NEUTROPHIL PHENOTYPES AND ACCELERATED AGEING AS A CAUSE OF DISEASE PATHOGENESIS, PROGRESSION AND MULTI-MORBIDITY IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE

by

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A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

Institute of Inflammation and Ageing

College of Medical and Dental Sciences

University of Birmingham

January 2021

Abstract

Chronic Obstructive Pulmonary Disease (COPD) remains one of the leading causes of mortality globally and places a high burden on patient quality of life. Neutrophils have been linked to multiple facets of disease pathology and are abundant in lung secretions of patients with COPD. However, recurrent infection and the lack of inflammatory resolution in COPD suggest neutrophil dysfunction. In addition, these patients also commonly present with other chronic diseases, termed multimorbidity, such as diabetes and cardiovascular disease and changes in circulating neutrophils may provide a link between these diseases.

This thesis aimed to investigate changes in peripheral neutrophil phenotype in healthy ageing and COPD, with a focus on how multimorbidity may influence these changes. Systemic neutrophil activation, senescence and maturity status appeared unaltered in stable COPD compared to healthy controls and did not suggest an accelerated ageing phenotype. However, expression of a key chemokine receptor, CXCR2, was shown to be reduced and impacted by the presence of multimorbidity in these patients and further reduced during exacerbations. Multiple changes in both surface and gene expression were also seen during an exacerbation, suggesting the neutrophil phenotype can be altered by both chronic and acute inflammation.

In addition, an activated phenotype was observed when neutrophils from healthy individuals were incubated with plasma from patients with COPD, suggesting systemic inflammation in patients with COPD was present and was able to activate neutrophils, further highlighting changes in the neutrophil phenotype due to chronic disease.

Differences in the neutrophil phenotype described here demonstrate the heterogeneity of neutrophils, as well as the different impact caused by multimorbidity. Altered phenotypes suggest a change in function, providing further understanding to the role of neutrophils in COPD.

Acknowledgements

Firstly, to my mentors and supervisors: Professor Elizabeth Sapey; Dr Helen McGettrick; and Professor Janet Lord. It has been a delight to learn from such knowledgeable scientists. To Liz, for the encouragement and belief in my abilities, welcoming me to the wonderful clinical world and providing the platform for me to present our work at conferences and through publications. To Helen for the office drop-ins, continual grounding and panic suppression – I also owe you a box of red pens. To Janet, for providing critical insight and a role model of leadership.

To the whole of Team Neutrophil past and present, without whom this PhD would not have been half as enjoyable. To Georgia Walton for running Neutrophil Academy and for endlessly assisting in troubleshooting everything from "you forgot to press 'go' on the centrifuge" to "burn all the reagents and start again". To Paul Newby for always knowing what to do and where everything is. To the rest of the Team Neutrophil and Team Respiratory (you know who you are) for all those coffee meetings, physical and virtual, pub trips and general good times – you have made this experience thoroughly enjoyable.

A special thanks to Helena Crisford, my PhD sister, who has provided endless encouragement, a listening ear and plenty of laughs – words cannot express my gratitude, or my apology for the distraction I provided (you'll never get those hours back).

A thanks also to Anita Pye, the clinical trial guru, who helped me through all the ethical hurdles, enabling me to recruit patients, and providing general wellbeing support. Thanks also to all the clinicians and support staff that made this PhD possible. To Dhruv Parekh for assisting in recruiting patients to the study and for teaching me how to handle criticism of

large error bars. To Farah for always being available to take blood for me. To Cath Thurlby and Jo Dasgin for supporting patient visits within the Inflammation Research Facility and of course to all the patients and volunteers without whom this thesis would end after the introduction.

Throughout it all, my wife Sarah, has stood by me through the highs and lows – especially through the writing stage. You have provided me with many memorable moments that have kept me sane during this PhD, always believing in me even when I did not believe in myself and being a great office companion during the almost year-long working from home routine. Also, to our baby, who (without knowing) has provided motivation for me to sit down and write up my PhD before their arrival. Thanks also to my family, who have provided continual support, encouragement and set me up to go on and pursue my dreams.

Lastly, I would like to thank my funders the Wellcome Trust who have set the bar incredibly high as a funding body for enabling the delivery of high-quality research and supporting the wellbeing of the people they fund.

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7AAD	7-Aminoactinomycin D
AAT	Alpha-1 anti-trypsin
AATD	Alpha-1 anti-trypsin deficiency
AECOPD	Acute exacerbation of COPD
AF700	Alexafluor700
AMPK	5' adenosine monophosphate-activated protein kinase
ANCA	Anti-neutrophil cytoplasmic antibody
ANOVA	Analysis of variance
AP	Autologous plasma
APC	Allophycocyanin
ApoE	Apolipoprotein E
ARDS	Acute respiratory distress syndrome
ARHGAP24	Rho GTPase-activating protein 24 (gene)
AS	Autologous serum
ATP	Adenosine triphosphate
BACE2	Beta-secretase 2 precursor (gene)
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
BV	Brilliant Violet
CACNG6	Calcium voltage-gated channel auxiliary subunit gamma 6 (gene)
CAD	Coronary artery disease
CAT™	COPD Assessment Test
CCAAT/enhancer	Enhancers binding the DNA sequence CCAAT
CCR	C-c chemokine receptor
CD	cluster of differentiation
CDK	Cyclin dependant kinase
CEACAM8	CEA Cell Adhesion Molecule 8
CGMP-PKG	cGMP-dependent protein kinase
CLC	Galectin-10 (gene)
COPD:	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CR	Complement receptor
CRP	C-reactive protein
CS	Cigarette smoke
CSE	Cigarette smoke extract
CSF	Colony-simulating factor
CVD	Cardiovascular disease
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor

CYSLTR2	Cysteinyl leukotriene receptor 2
DESeq2	Differential expression sequence version 2
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESAM	Endothelial cell adhesion molecule (gene)
FACS	Fluorescence-activated cell sorting
FBN1	Fibrillin-1 (gene)
FCγRIII	Fragment of crystallisation (Fc)-gamma receptor III
FCS	Flow cytometry standard
FEV_1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
FITC	Fluorescein isothiocyanate
FilGAP	Filamin A-binding rhoGTPase-activating protein
FMO	Fluorescence minus one
FSC (-A)/(-H)/(-W)	Forward scatter (-Area)/(-Height)/(-Width)
GENCODE	Gene encyclopedia of DNA elements
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
GO	Gene ontology
GOLD	Global initiative for COPD
GPCR	G-protein coupled receptor
GSK	GlaxoSmithKline
H_2SO_4	Sulphuric acid
HE	Healthy elderly
HLA-DR	Human leukocyte antigen – DR isotype
HT	High throughput
HY	Healthy young
IC_{50}	Inhibitor concentration at which 50% inhibition is achieved
ICAM-1	Intercellular adhesion molecule 1
ICU	Intensive care unit
IL	Interleukin
IL5RA	Interleukin 5 receptor alpha
IQR	Interquartile range
ITGA2B	Integrin subunit alpha 2b (gene)
ITGA7	Integrin alpha-7 (gene)
ITGB3	Integrin beta-3 (gene)
IV	Intravenous
JUP	Junction plakoglobin (gene)
K2EDTA	Potassium-Ethylenediaminetetraacetic acid

KEGG	Kyoto encyclopedia of genes and genomes
LAD	Leukocyte adhesion deficiency
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NHS	National Health Service
NIH	National Institute of Health
NOX	NADPH oxidase 1
NP	Neutrophil panel
NS	Non-smoker
NSAIDs	Non-steroidal anti-inflammatory drugs
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed-death receptor 1
PD-L1	Programmed-death ligand 1
PD-L2	Programmed-death ligand 2
PE	Phycoerythrin
PE/Cy7	Phycoerythrin/cyanine 7
PI3K	Phosphatidylinositol-3-kinase
Akt	Protein kinase B
PAF	Platelet activating factor
PDLIM1	PDZ and LIM domain protein 1 (gene)
PMA	Phorbol myristate acetate
PR3	Proteinase 3
PTEN	Phosphatase and tensin homolog
QC	Quality control
QEHB	Queen Elizabeth Hospital Birmingham
RA	Rheumatoid Arthritis
RBC	Red blood cell
REC	Research ethics committee
RFLNB	Refilin B (gene)
RIN	RNA integrity
RNA	Ribonucleic acid
RNase	Ribonuclease
RNASeq	RNA sequencing
ROS	Reactive oxygen species

RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute (in relation to cell culture media)
RT	Room temperature
SAA	Serum amyloid a
SDF-1	Stromal-derived factor 1
SIGLEC8	Sialic acid-binding Ig-like lectin 8
SIRS	Systemic inflammatory response syndrome
SIRT	Sirtuin (gene)
SLE	Systemic lupus erythematosus
SMPD3	Sphingomyelin phosphodiesterase 3 (gene)
SNE	Stochastic neighbour embedding
SPARC	Secreted protein acidic and cysteine rich (gene)
SYTL4	Synaptotagmin like 4 (gene)
SSC (-A)	Side scatter (-Area)
TIFF	Tag image file format
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TNFRII	Tumour necrosis factor receptor II
UHB	University Hospitals Birmingham
VEGFR1	Vascular endothelial growth factor receptor 1

Forwards

Chapter 1: *The material in Sections 1.1 – 1.5 of this introduction draws heavily on my previously published literature reviews* (Hughes, Sapey and Stockley, 2019) *and* (Hughes, McGettrick and Sapey, 2020b) *and have been adapted for this thesis. Original rights to figures retained upon publication (see Appendix for full author agreement).*

Chapter 4: *Chapter 4 is an expanded version of the data that was published as "The Importance of validating antibody panels: Anti-PD-L1 clone binds the AF700 fluorophore"* (Hughes, McGettrick and Sapey, 2020a).

Chapter 5: *The data relating to healthy volunteers and patients with COPD in this chapter are being submitted for publication at the time of writing this thesis. Some of these data are also published as conference abstracts:* (Hughes *et al.*, 2019; McIver *et al.*, 2019).

Chapter 6: *The data relating to patients with COPD and AECOPD in this chapter are being submitted for publication at the time of writing this thesis. In addition, some of the cell surface expression data collected from neutrophils from patients with AECOPD were done by William McIver, an intercalating medic in our group, under laboratory supervision of myself. In addition, migration data in patients with AECOPD and stable COPD were performed by William McIver and Georgia Walton, previous students within the group, and are for reference only within the introduction to this chapter.*

Some of these data are also published as conference abstracts: (Hughes *et al.*, 2019; McIver *et al.*, 2019).

CHAPTER 1:

INTRODUCTION

Why do some people, with a seemingly poor lifestyle and many of the risk factors for chronic lung disease, never develop lung disease, whilst others do? Is there a gene or a protein that is lost or broken, or a cell that does not work properly? What is it that means some people 'age' well? These questions form the basis of this thesis and seeking these answers is part of my personal motivation for undertaking this project. Whilst it is not possible to provide definitive answers to these questions in three years, this thesis aims to shed some light on what the answers might be by investigating the neutrophil, a white blood cell, and the role it plays in Chronic Obstructive Pulmonary Disease (COPD). This thesis focusses on neutrophil phenotypes based on the surface expression of key proteins linked to neutrophil functions, gene expression, and how these cells migrate towards chemical signals, known as chemotaxis. Within this, the question of "are all patients with COPD the same" is addressed in a novel way – highlighting differences in the underlying biology of the neutrophil in the context of multimorbidity.

1.1 The Neutrophil

The immune system is made of many different cells each performing specific, yet overlapping, functions to aid protection from infection and tissue damage; a system that develops throughout childhood into adulthood (Simon, Hollander and McMichael, 2015). The neutrophil is a white blood cell that is part of the first response to inflammation (Wang, 2018) and is collectively referred to as a granulocyte alongside eosinophils and basophils. Neutrophils form a major component of the immune system and account for over half of all circulating white blood cells in healthy individuals (Shah, Burg and Pillinger, 2017). It was previously thought that neutrophils, whilst providing an important function, were blunt

instruments against pathogens with limited functional heterogeneity (Smith, 1994). Since then, many studies have uncovered heterogeneity that exists within neutrophil populations (Christoffersson and Phillipson, 2018; Rosales, 2018), having implications for roles neutrophils play in both health and disease. There may, for instance, be a particular phenotype of cells that are more pro-inflammatory, less capable of controlling infection or cause more collateral damage – areas that require further exploration.

1.1.1 Neutrophil maturation

Neutrophils mature from haematopoietic stem cells in the bone marrow entering the circulation at a rate of around $1x10^9$ neutrophils/kg every day under homeostatic conditions (Dancey *et al.*, 1976). Upon release into the blood, they are terminally differentiated cells in permanent cell cycle arrest (Klausen *et al.*, 2004). The production of mature granulocytes is tightly controlled by transcription factors (such as CCAAT/enhancer-binding protein) and is influenced by cytokines (Ward *et al.*, 2000). The importance of cytokines, such as granulocyte-colony stimulating factor (G-CSF), have been demonstrated using mice lacking the G-CSF receptor, resulting in a reduction of mature neutrophils (Liu *et al.*, 1996).

Neutrophil development can be divided into three main stages: the pre-mitotic pool or stem-cell pool; the mitotic pool and the post-mitotic pool (Figure 1.1). Each stage can be identified by a combination of surface markers, cyclin-dependant kinase (CDK) expression and granule production (Bainton, Ullyot and Farquhar, 1971; Klausen *et al.*, 2004). Haematopoietic stem cells express high levels of a surface glycoprotein, cluster of differentiation (CD)34 (Civin *et al.*, 1984), that is subsequently lost during maturation (Figure 1.1). The pre-mitotic pool consists of myeloblasts, promyelocytes and myelocytes that are all able to undergo cell division.

Myeloblasts and promyelocytes express CDK6 and are self-replicating, developing into myelocytes that no longer express CDK6 and are limited to the production of neutrophils, monocytes and macrophage depending on the conditions within the bone marrow (Nakamura *et al.*, 1996). Development then enters the post-mitotic phase and marks the beginning of neutrophil-committed cell types as cells begin to lose all CDK expression and become unable to replicate. During this stage, cells continue to acquire the fundamental features of neutrophils, such as granules and a segmented nucleus (Borregaard and Cowland, 1997; Ward *et al.*, 2000; Carvalho *et al.*, 2015). Band cells, with a distinct banded nuclear morphology, are also referred to as immature neutrophils and directly proceed mature neutrophils. The appearance of band cells in the blood is usually only observed during acute inflammation, such as sepsis or systemic inflammatory response syndrome (SIRS) (Mare *et al.*, 2015). Finally, mature neutrophils develop a distinct multi-lobular nucleus, lose expression of C-X-C containing receptor (CXCR)4 and gain expression of an endopeptidase, CD10 (Elghetany *et al.*, 2004; Strydom and Rankin, 2013). These changes in phenotype during maturation enable neutrophils to respond appropriately to infection and tissue damage in the body once they enter the circulation.

