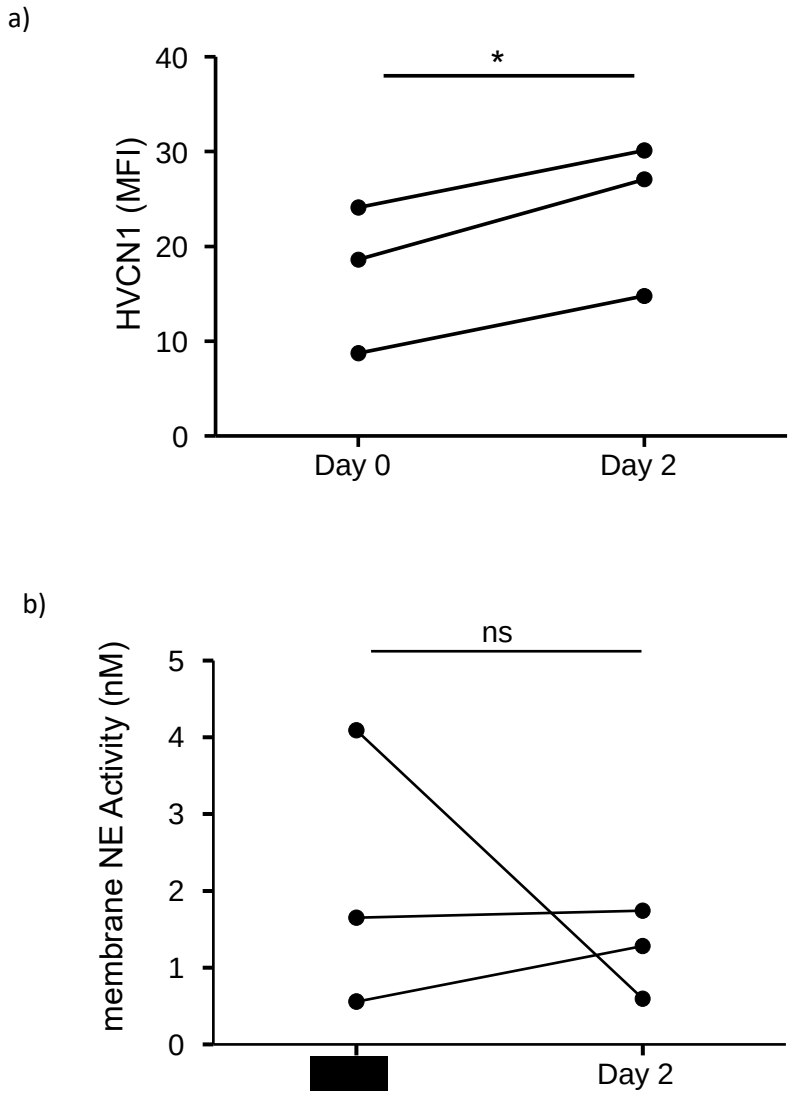


Figure 4-9: The effect of intravenous augmentation therapy on the abundance of HVCN1 and NE membrane activity.

a) A graph illustrating the increased abundance of neutrophil membrane HVCN1 in individuals receiving AAT augmentation therapy on day 0 and day 2 of therapy (n=3, p=0.01, Student's t test). HVCN1 was measured at the plasma membrane of isolated circulating neutrophils by flow cytometry analysis using anti-HVCN1 antibody. b) Membrane bound NE activity in AATD individuals receiving AAT augmentation therapy on day 0 and day 2 of treatment, as measured by FRET assay. Membrane NE activity was the same on day 0 and day 2, post infusion of AAT (n=5, p=0.07, Student's t test).

4.6 Neutrophil cytosol pH in AATD neutrophils at rest.

We have shown that the activity of NADPH oxidase of AATD neutrophils is increased. NADPH oxidase activity is associated with liberation of H^+ into the cytosol. AATD neutrophils may be unable to compensate for accumulating H^+ and their cytosols may acidify as a result of reduced HVCN1 on the plasma membrane. In the following set of experiments, we set out to measure the cytosolic pH of healthy control and AATD neutrophils at rest.

Cytosolic pH was measured using the fluorescent dye, SNARF-1. We used SNARF given it has a pK_a of 7.5 and a dynamic pH range of 6-11, therefore it is well suited for measuring the cytosolic pH of human neutrophils. Other measurement assays make use of fluorescein which is quenched by ROS. This does not occur with SNARF (52). Neutrophils were isolated as previously described. $1x10^7$ cells were made up to 1ml with dPBS and 50µl of SNARF-1 (12.5mg/ml). This was left on the bench for 10min at RT. The cells were then centrifuged at 470 x g for 5min at RT and resuspended in 500µl of fresh dPBS. pH standards, ranging from 5.8 to 8.6, were placed in a labelled 96 well plate. Cells ($1x10^5$ per well) to be used to form the pH standards were exposed to nigericin (10µl, 6.7nM) and then added to the various pH standards in the 96 well plate. Nigericin is an ionophore used to equilibrate the intracellular pH with the extracellular pH. The plate reader was set to read a single time point at 580nm and 640nm at RT.

Our results revealed that there was no difference in the cytosolic pH of resting AATD neutrophils (pH7.44, SD 7.26-7.61) compared to those of healthy controls (Figure 4-10) (pH7.46, SD 7.27-7.65) (n=12 subjects per group, p=0.73, Student's t test).

We have demonstrated, that despite decreased HVCN1 activity in AATD neutrophils, there is no effect on cytosolic pH in resting AATD cells. We have seen previously (Figure 3-4b) that stimulated healthy control or AATD cells produce O_2^- indicating increased NADPH oxidase activity. This would result in an increase in cytosolic pH and would require increased activity of HVCN1 to extrude protons and maintain the cytosolic pH. In our next set of experiments, we set out to assess whether stimulating NADPH oxidase activity in healthy control neutrophils would

result in acidification of their cytosols and whether this would be augmented by inhibiting HVCN1 with $ZnCl_2$.

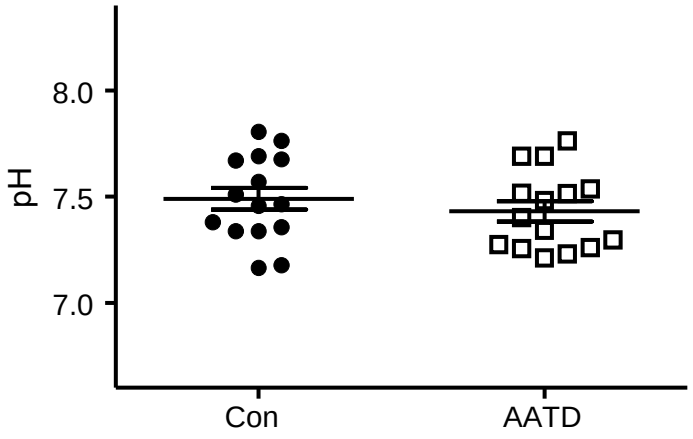


Figure 4-10: Cytosolic pH of circulating neutrophils from healthy control and AATD individuals.

This graph illustrates the cytosolic pH of circulating healthy control and AATD neutrophils at rest, as measured using the pH indicator SNARF. There was no difference in cytosolic pH between healthy control (Con) (7.49+/-0.2) and AATD neutrophils (7.42+/-0.2) at rest (n=15 subjects per group, p=0.4, Student's t test).

4.6.1 **Cytosolic acidification with NADPH oxidase activity and HVCN1 inhibition, in healthy control neutrophils.**

Despite having a lower abundance of HVCN1, AATD neutrophils at rest have a normal cytosolic pH. NADPH oxidase activity and H^+ liberation should be minimal in resting cells. It has previously been shown that stimulating NADPH oxidase activity results in a transient acidification of the neutrophil cytosol (250). We aimed to replicate these results in our next set of experiments. We also wanted to demonstrate the critical role of HVCN1 in compensating for cytosolic acidification due to NADPH oxidase activity. Zn^{2+} inhibits the activity of HVCN1 (51). It has been shown by others that inhibiting HVCN1 with Zn^{2+} in activated neutrophils results in a more profound and prolonged acidification of the neutrophil cytosol (251). We aimed to firstly reproduce these results.

Cells were prepared using the pH indicator SNARF as described in the previous section. $1x10^7$ cells were incubated with $ZnCl_2$ (100µM) for 5min, a further $1x10^7$ cells were suspended in dPBS only. Following this, TNFα (10ng/$2x10^7$ cells) and fMLP (10µM) were added immediately prior to the plate being read over 20min at 580nm and 640nm, RT.

Our results show that with the addition of TNFα and fMLP there was an acidification of the cytosol from 7.44 to 7.34 (Fig 4-11). The pH of resting cells did not change over time. In those cells that were pre-incubated with $ZnCl_2$, to inhibit HVCN1 activity, there was a more profound acidification of the cytosol to 7.23 (Fig 4-11) (n=3 biological repeats, p=0.0003, ANOVA, post Bonferroni test). The lowest pH occurred at 20sec post addition of the stimulants and the pH had not returned to baseline by 20min in either group.

These results mirror those of others (52, 251) and demonstrate the acidification of the neutrophil cytosol with activation of NADPH oxidase and the importance of HVCN1 in compensating for the accumulation of cytosolic H^+. We next wanted to examine the cytosolic pH of AATD neutrophils (with their lower abundance of HVCN1) in response to activation of NADPH oxidase.

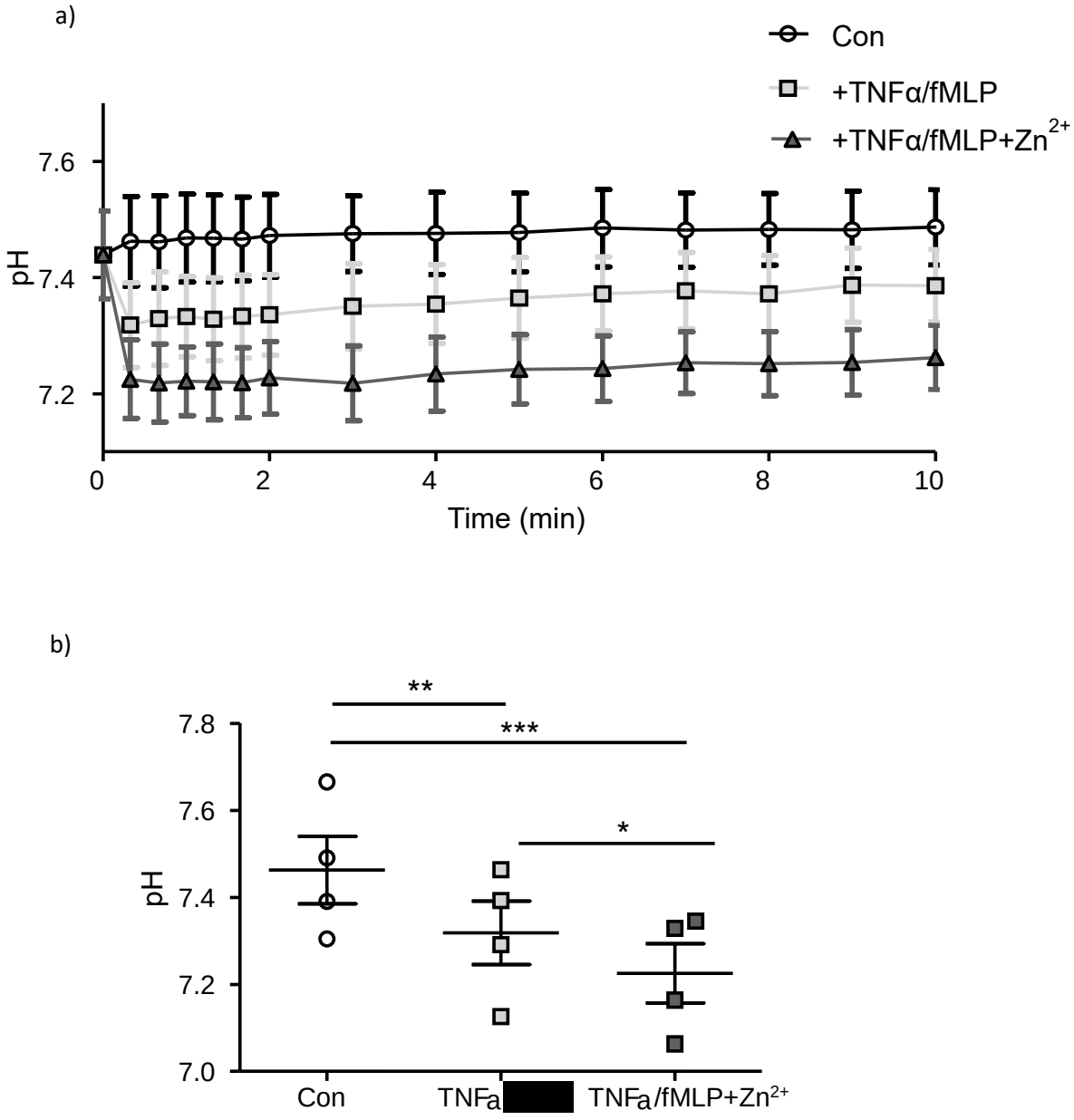


Figure 4-11: Neutrophil cytosol acidification in response to exogenous stimuli and inhibition of HVCN1

a) Shown is a 10 min time course of neutrophil cytosolic pH, measured using SNARF-1, following stimulation with TNFα/fMLP with or without prior incubation with $ZnCl_2$ (100µM). b) This graph illustrates 20 sec time point. This represents lowest cytosolic pH for all treatments. Following stimulation with TNFα/fMLP the cytosol of the neutrophils acidified slightly (from pH 7.44 to a nadir of 7.32). In those cells that were incubated with $ZnCl_2$ prior to being stimulated with TNFα/fMLP, the acidification was more prominent (from pH7.44 to a nadir of 7.23) (n=3 biological repeats, p=0.0003, ANOVA, post Bonferroni test).