1.1.2 Neutrophil release into the circulation

As with maturation, release into the circulation is regulated by cytokines and changes in the external environment. It is thought two main receptors control neutrophil release into the bloodstream: CXCR4 and CXCR2 (Suratt *et al.*, 2004); demonstrated *in vivo* both in mice (Eash *et al.*, 2010) and zebrafish (Isles *et al.*, 2019). Retention of immature neutrophils in the bone marrow is governed primarily by CXCR4, a receptor for C-X-C containing ligand (CXCL)12 (also

known as stromal-derived factor 1, SDF-1), which is highly expressed in haematopoietic cells and not in peripheral blood neutrophils (Patel *et al.*, 2001; Sugiyama *et al.*, 2006). As CXCR4 expression decreases with neutrophil maturation, neutrophils no longer respond to this retention signal (Suratt *et al.*, 2004). Concurrently, an increase in expression of CXCR2, the receptor for multiple cytokines in humans such as interleukin-8 (IL-8; CXCL8), CXCL1 and CXCL2, allows migration of neutrophils into the bloodstream (Martin *et al.*, 2003; Eash *et al.*, 2010). G-CSF also plays an important role in regulating neutrophil release (Semerad *et al.*, 2002) and mice injected with G-CSF show rapid neutrophilia, a response that is absent in CXCR2 knock-out mice (Eash *et al.*, 2010; Köhler *et al.*, 2011), demonstrating the dependence on CXCR2 for neutrophil mobilisation.

Whilst there has been some debate in the literature around the exact half-life of neutrophils in circulation (Pillay *et al.*, 2010, 2011; Tofts *et al.*, 2011; Tak *et al.*, 2013), it is generally accepted that an individual neutrophil persists for around 24-72 hours in the circulation (Dancey *et al.*, 1976; Suratt *et al.*, 2001). This short half-life, therefore, requires a large turnover of neutrophils in health to maintain circulating neutrophil numbers. Precisely what happens to neutrophils as they age either in the circulation, tissue and after responding to an inflammatory stimulus (referred to as senescence) is a topic of debate, however, this process and the associated intricacies within the context of COPD will be discussed later.

1.1.3 Sentinel role

With large numbers of circulating neutrophils in the blood, what is their function in health? Neutrophils are usually one of the first cells recruited to sites of inflammation (Aulakh, 2018) and play an important sentinel role because of the wide range of danger and pattern

recognition receptors they are capable of expressing (Hartl *et al.*, 2008). More recent findings have also revealed that phenotypically distinct neutrophils in mice - with higher expression of major histocompatibility class II (MHC-II) molecules - may also patrol healthy uninflamed tissue (Lok *et al.*, 2019), but the extent to which this happens in human is unknown.

1.2 Overview of Neutrophil Function

Neutrophils play an active role in protecting the body from pathogens in response to inflammatory signals and do this through several key functions (Hughes, Sapey and Stockley, 2019), including phagocytosis (van Kessel, Bestebroer and van Strijp, 2014), reactive oxygen species (ROS) production (El-Benna *et al.*, 2016) and NETosis (Delgado-Rizo *et al.*, 2017). Neutrophils are thought to exist in three states of activity dependant on their environment: (i) quiescent, where cells are in tissue or circulating in homeostasis; (ii) primed, where an initial activation signal is received; and (iii) activated, where effector responses can be deployed (Sapey and Stockley, 2014). A healthy response requires not only rapid activation but accurate migration, controlled effector functions and then the resolution of inflammation and tissue repair (Figure 1.2; Jones *et al.*, 2016). Understanding how neutrophils function in health provides an important lens to identify changes that occur with ageing and chronic disease. To this end, how neutrophils function in the context of healthy development and response to infection, with a focus on the lung, will now be explored.

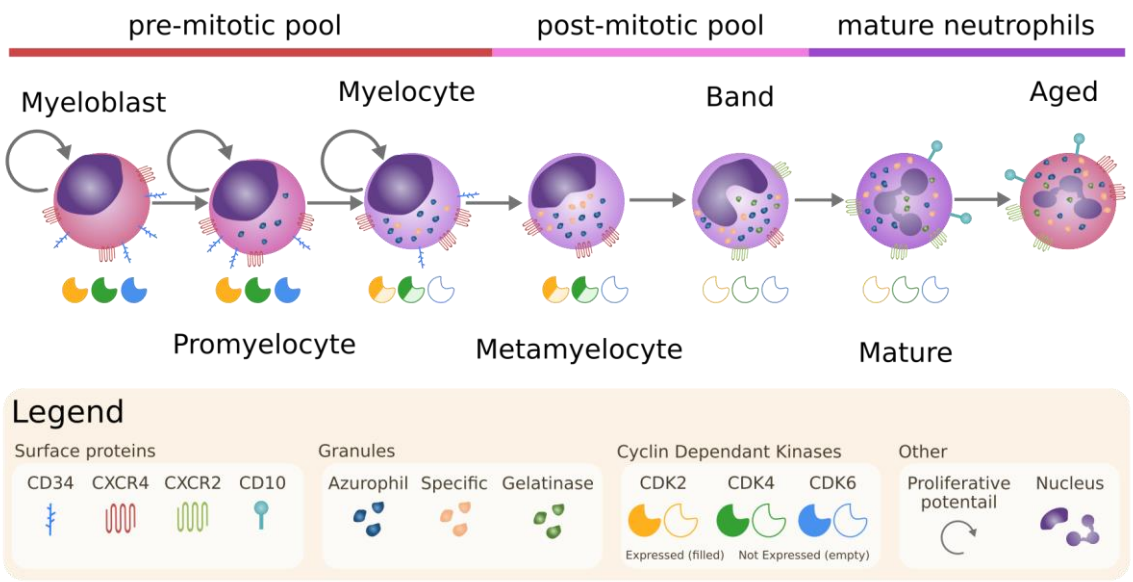

Figure 1.1: Overview of neutrophil maturation

As neutrophils mature, several distinct phenotypic changes occur. In the pre-mitotic pool, **myeloblasts** have high chemokine-motif CXC receptor 4 (CXCR4) and CD34 expression, along with cyclin-dependent kinase (CDK) 2, 4 and 6 expression, allowing proliferation. The **promyelocyte** is the first stage where granule production occurs, beginning with the primary or azurophil granules. **Myelocytes** still maintain replicative potential, but CDK expression is reduced and CDK6 is absent. In this stage, specific granule production also begins. Replicative potential is lost with **metamyelocytes,** despite residual CDK expression. This change is accompanied with the loss of CD34 expression and increased granule production. The next stage of development to **immature neutrophils (band cells)** includes the complete absence of CDK2,4 and 6 expression and production of gelatinase granules. Cells also begin to express CXCR2 and start to lose CXCR4 surface expression ready for release into the circulation. **Mature neutrophils** have high levels of CXCR2 and CD10 with very low CXCR4 expression. As neutrophils age in circulation, it is thought that CXCR4 expression begins to increase again allowing homing back to the bone marrow for clearance.

1.2.1 Activation and transmigration

Recruitment of neutrophils to tissue is a complex process, tightly regulated by both neutrophils and the vascular endothelium. Extensive research has slowly unravelled this process at a molecular level and can be described as a cascade broadly split into three stages: rolling, firm adhesion, and extravasation or transmigration (Ley *et al.*, 2007). Each of these stages has distinct groups of adhesion molecules that govern the interaction of neutrophils (and indeed other leukocytes) with the endothelium, including the crawling of neutrophils both inside and outside the vascular lumen (Voisin and Nourshargh, 2013). In the absence of inflammation, rolling of neutrophils on the endothelium is likely a rare event due to the lack of selectins (also referred to as CD62) or selectin receptors expressed at the endothelium plasma membrane (Zarbock *et al.*, 2011). However, studies in mice have revealed evidence of neutrophil transmigration in the absence of inflammation: fluorescent neutrophils transferred into the blood of recipient mice showed these cells were able to enter the lymph nodes via high-endothelial venules (Lok *et al.*, 2019) and were also found patrolling in the lungs absence of inflammation (Casanova-Acebes *et al.*, 2018).

Upon endothelial activation by inflammatory signals, such as histamine or cytokines, or by direct detection of lipopolysaccharide (LPS; a bacterial product) (Lorenzon *et al.*, 1998), Weibel–Palade bodies (a storage granule) containing intracellular stores of P-selectin (CD62P) fuse with the plasma membrane, leading to rapid expression of CD62P (Berman *et al.*, 1986; McEver *et al.*, 1989)**.** E-selectin (CD62E) is also expressed early on in endothelial activation from *de novo* production following transcriptional activation, demonstrated *in vitro* with human umbilical vein endothelial cells (Kansas, 1996). These two selectins can be bound by P-

selectin glycoprotein ligand-1 (PSGL-1) which, despite its name, can bind all three main selectins (CD62P, E and L) and is expressed on the surface of neutrophils (Zarbock *et al.*, 2011). CD62L is the only selectin expressed by neutrophils (Alon *et al.*, 1996) and is maintained on the plasma membrane (Gómez-Gaviro *et al.*, 2000). Notably, human neutrophils are also able to directly bind CD62E with CD62L (Zöllner *et al.*, 1997), demonstrated *in vitro* in affinity binding assays; highlighting the importance of CD62L in neutrophil recruitment to inflammatory sites. PSGL-1 is also expressed by activated endothelium (Martins *et al.*, 2007) and is, therefore, also capable of binding to CD62L on the surface of the neutrophil. Together, the expression of selectins and PSGL-1 results in many interactions between the vascular endothelium and neutrophils to mediate rolling – a process that occurs under shear stress in the circulation and indeed requires shear stress to function correctly (Marshall *et al.*, 2003).

Once rolling has been instigated, firm adhesion to the endothelium must occur to halt the neutrophil and allow extravasation (Figure 1.2). Integrins function as heterodimers, two of which are considered to be vital for neutrophil firm adhesion: lymphocyte function-associated antigen 1 (LFA-1) formed from integrin alpha L (CD11a)/β2 (CD18) and macrophage-1 antigen (Mac-1) formed from alpha M (CD11b)/CD18 (Ding *et al.*, 1999). Whilst these integrins are constitutively expressed by neutrophils, they are upregulated upon activation (Abdel-Salam and Ebaid, 2014), leading to the use of CD11b as an activation marker in many studies (Orr *et al.*, 2007; Costantini *et al.*, 2010). Whilst expression is important, a conformational change increased the affinity of these integrins for their binding partners, leading to firm adhesion (Anderson, Hotchin and Nash, 2000). Chemokines, such as CXCL8, produced by endothelial cells (Strieter *et al.*, 1989) and fibroblasts (Schröder *et al.*, 1990) are presented on glycosaminoglycans on the endothelial surface (Rot and Von Andrian, 2004). Chemokine

receptors, especially CXCR2 for neutrophils, then detect these chemokines and trigger integrin activation (Luu, Rainger and Nash, 2000; Lefort and Ley, 2012). LFA-1 and Mac-1 then link with the corresponding binding partner, Intercellular Adhesion Molecule-1 (ICAM-1, hereon referred to as CD54), on the endothelium surface that is similarly constitutively expressed at low levels and upregulated upon activation (Yang *et al.*, 2005). The binding of integrins and the detection of cytokines also provide signals that induce important changes intracellularly in the neutrophil, including cytoskeletal rearrangements, that contribute to cell activation and adhesion (Anderson, Hotchin and Nash, 2000), priming of the ROS response (Luo, Carman and Springer, 2007) and may also help prepare the nucleus to deform ready for transmigration (Manley, Keightley and Lieschke, 2018).

At the point of firm adhesion and rolling arrest, two processes can occur: crawling along the vascular lumen or transmigration into the tissue. Intraluminal crawling has been visualised *in vivo* in mice using intravital microscopy, identifying the reliance on LFA-1 for initial adhesion and Mac-1 for efficient crawling (Phillipson *et al.*, 2006). Transmigration (Figure 1.3) predominantly occurs paracellularly (between endothelial cell junctions) (Burns *et al.*, 1997), however, movement through the endothelial cytoplasm, known as transcellular migration (Gane and Stockley, 2012), has also been described *in vitro* with human endothelial cells and isolated neutrophils (Kvietys and Sandig, 2001; Carman and Springer, 2004) and *in vivo* in mice (Woodfin *et al.*, 2011).

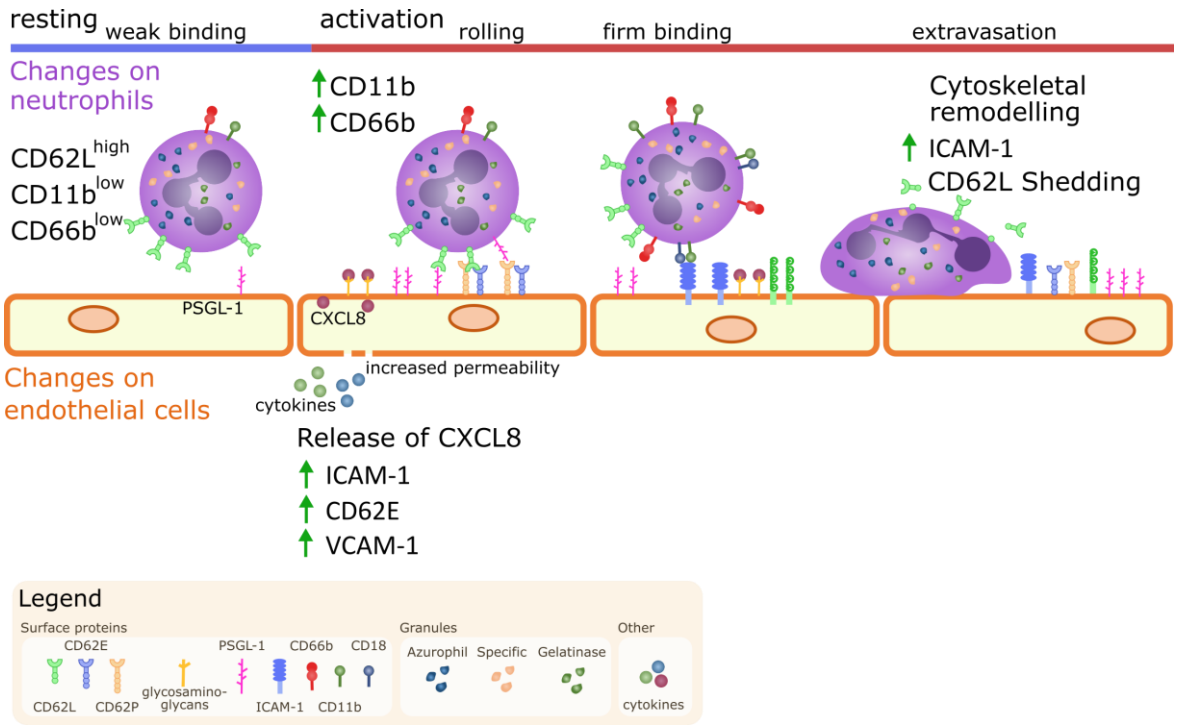

Figure 1.2: Overview of neutrophil adhesion cascade

At rest, circulating neutrophils express high levels of CD62L and low levels of adhesion molecules CD11b and CD66b. They undergo weak binding with the endothelium spontaneously through interactions of CD62L and P-selectin glycoprotein ligand-1 (PGSL-1). Activation in response to damage or chemokine detection causes several changes in both neutrophils and the endothelium. Neutrophils increase their surface expression of adhesion molecules CD11b and CD66b that increase the chance of firm binding. On the endothelium, release of chemokines such as CXC-motif ligand 8 (CXCL8) occurs, along with increased vascular permeability and the increase of adhesion molecules including intercellular cell adhesion molecule-1 (ICAM-1), E-selectin (CD62E) and vascular adhesion molecule-1 (VCAM-1). Tethering between CD62L, CD62E and CD62P slows the rolling of neutrophils prior to firm adhesion. Together these induce cytoskeletal changes in neutrophils in preparation for extravasation. During this process, neutrophils shed CD62L in order to assist migration through the extracellular matrix (ECM).

1.2.2 Migration and chemotaxis

After rolling, firm adhesion to the endothelium, crawling and transmigration, neutrophils are now able to enter the next phase in neutrophil migration: moving along chemotactic gradients within the tissue. It is important to note, neutrophils can also migrate along non-chemical gradients such as hydraulic pressure (Prentice-Mott *et al.*, 2013), but will not be discussed in detail here. CD62L is usually shed upon neutrophil activation and transmigration; blocking this process using mice expressing a mutant form of CD62L caused neutrophils to be retained in the tissue, but did not seem to impede the ability of neutrophils to successfully reach the site of inflammation (Venturi *et al.*, 2003). Neutrophils with different levels of CD62L expression could, therefore, impact on the clearance of activated neutrophils from the tissue.

For neutrophils to successfully migrate to the site of inflammation, they must navigate the complex milieu of different cytokines and damage-associated signals (Pittman and Kubes, 2013) and respond to these signals appropriately. A hierarchy of responses was observed using a competing migration system in agarose gel (Heit *et al.*, 2002), whereby neutrophils responded with lower priority to so-called 'intermediate' signals such as CXCL8 compared with 'end-stage' attractants such as N-Formylmethionyl-leucyl-phenylalanine (fMLP), a bacterial product (Heit *et al.*, 2002). The same hierarchy was observed in a more complex microfluidic system, again with human neutrophils *in vitro* (Kim and Haynes, 2012). Both studies highlight how complex signalling networks allow neutrophils to accurately migrate within the tissue microenvironment. Model organisms such as *Dictyostelium discoideum* that mimic neutrophil chemotaxis (Parent, 2004) also revealed migration is highly sensitive, as these cells could detect directionality in a gradient as shallow as 1% (Song *et al.*, 2006).

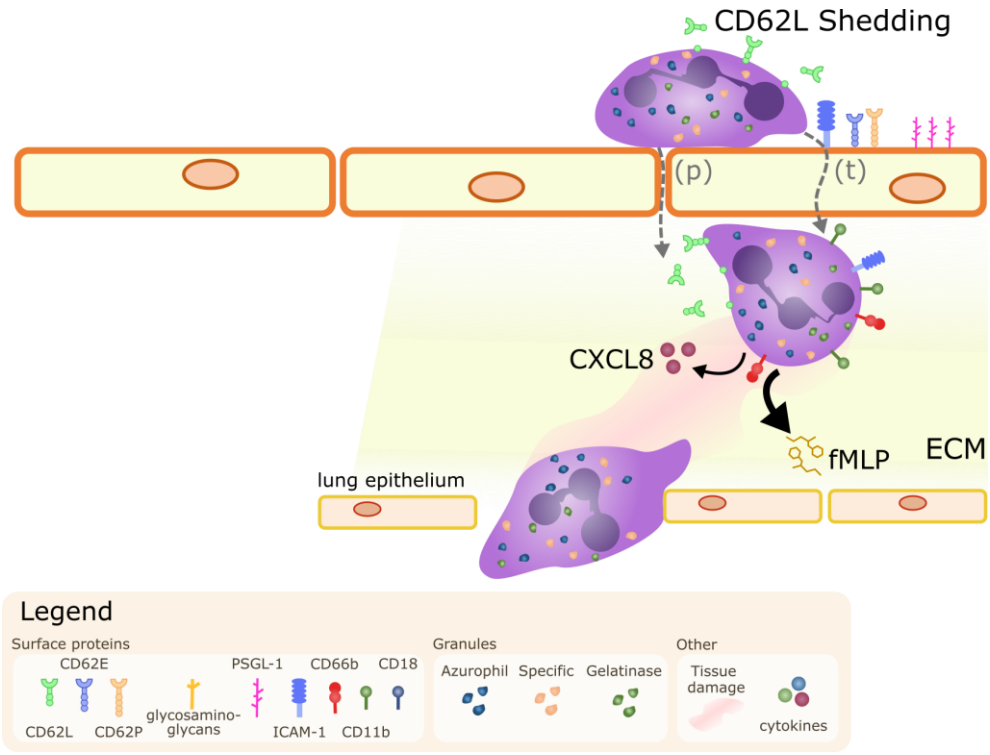

Figure 1.3: Overview of neutrophil chemotaxis

Neutrophils move from the blood stream through the extracellular matrix (ECM) before reaching the lung epithelium. This can occur by two routes, either paracellularly (p) or transcellularly (t). Through this migration, neutrophils cause some tissue damage due to release of enzymes that degrade ECM components. Receptors on the surface of the neutrophil are able to sense chemical gradients from bacterial products such as N-Formylmethionyl-leucyl-phenylalanine (fMLP) or inflammatory cytokines such as CXC-motif Ligand 8 (CXCL8).

Two major signalling pathways govern neutrophil chemotaxis: phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase MAPK (Heit *et al.*, 2002). Responses to intermediate chemoattractants are heavily reliant on the dual action of PI3K at the leading edge and phosphatase and tensin homolog (PTEN) at the lagging edge (Hirsch *et al.*, 2000; Kölsch, Charest and Firtel, 2008) – two enzymes that control the phosphorylation of phosphatidylinositol (Figure 1.4). In contrast, p38 MAPK co-ordinates neutrophil chemotaxis to end-point chemoattractants (Nick *et al.*, 1997). It is the PI3K pathway that has drawn attention in the context of ageing and disease, as there is a gradual decline in accurate migration with age that can be partly rescued by PI3K inhibition (Sapey *et al.*, 2014). Importantly, these neutrophils did not lack the ability to move, termed chemokinesis, but were not able to move as accurately in the model studied. The process of chemokinesis still requires cytoskeletal arrangements and may be activated by extracellular ATP (Isfort *et al.*, 2011). There is, therefore, an important distinction between chemotaxis and chemokinesis: chemotaxis is the ability to sense the environment and apply this motion directionally whilst chemokinesis demonstrates the correct function of cytoskeletal arrangements to migrate (Keller *et al.*, 1978).

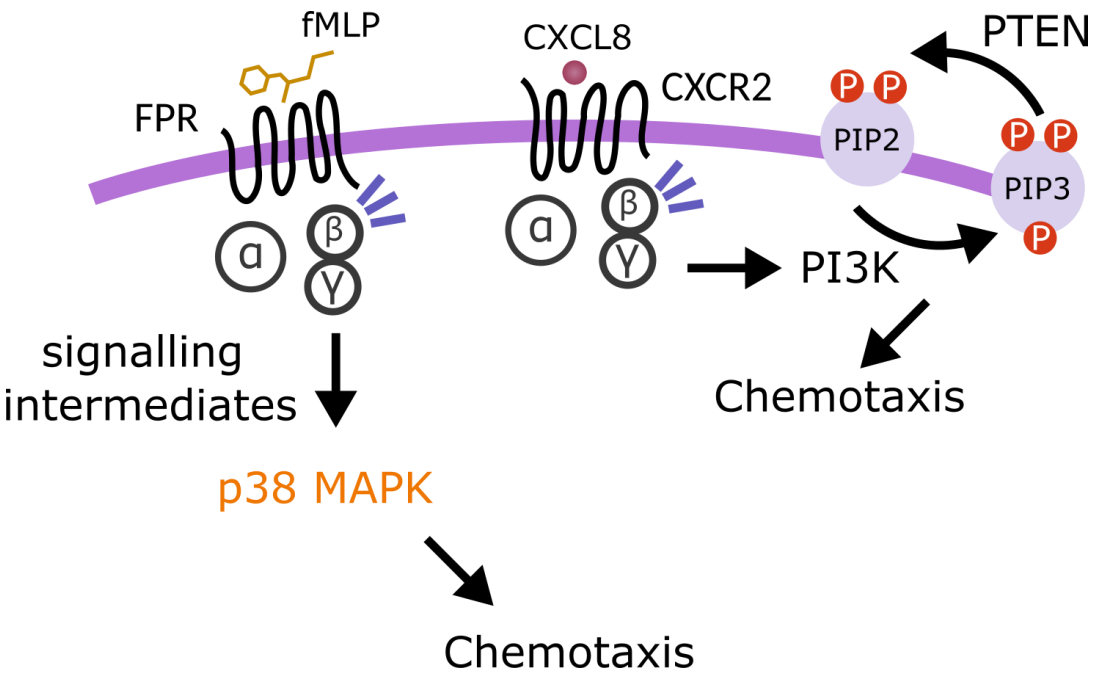

Figure 1.4: Simplified overview of PI3K and MAPK signalling pathways

N-Formylmethionyl-leucyl-phenylalanine (fMLP) binds the formyl peptide receptor (FPR), leading to activation of the G-protein coupled receptor and dissociation of G-protein subunits. Through several intermediaries, p38 mitogen-activated protein kinase is activated and results in activation of chemotaxis pathways. In a similar manner, binding of CXC-motif (CXC) ligand 8 (CXCL8) to the CXC receptors, such as CXCR2, leads to activation of phosphoinositide 3-kinase that catalyses the addition of a phosphate group to phosphatidylinositol (PI) 4,5 bisphosphate (PIP2) forming PI 3,4,5 triphosphate (PIP3). This leads to cell polarization and activation of chemotaxis pathways. PIP3 can be dephosphorylated by phosphatase and tensin homolog (PTEN).

1.2.3 Phagocytosis

Upon reaching the site of injury or infection, neutrophils are capable of destroying micro-organisms by phagocytosis and the production of lethal ROS. These two processes together enable micro-organism specific damage, however, ROS production can also be highly damaging to local tissue and, therefore, needs to be tightly regulated to minimise collateral damage (Dupré-Crochet, Erard and Nüβe, 2013; El-Benna *et al.*, 2016). Bacterial phagocytosis begins with the binding of the bacterial surface by specific cell membrane receptors on the neutrophil, including those for immunoglobulins bound to opsonised bacteria and non-specific receptors such as complement receptors (CR)1 and 3 (van Kessel, Bestebroer and van Strijp, 2014). However, binding of the bacteria is only the first step and major restructuring of the cytoskeleton of the neutrophil is still required to pull the bacteria into the cytoplasm of the neutrophil (Herant, Heinrich and Dembo, 2006). Once bacteria are contained within the phagosome, fusion with lysosomes containing various cytotoxic compounds and Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase – the main producer of ROS (Dupré-Crochet, Erard and Nüβe, 2013) - leads to bacterial killing (Lee, Harrison and Grinstein, 2003).

1.2.4 Neutrophil granules

ROS also have a role outside bacterial killing including mediating the release of proteases held within several types of neutrophil granules (Faurschou and Borregaard, 2003). A variety of proteases are held within neutrophil granules ready for rapid release, allowing neutrophils to degrade proteins extracellularly and have powerful antimicrobial effects. They have distinct protein content (Falloon and Gallin, 1986), primarily determined by protein expression variations during neutrophil development (Figure 1.1) (Faurschou and Borregaard, 2003).

Azurophil (primary) granules are produced earliest in neutrophil development and contain many enzymes that aid bacterial killing such as neutrophil elastase (NE) and proteinase-3 (PR3) (Faurschou and Borregaard, 2003; Segal, 2005; Crisford, Sapey and Stockley, 2018). Both specific (secondary) and gelatinase (tertiary) granules are produced from myelocytes onwards but have very different protein compositions: specific granules contain multiple antimicrobial proteins, whereas gelatinase granules contain enzymes used for digesting the extracellular matrix (ECM) during transmigration (Faurschou and Borregaard, 2003). These proteases aid in bacterial clearance in the lung (Craig *et al.*, 2009) and are usually held in check by anti-proteases to prevent damage to healthy lung tissue (Stockley, 1999; Sallenave, 2015). The unregulated activity of proteinases are key in the pathogenesis of COPD and will be expanded on later.

1.2.5 Neutrophil Extracellular Traps (NETs)

The final mechanism neutrophils can deploy is the release of nuclear and mitochondrial DNA complexed with proteases to trap and destroy bacteria, viruses, fungi and parasites extracellularly (Papayannopoulos, 2017). It is thought that NET formation occurs either in the presence of larger microbes or once phagocytic capacity is overwhelmed (Manfredi *et al.*, 2018). The exact role *in vivo* is still subject to intense investigation as there is currently no consensus on how best to study their formation and function *in vivo* or *in vitro* (Boeltz *et al.*, 2019). It is also emerging that NETs are cytotoxic to the lung epithelium, suggesting that NETs within the lung microenvironment may cause more harm than benefit, leading to the association of NETs with several lung diseases (Twaddell *et al.*, 2019).

1.2.6 Neutrophil senescence and removal from tissues and circulation

The final stage of the neutrophil life cycle is appropriate apoptosis and removal from either the tissue or circulation. It was initially assumed neutrophils that entered tissue were cleared exclusively by efferocytosis – phagocytosis by macrophage – after apoptosis and during inflammation resolution (Savill and Haslett, 1995). Efferocytosis likely remains the primary mechanism of neutrophil clearance following inflammation and is an important mechanism to stimulate an anti-inflammatory response by macrophage and neutralises proteases (Fadok *et al.,* 2001). However, early studies using radiolabelled neutrophils in rats revealed that neutrophils could indeed return to the circulation (Hughes *et al.*, 1997). Nearly 10 years later, this process was visualised in zebrafish (Mathias *et al.*, 2006) and from sterile hepatic injury in mice (Wang *et al.*, 2017), with a clear reverse transmigration phenotype exhibited in human neutrophils involving increased CD54 expression (Buckley *et al.*, 2006). CD54, therefore, has emerged as a useful tool for identifying neutrophils that have undergone reverse transmigration.