4.6.2 **Cytosolic pH of AATD neutrophils in response to increased NADPH oxidase activity.**

We have demonstrated that resting AATD neutrophils have a normal cytosolic pH. NAPDH oxidase activity results in cytosolic acidification that is exaggerated with inhibition of HVCN1 by $ZnCl_2$. Given that we have described a reduced abundance of HVCN-1 on AATD neutrophils we hypothesised that these cells would exhibit exaggerated acidification of their cytosols in response to activation of the NADPH oxidase.

Isolated neutrophils from AATD and healthy control individuals were prepared as previously described with SNARF-1, to determine cytosolic pH. A standard curve using pH standards and cells permeabilised with nigericin were used for each experiment. For both healthy control and AATD neutrophils, aliquots of cells were untreated and others treated with TNFα (10ng/2x10^7 cells) and fMLP (10μM) immediately prior to being recorded at 580nm and 640nm.

When healthy control and AATD neutrophils were stimulated (to activate NADPH oxidase and therefore create an intracellular H^+ burden), AATD neutrophils exhibited an acidification of the cytosol that was not different from that seen in healthy control neutrophils. In unstimulated, healthy control neutrophils the starting pH was 7.48 and this became marginally more alkaline over time. Healthy control neutrophils that were stimulated with TNFα and fMLP had a starting pH of 7.48 and reached a nadir of 7.38 at 60sec. AATD neutrophils that were stimulated in the same manner had a similar starting pH of 7.52 and the nadir of 7.4 was not significantly different from the healthy control stimulated cells (Figure 4-12) (n=7, p=0.5, Student's t test).

These results suggest, that the lower abundance of HVCN1 in AATD individuals does not affect the cells ability to compensate for an increased accumulation of H^+, such as occurs with activation of NADPH oxidase. This correlates with the results of our O_2^- experiments in the previous chapter, as the lower abundance of HVCN1 does not appear to affect the production of ROS, by NADPH oxidase either. In our next experiments we would examine the ability of AATD neutrophils to mobilise internal stores of HVCN1 to the plasma membrane in response to exogenous stimuli.

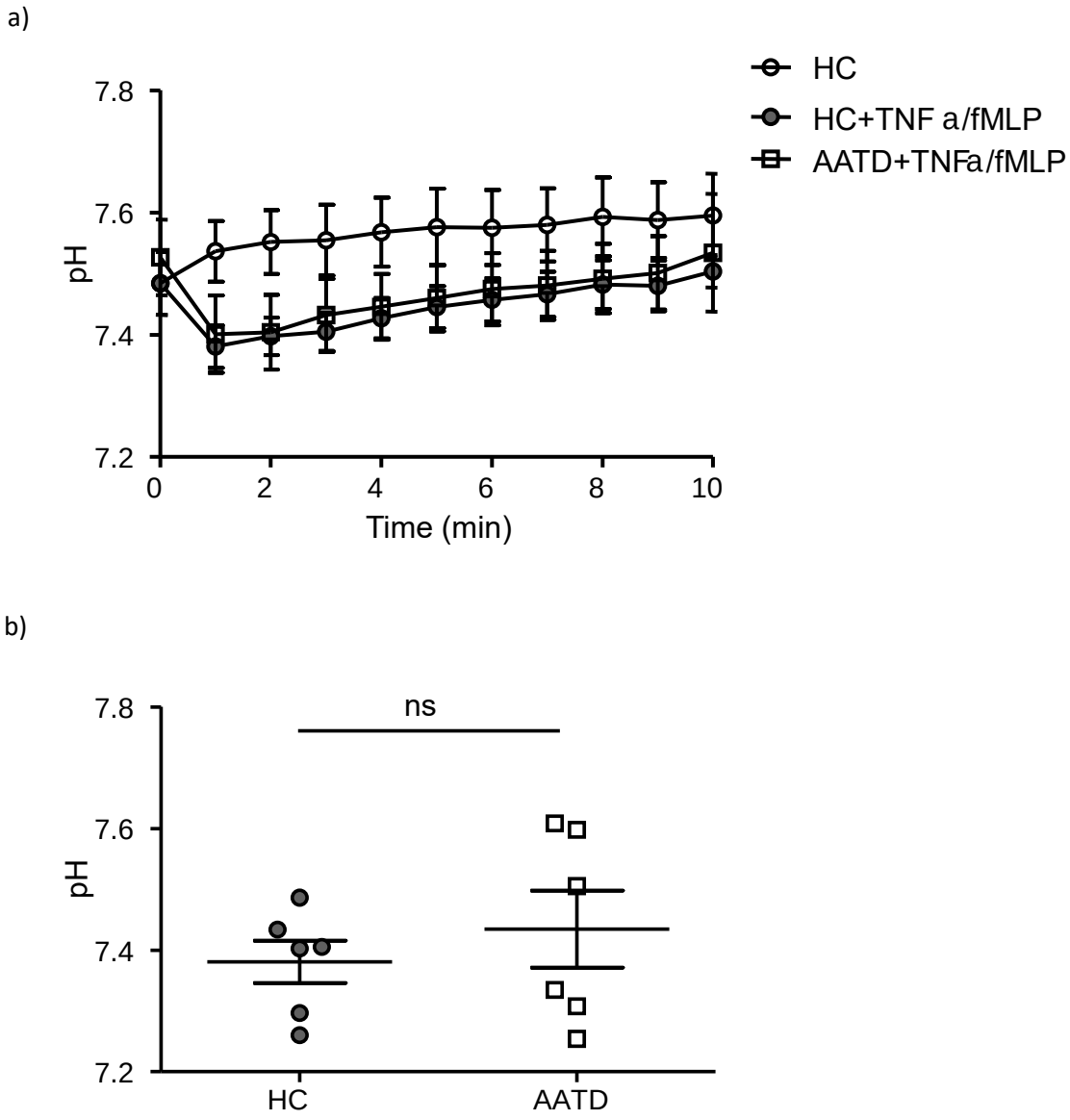


Figure 4-12: Cytosolic pH of AATD neutrophils in response to NADPH oxidase activation, in healthy control and AATD neutrophils.

a) A time course is shown of neutrophil cytosolic pH for healthy control and AATD neutrophils. Cells were untreated or exposed to TNFα/fMLP. b) A graph illustrating cytosolic pH at 60sec, which represented the lowest pH for all cells. Healthy control neutrophils that were stimulated with TNFα/fMLP had a starting pH of 7.48 and reached a nadir of 7.38 at 60sec. AATD neutrophils that were stimulated with TNFα/fMLP had a similar starting pH of 7.52 and the nadir of 7.4. No significant difference was seen between healthy control and AATD following stimulation (n=6 subjects per group, p=0.53, Student's t test).

4.7 AATD neutrophils translocate internal stores of HVCN1 to the plasma membrane more readily than healthy controls.

The lower abundance of HVCN1 in AATD neutrophils does not affect the cells ability to compensate cytosolic H^+ accumulation. We have shown that AATD neutrophils more readily release primary granule contents in response to TNFα and fMLP than healthy control cells. We hypothesised that AATD neutrophils, being in a primed state in the circulation, would exhibit more robust mobilisation of HVCN1 from internal stores to the plasma membrane in response to exogenous stimuli. We aimed to measure the surface expression of HVCN1 on circulating neutrophils of AATD and healthy control individuals in response to the exogenous stimuli, PMA or TNFα and fMLP used in combination.

Neutrophils were isolated from the circulation of AATD and healthy control individuals. These cells were then exposed to either PMA (200nM) or a combination of TNFα (10mg/2x10^7 cells) and fMLP (10µM). At 20min post addition of the various stimulants, 2x10^6 cells from each treatment were removed and fixed with 4% (w/v) paraformaldehyde. In parallel, reaction cells were not treated with any stimuli and were fixed in the same way. The surface expression of HVCN1 was quantified by flow cytometry, as previously described.

Our results show that, as expected, there was a robust increase in HVCN1 expression in response to PMA by both AATD and healthy control cells (Figure 4-13). The healthy control cells showed a 50% increase in HVCN1 expression on the cell surface in response to PMA while the AATD cells showed a 70% increase in HVCN1 expression in response to PMA ($p<0.01$, $P<0.001$). In response to TNFα and fMLP, healthy control cells showed only a 5% increase in surface HVCN1 expression, which did not reach statistical significance. However, AATD neutrophils demonstrated a 47% increase in surface HVCN1 expression in response to TNFα and fMLP ($p<0.05$).

We have shown here, that in AATD neutrophils, HVCN1 is more readily mobilised to the plasma membrane in response to exogenous stimuli. This supports our previous results that point to circulating AATD neutrophils being in a ‘primed’ state with a more ready release of granular and secretory vesicle contents. This result supports our hypothesis that AATD cell are able to mobilise HVCN1 to the

membrane, thus negating any deleterious effect of low HVCN1 in AATD neutrophils as we found on the plasma membrane.

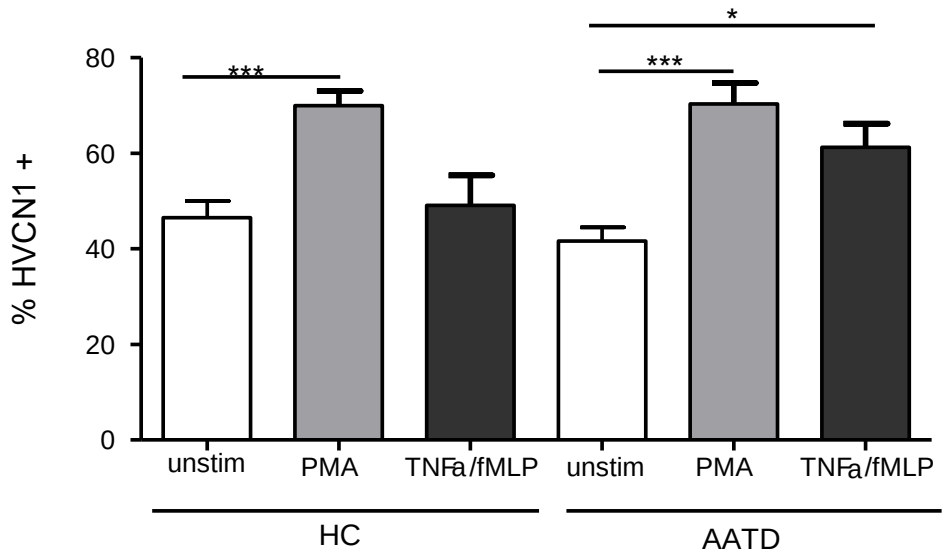


Figure 4-13: HVCN1 recruitment to the plasma membrane from internal stores.