As not all neutrophils are removed in the tissue, other mechanisms of neutrophil clearance exist. Senescence, a term originally coined by Hayflick and Moorhead (Hayflick and Moorhead, 1961) to describe the limited replicative capacity of cells in culture as they aged, is a process that cells go through as they age. There are two key mechanisms described that are associated with and identify senescence of individual cells: the change in β-galactosidase activity, termed senescence-associated β-galactosidase (SAβG) activity; and the senescence-associated secretory phenotype (SASP), a unique pattern of proteins secreted by cells undergoing senescence (reviewed in the context of cancer by Coppé *et al.*, 2010). The detection of β-

galactosidase activity in senescent fibroblasts and not quiescent fibroblasts or terminally differentiated keratinocytes (Dimri *et al.*, 1995) led to this biomarker of senescence being generally adopted. *In vitro* studies show an increase in proteins responsible for cell cycle arrest in combination with SAβG in human lung fibroblasts exposed to cigarette smoke (Nyunoya *et al.*, 2006) and from lung fibroblasts from patients with emphysema (Müller *et al.*, 2006), potentially indicating senescence. However, crucially as mature neutrophils do not replicate, neutrophil 'senescence' requires a more complex interpretation and definition (Klausen *et al.*, 2004), yet may still occur as a process allowing clearance from the circulation. Studies in mice showed that neutrophils increased CXCR4 expression – the receptor that is lost during neutrophil maturation – with cellular age, allowing homing of neutrophils back to the bone marrow (Martin *et al.*, 2003; Adrover, Nicolás-Ávila and Hidalgo, 2016). There is also evidence of this process occurring in human *in vitro* as neutrophils cultured for 12-18h showed reduced CXCR2 expression, increased CXCR4 expression and an increase in migration towards the CXCR4 ligand CXCL12 (Yildirim *et al*., 2005). The use of green fluorescent protein (GFP) reporter mice revealed that neutrophils were removed from the circulation at distinct rates in different organs including the spleen, liver, lung, bone marrow and skeletal muscle (Casanova-Acebes *et al.*, 2018). In COPD, where significant numbers of neutrophils are found in the lung, clearance may also occur by migration into the airways and expectoration of sputum (Singh *et al.*, 2010). There appears, therefore, multiple routes for neutrophil clearance that maintain a turnover of functional neutrophils.

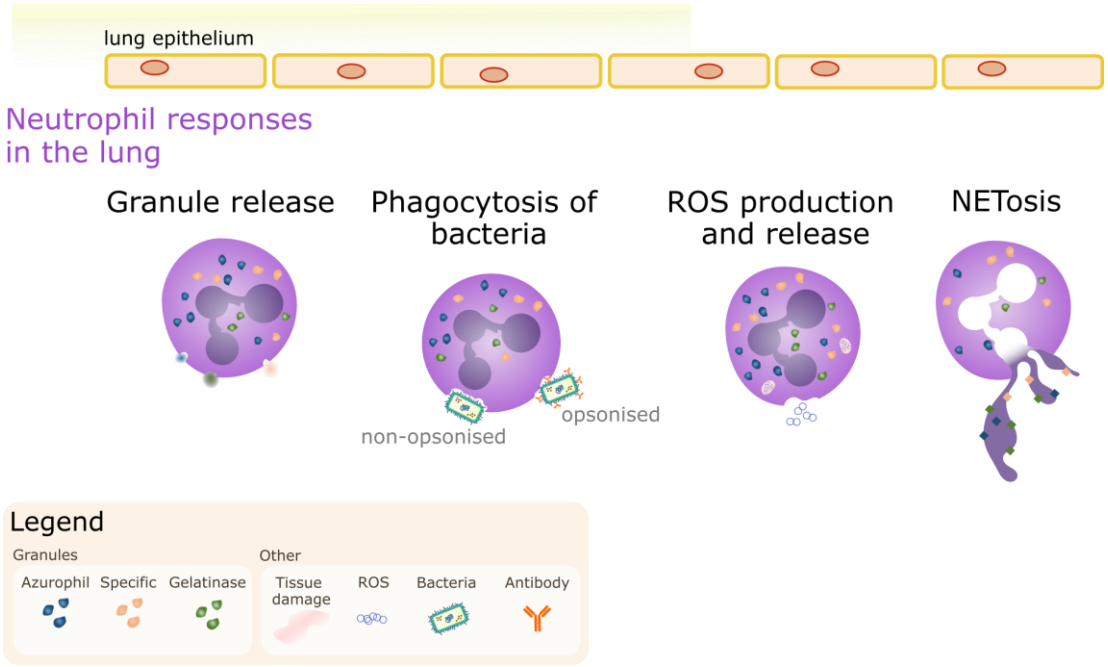

Figure 1.5: Neutrophil functions within the lung

Upon reaching the source of inflammation or infection, neutrophils have multiple effector functions that include the release of proteases from granules; phagocytosis of both opsonised and non-opsonised bacteria; production and release of reactive oxygen species (ROS) and, usually as a last resort, the expelling of nuclear material coated in various proteases called neutrophil extracellular traps (NETs).

1.3 Neutrophil phenotypes in health and ageing

The concept of phenotype within the context of neutrophils must be considered carefully. It does not describe 'subtypes' of neutrophils in the same way that distinct types of T cell exist (Raphael *et al.*, 2015). Cellular phenotype can be described by any observable characteristic, such as gene and protein expression, that contributes to the morphology and function of the cell. The surface expression of receptors, integrins and ligands by neutrophils are involved in many of the functions discussed, such as activation, chemotaxis and transmigration, and can be used to identify the phenotype of the cell. This has a clear advantage – flow cytometry can be used to identify surface expression of these markers to gain an insight into the function of these neutrophils (Borregaard *et al.*, 1994). However, as neutrophils alter the surface expression of proteins to perform different functions, an ongoing debate has questioned if neutrophils are just highly plastic heterogeneous cells, or if they do indeed have distinct roles (Rosales, 2018). Differences in the functional responses of neutrophils, such as migration accuracy and antimicrobial actions can also be measured to add another dimension to the neutrophil phenotype that may not necessarily be gained from measuring surface expression (Muinonen-Martin *et al.*, 2010). Some of the key phenotypes that can be seen in health will now be discussed briefly, including how these may change with healthy ageing and are relevant to disease processes. These phenotypes are also linked to COPD and will be expanded upon later.

1.3.1 Maturity and activation

Multiple changes occur during neutrophil maturation (Section 1.1.1) and studies with peripheral blood neutrophils have shown that expression of CD10 reliably identified mature

neutrophils (Brandau and Hartl, 2017; Marini *et al.*, 2017). In health, all neutrophils within the circulation should be mature and express CD10. However, acute inflammation, such as that caused by invasive surgery (Orr *et al.*, 2005), can cause the release of immature neutrophils into the circulation that do not express CD10 on their surface. Maturity is likely an important feature for correct neutrophil function as immature neutrophils from patients with sepsis carried out phagocytosis and ROS production less efficiently than their mature counterparts (Drifte *et al.*, 2013).

Likewise, neutrophil activation results in the increased expression of CD11b, another adhesion molecule, CD66b, and the shedding of CD62L (see Section 1.2.1) – leading to these proteins being commonly used to identify neutrophil activation (Kinhult *et al.*, 2003; Costantini *et al.*, 2010; Lakschevitz *et al.*, 2016). Whilst neutrophil activation would not be expected in the absence of pathogenic or damage signals, one report suggested an increased proportion of activated neutrophils may be present in the circulation after strenuous muscle exercise (Pizza *et al.*, 1996), although the intensity of exercise and population group may influence this finding, as another study did not find any significant changes in neutrophil activation following downhill running in athletes (Peake *et al.*, 2005).

Increased inflammation observed in an age-dependent manner, so-called inflammaging (Franceschi *et al.*, 2000), may also impact on neutrophil activation. Increased circulating cytokines, including CXCL8, in elderly individuals demonstrated an increase in activation signals for neutrophils (Franceschi and Campisi, 2014). As activation primes neutrophils for a functional response, detecting activation gives an insight to the neutrophil activity in health, damage and disease.

1.3.2 Chemokine sensing and migration

Neutrophils also alter the surface expression of chemokine receptors during maturation and these play a vital role in controlling neutrophil release and responses to inflammatory stimuli. As discussed, CXCR2 and CXCR4 are thought to govern neutrophil release from the bone marrow and return during senescence (Section 1.1.2 and 1.2.6). In the circulation, therefore, neutrophils expressing CXCR2, but lacking CXCR4, indicates mature functional neutrophils (De Filippo and Rankin, 2018). As CXCR2 is a major chemokine sensing receptor on neutrophils, expression is vital for the ability of neutrophils to migrate towards sites of inflammation and links closely to functional neutrophil responses.

Several previous studies have suggested that neutrophil migration is not substantially altered with age (Butcher, Chahel and Lord, 2000). However, whilst the overall speed that neutrophils can travel might not be altered, neutrophils isolated from elderly individuals did not travel as accurately towards chemokines compared with neutrophils from younger individuals (Sapey *et al.*, 2014). Murine models have also revealed aspects of age-related changes in chemotaxis. Fewer neutrophils in older mice (18-20 months, equivalent to humans in their 60s; Dutta and Sengupta, 2016) were recruited *in vivo* into tissue injected with the murine homologue of CXCL8 compared to younger mice (3-4 months, equivalent to humans in their mid-20s; Brubaker *et al.*, 2013). The same trend was observed by the same group using a cutaneous wound model combined with *Staphylococcus aureus* infection (Brubaker *et al.*, 2013). However, a model using 26-28-month-old mice compared to 8-10-month-old mice (equivalent to 70+ years vs 40-50 years in human) found that *in vitro* neutrophils showed no difference in their response to fMLP, but neutrophils from older mice actually responded in greater

numbers to a common respiratory bacterium, *Streptococcus pneumoniae* (Esposito, Poirier and Clark, 1990).

There are, however, important differences between these human and murine studies. Both murine studies looked at numbers of neutrophils responding to stimuli rather than the accuracy of the response. In addition, aged mice do not directly represent older humans, as environmental exposure to infection and harm is usually tightly controlled in murine models. Nevertheless, together these data suggest neutrophils may respond in greater numbers and less accurately with increased age.

1.3.3 Senescence, apoptosis and cell death

It is important to understand that two distinct ageing processes exist: the ageing of the host and the ageing of an individual cell. When studied *in vitro*, human neutrophils upregulated CXCR4 during the ageing process, detected in both mRNA and surface expression (Weisel *et al.*, 2009). CD62L expression is also reported to be reduced as CXCR4 levels increase on the surface of ageing neutrophils in mice (Casanova-Acebes *et al.*, 2013). Further to these, a murine study reported that aged neutrophils, determined by detection of neutrophil precursors labelled 48 hours before experimental procedures, responded faster to intraperitoneally injected LPS than non-aged neutrophils (Uhl *et al.*, 2016).

However, homing of neutrophils expressing CXCR4 back to the bone marrow has only really been shown in mice (Martin *et al.*, 2003; De Filippo and Rankin, 2018) with data on the process in humans lacking and reliant on artificial *in vitro* ageing methodology (Nagase *et al.*, 2002). Caution must also be taken in translating murine studies on CXCR4 into human, as whilst functional CXCR4 (that responds to CXCL12) can be expressed on human neutrophils, CXCR4

was not involved in sequestering neutrophils in the human lung in contrast to murine studies (Pillay *et al.*, 2018). Whilst detection of CXCR4 neutrophils in the circulation could indicate an increase in senescent neutrophils, it is necessary to place this in the context of a functional alteration if it is to be of use clinically.

Apoptosis is also an important process to allow the clearance of neutrophils by efferocytosis and facilitate the rapid turnover of neutrophil number in health (McCracken and Allen, 2014). Importantly, neutrophil apoptosis is associated with functional decline and, whilst a completely healthy process, it is vital apoptotic neutrophils are removed to maintain homeostasis of functional neutrophils in the body (Whyte *et al.*, 1993; Kebir and Filep, 2013). There is not a wealth of evidence about how host ageing impacts on neutrophil apoptosis, maybe in part due to the lack of changes observed in published data. Neutrophils from older individuals showed no difference in apoptosis over a 24 hour period *in vitro* compared to younger individuals, nor was it found that expression of receptors allowing cells to respond to pro-apoptotic signals was changed in older individuals (Tortorella *et al.*, 1998). However, in response to inflammatory challenge, subtle changes were noted as apoptosis in neutrophils from older individuals was not prevented to the same degree when exposed to common stimulants such as LPS (Fülöp *et al.*, 1997). Determining levels of cellular apoptosis may give an insight into the function of neutrophils and the rate of clearance from the body and provide a disease-related signature.

Understanding the normal behaviour of neutrophils and the variety of functions and phenotypes that neutrophils have is vital to identify any aberrant behaviour within disease settings. Neutrophils do, therefore, have multiple phenotypes that alter depending on

developmental stage and contribute to their function, and how they may be altered in the context of disease will be expanded upon later.

1.4 Chronic Obstructive Pulmonary Disease

One of the major chronic respiratory diseases is Chronic Obstructive Pulmonary Disease (COPD), defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as "a common, preventable and treatable disease that is characterised by persistent respiratory symptoms and airflow limitation" (GOLD, 2021). COPD tends to present later in life and is commonly described as an age-related disease (Cosio, Cazzuffi and Saetta, 2014), with the exception of a specific genetic deficiency in alpha-1 anti-trypsin (AAT) that predisposes to the development of COPD at a younger age (R. A. Stockley, 2015). COPD ranks in the top five causes of mortality worldwide (Lozano *et al.*, 2012) and remains the most prevalent chronic respiratory disease worldwide (Soriano *et al.*, 2020), thus presenting a substantial socioeconomic burden on society.

1.4.1 Diagnosis

The clinical diagnosis of COPD is based on the presence of symptoms (e.g. shortness of breath or a cough) and fixed airflow obstruction, determined by the ratio of the post-bronchodilator forced expiratory volume in 1 second (FEV_1) to the forced vital capacity (FVC) being less than 0.7 (GOLD, 2021). Many patients with these symptoms and airflow obstruction will also present with emphysema - the destruction of the alveoli leading to a reduction of oxygen transfer into the bloodstream (Turino, 2006) - small airways abnormalities and mucus hypersecretion (Barnes, Shapiro and Pauwels, 2003). However, these features are interlinked

with the underlying biology (O'Donnell *et al.*, 2004) and provide a landscape for a highly heterogeneous disease that will be explored in more detail in the following sections.

1.4.2 Associated risk factors and susceptibility

Whilst the exact pathogenesis of COPD is still under investigation, it is likely to be multifactorial and involve both inflammation and environmental exposures (MacNee, 2005). In western countries, the largest risk factor for developing COPD remains smoking and exposure to cigarette smoke (CS) plays a key role in introducing ROS into the lung, causing damage and an inflammatory response known to be implicated in the development of COPD (McGuinness and Sapey, 2017). However, even if patients do not continue to smoke, patients with COPD have persistent lung inflammation (Barnes, 2008a) and a large influx of neutrophils (Hogg, 2004) and monocytes (Traves *et al.*, 2004) into the lung tissue. It is also true that not all smokers develop COPD that may, in part, be explained by differential cellular responses to smoke exposure in healthy individuals (Franciosi *et al.*, 2014) leading to different levels of damage caused in the lungs by smoking (Cosio Piqueras and Cosio, 2001).

A clear genetic-associated risk factor comes from people with alpha-1 antitrypsin deficiency (AATD), where mutations in the AAT gene prevent the release, or complete absence, of this key anti-proteinase (Stockley, 2016). Several genome-wide association studies have been performed in large cohorts of both patients and generally people with a substantial smoking history (Pillai *et al.*, 2009; Cho *et al.*, 2010; W. Chen *et al.*, 2015), but results reveal very few gene associations with variation based on factors such as the ethnicity of population group used. Several other factors are also in play in determining if individuals will develop COPD. The role of immune cells, such as neutrophils and macrophage, as well as epithelial cells, are

known to play a role in established COPD (Barnes, Shapiro and Pauwels, 2003), so dysfunction in any of these cell types may make the development of COPD more likely. There is also increasing evidence that healthy early childhood lung development plays a crucial role in preventing lung disease in later life (Stocks and Sonnappa, 2013). Changes in the lung microbiome, including colonisation with pathogenic bacteria (Dickson *et al.*, 2016), may play a role in determining the extent of lung damage caused in COPD (Wang *et al.*, 2019).

1.4.3 Disease severity and clinical phenotypes

The categorisation of COPD is routinely used to inform what therapies are offered to patients (Montuschi, 2006), but also has important considerations when studying COPD (Chung and Adcock, 2008). Previously, GOLD COPD stages (I-IV) were used to classify patients based on the FEV_1 percent predicted (GOLD, 2017). However, as this approach only reflects an aspect of the disease and more recent global guidelines now include an assessment of the patient's exacerbation history and a symptom score to determine a grade and group (Table 1.1). However, in this thesis, as it was not always possible to conduct or access results from an exacerbation history and symptom score. Therefore, only GOLD Grades, equivalent to the National Institute for Health and Care Excellence (NICE) stage (NICE, 2019) was used to classify COPD severity (Table 1.1).

Smoking also has a profound impact on both disease pathology and immune cell function, and is an important factor to consider when investigating COPD. Smoking history is routinely recorded as a pack-year, where one pack-year is equivalent to smoking 20 cigarettes (one pack) a day for a year (NIH, 2020). This measure allows a comparison of total smoking

exposure, as a person who has smoked 10 cigarettes (half a pack) a day for 10 years will have the same history as someone who has smoked 20 cigarettes for 5 years.

In addition to these measures of severity, it is now well established that there are many clinical phenotypes of COPD formed by differing contributions of the underlying pathology that have been referred to as 'treatable traits' (Agusti *et al.*, 2016). These traits include pulmonary features of COPD such as emphysema and airway smooth muscle contraction, but also other factors such as the presence of obesity, persistent systemic inflammation and lifestyle factors (Agusti *et al.*, 2016). Together, these traits lead to other phenotypes such as frequent exacerbators (patients that routinely have more than 2 exacerbations a year), COPD-asthma overlap where patients have some reversible airflow obstruction (Miravitlles, Calle and Soler-Cataluña, 2012) and those who see a rapid decline in FEV_1 (Lange *et al.*, 2016). Patients with COPD that do not experience exacerbations and have fairly stable lung function are likely to have very different underlying cellular mechanisms to those experiencing frequent exacerbations and rapid decline in FEV_1. Whilst it is not always possible to ascertain these aspects of COPD for every patient in a study, it is important to understand heterogeneity within COPD – an aspect that directly links to the clinical care of patients and has potentially limited therapeutic development.

1.4.4 Current therapeutics

Unfortunately, current therapies for COPD are limited to elevating symptoms and slowing disease progression rather than reversing or repairing lung damage. A review in 2005 highlighted several therapies currently available for COPD, including smoking cessation, the use of several inhaled drugs such as long-acting β2-agonists that enable bronchodilation,

inhaled steroids and pulmonary rehabilitation (Barnes and Stockley, 2005) – all of which primarily elevate symptoms to varying degrees of success. Limited progress has been made since, with therapeutics aiming to tackle the underlying biology of COPD, even if showing promise *in vitro* or in animal models, showing limited benefit in patients. Examples include anti-TNFα therapies showing no benefit (Rennard *et al.*, 2007) and antioxidant therapeutics aiming to increase antioxidant genes that worked in murine models (Rangasamy *et al.*, 2004) showed no effect in patient groups (Wise *et al.*, 2016).

Even repeated promising data showing CXCR2 (and in some cases both CXCR2 and CXCR1) inhibition reduced neutrophil influx to the lungs and reduced inflammation in animal models (Lomas-Neira *et al.*, 2004; Gordon, 2005; Stevenson *et al.*, 2005; Mattos *et al.*, 2020) have failed to provide benefits to patients with acceptable safety profiles (Rennard *et al.*, 2015; Lazaar *et al.*, 2020). These have created a somewhat graveyard for failed COPD therapies, potentially caused by lack of understanding of the underlying biology or the heterogeneity of COPD where benefits may be seen on a more discrete cohort of patients. Repurposing existing therapeutics to help reduce the lung decline seen in COPD have showed some positive results (Walton *et al.*, 2016) but are yet to be proven in larger-scale clinical trials.

Together, these provide a strong need to further understand COPD to guide and improve the development of effective therapies for these patients.

1.4.5 Acute exacerbations of COPD

As well as heterogeneity within stable disease, exacerbations represent another element of COPD. At the simplest level, an acute exacerbation of COPD (AECOPD) is defined as “an acute worsening of respiratory symptoms that results in additional therapy” (GOLD, 2021).

However, it has been highlighted that no biomarkers exist to determine a COPD-specific exacerbation and that heterogeneity in both clinical aspects and underlying biological processes exist in AECOPD (Celli, 2017; Sapey *et al.*, 2019). Studies of cohorts of hospitalised (or previously hospitalised) patients with COPD have identified Serum Amyloid A (SAA; Bozinovski *et al.*, 2008) and C-reactive protein (CRP; Hurst, Donaldson, *et al.*, 2006) to be potential markers of COPD exacerbations, however, these were not able to predict the severity of the exacerbation. In addition, there is likely a large selection bias in both these and many other studies (including this thesis) investigating AECOPD, as patients presenting at hospital are likely to be those experiencing the most severe AECOPD – balanced by the fact these patients are the ones most at risk from the most adverse health outcomes and require the most help (Antoniu and Carone, 2013).

There is often a strong inflammatory component to AECOPD (Sapey and Stockley, 2006) and patients with AECOPD were found to have higher levels of CXCL8, IL-6 and CRP in serum, sputum and nasal washes (Hurst, Perera, *et al.*, 2006) compared with those with stable COPD – indicating systemic inflammation as well as localised airway inflammation. Patients with AECOPD are also far more likely to have cardiovascular complications, including ischemic heart disease (Malo de Molina *et al.*, 2018), pulmonary hypertension, and heart failure (El-Shabrawy and Eldamanhory, 2017), leading to a significant healthcare burden, morbidity and mortality. Heterogeneity also exists within exacerbations and a key study following patients over a year measuring biomarkers in both sputum and serum identified four distinct phenotypes of exacerbation (Bafadhel *et al.*, 2011) – these are summarised in Table 1.2.

Table 1.1: GOLD assessment of patients with COPD based on spirometry, exacerbation history and symptoms

GOLD Grade	**FEV_1 % predicted**	**NICE Stage**	
1	≥80	1 – Mild	
2	50-79	2 – Moderate	
3	30-49	3 – Severe	
4	< 30	4 – Very Severe	
GOLD Group	**Exacerbations (past year)**	**mMRC score**	**CAT score**
A	0 or 1 (not hospitalised)	0-1	<10
B	0 or 1 (not hospitalised)	≥2	≥10
C	≥2 or ≥1 hospitalised	0-1	<10
D	≥2 or ≥1 hospitalised	≥2	≥10

Legend: Global Initiative for Chronic Obstructive Lung Disease (GOLD) grade and National Institute for Health and Care Excellence (NICE) stage assigned based on the percent predicted forced expiratory volume in one second (FEV_1). GOLD group assigned based on both the number and type of exacerbations in the previous year followed by either the modified Medical Research Council (mMRC) score or COPD Assessment Test (CAT) score.

Table 1.2: Phenotypes of AECOPD

Phenotype	Features
Bacterial-dominant	Associated with positive bacterial pathogen sputum cultures, raised neutrophil counts in both blood and sputum and high levels of sputum IL-1β and TNFRII
Viral-dominant	Associated with viral infection (commonly rhinovirus) and serum CXCL10. Also showed the greatest decline in FEV_1
Eosinophilic-dominant	Associated with high numbers of blood eosinophils
Pauciinflammatory	Generally low levels of sputum mediators with no clear dominant cause, with a mixture of the other three phenotypes

Legend: A summary of exacerbation phenotypes identified using cluster analysis of multiple sputum and serum biomarkers (Bafadhel *et al.*, 2011).

IL-1β, interleukin 1-beta; TNFRII, tumour necrosis factor receptor II; CXCL10, C-X-C containing ligand 10; FEV_1, forced expiratory volume in 1 second.

1.4.6 Multimorbidity

Several other chronic diseases occur in patients with COPD more commonly than the general population, including type-2 diabetes mellitus (T2D) and cardiovascular diseases (CVD); even after accounting for common risk factors such as smoking and poor diet (Lee *et al.*, 2013; Mullerova *et al.*, 2013; Martín-Timón *et al.*, 2014). The presence of multiple chronic diseases in a single person is referred to as multimorbidity and has a significant impact on mortality and healthcare costs (Barnett *et al.*, 2012; Sambamoorthi, Tan and Deb, 2015), affecting around 30% of individuals in the UK, rising to over 50% in over 65 year-olds (Cassell *et al.*, 2018). Not only does this place importance in understanding multimorbidity, but also on the potential of unified underlying mechanisms linking COPD, T2D and CVD (Hughes, McGettrick and Sapey, 2020b).

There is great interest in understanding why multi-morbidities cluster in individuals (Ording and Sørensen, 2013) and there are several common threads that might link these diseases together, including inflammation, age and changes in neutrophil function (Hughes, McGettrick and Sapey, 2020b). It is increasingly recognised that inflammation is both caused by and drives many chronic non-communicable diseases. Furthermore, there are increased levels of acute-phase proteins and inflammatory cytokines in the bloodstream of multi-morbid patients compared with those with a single disease or healthy volunteers (Martín-Timón *et al.*, 2014; Wei *et al.*, 2015; Min *et al.*, 2017), suggesting a potentially cumulative inflammatory effect with increasing numbers of diseases.

Atherosclerosis (AS) is often a precursor to cardiovascular disease and the use of atherosclerosis-prone apolipoprotein E-deficient ($ApoE^{-/-}$) mice has provided evidence to

support the inflammatory basis of AS (Demer, 2002; Lo Sasso *et al.*, 2016). Equally, T2D has an important inflammatory component. Raised levels of soluble TNF-α in patients with T2D has been linked with insulin resistance (Nilsson *et al.*, 1998; Moller, 2000) as well as being a potential mediator of murine pancreatic β-cell destruction in insulin-dependent diabetes mellitus *in vitro* (Stephens *et al.*, 1999).

It is also important to recognise that multimorbidity itself may be a source of heterogeneity among patients with COPD. Heterogeneity within COPD has implications for both the clinical management of the disease (Barrecheguren and Miravitlles, 2016) and research that underlies clinical trials to provide treatment options for these patients as it is likely one size does not fit all (Sidhaye, Nishida and Martinez, 2018). Whilst many trials separate patients with COPD based on clinical characteristics such as FEV_1 and annual exacerbation frequency (De Soyza and Calverley, 2015), multimorbidity and more subtle underlying neutrophil biology may be equally as important.

1.4.7 Inflammatory mechanisms in COPD

Several inflammatory processes are known to be implicated in COPD, contributing to disease progression and pathogenesis (King, 2015). Macrophages present in the lung are markedly increased in COPD and they also associate with regions of emphysema (Retamales *et al.*, 2001). Macrophages are also capable of producing cytokines that recruit more immune cells to the lung and perpetuate the inflammatory response (Barnes, Shapiro and Pauwels, 2003). In addition, defective clearance of bacteria and apoptotic neutrophils by macrophages in COPD may further shift the balance to increased damage in the lungs (Belchamber *et al.*, 2019). Other cell types are also linked with COPD such as T cells, as the number of T cells in

the lung and circulation increases in COPD and has been correlated with increased alveolar destruction (Majo, Ghezzo and Cosio, 2001).

Neutrophils are the most abundant immune cells found in the lungs of patients diagnosed with COPD, and elevated levels of CXCL8, a potent neutrophil chemoattractant, in the lungs of patients with COPD perpetuates this response (Hollander *et al.*, 2007). The chronic presence of neutrophils is also likely to be direct a contributor to the symptoms seen in COPD such as increased mucus production and tissue destruction through the release of neutrophil elastase (Nadel, 2000). The role of neutrophils, and their potential dysfunction, in the pathogenesis of COPD will now be discussed in more detail.

1.5 The neutrophil in COPD and multimorbidity

Multiple facets of neutrophil biology have been linked to COPD (Jasper *et al.*, 2019) and it is well-established neutrophils are present in large numbers in the lungs of patients with COPD (Tregay *et al.*, 2019). A decline in lung function (Stănescu *et al.*, 1996), development of emphysema (Shapiro *et al.*, 2003) the severity of COPD (Singh *et al.*, 2010) and association of small airways disease and exacerbation frequency (Day *et al.*, 2020) have all been linked to neutrophil products or numbers, cementing the role for neutrophils in the pathogenesis of COPD. The persistence of neutrophils in the lung of patients with COPD also makes them prime candidates for causing collateral damage in the lung (Stockley, 2002). However, attempts to block neutrophil recruitment to the lungs using CXCR2 antagonists did not universally lead to clinical benefits to patients with COPD and led to increased susceptibility to infection (Rennard *et al.*, 2015) – indicating it is not simply about the number, but also the function of neutrophils in COPD.

1.5.1 Neutrophil proteinases

Neutrophils in COPD are thought to disrupt the balance between proteinases and anti-proteinases in the lung. This proteinase/anti-proteinase imbalance has long been suspected (Stockley, 1999) and is especially important in AATD where levels of natural anti-proteinases are lower. Neutrophils, when active, release large quantities of proteases (Shamamian *et al.*, 2001) that can also be bound on the cell surface of neutrophils (Csernok *et al.*, 1994). Some of the key proteinases, collectively known as neutrophil serine proteinases, including neutrophil elastase (NE) and proteinase 3 (PR3), and the role they play in lung damage are summarised in Table 1.3. Local injection of NE and PR3 in hamsters, or mice deficient in AAT, resulted in the development of emphysema (Kao *et al.*, 1988; Borel *et al.*, 2018) – supporting the role of the proteinase/anti-proteinase balance in maintaining healthy lung tissue. NE activity in both human and murine models has also been linked with insulin resistance and an NE inhibitor (GW311616A) reversed insulin resistance and body weight gain in mice (Mansuy-Aubert *et al.*, 2013), indicating roles for neutrophil proteinases in the development of T2D.

1.5.2 The role of systemic inflammation and neutrophil retention by the lung

Systemic inflammation - the presence of inflammatory markers within the blood that can lead to inflammation in multiple organs (Jaffer, Wade and Gourlay, 2010) - has been linked to COPD in various studies (Garcia-Rio *et al.*, 2010; Singh *et al.*, 2018) and provides a potential link between COPD and other chronic inflammatory conditions (Hughes, McGettrick and Sapey, 2020b). A study looking at both smokers and ex-smokers with and without COPD found current smokers had increased levels of systemic TNF-α compared to ex-smokers with COPD (Tanni *et al.*, 2010). Higher levels of IL-6 and CRP were found systemically in patients with

COPD compared to both smokers and non-smokers without COPD, suggesting systemic inflammation is not only due to smoking status (Tanni *et al.*, 2010). However, another study of 50 patients with COPD observed poor correlations between systemic levels of CXCL8 and IL-6 disease severity in ex-smokers. It is, therefore, complex to separate the cause and effect relationship between systemic inflammation and smoking in the context of chronic inflammation observed in COPD.