Shown is a graph depicting the abundance of HVCN1 on the surface of circulating neutrophils as measured by flow cytometry. Neutrophils were isolated from healthy control and AATD individuals, following exposure to various stimuli (PMA (200nM), TNFα (10ng/2x10^7 cells) and fMLP (10μM)) the cells were fixed with paraformaldehyde (4% (w/v)) and examined for the abundance of HVCN1 using an anti-HVCN1 antibody. There was increased abundance of HVCN1 on the cell surface in both healthy control and AATD neutrophils compared to unstimulated (unstim) cells in response to PMA (n=3 biological repeats, **p<0.01, ***p<0.001, ANOVA, post Dunn's test). Following stimulation with TNFα and fMLP there was increased abundance of HVCN1 on the cell membrane at 20min in the AATD but not healthy control neutrophils (n=3, ANOVA, Dunn's multiple comparison test). Only significant differences are shown.

4.8 Discussion

The first aim of this chapter was to examine the abundance of HVCN1 in neutrophils of individuals with AATD. We have shown in the previous chapter that the assembly and activity of NADPH oxidase is increased in circulating AATD neutrophils. As NADPH oxidase activity is associated with cytosolic accumulation of protons, we hypothesised that AATD neutrophils must increase the abundance or activity of proton channels on their cell surface to prevent excess cytosolic acidification. HVCN1 is responsible for at least 85% of proton currents in neutrophils (251), we therefore focused our experiments on this proton channel.

We found HVCN1 to be reduced on the plasma membrane of AATD neutrophils. We demonstrated this by measuring the surface abundance of HVCN1 by flow cytometry and we also performed Western blot analysis of whole cell neutrophil lysates. Further experiments were performed to establish a mechanism by which AATD neutrophils express lower levels of HVCN1. We demonstrated that stimulating cells to release their primary granule contents resulted in increased NE activity on the plasma membrane. Application of the same exogenous stimuli also resulted in a significant reduction in the abundance of HVCN1 on the cells surface. This led us to consider that, in AATD neutrophils, HVCN1 was being cleaved from the neutrophil surface by a protease within neutrophil granules. We have shown, by use of a spectrum of protease inhibitors that a serine protease inhibitor was most likely to be responsible for HVCN1 cleavage. We also demonstrated that cysteine and metalloproteases are unlikely to be culprits by using their relevant inhibitors and showing that they do not prevent the cleavage of HVCN-1 from the surface of neutrophils. NE, PR-3 and cath G are all serine proteases found in neutrophil primary granules (10). Exogenous NE was shown to cleave HVCN1 from the surface of neutrophils, in vitro.

We found that circulating AATD neutrophils have a greater abundance of membrane bound NE than healthy controls, which supports previously published data (156). This, previously demonstrated finding and our finding that exogenous NE can reduce the surface abundance of HVCN1, supports our theory that NE is responsible for the lower abundance of HVCN1 on the plasma membrane in circulating AATD neutrophils.

Having demonstrated a lower abundance of HVCN1 on AATD neutrophils we questioned what the physiological consequence of this would be for the neutrophil. As discussed, the activity of NADPH oxidase generates electrons to form O_2^- and also generates H^+. When H^+ accumulates in the cytosol it can cause acidification, with potential knock on affects for many enzymatic reactions. For example, NADPH oxidase will cease to function if the cytosolic pH is not maintained within a narrow range. The function of many enzymes have optimal activity at various pH ranges. In healthy individuals, the accumulation of H^+ in the cytosol as a result of NADPH oxidase activity is compensated for predominantly by HVCN1. With the reduction in HVCN1 on AATD neutrophils we hypothesised that the cytosolic pH of AATD neutrophils might be affected.

In this regard, we measured the cytosolic pH of healthy and AATD neutrophils. In AATD neutrophils, we found that the cytosolic pH of resting cells was not different to healthy control neutrophils. We also measured the cytosolic pH of healthy control neutrophils after stimulation with soluble stimulants. We aimed to demonstrate that the activation of NAPDH oxidase resulted in acidification of the cytosol and that this would be augmented by inhibiting HVCN1. We found that stimulation with TNFα/fMLP resulted in acidification of the cytosol of neutrophils and when Zn^{2+} was added to these cells to inhibit HVCN1 there was a more profound acidification. This replicated the findings of many others and the findings have been further validated in HVCN1 knock-out models (52, 251).

Although our results demonstrate that the resting pH of AATD neutrophils was unaffected by lower abundance of HVCN1, we questioned how they would behave at times of increased H^+ load. For example, in the setting of neutrophils recruitment to a site of infection, these neutrophils become activated and will have increased NADPH oxidase activity. We hypothesised, that due to their lower abundance of HVCN1, at times of increased cell activation AATD neutrophils would have impaired handling of protons and thus would demonstrate cytosolic acidification. Interestingly, the AATD cells, despite having a lower abundance of plasma membrane HVCN1 exhibited acidification to the same extent as healthy control neutrophils, not greater.

An explanation for the ability of AATD neutrophils to compensate for cytosolic acidification at times of stress can be explained by our final experiment. In response to TNFα and fMLP, AATD neutrophils demonstrate significantly greater translocation of HVCN1 from internal stores to the plasma membrane than do control cells. This supports our conclusion from the previous chapter that circulating neutrophils from AATD are in a primed state. From our healthy control experiments we know that TNFα/fMLP causes release of primary granules that is significant at 60min post exposure. Increase in HVCN1 in AATD neutrophils, in response to TNFα/fMLP occurred by 20min. This is likely due to the sequential exocytosis of granule contents. HVCN1, contained in secretory vesicles, would be released prior to NE being released from primary granules. From our healthy control experiments we know that NE causes degradation of HVCN1 by 30min but not at 15min. It is likely then, that at times of stress or increased NAPDH oxidase activity that AATD neutrophils have a more robust recruitment of HVCN1 to the plasma membrane that occurs before NE release and prior to significant degradation of HVCN1.

HVCN1 can be present on the plasma membrane as a monomer or a dimer (48). In both forms it maintains its activity as a proton channel. Interestingly, HVCN1 can demonstrate an ‘enhanced gating mode’, whereby, in response to certain stimuli proton currents are larger, activated more quickly and deactivated more slowly (51). Agonists that induce enhanced gating of HVCN1 also activate NADPH oxidase (including fMLP). The enhanced gating of HVCN1 may be as a result of phosphorylation (254). We did not measure HVCN1 phosphorylation in our experiments. It is possible that AATD circulating neutrophils in their primed state may express a higher abundance of phosphorylated HVCN1 than healthy control cells, but this would need further experimentation to prove.

Another consideration is the efficiency of a single HVCN1 channel. The rate of O_2^- production by NADPH oxidase is very large. As discussed in section 1.1.6 of the introduction, one proton channel can compensate completely for the activity of 100 NADPH oxidase molecules. This may also explain why our finding of a 20% reduction in the surface expression of HVCN1 in AATD neutrophils does not affect the cells ability to produce O_2^- or maintain its cytosolic pH.

While HVCN1 is the most important proton channel and contributes more than 85% of proton currents, there are other ion channels and proton channels present in neutrophils that can provide assistance in normalising the cytosol pH when needed and may play a role in the AATD neutrophil. For example V-ATPase on the surface of intracellular organelles can also act to extrude H^+ from the cytosol to prevent excess or prolonged acidification (255).

Overall, this data has demonstrated that AATD neutrophils demonstrate a reduced abundance of HVCN1 that is likely due to cleavage by excessive membrane activity of NE. There appear to be no physiological consequences to the AATD neutrophil of having a 20% reduction in HVCN1 abundance. NAPDH oxidase activity is increased but cytosolic pH is maintained at rest and at times of increased demand in the AATD neutrophil.

Chapter 5: A mechanism of modulation of neutrophil O_2^- production by AAT.

5.1 Introduction

The previous results chapters revealed the ability of AAT to decrease the assembly and activity of NADPH oxidase in circulating neutrophils, in vitro. We also demonstrated, that circulating AATD neutrophils exhibit enhanced NADPH oxidase activity in response to both fMLP and IL-8, suggesting that they are in a primed state in the circulation. The mechanism by which AAT modulates the neutrophil's response to fMLP, via its cognate receptor FPR1, will be the focus of this chapter.

AAT is the prototypical serpin. Its predominant function is to inhibit neutrophil elastase and other proteases. However, the serpin family have a broad range of functions and some members of this family function solely as carrier proteins. AAT can act as a binding partner to a host of plasma proteins and lipids. It can achieve this binding interaction by a number of different mechanisms. AAT can directly bind defensins and LTB_4 (156, 256). AAT binds to LDL and HDL. Complexes of oxidised AAT and LDL are found in higher abundance in smokers and decrease after smoking cessation (257). Some authors suggest that AAT-LDL complexes may be taken up by the arterial endothelial cells and contribute to atherosclerosis formation (258). The binding of AAT to HDL may explain the anti-elastase activities of HDL that have been demonstrated (259) and some studies suggest that HDL binding to AAT may potentiate the anti-protease effects of AAT therapy.

In chapter 3, we have examined neutrophil NADPH oxidase response to two soluble stimuli, IL-8 and fMLP and revealed the ability of AAT to modulate O_2^- production in response to IL-8 and fMLP or to fMLP alone. The mechanism by which AAT modulates IL-8 signalling and the interaction of AAT with IL-8 has been studied in some detail (157). IL-8 binds to the GPCR, CXCR1, on the neutrophil membrane (157). This interaction results in the release of secondary granules, upregulation of integrin expression on the surface of neutrophils and an increase in intracellular Ca^{2+}, which facilitates cytoskeletal rearrangements to promote neutrophil migration (260). Bergin *et al.* demonstrated that AAT blocked the binding of IL-8 to CXCR1. These authors showed that IL-8 was binding directly to AAT as a result of electrostatic interactions owing to the glycosylated moieties of AAT (non-glycosylated AAT failed to bind IL-8) (154).

AAT has been shown to inhibit fMLP-induced neutrophil adhesion and chemotaxis (261, 262) but the mechanism of this inhibition has not been fully elucidated. The main ligands for FPR1 are bacterial formylated peptides that are actively secreted by invading microorganisms or mitochondrial formylated peptides that are released from host cells after tissue injury (263). Activation of FPR1 can trigger neutrophil chemotaxis, degranulation, phagocytosis and ROS production (263-265). The consequences of FPR1 activation is a complex chain of intracellular signalling events that includes; activation of PI3Kγ, intracellular Ca^{2+} release, PKC activation, ERK 1/2 and p38 MAPK activation and the activation of RhoGTPases (264).

Our results from chapter 3 demonstrate the downstream responses of AATD cells to fMLP and the effects of exogenous AAT on fMLP induced cell responses. The mechanism by which AAT modulates these fMLP induced cellular responses (i.e. O_2^- production) is not known. We know that AAT can act as a carrier protein and has the potential to interact and bind to a vast array of proteins and peptides via various different mechanisms. We theorised that AAT may have an affinity for fMLP. To date no information is available on the interaction of AAT with fMLP and that will be the focus of this chapter.

5.2 Aims of this chapter

Both IL-8 and fMLP have been demonstrated in previous results chapters to induce increased NADPH oxidase activity in circulating neutrophils of AATD individuals. In vitro, AAT modulated the activity of NADPH oxidase in response to both of these stimuli. Our hypothesis was that by binding fMLP, AAT inhibits its ability to engage with its receptor, resulting in decreased downstream signalling and cellular responses (i.e. NADPH oxidase activity). Our aim was to determine the ability of AAT to bind fMLP and to inhibit NADPH oxidase activity ex vivo. To fulfil this aim the following objectives were set;

1. To explore the binding interaction of AAT with fMLP in vitro
2. To examine the ability of AAT to inhibit fMLP interaction with FPR1 on neutrophils
3. To determine the impact of AAT therapy on neutrophil O_2^- production, in response to fMLP, in individuals with AATD

5.3 The binding capacity of AAT for fMLP, in vitro, by flow cytometry.

We have demonstrated the enhanced cellular responses of AATD neutrophils to fMLP and the effects of exogenous AAT on fMLP induced cellular responses in vitro. In both situations, AAT prevented the assembly and reduced the activity of neutrophil NADPH oxidase. This results chapter is concerned with establishing if AAT binds to fMLP thereby modulating FPR1 binding. As we have discussed, AAT is a 345 residue, 52kDa glycoprotein. The glycobiology of AAT is important in relation to AAT's immunomodulatory functions, outside of its ability to inhibit NE. AAT possess many potential binding mechanisms including; glycan binding, cysteine binding and hydrophobic binding (116). fMLP is an *N*-formylated tripeptide that binds to FPR1 to cause enhanced neutrophil NADPH oxidase activity, as well as other cellular responses. Therefore, the aim of our next set of experiments was to demonstrate binding of fMLP to AAT, in vitro, by flow cytometry.

For flow cytometry analysis, initial experiments required generation of AAT coated polystyrene beads. In brief, 10µm polystyrene beads (Polysciences, Europe) were suspended in a saline solution (0.9% NaCl (w/v)) with added AAT (27.5µM) for 24 h at 4^{o}C with constant, gentle rotation. An aliquot of beads were suspended in saline solution only. The beads were centrifuged (15,000 × g for 4 min at 4°C) and the amount of AAT binding to the beads was indirectly determined by measuring the unbound levels by UV spectrometry. Based on this calculation 12% of the AAT in suspension bound 50µl packed bed of polystyrene beads equating to 3.3µM of AAT.

Our initial flow cytometry analysis was performed to confirm that the polystyrene beads were indeed coated with AAT (Figure 5-1). Polystyrene beads that were coated with AAT and beads that were not coated with AAT (control) were incubated with a FITC-conjugated goat anti-AAT antibody for 1 h in the dark. After washing, the beads were analysed by flow cytometry. These results confirmed the presence of AAT on polystyrene beads following incubation in AAT protein overnight (n=3 technical repeats, p=0.0007, Student's t test).

Subsequent to producing a polystyrene bead with the confirmed presence of AAT, we wanted to next examine the ability of the AAT-coated bead to bind fMLP. We made use of a FITC-labelled fMLP peptide. Therefore, aliquots of the AAT beads

and control beads were incubated with 20nM FITC-labelled fMLP peptide for 1h at RT in the dark. After washing, the abundance of FITC-labelled fMLP on the beads was determined by flow cytometry analysis (Figure 5-2). We found that the AAT bead bound significantly more AAT than the uncoated beads (Figure 5-2) ($n=3$, $p<0.0001$, Student's t test).

Collectively, these results confirm the successful generation of AAT coated polystyrene beads (Figure 5-1) and these beads can be used to assess fMLP:AAT interaction. The subsequent results clearly demonstrate that there is a binding potential between AAT and fMLP. In our next set of experiments, we would examine this interaction further using a slot blot technique.

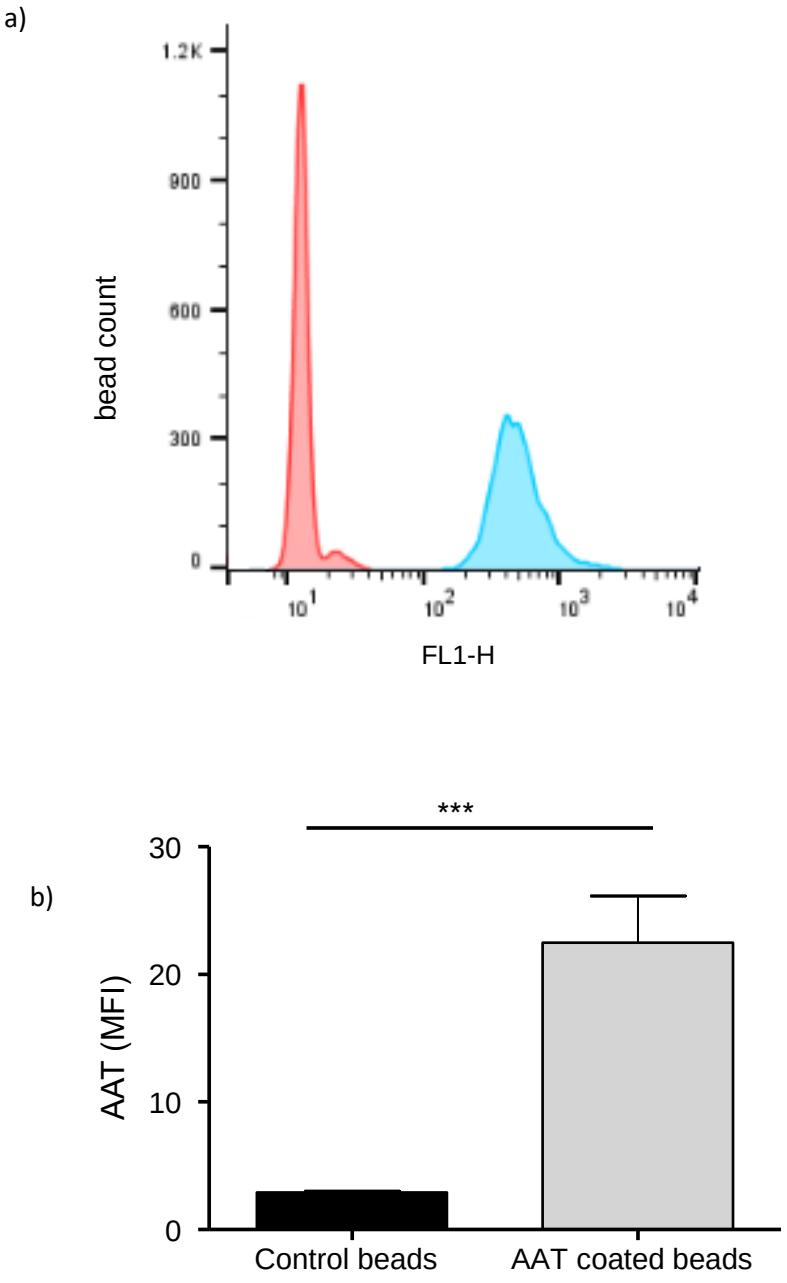


Figure 5-1: Establishment of AAT coated polystyrene beads

a) A histogram from flow cytometry analysis of 10µm polystyrene beads incubated with AAT (27.5µM) (blue) or just saline (0.9% w/v) (red). 3.3µM AAT bound to the beads based on UV spectroscopic analysis of the unbound supernatants. Both aliquots of beads were probed with goat anti-AAT FITC-conjugated antibody. b) AAT coated beads exhibited greater fluorescence than those not coated with AAT (control beads) (n=3 technical repeats, p=0.0007, Student's t test).

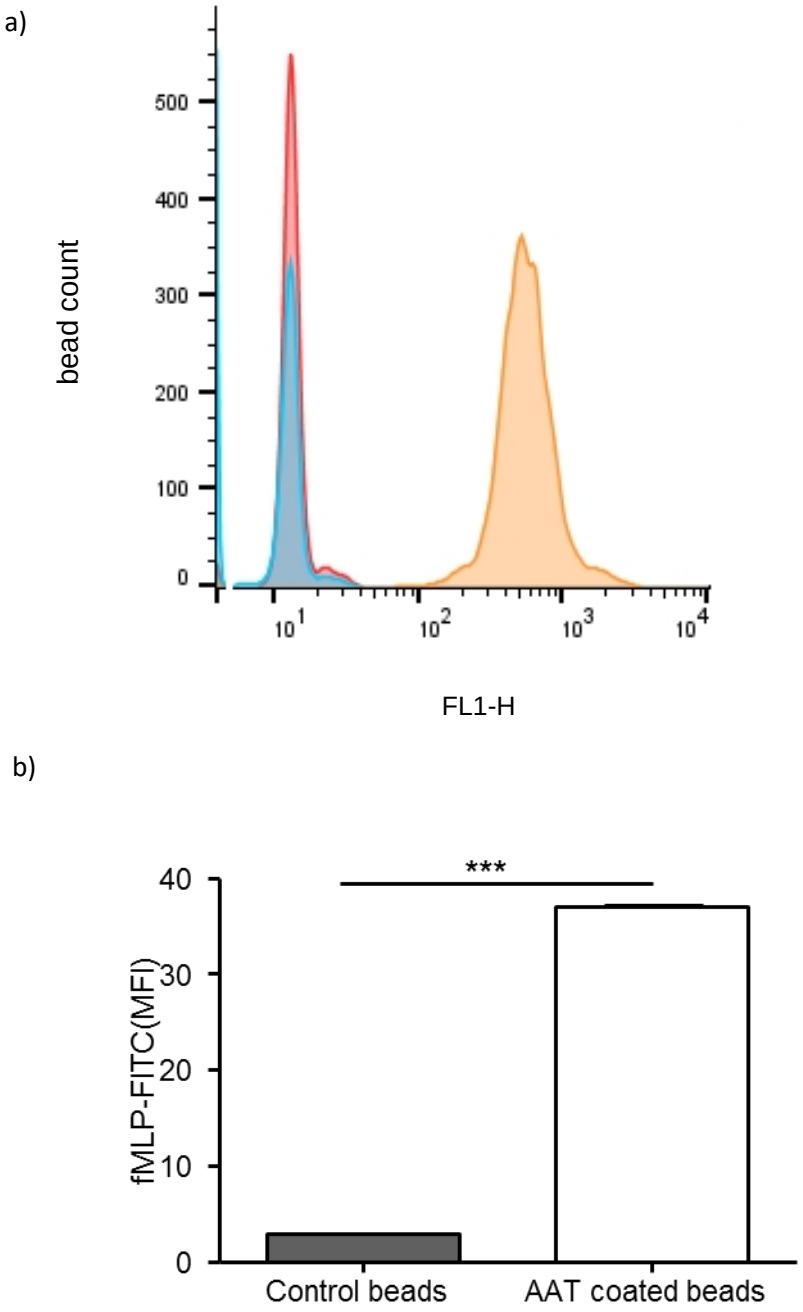


Figure 5-2: fMLP binds to AAT coated polystyrene beads.

a) Histogram of flow cytometry analysis showing detection of fMLP FITC-labelled peptide on 10µm polystyrene beads that were coated with AAT (orange) or not coated with AAT (red) (i.e. control beads). Blank beads (blue), which were not incubated with AAT or fMLP FITC-labelled peptide are also shown. b) Those beads coated with AAT showed greater abundance of fMLP FITC-labelled peptide, as analysed by flow cytometry, compared to uncoated beads (n=3 technical repeats, $p<0.0001$, Student's t test).

5.4 Confirmation of increased binding of AAT to fMLP

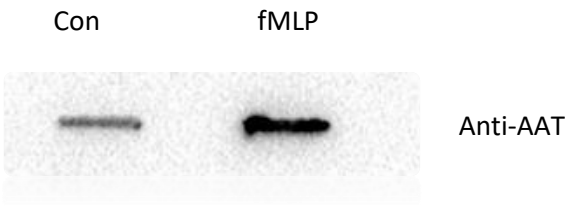
We have demonstrated that fMLP binds to AAT by flow cytometry analysis of polystyrene beads. We wanted to confirm this interaction by employing an additional experimental technique. In this set of experiments, the binding capacity of AAT for fMLP was determined using slot blot analysis.

In brief, fMLP (10μM) was loaded into the wells of a Manifold Slot Blot System and filtered on to PVDF membrane under vacuum. AAT (27.5μM) was then incubated in the wells for 1 h to allow for potential binding interaction to occur. The unbound AAT was discarded and the membrane was washed with dPBS. The PVDF membrane was blocked with 5% (w/v) milk marvel in Tris-Buffered Saline with Tween 20 (TBST) (50mMTRIS, 0.14M NaCl, 0.05% (v/v) Tween 20, pH 7.6) for 1 h at RT. Following this, the membrane was incubated with goat anti-AAT HRP-conjugated antibody (Abcam, ab191350) for 1 h and washed three times with dPBS. Protein bands were detected by measuring chemiluminesence with the Chemidoc system following application of chemiluminescent HRP-substrate.

Our results show, that in those wells in which fMLP was present, there was a 2-fold greater abundance of AAT detected (Figure 5-3) (n=3, p=0.0035, Student's t test). This result suggests, and strengthens our flow cytometry findings, that AAT has the potential to bind directly to fMLP.