The presence of systemic inflammatory cytokines, such as IL-1β and CXCL8, has several implications for neutrophils in patients with COPD. Some studies have suggested that neutrophil retention in the lung capillaries is increased with systemic inflammation – a phenomenon that could partly explain the influx of neutrophils in patients with COPD (Wozniak *et al.*, 1993; Summers *et al.*, 2014; Tregay *et al.*, 2019). Neutrophils are required to deform to pass through capillaries, including the lung capillaries (Doerschuk *et al.*, 1993), but despite this, *in vivo* studies whereby healthy volunteers were infused with autologous radiolabelled neutrophils have shown that in health, human neutrophils pass through the pulmonary capillaries only marginally slower than red blood cells (Summers *et al.*, 2014). Studies using radiolabelled neutrophils injected into patients with COPD showed increased retention of neutrophils in the lung, similar to that of healthy volunteers that inhaled LPS as a priming agent (Tregay *et al.*, 2019). The *ex vivo* priming of radio-labelled neutrophils with either granulocyte-macrophage colony-stimulating factor (GM-CSF) or platelet-activating factor (PAF) before injection into the right jugular vein of healthy volunteers demonstrated the retention of neutrophils by the lung was steadily lost, recorded by external imaging of the thorax, suggesting these cells are either cleared from the body or are deprimed (Summers *et al.*, 2014). Of note, the retention time in the lung vasculature was dependant on the priming

agent, as GM-CSF had a greater effect on neutrophil retention compared with PAF (Summers *et al.*, 2014). Forced mechanical deformation *ex vivo* of neutrophils has been shown to deprime neutrophils (Ekpenyong *et al.*, 2017). It has been suggested that the lungs may form an important site where neutrophils, primed through exposure to circulating inflammatory mediators, may return to the quiescent state because of mechanical modulation in the tight pulmonary capillaries (Ekpenyong *et al.*, 2017). The narrow pulmonary vasculature might therefore have important functions for immunomodulation in systemic inflammation (Summers *et al.*, 2014).

In patients with COPD, increased systemic inflammation in combination with inflammatory signals in the lung may explain the sustained influx in neutrophils. It has also been shown that persistent systemic inflammation leaves patients with COPD at a higher risk of developing CVD and diabetes (Agusti *et al.*, 2012). Furthermore, there appears to be an additive effect of multiple chronic diseases in a single patient: the ECLIPSE study with over 2000 patients with COPD demonstrated higher circulating IL-6 and CXCL8 levels in patients with both COPD and heart disease; higher CRP levels in patients with COPD and diabetes and both were associated with poorer clinical outcomes (Miller *et al.*, 2013).

Table 1.3: Overview of key neutrophil proteinases and their main inhibitors along with their potential role in lung damage

Proteinase	Anti-proteinase	Role in lung damage	Reference
Neutrophil elastase	α1-antitrypsin	Degradation of elastin and development of emphysema. Also evidence it can cause increased activity of macrophage-derived proteinases.	(Owen, 2008; Guyot *et al.*, 2014)
Proteinase 3	α1-antitrypsin Secretory leukocyte protease inhibitor	Linked with development of emphysema, impaired mucus clearance and amplifying inflammation by attenuating cytokine activation states.	(Owen, 2008; Guyot *et al.*, 2014; Crisford, Sapey and Stockley, 2018)
Cathepsins	Cystatins	Contributes to elastin degradation and alveolar enlargement, but also can cause epithelial apoptosis.	(Owen, 2008; Guyot *et al.*, 2014; Brown *et al.*, 2020)

Legend: A brief overview of proteinases associated with lung damage in COPD, including their major inhibitors.

1.5.3 Defective chemotaxis

Accurate chemotaxis is an important attribute of neutrophil function, enabling neutrophils to reach a site of infection or injury (Iglesias and Devreotes, 2008; White *et al.*, 2018). Multiple factors can affect neutrophil chemotaxis, including the type of chemoattractant and the age of the neutrophil (McDonald *et al.*, 2010; Uhl *et al.*, 2016). CS, whilst altering the expression of adhesion molecules by endothelial cells (Scott and Palmer, 2003), has also been shown to alter migratory dynamics as neutrophils exposed to CS extract (CSE) showed a reduction in the speed and accuracy of chemotaxis (White *et al.*, 2018). However, neutrophils are still present in large numbers within the lungs of smokers (Higuchi *et al.*, 2016), demonstrating neutrophils are still recruited to the lungs, even if they move through the tissue less accurately. In addition, isolated neutrophils exposed to CSE also produced more CXCL8, further enhancing recruitment to the lungs (Mortaz *et al.*, 2010).

Neutrophil chemotaxis is also impaired in patients with COPD. Isolated blood neutrophils from patients with COPD demonstrated a significant reduction in accuracy *in vitro* to both individual chemoattractants, such as fMLP and CXCL8 (Sapey *et al.*, 2011), as well as sputum from patients with COPD (Yoshikawa *et al.*, 2007). These studies expanded on earlier work suggesting isolated neutrophils from patients with emphysema digested larger amounts of fibronectin, a component of the ECM (Burnett *et al.*, 1987). Together these studies build a picture of altered function in COPD: active neutrophils that are responsive to chemoattractants but move less accurately in the tissue leading to enhanced tissue destruction.

Both a reduction in accuracy and a reduced number of pseudopodia being extruded from migrating COPD neutrophils have been described *in vitro* (Sapey *et al.*, 2011). In this study, PI3K inhibitors were able to restore migratory accuracy of these cells to that of neutrophils from healthy volunteers, demonstrating a role for PI3K in controlling neutrophil chemotaxis (Sapey *et al.*, 2011). A reduction in neutrophil migratory accuracy has also been described using the same experimental technique in peripheral neutrophils taken from patients with severe acute infections such as pneumonia in aged but not younger adults (Sapey *et al.*, 2017), suggesting COPD changes might reflect, in part, an accelerated ageing phenotype.

In COPD, the impact of smoking, lung damage and age must all be considered. There is a difference between an aged cell and cells taken from an aged host (who will contain neutrophils of different ages). This concept is particularly important in the context of accelerated ageing and understanding the loss of function that occurs with cellular senescence and with advancing age of the host.

1.5.4 Accelerated ageing as a theory of COPD

There are many parallels between age and chronic disease, giving rise to the theme of COPD being a disease of accelerated ageing that has been reviewed multiple times in the literature (Ito and Barnes, 2009; MacNee, 2009; Mercado, Ito and Barnes, 2015; Hughes, McGettrick and Sapey, 2020b). One aspect is based on the hallmarks of ageing, including areas such as senescence, genetic instability and alteration, mitochondrial dysfunction and the loss of proteostasis (López-Otín *et al.*, 2013) that may be accelerated in COPD. Features such as DNA instability (Samara *et al.*, 2010; Palazzo *et al.*, 2011; Cervelli *et al.*, 2012), epigenetic modifications (Barnes, 2008b; Kong *et al.*, 2009; Noh *et al.*, 2009) and the loss of proteostasis

and autophagy (Mukherjee *et al.*, 2015; Bodas *et al.*, 2017; Hofmann, Katus and Doroudgar, 2019) have all been linked with COPD, CVD and T2D.

In addition, lung function declines even in healthy ageing (Janssens, Pache and Nicod, 1999) and some people will develop emphysema in old age (Lamb, Gillooly and Farrow, 1991), further leading to the concept that COPD may represent an acceleration of the processes that occur with ageing – although this observation alone does not identify if the mechanisms underlying the ageing lung are similar.

Molecular mechanisms such as expression of sirtuins (SIRT), so-called anti-ageing histone-deacetylases (Chun, 2015), and release of cytokines by senescent cells, known as the senescence-associated secretory phenotype (SASP), have also been implicated in COPD (Rajendrasozhan *et al.*, 2008; Baker, Donnelly and Barnes, 2020) – although not directly with neutrophils. Decreased SIRT1 expression by lung tissue was identified in patients with COPD compared to non-COPD controls (Rajendrasozhan *et al.*, 2008). The SASP also contains multiple chemokines that attract neutrophils, linking senescence in the lungs with an increased influx of neutrophils (Prata *et al.*, 2018)

Parallels between COPD and ageing are also observed in neutrophil function. As mentioned in Section 1.3.2, both human and murine studies have suggested an age-related decline in the accuracy of neutrophil chemotaxis (Sapey *et al.*, 2014). Indeed, reduced neutrophil migratory accuracy is a feature that appears to be exaggerated in patients with COPD beyond that of age-matched volunteers without COPD (Sapey *et al.*, 2011). Reduced neutrophil migratory is linked with increased tissue damage through migration associated release of proteases and ROS (Naccache and Lefebvre, 2014; Sapey *et al.*, 2014). In turn, this leads to the accelerated

development of emphysema (Barnes, Shapiro and Pauwels, 2003) and further observations of an accelerated ageing effect. Therefore, migratory inaccuracy in COPD may represent an exaggeration of the ageing process.

Oxidative stress and ROS production are also linked to inflammation, ageing and senescence as described by the free radical theory of ageing originally proposed by Professor Denham Harmen (Harman, 1956, 2006). Whilst only one aspect of the damage-induced ageing hypothesis (Gladyshev, 2014), damage from increased ROS responses provides a potential link between the neutrophil and accelerated ageing in COPD. Successful bacterial killing in neutrophils relies on the ability to mount an appropriate ROS response (Nguyen, Green and Mecsas, 2017), but the generation and extracellular release of ROS by neutrophils can also contribute to collateral lung tissue damage (McGuinness and Sapey, 2017). Furthermore, mouse models have demonstrated that oxidising environmental agents (such as ozone) cause neutrophilia and airway damage, leading to emphysema (Williams *et al.*, 2007; Wiegman *et al.*, 2014). Evidence of oxidative stress has been identified in both the sputum and plasma of patients with COPD, related to both the increased ROS challenge and decreased anti-oxidant activity (Zeng *et al.*, 2013).

1.5.5 Neutrophils in AECOPD

Increases in neutrophil chemoattractants in the lungs during exacerbation causes an increased number of neutrophils within the lung (Crooks *et al.*, 2000). The influx of neutrophils further increases the presence of neutrophil proteinases, tipping the balance towards increased lung damage. Recent cohorts of patients with AECOPD have identified that exacerbations with a

neutrophilic phenotype were more severe and may impact on mortality (Kandemir *et al.*, 2020).

In addition, multiple aspects of neutrophil function are also thought to be altered during an exacerbation of COPD. Peripheral blood neutrophils isolated from patients hospitalised with AECOPD showed reduced chemotaxis towards CXCL8 and fMLP compared with neutrophils from stable COPD – a phenomenon that was sustained at 56-day follow up (McIver *et al.*, 2019). In a small cohort of patients with AECOPD, neutrophils also demonstrated reduced phagocytosis of *Staphylococcus Aureus* bacteria *in vitro* (Lavinskiene *et al.*, 2011). However, it is difficult to determine if changes in neutrophil behaviour predispose to AECOPD or are as a result of the respiratory insult. Further investigation of neutrophil phenotypes may shed light on the increased disease burden in these patients.

1.5.6 Other functional defects

Other key neutrophil functions discussed in Section 1.2, including phagocytosis, ROS and NET production are also implicated in COPD, CVD and T2D.

Neutrophils exposed to CSE *in vitro* were capable of releasing more ROS compared with unexposed cells in a TNF-α-dependant manner (Friedrichs *et al.*, 2014), especially important given raised TNF-α levels in the lungs of patients with COPD. In addition, TNF-α increases endothelium susceptibility to ROS (Ishii *et al.*, 1992), presenting another mechanism of enhanced lung damage in patients with COPD. There is also evidence of systemic ROS activity in patients with COPD, potentially predisposing to events seen in the pathogenesis of AS and T2D (Rahman *et al*., 1996; Zeng *et al*., 2013). ApoE$^{-/-}$ mice with an absence of functional NADPH Oxidase (NOX)1/2, modelling a reduction in ROS production, showed reduced lesion size and

reduced ROS production by smooth muscle cells *in vitro* (Barry-Lane *et al*., 2001; Madamanchi, Vendrov and Runge, 2005). ROS production can also contribute to pancreatic β-cell destruction – as these cells are particularly sensitive to ROS-induced apoptosis (Lenzen, 2008). However, comparisons of ROS production between mice and humans are complicated by the fact that TNF-α serves as an important priming agent for the oxidative burst in neutrophils in humans, but this mechanism does not exist in mice or rats (El-Benna *et al.*, 2016). These studies suggest how ROS could be a mechanism linking COPD, CVD and T2D; one that increased ROS production by neutrophils may play an important role.

Studies of neutrophils from patients with COPD have demonstrated these neutrophils maintain the molecular mechanisms to carry out phagocytosis as neutrophils were able to phagocytose fluorescently-labelled polystyrene beads (Thomas, Barnes and Donnelly, 2012). However, the uptake of *Candida*, *Haemophilus influenzae* and *Streptococcus pneumoniae* by neutrophils from patients with COPD was reduced compared to neutrophils from both healthy smoker and non-smoker controls – indicating changes in the ability to sense bacteria and fungi (Thomas, Barnes and Donnelly, 2012; Shanmugam *et al.*, 2015). Not all studies, however, agree with these findings as neutrophils isolated from a small cohort of 10 patients with COPD found no difference in phagocytosis compared to healthy age-matched controls (Walton *et al.*, 2014). Potential confounders, such as alcohol consumption, that may also reduce phagocytic capacity by neutrophils (Chiu *et al.*, 2018) could skew these results.

A putative role for NETs in chronic disease is beginning to emerge, with *in vitro* evidence of a transiently reduced ability of circulating neutrophils to produce NETs in patients with COPD (Pullan *et al.*, 2015), but increased NET production identified in the sputum (Dicker *et al.*, 2018)

that may link to collateral lung damage (Trivedi *et al.*, 2021). Despite this potential location-dependent difference, both studies postulate that altered NET function is linked to a reduction in effective bacterial clearance and a mechanism of damage in COPD. Induction of NETosis in the lungs of mice using ovalbumin sensitisation and exposure showed a reduction in ROS and oxidative stress in the lung after treatment with DNase (that breaks down extracellular DNA, the primary structural component of NETs), demonstrating not only a potential detrimental role of NETs in the lung but also a potential therapeutic option and a link between oxidative stress and extracellular DNA (da Cunha *et al.*, 2016). These studies suggest an involvement of NETs in lung disease, but the variability in the way NETs are measured both *in vitro* and *in vivo* (Boeltz *et al.*, 2019) presents a challenge in interpreting these data.

All the factors discussed in this section influence disease progression and neutrophil function: bacterial infection or colonisation, smoking status, age and lung damage. Investigating the phenotype of neutrophils based on the surface expression of receptors and adhesion molecules has begun to change the way neutrophils are viewed in the context of health and disease, especially as these may link to the aforementioned functions. The next section will address the topic of neutrophil phenotypes and subtypes, and the key molecules that have been linked to key neutrophil functions within a disease setting.