This is a novel finding and may explain the ability of AAT to modulate fMLP induced cellular responses such as NADPH oxidase activity. Next we wanted to examine whether AAT binding of fMLP alters the interaction of fMLP with the FPR1 receptor on the neutrophil membrane.

a)



b)

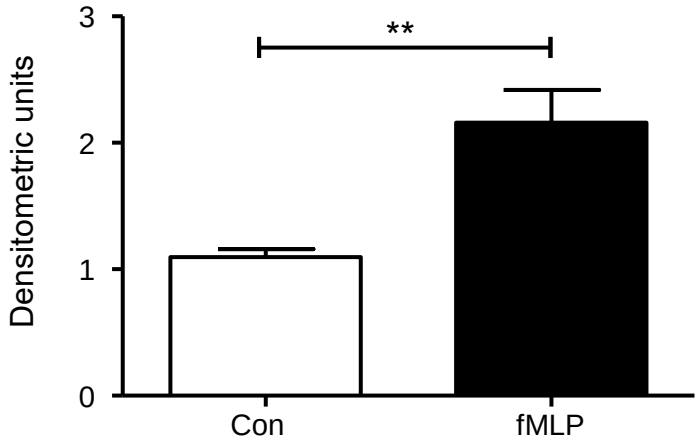


Figure 5-3: Binding of AAT to fMLP by slot blot analysis.

a) Slot blot analysis of the binding of AAT to fMLP. Representative image of n=3 experiments, the membrane was probed with goat polyclonal anti-AAT HRP conjugated antibody. Control wells (con) lacked fMLP. b) Densitometry of immunobands demonstrated binding affinity of fMLP for AAT (n=3 technical repeats, p=0.0035, Student's t test).

5.5 The interaction of AAT/fMLP complex with FPR1 on circulating neutrophils.

We have demonstrated, in vitro, that AAT can bind to fMLP. Our next goal, was to determine whether AAT binding to fMLP would affect the interaction of fMLP with FPR1 on the surface of circulating neutrophils.

In order to examine the interaction of fMLP with FPR1, we used an fMLP peptide that was FITC-labelled. In this way, we could examine the interaction of FITC-labelled fMLP with FPR1 on the neutrophil surface, in the presence or absence of AAT. Isolated healthy control neutrophils ($2x10^6$) in dPBS containing 5mM glucose (control) were incubated with fMLP (10µM) in the presence or absence of AAT (27.5µM) for 10 min. Neutrophils that were incubated with 'cold' fMLP (i.e. without FITC label) would act as a negative control, as FPR1 engagement would be saturated. Cells were then fixed with 4% (w/v) paraformaldehyde and washed three times. FITC-labelled fMLP (10nM) was added to the control and fMLP treated cells. In a further set of experiments, FITC-labelled fMLP that had been incubated with AAT for 1h was added to the AAT treated cells. All cells were left to incubate for 30 min at RT, in the dark, with gentle rotation. All samples underwent one further washing step and were analysed by flow cytometry.

FITC–labelled fMLP bound to the neutrophil in the control sample (black bar) confirming the presence of FPR1 on the cell surface and the engagement of the FITC-labelled fMLP to FPR1. Pre-exposure of cells to fMLP prevented further engagement of FITC-labelled fMLP to its receptor (Figure 5-4) (light grey bar). Pre-incubating the cells with AAT prevented engagement of FITC-labelled fMLP with its receptor resulting in greatly reduced detection on the surface of the neutrophil (dark-grey bar) ($n=3$, $p<0.05$, ANOVA post Bonferroni).

This experiment demonstrates the ability of AAT to modulate engagement of fMLP with its receptor on the surface of circulating neutrophils.

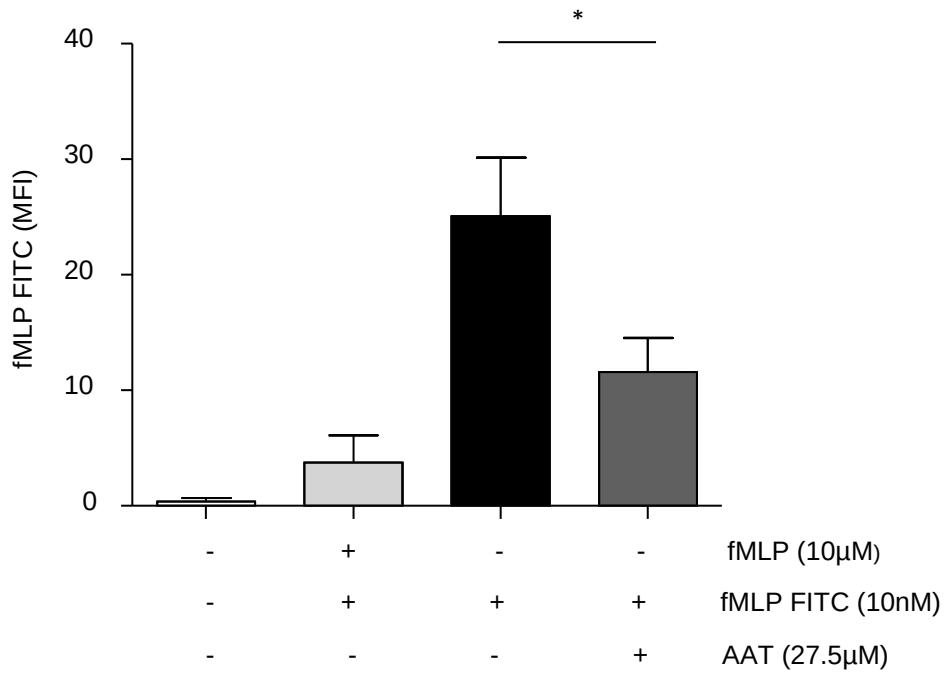


Figure 5-4: AAT modulates engagement of fMLP with its receptor on the neutrophil plasma membrane.

Isolated neutrophils were analysed for the abundance of fMLP-FITC (10nM) on the cell surface of cells. The white bar represents isolated neutrophils that were not treated in any way to exclude autofluorescence. The light grey bar represents neutrophils that were exposed to fMLP, in order to saturate FPR1 engagement, prior to the addition of FITC-labelled fMLP (i.e. negative control). Results demonstrate reduced engagement of FITC-labelled fMLP with neutrophils in the presence of AAT (n=3 biological repeats, $p<0.05$, ANOVA post hoc Bonferroni test).

5.6 The effect of intravenous AAT augmentation therapy on neutrophil O_2^- production.

We have shown that AAT has the ability to bind fMLP and to modulate fMLP engaging with its receptor on the neutrophil plasma membrane. Previously (Chapter 3), we have demonstrated that ROS production in AATD neutrophils is exaggerated in response to fMLP and that AAT can reduce ROS production initiated by fMLP. We next wanted to examine, ex vivo, the effects of AAT augmentation therapy on the production of neutrophil O_2^- in AATD individuals. To this end, we studied our cohort of patients receiving intravenous AAT augmentation therapy. AAT augmentation therapy is the only approved treatment for lung disease due to AATD. It has been shown in a randomised control trial to slow the loss of lung tissue in individuals with AATD and moderate to severe airflow obstruction (186). AAT augmentation therapy has also been shown to have significant immunomodulatory effects and has been studied in diseases other than AATD (146, 154, 156, 266). Individuals with AATD receive intravenous AAT augmentation therapy (60mg/kg) once a week. This group of individuals offer a unique opportunity to examine the effects of AAT on neutrophil O_2^- production ex vivo. It has previously been shown that AAT post augmentation therapy binds the circulating neutrophils in AATD patients (161) and we hypothesised that AAT augmentation therapy would reduce the production of O_2^- from AATD neutrophils.

Neutrophils were isolated from AATD individuals receiving AAT augmentation therapy on day 0 and day 2 of treatment. Blood was drawn on day 0, immediately prior to AAT augmentation therapy infusion and this represents trough plasma AAT levels in these individuals. The day 2 samples were taken two days after the day 0 samples for all of the patients. Blood drawn on day 2 post AAT augmentation therapy represent an environment, in the circulation, where neutrophils have spent the majority of their short life span in an environment of abundant high AAT. All of the patients studied were ex-smokers, with obstructive lung disease. None had experienced an exacerbation in the preceding 8 weeks of having blood drawn. A paired healthy control blood sample was taken with each AATD patient sample and neutrophils were isolated. AAT levels were measured by nephelometry. O_2^- production was measured using a cytochrome c reductase assay. An aliquot of

cells was left untreated and further aliquots treated with fMLP (10μM) for 10 min prior to measurement of O_2^- production.

Our results show that plasma AAT levels were higher on day 2 (1.3g/L) than day 0 (0.78g/L) (Figure 5-5) (n=3, p=0.01, Student's t test). Interestingly, when we compared O_2^- production by AATD neutrophils on day 0 of treatment to healthy control neutrophils, we found that basal levels of O_2^- production were significantly increased in AATD neutrophils (Figure 5-6a) (n=3, p=0.049, two-way ANOVA). On day 2 of treatment there was no difference in the levels of O_2^- produced between AATD neutrophils and controls (i.e. AATD neutrophil O_2^- production returned to healthy control levels following treatment with AAT augmentation therapy) (Figure 5-6b) (n=3, p=0.065, two-way ANOVA). In resting, freshly isolated neutrophils O_2^- production was significantly reduced from 3.7nmol/1x10^6 cells on day 0 to 2.6nmol/1x10^6 cells on day 2. This represents a significant reduction of 30% in maximal O_2^- production on day 2 of treatment (Figure: 5-6c) (n=3, p=0.008, Student's t test). We saw no significant difference in fMLP treated AATD neutrophils on day 0 compared to day 2 (n=3, p=0.5, Student's t test).

We also assessed the effect of AAT augmentation therapy on the response of AATD neutrophils to fMLP, compared to that of healthy control cells treated in the same way. We found no difference in O_2^- production by AATD neutrophils isolated on day 2 compared to day 0 of treatment, in response to fMLP (Figure 5-7)(n=3, p=0.23, Student's t test).

We demonstrate here that, in vivo, AAT can modulate the production of O_2^- by circulating neutrophils. On day 2 of treatment when AAT levels reach 1.3g/L there is significantly less O_2^- production by AATD neutrophils. The enhanced fMLP-induced O_2^- production seen in AATD neutrophils (chapter 3) appears to be reduced by the administration of augmentation therapy.

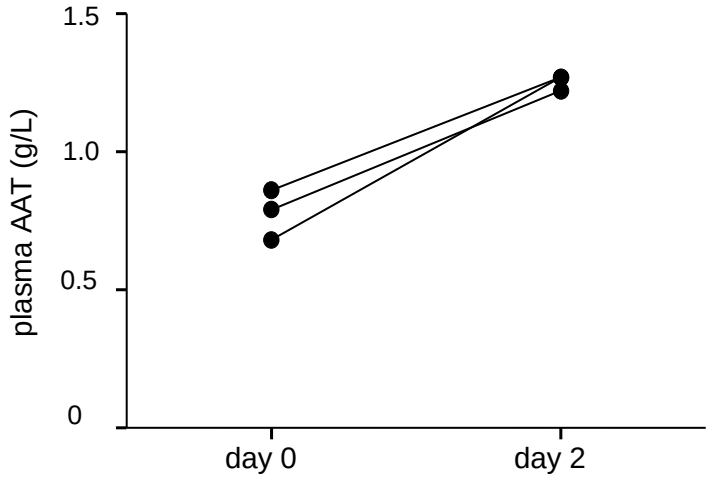


Figure 5-5: Plasma AAT on day 0 and day 2 of AAT augmentation therapy compared to healthy control cells.

Plasma AAT levels on day 0 and day 2 of treatment with AAT augmentation therapy, as measured by nephelometry (n=3, p=0.01, Student's t test).

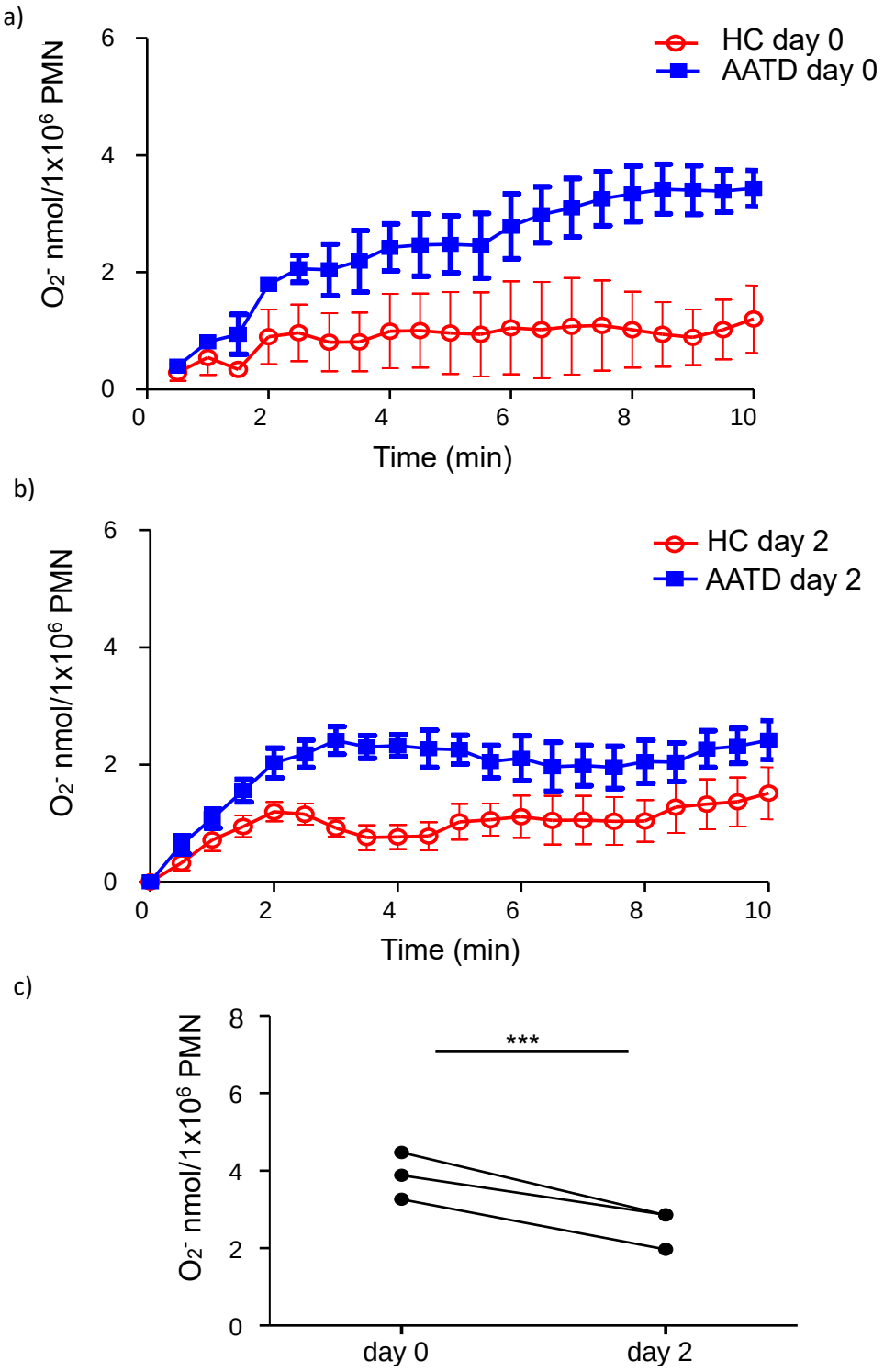


Figure 5-6: Time course of O_2^- production by neutrophils isolated on day 0 and day 2 of AAT augmentation therapy compared to healthy control cells.

a) A time course of basal O_2^- production by AATD neutrophils (blue squares) on day 0 of augmentation therapy with paired healthy control cells (HC) (red circles). There is greater production of O_2^- by AATD cells (n=3, p=0.049, two-way ANOVA). b) O_2^- production over a 10min time course in healthy controls and AATD neutrophils on day 2. There is no difference between the two groups (n=3, p=0.065, two-way ANOVA) No stimuli were added to these cells. c) O_2^- production by circulating neutrophils of individuals with AATD was reduced on day 2 post intravenous infusion of AAT, as measured by a cytochrome c reduction assay (shown is max O_2^- production) (n=3 biological repeats, p=0.008, Student's t test).

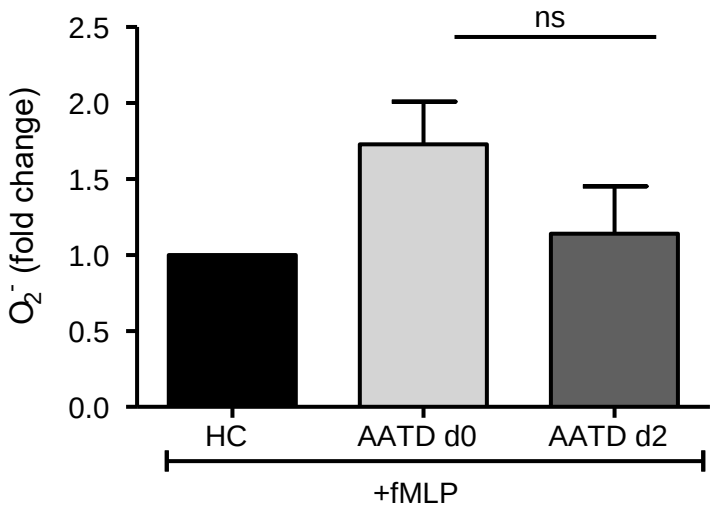


Figure 5-7: fMLP induced O_2^- production on day 0 and day 2 of AAT augmentation therapy by AATD neutrophils compared to healthy control cells.

O_2^- production at 10 min in healthy control cells (HC) (with HC normalised to 1, for day 0 and day 2) and AATD cells on day 0 (d0) and day 2 (d2) of treatment (presented as fold change in relation to normalised HC). All cells were treated with fMLP (10µM). There was no significant difference in O_2^- production between AATD and control cells or from day 0 to day 2 (n=3, p=0.23, Student's t test).

5.7 Discussion

AAT has a high affinity for NE, and inhibits the function of this protease. Moreover, it demonstrates many immunomodulatory effects that are unrelated to its anti-NE capacity, as we have discussed already in some detail. In the previous chapters, we have demonstrated that AAT inhibits the assembly of NAPDH oxidase and the production of ROS in response to certain soluble stimuli. Leading on from this we demonstrated that circulating neutrophils of AATD individuals have increased assembly of NADPH oxidase at the cell membrane in response to fMLP and this leads to increased ROS generation, relative to control cells. We know, from previous studies (154), that IL-8 and AAT bind by direct electrostatic interaction thereby preventing CXCR1 engagement. This finding may, in part, explain our findings of AAT's effects on IL-8 and fMLP induced ROS production. IL-8 is a priming agent of NADPH oxidase activity (36). Loss of IL-8 engagement with CXCR1 on the surface of neutrophils, due to an interaction with AAT, would result in a less robust activation of NADPH oxidase in response to a further stimulus (e.g. fMLP). In the present chapter, we wanted to further characterise the interaction of AAT with fMLP and its cognate receptor FPR1. This interaction has not been studied before, to the best of our knowledge. Further to this, we aimed to examine the effects of AAT augmentation therapy on ROS production by circulating neutrophils in AATD individuals. We postulated that, the effects of AAT on NADPH oxidase activity that we had demonstrated, in vitro, would be seen in our in vivo model.

In this chapter, we have shown that AAT has the ability to bind fMLP. This was demonstrated, in vitro, with the use of AAT coated polystyrene beads assessed by flow cytometry, and additionally by slot blot analysis. fMLP is a tripeptide and there is no commercially available antibody. Therefore we used a FITC-labelled fMLP to evaluate whether AAT has the ability to bind fMLP and disrupt interaction with its cognate receptor on the neutrophil plasma membrane. We were able to confirm that AAT was able to prevent binding of fMLP to FPR1. We used freshly isolated circulating neutrophils from healthy control individuals. When neutrophils were pre-incubated with AAT and then incubated with FITC-labelled fMLP (that had been pre-incubated with AAT) there was near complete inhibition of engagement of the

tagged fMLP with the neutrophil. While this did not define the nature of the interaction between AAT, fMLP and its receptor, it did provide confirmation of our hypothesis and a possible mechanism by which AAT inhibited fMLP-induced cellular responses, as demonstrated in chapter 3.

There are a number of ligands that bind the FPR1 receptor of neutrophils. In fact, FPRs are known for their molecular promiscuity in terms of ligands (267). Some other ligands of FPR, that bear little homology in either common amino acid sequence or their natural origins, include; N-formylated, C-amidated, and unmodified peptides derived from bacterial pathogens as well as endogenous host derived mitochondrial peptides and even several non-peptide agonists, such as lipoxin A4 and resolvin D1 (43, 267, 268). Patients with COPD (including those with AATD) experience exacerbations and they show airway colonisation with *Haemophilus influenzae* and *Moraxella catarhallis* (269). With the presence of bronchiectasis, AATD individuals can demonstrate airway colonisation with *Pseudomonas aeruginosa* and *Staphylococcal aureus* (171). There is an over representation of NTM disease in those with abnormal AAT genotypes, however, NTM is still not a common occurrence in AATD (172). Based on these findings, bacterial peptides may be present in the airway of individuals with AATD associated lung disease to act as ligands for FPR1. Mitochondrial derived peptides are also of interest when studying individuals with AATD associated lung disease. These are autologous mitochondrial break down products that may be present in the airways or in the circulation and have the ability to act as DAMPs and may activate FPR1 of neutrophils. In ‘normal’ COPD it has been demonstrated that there is an increase in endothelial, alveolar and epithelial cell apoptosis (270, 271), this is likely also the case in individuals with COPD related to AATD although it has not been directly assessed, to our knowledge. Neutrophil apoptosis has been shown to be increased in AATD (146). There has been some work performed in this regard on animal cell lines. An example of this is work by Sohrab *et al*. demonstrating that rat endothelial cells were capable of AAT endocytosis and that AAT inhibited cell apoptosis (272). Serban *et al.* have shown that AAT improves the ability of alveolar macrophages to clear apoptotic cells (efferocytosis) in individuals with COPD and this therefore may be impaired in individuals with AATD (273). All of these may be sources of mitochondrial formylated peptides for FPR1

agonism. Mitochondrial formylated peptides have been shown to be present in the circulation and BAL fluid of humans with ARDS and are felt to promote sterile inflammation through interaction with FPR1 (263). In conclusion, bacterial colonization of the lower respiratory tract and lung cell damage may represent sources of formyl peptides in patients with COPD and AATD.

In this chapter, we have also shown for the first time, that AAT augmentation therapy reduces neutrophil ROS production in patients with AATD. Our cohort of patients receiving AAT augmentation therapy had been receiving treatment for many years and had stable disease in the previous 8 weeks, without exacerbation. There was a 30% reduction seen in O_2^- production on day 2 of treatment that was associated with higher levels of circulating AAT in plasma at this time. When we assessed neutrophils on day 0, we found that AATD neutrophils produced more O_2^- than healthy control neutrophils. However, on day 2 of treatment there was no difference in O_2^- production between AATD and control neutrophils. This result suggests that, in resting unstimulated cells, AAT therapy modulates O_2^- production by neutrophils (which is higher on day 0) to healthy control levels.

We also examined fMLP induced ROS production on day 0 and day 2 of treatment with AAT augmentation therapy. We found that, following treatment with fMLP, neutrophils of AATD individuals there was no significant difference in O_2^- production on day 2 of treatment compared to day 0. Circulating AAT levels, in individuals receiving AAT augmentation therapy, remained above the putative protective threshold of 11μM throughout their treatment (Figure 5-7a).

The reduced O_2^- on day 2 of AAT augmentation therapy treatment may be explained, in part, by the capability of infused M-AAT to scavenge O_2^-. We know from the work of others, that AAT binds IL-8 and that AAT augmentation therapy reduces neutrophil chemotaxis in response to IL-8 in AATD individuals (154). The reduced interaction of the chemokine IL-8 with CXCR1 as demonstrated by others (154), may in part explain our findings of reduced O_2^- in AATD individuals on day 2 of AAT augmentation therapy.