1.6 Neutrophil phenotypes in disease

The many functions of neutrophils that have been discussed may have important implications in chronic lung disease. The question remains regarding the multi-faceted nature of neutrophil function: does this reflect a highly plastic population with adaptive responses which are environment-dependent or reflect distinct subtypes of cells each with a specific role?

The balance of evidence currently suggests that neutrophils change in response to signals such as cytokines, physical pressure or bacterial products, resulting in different functional phenotypes, supported by neutrophil heterogeneity observed *in vitro* in pro-inflammatory culture conditions (Chakravarti *et al.*, 2009; Takashima and Yao, 2015; Evrard *et al.*, 2018). These changes can be subtle and may relate to the immediate cellular environment. To further complicate investigations of neutrophil phenotypes, experimental methods may inadvertently alter the neutrophil phenotype (Pallister *et al.*, 2006). Examining conditions and diseases where neutrophils have a different phenotype may help identify differences between individuals that are healthy, or with COPD and the associated co-morbidities. An overview of these key phenotypes, and the markers used to identify them will now be discussed and are summarised in Figure 1.6.

1.6.1 Activation and adhesion

1.6.1.1 CD11b

Activation markers may play an important part in identifying potentially aberrant neutrophil responses. CD11b epitomises the fine balance that exists in neutrophil responses: patients constitutively lacking CD11b have neutrophils with a severely hampered phagocytic response (Arnaout, 1990) and neutrophils from patients with COPD with higher expression of CD11b that may be linked to airflow limitation (Yamagata *et al.*, 2007). The latter study does, however, make a leap from CD11b overexpression to the causative role in airflow limitation (Yamagata *et al.*, 2007), yet warranting further investigation. Trauma and sterile injury have also been linked with increased neutrophil activation, with one study demonstrating

neutrophil activation in trauma patients with a systemic inflammatory response syndrome (Hazeldine *et al.*, 2015).

1.6.1.2 CD62L

CD62L expression is also in a fine balance, requiring temporal expression and shedding for normal neutrophil function. A study utilising mice with a mutant CD62L molecule resistant to cleavage revealed blocking CD62L cleavage resulted in an increased number and prolonged presence of neutrophils migrating into inflamed peritoneum compared to wild-type mice (Venturi *et al.,* 2003). The same study also showed, however, neutrophils lacking cleavable CD62L were less capable of migrating large distances (>50µm) from post-capillary venules compared to wild-type following stimulation with the murine CXCL8 homologue (Venturi *et al.,* 2003), demonstrating stimuli-dependant differences in neutrophil migration following CD62L cleavage. In addition, another murine model utilising a mutant CD62L molecule with enhanced catch-bond lifetime and engagement resulted in increased sensitivity of neutrophils to activation signals (Z. Liu *et al.*, 2017), suggesting increased CD62L expression may confer a similar outcome.

Turning attention to CD62L expression from neutrophils in humans, a study demonstrated, both *in vitro* and *in vivo,* the use nonsteroidal anti-inflammatory drugs (NSAIDs) resulted in enhanced CD62L shedding and that maintaining CD62L expression was an energy-dependant process, suggesting common drugs can alter neutrophil behaviour (Gómez-Gaviro *et al.*, 2000). Indeed, blood neutrophils from long-term smokers and patients with COPD have been linked with lower CD62L expression compared with healthy non-smokers (Stockfelt *et al.*, 2020). This phenotype of reduced CD62L expression has also been demonstrated in blood

neutrophils from patients with COPD compared with asthmatics and healthy volunteers (Lokwani, Wark, Baines, Fricker, *et al.*, 2019) and linked with reduced lung function (Lokwani, Wark, Baines, Barker, *et al.*, 2019). Preceding these observations, it has been suggested lower CD62L expression denotes hypersegmented neutrophils, so named because of the increased in nuclear lobes (Pillay *et al.*, 2012; Tak *et al.*, 2017). Whilst the exact function, or dysfunction, of hypersegmented neutrophils remains unknown, they do not typically exist in the circulation of healthy individuals (van Grinsven *et al.*, 2019), do not exhibit altered migratory function (van Grinsven *et al.*, 2019), are less able to adhere to endothelial cells (Kamp *et al.*, 2012) and can be seen in the circulation following systemic LPS challenge in humans (Pillay *et al.*, 2012; van Grinsven *et al.*, 2019) – although the reduction in endothelial adhesion may be purely due to the role CD62L plays in initial endothelial attachment (see Section 1.2.1).

1.6.2 Chemokine sensing and migration

As discussed (Sections 1.2.2 and 1.3.2), the ability to detect and migrate accurately to chemical signals is vital to the role neutrophils play in the immune response. Several receptors and surface proteins co-ordinate this response and may provide insight into the dysfunction observed within disease. Neutrophils express a wide range of chemokine receptors that enable responses to lipid mediators (such as leukotriene B4), complement proteins (such as C5a), formyl peptides (such as fMLP) and a variety of CXC and CC containing ligands, leading to some redundancy between receptors, but also with nuanced roles for neutrophil activation and migration (Metzemaekers, Gouwy and Proost, 2020), As mentioned, CXCR2 is one such receptor expressed by neutrophils that is able to bind to CXCL1-3 and CXCL5-8 (Bachelerie *et al.*, 2014) with overlapping function with CXCR1 that also binds CXCL6 and CXCL8 (Bachelerie

et al., 2014). Given the importance of CXCR2 in binding the most potent chemoattractant CXCL8, ongoing trials for pharmacological blockade in therapies and the role in controlling neutrophil release from the bone marrow, CXCR2 was chosen as a chemokine receptor to investigate further.

1.6.2.1 CXCR2

In mice, a model of severe sepsis demonstrated both a reduction in the number of neutrophils in the peritoneal cavity (the source of the bacterial insult) and a reduction in CXCR2 expression compared to non-severe sepsis (Rios-Santos *et al.*, 2007). Furthermore, blockade of CXCR2 recapitulated the neutrophil phenotype observed in severe sepsis mice in those with non-severe sepsis (Rios-Santos *et al.*, 2007). Reduction in CXCR2 expression and impaired migration has also been linked with anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (Hu *et al.*, 2011). The same study also revealed that pharmacological blockade of CXCR1 and CXCR2 exhibited altered migration (Hu *et al.*, 2011).

The complexities of neutrophil CXCR2 expression are highlighted in COPD, as neutrophils isolated from the sputum had reduced CXCR2 expression and increased CD11b expression compared with peripheral neutrophils, both of which were reduced compared to peripheral neutrophils from healthy individuals (Pignatti *et al.*, 2005). It is important to note, however, that a reduction in CXCR2 expression is also observed after bacterial phagocytosis, a potential confounder in experiments where phagocytosis is possible but not measured (Doroshenko *et al.*, 2002).

These studies demonstrate that CXCR2 expression and migration accuracy are key neutrophil phenotypes, but also raise the potential that modulation of CXCR2 may be a therapeutic avenue to reduce neutrophil infiltration to inflamed tissue (Konrad and Reutershan, 2012).

1.6.2.2 CXCR4

As CXCR4 has also been linked with neutrophil senescence, the role of CXCR4 will be discussed in Section 1.6.3.

1.6.2.3 CD54

As highlighted with CXCR2, different phenotypes of neutrophils may be somewhat restricted by the microenvironment, especially regarding neutrophils within the lung compared with those in the circulation. However, human neutrophils can reverse migrate, demonstrated *in vitro* with neutrophils moving through endothelium monolayers, modelling movement back into the circulation (Buckley *et al.*, 2006). These neutrophils maintained a pro-inflammatory and apoptotic-resistant phenotype, as well as upregulating CD54 expression (Buckley *et al.*, 2006). If neutrophils are capable of migrating from the lung tissue back into the circulation, this could provide a link between chronic inflammation and systemic damage in COPD, AS and T2D, especially as increased numbers of circulating CD54+ neutrophils were found in patients with AS (Buckley *et al.*, 2006).

Other scenarios where neutrophils display increased CD54 expression also exist. Hypersegmented neutrophils, in the same study that identified these neutrophils displaying a $CD62L^{dim}$ phenotype, also upregulated CD54 in response to LPS injection *in vivo* with human volunteers (Pillay *et al.*, 2012). The authors also note that this phenotype could not be recapitulated *in vitro*, highlighting both limitations of *in vitro* models, but also that other cells

and processes are involved in regulating neutrophil phenotypes. The same group also identified that increased CD54 expression, along with an increase in CD11b and reduction of CD62L, occurs when neutrophils enter the lung tissue irrespective of disease (Fortunati *et al.*, 2009). Together, these studies highlight that CD54 provides a useful tool to identify transmigrated neutrophils.

1.6.3 Senescence and apoptosis

In both inflammation and chronic lung conditions such as COPD, the lifespan of neutrophils is prolonged due to resistance to apoptosis (Uddin *et al.*, 2010; Geering *et al.*, 2013), but is this response helpful or harmful in the context of chronic inflammation?

1.6.3.1 CXCR4

CXCR4 is the major receptor for CXCL12 and a potential marker of neutrophil senescence as discussed (Section 1.2.6). CXCR4 functionality in health is partly revealed by a genetic condition whereby CXCR4 signalling is enhanced, leading to neutrophil retention in the bone marrow and peripheral neutropenia (Hernandez *et al.*, 2003). It might also be expected that reductions or enhancements of CXCR4 in peripheral neutrophils would also impact their ability to migrate effectively.

Neutrophils taken from the lungs of patients with fungal colonisation and cystic fibrosis (Carevic *et al.*, 2015), and from mice with LPS-induced lung injury (Yamada *et al.*, 2011), had increased CXCR4 expression – indicating lung damage may increase neutrophil CXCR4 expression. Peripheral damage may also increase CXCR4 expression as blood neutrophils isolated from patients with ischemic stroke revealed higher CD11b expression and lower CD62L expression in conjunction with raised CXCR4 expression – a phenotype termed

'overactive senescent' (Weisenburger-Lile *et al.*, 2019). CXCR4 expression has also been linked with a pro-angiogenic phenotype in both mice and human (Massena *et al.*, 2015), but this phenotype may be more dependent on Vascular Endothelial Growth Factor Receptor 1 (VEGFR1) expression than CXCR4. Together these data highlight scenarios where neutrophils express higher levels of CXCR4, however, the disparate nature of these conditions still leaves many questions around neutrophil CXCR4 expression and regulation unanswered.

It is important to also note that CXCL12 is present outside the bone marrow with concentrations in healthy humans reported anywhere between 0.2ng/mL (Yang *et al.*, 2020) to 40ng/mL (Soriano *et al.*, 2002). Patients with COPD who were current or ex-smokers have been reported to have raised serum levels of CXCL12 compared to people without COPD (Kuźnar-Kamińska *et al.*, 2016). Elevated CXCL12 levels have also been linked to the pathogenesis of T2D as diet-induced obese mice showed raised CXCL12 levels in the adipose tissue and blocking CXCR4 pharmacologically improved insulin sensitivity (Kim *et al.*, 2014). It is unclear, however, if raised CXCL12 levels interfere with the return, or indeed the release, of neutrophils to or from the bone marrow. These data indicate CXCR4 can be altered by chronic inflammation, but the exact role and functional impact requires further investigation.

1.6.3.2 Phosphatidylserine and annexin V

It has long been known that phosphatidylserine, a membrane phospholipid that is usually contained to the inner plasma membrane, is exposed on the outer plasma membrane during cellular apoptosis and can be readily detected by the binding of annexin V (Darzynkiewicz *et al.*, 1997; Wlodkowic, Skommer and Darzynkiewicz, 2009). Apoptosis of neutrophils is not only a normal physiological process but also aids the resolution of inflammation – particularly after

phagocytosis of bacteria (Greenlee-Wacker, 2016). Neutrophils, like other cells, have a variety of mechanisms that trigger apoptosis and have been well documented (Simon, 2003; Geering and Simon, 2011). An extensive review has drawn together how neutrophil apoptosis is influenced by various bacterial pathogens (DeLeo, 2004) – of note, a common respiratory bacteria *S. pneumoniae* accelerated neutrophil apoptosis when cultured with isolated neutrophils *in vitro* (Zysk *et al.*, 2000), a process that was not altered by co-incubation with GM-CSF (Engelich, White and Hartshorn, 2001). In combination with a study suggesting neutrophils from patients with ARDS showed increased apoptotic resistance (Matute-bello *et al.*, 1997), partially due to increased G-CSF and GM-CSF, neutrophil apoptosis appears fine-tuned by the microenvironment.

It is possible, therefore, fine-tuning of apoptosis could provide benefits in either neutrophil clearance or inflammation resolution. Sputum neutrophils from patients with COPD were shown to be no more resistant to apoptosis than sputum neutrophils from healthy smokers and non-smokers (Rytilä *et al.*, 2006), suggesting neutrophilia seen in the lungs of patients with COPD may be linked with prolonged survival. It is important to note, however, that neutrophils are not generally present at any large numbers in healthy lungs, making direct comparisons between COPD and healthy sputum challenging. A small study suggested that blood neutrophils from patients with COPD exhibited increased activation during apoptosis, although the rate of apoptosis itself was not altered (Noguera *et al.*, 2004), however, this may represent an increased sensitivity to activation during isolation – especially relevant with *in vitro* experimental procedures.

Altered apoptosis, either accelerated or inhibited is likely to impact on both neutrophil function and the resolution of inflammation, but it is unclear the exact role this may play in COPD. In diabetes, again, neutrophils isolated from patients with T1D demonstrated normal spontaneous apoptosis but were unable to delay apoptosis in response to LPS, in contrast with neutrophils from healthy volunteers (Tennenberg, Finkenauer and Dwivedi, 1999). Neutrophil apoptosis has been reported to be altered in a variety of other inflammatory diseases, including inflammatory bowel disease (Brannigan *et al.*, 2000) and cystic fibrosis (Gray *et al.*, 2018), highlighting it is an important parameter to measure and investigate in the context of disease.

1.6.4 Maturity

1.6.4.1 CD10

As discussed, CD10 is a useful marker to identify neutrophil maturation status (Section 1.1.1 and 1.3.1), however, it may also reveal important information about other neutrophil functions. CD10 is an endopeptidase and incubation of human neutrophils with fMLP resulted in the degradation of fMLP that was blocked with a CD10 inhibitor, phosphoramidon (Painter *et al.*, 1988). Importantly, a study using monoclonal antibodies to CD10 reported inhibition of neutrophil chemotaxis towards fMLP with no impact on chemokinesis (McCormack, Nelson and LeBien, 1986). Conversely, two other studies report the use of CD10 inhibitors (thiorphan (Shipp *et al.*, 1991) and thiorphan/phosphoramidon (Hofman *et al.*, 1998)) resulted in enhanced chemotaxis across an acellular membrane towards fMLP. The first of these studies also highlighted that stimulation of neutrophils with PMA resulted in decreased CD10 activity, whereas activation with TNF-α increased CD10 activity (Shipp *et al.*, 1991). Together these

results suggest CD10 not only has enzymatic activity but may also participate in intracellular signalling events involved in regulating chemotaxis. Indeed, a prostate cancer cell line capable of performing cellular migration revealed CD10 engagement was capable of directly blocking migration via focal adhesion kinase (Sumitomo *et al.*, 2000) – suggesting an explanation for the reduced chemotaxis response observed with antibody blocking of CD10 (McCormack, Nelson and LeBien, 1986). CD10 expression, therefore, may be an important marker of both maturity and neutrophil bacterial responses in patients.