We have shown here that AAT binds to fMLP and can prevent fMLP engagement with its receptor on the plasma membrane. We have also shown that AAT augmentation therapy reduces O_2^- production significantly between day 0 and day

2 of treatment and compared to control cells. These findings describe a novel effect of AAT augmentation therapy in individuals with AATD. Given the role of oxidative stress in other disease states, such as COPD, these findings may have further reaching implications and warrant further investigation.

Chapter 6: General Discussion

The field of AAT research has for many years examined the ability of AAT to modulate the immune response. It is well established that AAT functions are beyond solely inhibiting NE, although this is a function that in its native form it performs extremely efficiently (113). There have been many studies in the recent past demonstrating the role that AAT plays as an anti-inflammatory molecule, particularly in relation to neutrophil function, including; apoptosis, chemotaxis, adherence and TNFα release (146, 154, 156, 262). From these studies we have been informed of novel functions of AAT and we have gained insights into potential pathogenic mechanisms of AATD related disease. Moreover, some of these studies have revealed new functions of AAT augmentation therapy as an immune modulating treatment. Indeed, the potential use of AAT as a therapeutic has expanded to include rheumatoid arthritis (274), diabetes mellitus (275), graft-versus-host disease (276) and post lung transplant graft rejection (277).

The greatest morbidity and cause of mortality in AATD is related to COPD. There is evidence of exaggerated markers of oxidative stress and reduced antioxidant defence in the airways and circulation of individuals with COPD (63). This oxidative stress is likely initiated by the inhalation of exogenous oxidants (i.e. cigarette smoke) that then recruit immune cells to the airway and, in susceptible individuals, these cells then become an endogenous source of ROS which self-propagates in a process of chronic inflammation. There are no therapies available that significantly modify the course of the disease in COPD, and this remains a significant unmet need in COPD treatment. Moreover, the negative effects of oxidative stress are not confined to the lung. Oxidative stress in the brain has been linked with neuronal degeneration and the development of Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (278). Oxidative stress has also been linked to the development of retinal disease, rheumatoid arthritis and psoriasis (279, 280). Oxidised AAT has reduced capacity to inhibit NE and this has been implicated in the pathogenesis of emphysema in AAT replete individuals. One of the aims of this project was to examine the production of ROS by neutrophils in individuals with AATD.

Neutrophils are known to play an important role in the pathogenesis of lung disease in AATD and the current study adds to this field. We demonstrate that AAT can modulate neutrophil ROS production and elucidate a mechanism by

which AAT deficiency leads to enhanced ROS production by neutrophils of AATD individuals.

Bucurenci *et al.* demonstrated in the early 1990's that neutrophil O_2^- production in response to various stimuli could be inhibited by AAT in vitro (160). Bergin *et al.* found there to be anti-lactoferrin antibodies in the plasma of AATD individuals and these antibodies can target antigen that is bound to the neutrophil cell surface to induce extracellular ROS production (161). In addition to this, Dasi *et al.* reported serum markers of oxidative stress to be elevated in the serum of AATD adolescents, possibly related to reduced abundance of glutathione and superoxide dismutase (281). There have been no further studies, that we are aware of, examining the role of AAT in ROS production or examining more closely the role of neutrophil O_2^- production in AATD lung disease. The information available to date on the role of AAT in ROS production and the presence of oxidative stress in AATD individuals is limited. The aim of this study was to further investigate the role of AAT in neutrophil ROS generation and explore the production of ROS by neutrophils of individuals with AATD.

In this study, we confirmed the findings of Bucurenci *et al.* that, in a dose-dependent manner, AAT modulates neutrophil O_2^- detection in response to the soluble physiological stimuli, IL-8 and fMLP. Further to this, we were able to demonstrate that AAT directly affected NADPH oxidase activity by assessing the assembly of its cytosolic components ($p67^{PHOX}$ and $p47^{PHOX}$) at the plasma membrane and by measuring the O_2 consumption of neutrophils in response to these soluble stimuli, in the presence or absence of AAT. In health, neutrophil derived ROS functions to kill invading pathogens, and is generally localised to the site of infection (e.g. the lungs) and within the phagosome (29). In the absence of an invading pathogen NADPH oxidase of neutrophils is quiescent. Upon encountering a pathogen, surface receptors recognise PAMPs and phagocytosis ensues. FPR1 may be activated by bacterial derived peptides, stimulating extracellular ROS production, degranulation and chemotaxis. In the setting of infection/inflammation, circulating levels of AAT increase. In healthy individuals and once bacterial killing has occurred, AAT may serve to modulate extracellular ROS production in response to an invading pathogen, via its modulation of fMLP and FPR1 interaction that we have shown, thereby preventing excessive and

sustained inflammation. Thus protecting against excessive tissue damage by extracellular O_2^-. The high abundance of AAT will act to prevent further neutrophil chemotaxis and also to scavenge O_2^- via oxidation of specific methionine residues. This further supports the role of AAT in the resolution of inflammation (112).

Neutrophil priming in AATD

We found that circulating neutrophils in AATD exhibited increased NADPH oxidase activity in response to fMLP. We confirmed that this was due to increased assembly of NADPH oxidase cytosolic components, $p67^{PHOX}$ and $p47^{PHOX}$, at the plasma membrane. This provides further evidence that circulating neutrophils from individuals with AATD are primed. There is a large amount of work to date describing circulating neutrophil abnormalities in AATD individuals (116, 146, 154, 156, 161, 261, 262, 282). It appears now to be well established that circulating neutrophils in AATD are primed. Our findings add to this large body of work. Neutrophil priming is evident when exposure of a neutrophil to an activating agent results in a more robust response. This may be in the form of migration, degranulation, rate of apoptosis, NET formation or ROS production. A typical example of NADPH oxidase priming, in vitro, would be the treatment of cells with TNFα, IL-8 or GM-CSF resulting in a more robust ROS production in response to fMLP (240). fMLP alone triggers only low amounts of O_2^-, but when the neutrophil is primed there is an enhanced response, as we have found. There are a large host of substances that can act as priming agents in vitro. These can be largely separated into three groups; chemoattractants, cytokines and TLR agonists. This array of priming agents may also cause in vivo priming and there may be interplay of multiple priming agents in an in vivo setting. In relation to NADPH oxidase, priming typically results in phosphorylation of $p47^{PHOX}$ (228) with the translocation of $p47^{PHOX}$, $p67^{PHOX}$, $p40^{PHOX}$ and Rac2 to the plasma membrane, as we found in our fMLP treated neutrophils from individuals with AATD.

Neutrophil activation in health must be tightly regulated given the arsenal of potentially cytotoxic substances contained within them. Uncontrolled neutrophil activation can result in these antimicrobial responses damaging host cells, which can lead to pathologic changes to tissues and the development of autoimmune and inflammatory diseases. Primed neutrophils have been identified in humans

with ARDS (283), rheumatoid arthritis (284), diabetes (285), sepsis (286), atherosclerosis (287), chronic kidney disease (288), trauma and infections (289).

The effects of primed neutrophils have been studied using in vitro cell models and in animal models. Priming of neutrophils correlates with neutrophil mediated endothelial cell injury in a model using neutrophils from haemodialysis patients (290) and in another study looking at LPS and Lys-PC primed healthy control neutrophils (291). ROS increases the ability of ICAM-1 to bind to neutrophils promoting neutrophil arrest (292). In a mouse model, TNFα priming promotes FcγR interaction with intravascular immune complexes, with increased binding affinity of Mac-1 for ICAM-1 and subsequent neutrophil arrest (293). In mice, GM-CSF induced priming plays a key role in high-dose LPS induced, or haemorrhage induced, acute lung injury model (294).

Of interest, neutrophils that are primed in the circulation are not committed to activation. It has been shown that primed neutrophils can become de-primed. Chilvers *et al*. demonstrated that in response to PAF neutrophils could be primed, then after a period became de-primed and could then become primed again (295). Summers *et al.* proposed that the healthy pulmonary vasculature can retain neutrophils and facilitate their de-priming (296). In certain disease states, however, (e.g. ARDS) the lung may become the site of priming. This has been examined in a study that looked at a transpulmonary gradient of neutrophil priming (297). Arterial blood samples and mixed venous blood samples were taken from patients with sepsis with or without co-existent lung injury. In patients with sepsis alone the neutrophils entered the lungs in a primed state and left the lungs in a deprimed state. However, in patients with lung injury this gradient was reversed and neutrophils leaving the lungs were more primed than those in the peripheral circulation prior to entering the pulmonary vasculature. The pulmonary endothelium or a factor released by the pulmonary endothelium, such as ADP, has been suggested as a source of depriming in the lung (298, 299). AAT has been shown to have numerous effects on lung endothelial cells. AAT can be endocytosed by endothelial cells serving a protective mechanism against cigarette smoke induced changes (272). Furthermore, AAT can inhibit TNFα release from endothelial cells (300) . The endocytosis of AAT by endothelial cells suggests their

role in the modulation of inflammatory responses. Perhaps a deficiency in AAT results in a pulmonary endothelium less well able to deprime neutrophils.

Hurley *et al*. have demonstrated that in AATD, Z-AAT accumulates in the endoplasmic reticulum (ER) of neutrophils leading to ER stress that results in increased production and release of TNFα in the circulation. The effect of TNFα is further enhanced due to the increased activity of ADAM-17 (146). This soluble TNFα is a potential priming agent. Similarly, Bergin *et al.* have shown that AAT can bind to the NADPH oxidase priming agent IL-8 preventing its interaction with CXCR1 on the neutrophil surface (154). These are two potential contributors to priming of circulating neutrophils in AATD. We have shown a novel mechanism by which AAT may inhibit neutrophil priming. We demonstrated the ability of AAT to prevent the interaction of fMLP with its neutrophil receptor FPR1 and have demonstrated that this protective mechanism is lost in AATD.

AAT modulates FPR1 activation

Our finding of the disruption of the interaction of fMLP and FPR1 by AAT is an important and novel finding. Given the diverse activity of this GPCR it is an important interaction to consider in more detail. There has been much interest in the role of FPR1 and its ligands in inflammatory processes (43, 264) and there is a suggestion from our results that this is an interaction of interest in AATD. We have shown that AATD neutrophils have an enhanced respiratory burst in response to activation of FPR1. Activation of FPR1 triggers a wide variety of functions that include not only ROS production but chemotaxis, phagocytosis and IL-8 release also. The ligands of FPR1 include bacterial formylated peptides, such as fMLP and mitochondrial formylated peptides (263). Mitochondria represent a rich source of danger associated molecular patterns (DAMPs), probably because of their shared ancestry with bacterial DNA (301, 302). DAMPs are intracellular molecules that are released from various cells following injury. Protein synthesis by both bacteria and mitochondria use N-formyl-methionine as a common residue and it is in this way that mitochondrial N-formylated peptides arise, with the potential to act as ligands for FPR1 following cell death. Mitochondrial formylated peptides have been found in BAL fluid and serum of patients with ARDS, as measured using mass spectrometry (263) and were found to drive neutrophil activation and chemotaxis

(303). In a mouse model of lung injury, *FPR*$^{-/-}$ mice exhibit reduced inflammation. In the same lung injury mouse model, an FPR1 antagonist was found to attenuate lung injury even when administered after the onset of lung injury (263). The levels of mitochondrial formylated peptides in AATD airways or serum have not been assessed, to the best of our knowledge. There is some evidence, however, for the presence of mitochondrial degradation products in 'normal' COPD. For example, ATP is an energy coenzyme for mtDNA and is found with higher abundance in COPD BAL fluid (304), moreover mtDNA has been found in BAL fluid of mice exposed to cigarette smoke (305). Our novel discovery, that AAT modulates the interaction of fMLP and FPR1 has implications for both sterile and infective inflammation and warrants further study. Modulation of this ligand receptor interaction by AAT may represent a further potential therapeutic application for AAT augmentation therapy.

O_2^- in excess: the biological consequences

The potential consequences of extracellular O_2^- include the oxidation of proteins, lipids and DNA. Pro-inflammatory effects of O_2^- can also be mediated through neutrophil TLR4 with nuclear translocation of NF-κB which can induce the production of TNFα and MIP-2 from neutrophils (306). ROS, particularly O_2^-, is also known to regulate autophagy (307). Dependent on the extent of cellular degradation autophagy may be a cell survival mechanism or a cell death pathway. In AATD, we have shown that the neutrophil is primed in the circulation to produce enhanced O_2^- and this may lead to accelerated degradation of cytosolic proteins and organelles essential for basic homeostasis and cell survival culminating in cell death. We have measured extracellular O_2^- in our experiments, which does not permeate the membrane. However, O_2^- rapidly dismutates to form H_2O_2 which is capable of permeating cell membranes to exert an effect intracellularly. In this way ROS produced extracellularly can exert effects on intracellular processes directly, as would occur with regulation of autophagy. Perhaps, AATD neutrophils are primed towards this autophagic death pathway given their enhanced propensity to produce O_2^-.

FPR1 as a therapeutic target

Our results suggest that FPR antagonism may represent a novel therapeutic target for modulating host defence and innate immunity. FPR1 is a relevant receptor within the acute inflammatory process, both infectious and sterile, that has the capacity to respond to unique bacterial and host-derived factors. *FPR*1$^{-/-}$ mice are protected from cigarette smoking induced emphysematous changes, with an associated reduction in neutrophil and macrophage number (308). A variety of molecules have been identified and studied in vitro. These include cyclosporine A and H, Boc-1 and -2, and fungal hydrophobic cyclic peptides (309) and in recent years there has been a search for a novel small-molecule FPR antagonist.

Interestingly, we found that there was significant variation in neutrophil O_2^- production over the course of treatment with AAT augmentation therapy (i.e. between day 0 and day2) in individuals with AATD. This is a novel finding and represents an exciting result given our earlier in vivo findings. The clinical implications of excessive neutrophil NADPH oxidase activity in such individuals have not been studied in any great detail. Our findings do support the findings of others, that AAT augmentation therapy has immune-modulatory actions, it also raises the question as to whether this treatment is sufficient and whether there is potentially a role for higher dosing of AAT augmentation therapy. Campos *et al.* have shown that double dosing (120mg/kg) AAT augmentation therapy in AATD individuals reduces serine protease activity in BAL fluid and increases trough AAT levels to normal compared to the standard dose therapy. It would be interesting to examine whether double dose therapy could reduce neutrophil O_2^- generation even further in AATD individuals (193). It may be that AAT serves to dampen but not completely reverse an already well established inflammatory process. Perhaps initiation of treatment would be more effective prior to the onset of significant lung disease as neutrophil priming may not be fully reversible in AATD once initiated. This would be challenging to achieve given the heterogeneity of disease burden in AATD and the costs associated with AAT augmentation therapy. There may then be a role for adjuvant therapy in the form of antioxidants in individuals with AATD on augmentation therapy and this warrants further study given our findings.

Any new insights that we gain into the pathogenesis of lung disease in AATD are invaluable. AATD is a heterogeneous disease and even in its severe form (i.e. PI*ZZ) there is a wide spectrum of disease presentations; from asymptomatic individuals with normal life expectancy to neonatal hepatic failure to early onset emphysema with early death or requirement for lung transplantation (165). This heterogeneity can pose a challenge to investigators and may explain some of the biological variability seen in our in vivo experiments presented here. However, the study of AAT can benefit not only individuals with AATD but those with disorders predominated by neutrophilic inflammation. Our study provides further evidence of the role of AAT in the modulation of neutrophil driven inflammation in AATD (Figure 6.1). We have shown here a further mechanism by which AAT therapy exerts immune modulating effects in AATD individuals, pointing to the potential role of fMLP and FPR1 in the pathogenesis of AATD lung disease. This study has implications for AATD, as presented here, and potentially other neutrophil driven chronic inflammatory conditions. Our findings have exciting implications for the use of AAT augmentation therapy in conditions in which neutrophil derived ROS play a role.

The clinical heterogeneity of our AATD population and the small numbers of patients within individual experiments is a limitation of this study. This should be taken into account when considering the results of the work presented here. Future work would aim to increase the number of patients recruited to be better able to phenotype individuals, to take into account variables other than genotype that may be influencing our results. Such analysis was not possible with the limited number of patients recruited during this study. A strength of this thesis was access to patients who were receiving intravenous weekly augmentation therapy. This allowed comparison of individual patients before and after this therapeutic intervention. Future work would look to examine further time points and larger numbers of patients receiving AAT augmentation therapy.

a)

b)

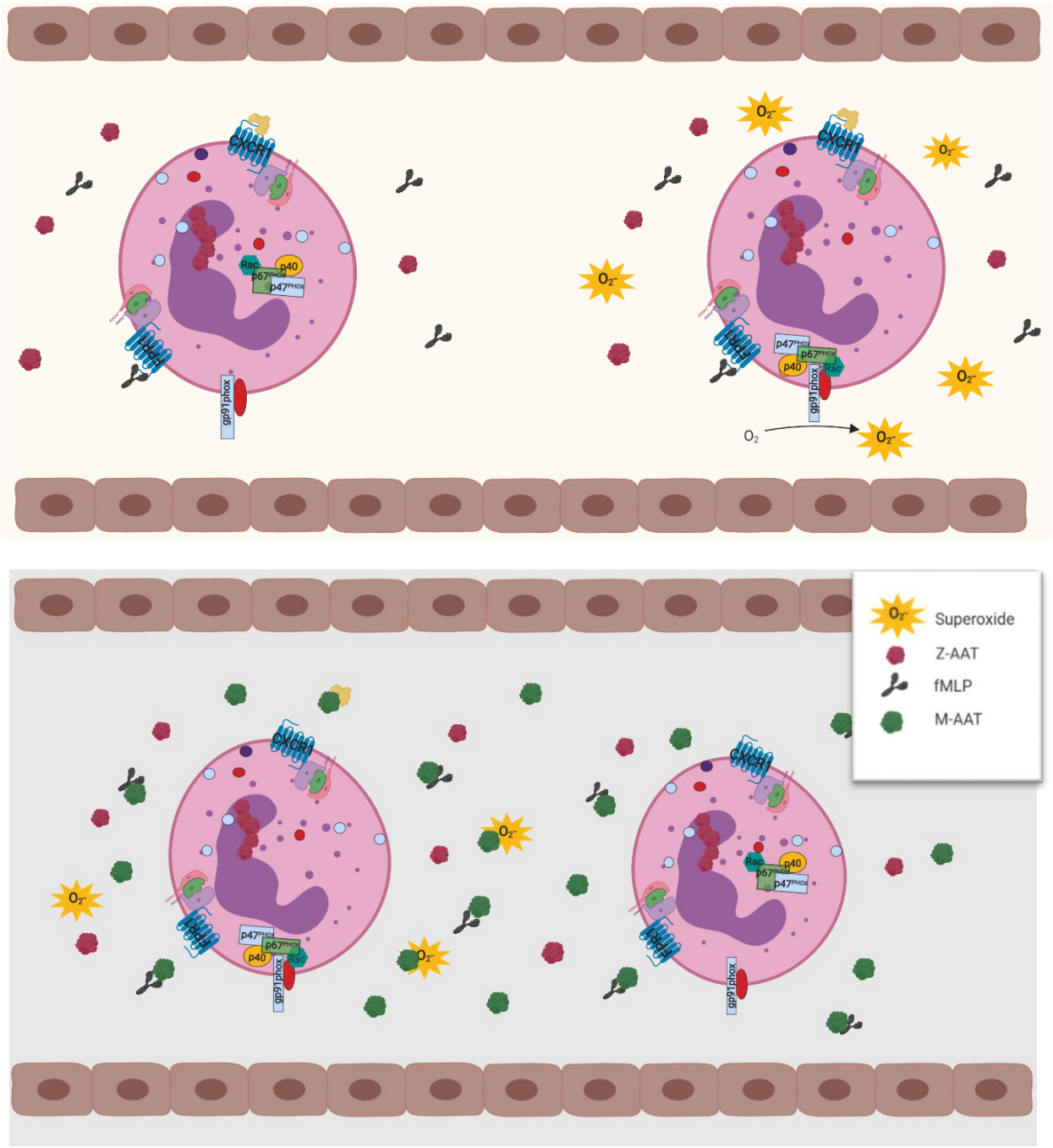


Figure 6-1: AAT modulates NADPH oxidase activity by interaction with fMLP and FPR1. Schematic illustration summarising the results of the project. a) In AATD individuals, circulating neutrophils show enhanced O_2^- production in response to fMLP with the assembly of the cytosolic components of NADPH oxidase ($p67^{PHOX}$ and $p47^{PHOX}$) at the plasma membrane. b) Following AAT augmentation therapy there is a reduction in the production of O_2^- by neutrophils of individuals with AATD. AAT was shown to directly bind to fMLP and prevent binding to FPR1.

Chapter 7: Future Directions

The aim of this study was to examine the effects of AAT on neutrophil NADPH oxidase. We demonstrated the ability of AAT to modulate the assembly and activity of NADPH oxidase of neutrophils in response to fMLP. We have also shown that neutrophil NADPH oxidase activity is enhanced in individuals with AATD and that this can be corrected with AAT augmentation therapy. Our results raise a number of interesting questions and potential avenues of further investigation.

The cohort of AATD patients receiving augmentation therapy represent a unique and valuable population to measure the in vivo effects of AAT. The in vivo effects of AAT can be examined in these individuals due to the rise and fall in plasma AAT levels. There are few other disease states in which such biological samples (pre and post treatment) are available for experimentation. It would be interesting to study this population further in relation to reactive oxygen species and the role of oxidative stress in AATD lung disease. Further experiments would aim to quantify the abundance of O_2^- and antioxidants in airway samples of these individuals and assess for fluctuations during the course of their treatment with AAT augmentation therapy.

fMLP is not known to occur in the circulation of individuals with AATD, to the best of our knowledge. Future experiments would aim to identify other FPR1 ligands that may be found in altered abundance in the circulation and/or airways of individuals with AATD. Annexin A1 and cath G are both known ligands for FPR1. Mitochondrial break down products would be another FPR1 ligand that would be of interest in AATD. Mitochondrial products have been found in the circulation of COPD individuals, as discussed previously. We also know that individuals with AATD have increased neutrophil apoptosis (146) and that AAT is required for effective alveolar macrophage efferocytosis (273). It would be of interest to examine serum and airway samples of AATD individuals for the presence of mitochondrial formylated peptides. It would be important to quantify these mitochondrial products and study their relationship to smoking status, FEV_1 and the effects of treatment with AAT augmentation therapy.

We have examined the binding of AAT to fMLP. Future experiments would examine neutrophil cellular responses to mitochondrial formylated peptides,

including O_2^- production, chemotaxis and degranulation. Leading on from this it would be of interest to examine the effect of AAT on any mitochondrial formylayed peptide induced cellular responses seen. Mitochondrial formylated peptides can be synthesised and purified using high performance liquid chromatography (263, 310). Any interaction with AAT and these mitochondrial products should be well characterised. It would be important to determine the nature of the interaction and whether binding effects NE inhibitory capacity of AAT. Perhaps different glycoforms of AAT may bind with greater affinity to mitochondrial derived products.

In chapter 3, we demonstrated that HVCN1 was found in reduced abundance in neutrophils of individuals with AATD. This did not affect the neutrophils ability to compensate cytosolic pH in response to activation of NADPH oxidase. There are some reports of impaired bacterial killing by neutrophils of individuals with AATD (146). Future experiments would aim to examine the neutrophil phagosome in this regard. The phagosome has a much smaller volume with higher concentration of proteins and the potential for greater shifts in pH compared to the relatively large volume of the neutrophil cytoplasm (250, 252). The cytoplasm has a variety of mechanisms of charge compensation that are not available at the phagosome (e.g. lysosomes and granules). An optimal pH is required in the phagosomal lumen to ensure optimal activity of neutrophil proteases (52). Given the opportunity, future experiments would quantify HVCN1 abundance on the phagosomal membrane in neutrophils of individuals with AATD and examine the vacuolar pH. Once again, the study of AATD individuals receiving augmentation therapy could be vital in examining these changes and their relationship to the effects of AAT in vivo.

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