1.6.5 Inflammatory phenotypes

Potentially pro-inflammatory and anti-inflammatory phenotypes of neutrophils have also been reported in the literature. Aside from surface marker expression, regulatory or 'anti-inflammatory' neutrophils have been reported in patients with melanoma and from wild-type mice based on production of the anti-inflammatory cytokine IL-10 (De Santo *et al.*, 2010). However, another study has suggested that the IL-10 locus is epigenetically inactive in human neutrophils and, therefore, these results might arise from contaminating monocytes in human neutrophil preparations (Tamassia *et al.*, 2013).

The divergence between pro- and anti-inflammatory neutrophils is also demonstrated in patients receiving peripheral blood stem cell therapy in conjunction with G-CSF infusions (Marini *et al.*, 2017). In this patient population, two neutrophil phenotypes were observed: CD66b+CD10+ neutrophils – mature neutrophils that inhibited T cell responses and CD66b+CD10- neutrophils with an immature morphology and the promotion of T cell function (Marini *et al.*, 2017). However, the functional difference was only observed in low-density granulocytes – a specific subset of granulocytes that exist in primarily inflammatory conditions

(Hacbarth and Kajdacsy-Balla, 1986; Hassani *et al.*, 2020), are of interest in their own right, but will not be covered in detail here. Yet, these observations set the premise for pro- and anti-inflammatory phenotypes of neutrophils.

1.6.5.1 CD11c

CD11c, or integrin alpha X, is one of the four α-subunits expressed on the surface of neutrophils that pairs with CD18 and has over 50% homology with CD11b (Frick *et al.*, 2005). CD11c binds fibrinogen; demonstrated by blocking the binding of stimulated neutrophils with TNF-α *in vitro* to fibrinogen with anti-CD11c antibodies (Loike *et al.*, 1991). Neutrophils expressing higher levels of CD11c *in vivo* has been linked to an immunosuppressive phenotype, as volunteers injected intravenously with LPS resulted in neutrophils with higher CD11c expression that *in vitro* suppressed T cell activation (Pillay *et al.*, 2012). Neutrophils with higher CD11c expression have also been found in the circulation of patients with sepsis, of note without the loss CD62L or increase in CD11b expression (Lewis *et al.*, 2015). Another study reported increases in neutrophil expression of both CD11b and CD11c in patients with sepsis (Muller Kobold *et al.*, 2000) – the differences in isolation procedures may be responsible for the discrepancy in CD11b expression.

Peripheral blood neutrophils from patients with T2D have also been reported to have increased surface expression of CD11c (Caimi *et al.*, 2002), including a blunted upregulation of CD11b expression in response to fMLP, adding to the notion that CD11c expression may indicate an immunosuppressive phenotype.

1.6.5.2 PD-L1

Programmed-death receptor 1 (PD-1) is predominantly expressed on T cells and engagement of PD-1 inhibits T cell proliferation and activation, maintaining immune tolerance in health (Keir *et al.,* 2008; Wei *et al.,* 2013). The PD-1 receptor has two natural ligands, PD-L1 and PD-L2. PD-L1 has gained attention because of its role in cancer, as expression by tumour cells inhibits T cell-mediated killing through engagement of PD-1, and there is an evolving clinical programme to block PD-L1 as a therapeutic (Dong, Sun and Zhang, 2015; Huang *et al.*, 2015; J. Chen *et al.*, 2016; Hahn *et al.*, 2017).

Neutrophils can also express PD-L1 and this is implicated in a variety of disease settings. Neutrophils isolated from patients with systemic lupus erythematosus (SLE) showed an increased proportion of PD-L1 expressing neutrophils in the circulation that correlated with disease severity (Luo *et al.,* 2016). Another study also found increased PD-L1 expression on peripheral blood neutrophils isolated from patients with active tuberculosis infection compared to healthy volunteers (McNab *et al.,* 2011). Human neutrophils exposed *in vitro* to cancer-associated fibroblast conditioned media also up-regulated PD-L1 leading to inhibition of T cell proliferation, and reduced neutrophil apoptosis (Cheng *et al*., 2018). It is yet unclear if these represent advantageous compensatory changes or pathogenic changes in neutrophil function.

The PD-1/PD-L1 axis also has links to COPD as CD8+ T cells isolated from lung sections of patients with COPD have increased PD-1 expression that has been linked with reduced anti-viral responses (McKendry *et al.,* 2016). However, the impact of altering the PD-1/PD-L1 axis in patients with COPD is not yet well understood (Stoll, Virchow and Lommatzsch, 2016).

These studies suggest that neutrophil expression of PD-L1 may indicate an immunosuppressive function, one that may be of future therapeutic benefit.

1.6.5.3 HLA-DR

A third surface molecule linked with neutrophil inflammation is Human Leukocyte Antigen (HLA)-DR, a molecule that presents antigen to the T Cell Receptor (Jendro, Goronzy and Weyand, 1991). HLA-DR is a class II Major Histocompatibility Complex (MHC) molecule more commonly linked to functions of dendritic cells, macrophage and B cells (Ting and Trowsdale, 2002) rather than neutrophils (Lin and Loré, 2017).

HLA-DR expression may be linked to neutrophil activation: *in vitro* stimulation of synovial neutrophils with LPS and fMLP, among others, induced HLA-DR surface expression (Sandilands *et al.*, 2006), a finding supported by the expression of HLA-DR by synovial neutrophils in patients with RA (Cross *et al.*, 2003). Is this feature limited *in vivo*, therefore, to synovial neutrophils? A growing body of evidence suggests that peripheral blood neutrophils can express HLA-DR, at least *in vitro*, as isolated neutrophils from healthy volunteers could present antigen via HLA-DR, but importantly required antigen-specific or activated T cells as co-stimulation (Vono *et al.*, 2017). Another study demonstrated that neutrophils can indeed express HLA-DR by incubating isolated neutrophils with IgG-opsonised erythrocytes (Meinderts *et al.*, 2019). Furthermore, this study indicated these neutrophils could indeed present antigen and induce T cell proliferation (Meinderts *et al.*, 2019), albeit in an artificial *in vitro* system.

The role HLA-DR expression on human neutrophils in the context of COPD, T2D and CVD still requires further investigation. An abstract submitted for the European Respiratory Society

conference suggested circulating neutrophils from patients with COPD showed increased HLA-DR expression (Scrimini *et al.*, 2013), however, the full data was not published.

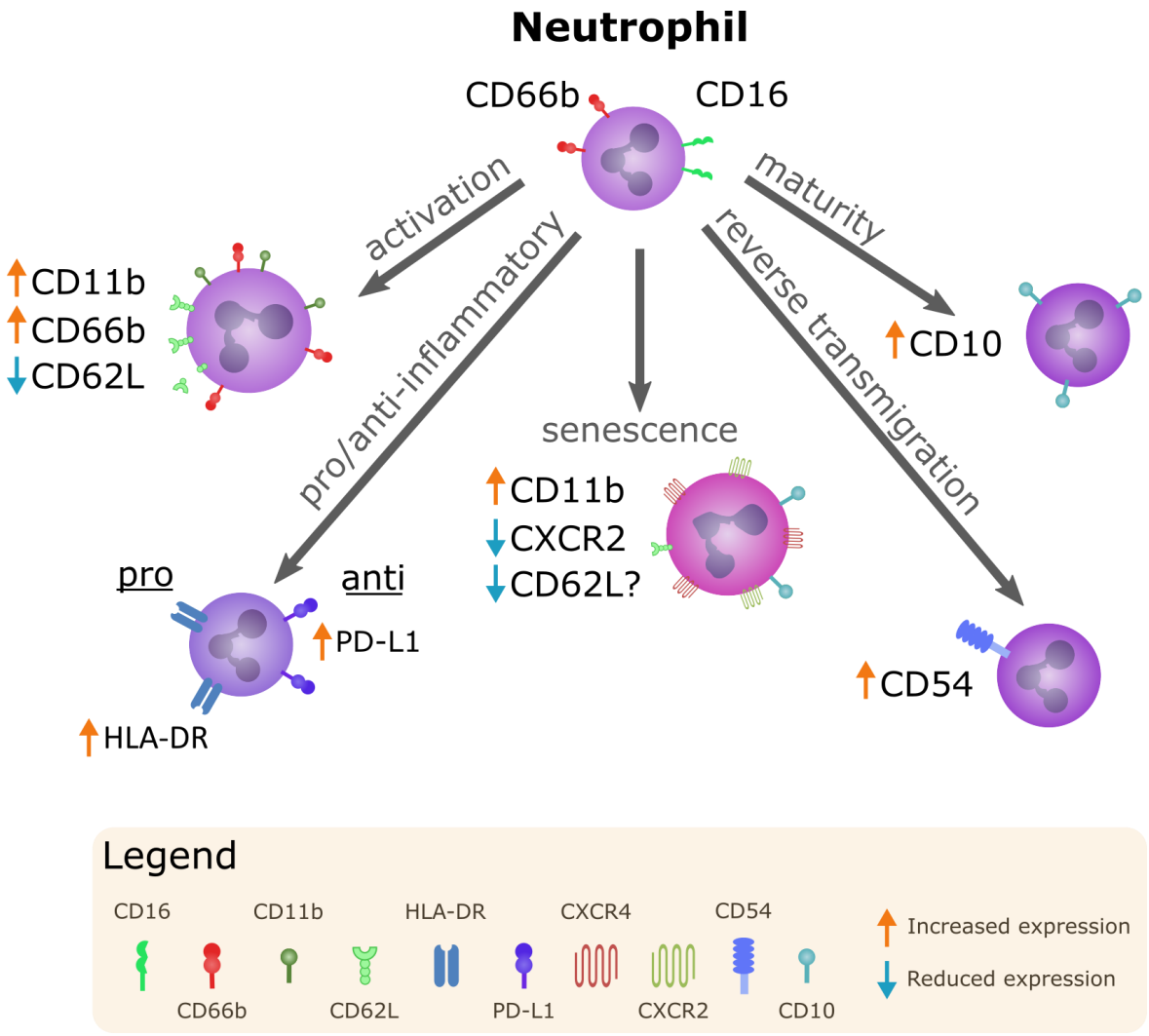

Figure 1.6: Overview of several described neutrophil phenotypes in health and disease

Several phenotypes of neutrophils have been described in health and disease and may potentially be altered in COPD. Neutrophils constitutively express CD16 and CD66b and are commonly used to identify human neutrophils. During activation, expression of CD11b and CD66b increase and can be accompanied by shedding of CD62L. Neutrophils may acquire expression of HLA-DR or PD-L1, potentially with pro- or anti-inflammatory effects respectively. Neutrophil senescence has previously been described by the increase of CXCR4 and decline of CXCR2 expression, potentially with the loss of CD62L expression. Neutrophil transmigration causes an increase in CD54 expression and, therefore, CD54 expression in the circulation is an indicator of reverse transmigration. Neutrophils increase CD10 expression during maturation.

1.7 Aims and Hypothesis

COPD is a disease with a considerable healthcare burden and substantial morbidity and mortality both in the UK and worldwide (World Health Organisation, 2018). Among others, the neutrophil plays a wide-ranging role in the pathogenesis of COPD, and changes in the phenotype and function of neutrophils may improve our understanding of both the mechanism of disease progression and heterogeneity in COPD linked with multi-morbidity.

It was hypothesised that altered functions of neutrophils in COPD would be revealed by changes in surface expression and gene expression, consistent with an activated, pro-inflammatory and senescent phenotype, with changes due to healthy ageing, accelerated in patients with COPD. These changes would be exaggerated in patients with multimorbidity and further increased during AECOPD.

Therefore, the main aims of this thesis are as follows:

1. To validate a novel panel of antibodies in order to investigate potentially important phenotypes of circulating neutrophils (Chapter 3 and 4).

2. To assess the phenotype of neutrophils from healthy young and old volunteers, patients with COPD, including the impact that multimorbidity may have on causing changes in the neutrophil phenotype, specifically T2D and CVD (Chapter 5).

3. To assess changes in the circulating neutrophil phenotype during AECOPD compared with stable disease (Chapter 6).

4. To determine the potential for neutrophils from healthy volunteers to be altered with pooled plasma from patients with COPD (Chapter 7).

5. The gene expression profile of neutrophils from healthy volunteers, patients with COPD and AECOPD will be investigated to further support the identification of phenotypes by surface expression (Chapter 5 and 6).

6. The ability of neutrophils to accurately migrate to chemokines both from patients and healthy volunteers will be investigated, including the response to CXCL12 and how chemotaxis may be altered when neutrophils from healthy elderly volunteers are incubated with plasma from patients with COPD (Chapter 7).

CHAPTER 2:

MATERIALS AND METHODS

2.1 Ethical Approval

All participants provided informed written consent before biological samples were obtained and used in this study. The study was sponsored by the University of Birmingham and approved by University Hospitals Birmingham (UHB) NHS Trust under approval from the West Midlands – Solihull Research Ethics Committee (REC 18/WM/0097). Research was conducted following Good Clinical Laboratory Practice and in accordance with the Declaration of Helsinki with Research and Development support from UHB NHS Trust.

2.2 Participant Recruitment

2.2.1 Healthy volunteers

Healthy volunteers were recruited from the Centre for Translational Inflammation Research or from the Birmingham 1000 Elders cohort. All participants were seen at the Queen Elizabeth Hospital Birmingham (QEHB; Birmingham, UK) where a clinical assessment was performed and a blood sample obtained. Volunteers must have been over 18, with no significant respiratory symptoms and no previous diagnosis of COPD, asthma, interstitial lung disease, bronchiectasis, lung cancer or previous lung resection.

2.2.2 Stable COPD

Patients with a clinical diagnosis of COPD, defined as an FEV_1/FVC ratio <0.7 (GOLD, 2019), were recruited from the Chronic Disease Resource Centre COPD cohort and from routine respiratory clinic outpatient appointments, both at the QEHB. Briefly, patients must have had at least a 10 pack-year smoking history, be over 40, be able to provide informed consent, not

have a diagnosis of asthma, interstitial lung disease or lung cancer and be free from exacerbation in the past 42 days.

2.2.3 Exacerbations of COPD

Patients hospitalised with an acute exacerbation of COPD (AECOPD), determined by a worsening of respiratory symptoms with an existing diagnosis of COPD (GOLD, 2019), were recruited from the Acute Medical Unit at QEHB. Briefly, patients must have had at least a 10 pack-year smoking history, be over 40, be able to provide informed consent, the first blood sample taken within 48 hours of admission with AECOPD being the primary reason for admission, and not have a diagnosis of asthma, interstitial lung disease or lung cancer.

The full inclusion and exclusion criteria for each group can be found in Appendix 1.

2.3 Isolation of human neutrophils from whole blood

All centrifugation steps were carried out at room temperature (RT) with acceleration and brake unless otherwise specified. Human blood was collected via venepuncture using the vacutainer system (BD Biosciences, UK) into 6 mL lithium heparin-containing (17 international units/mL) tubes. Neutrophils were isolated as previously described (Jepsen and Skottun, 1982). Briefly, the red blood cells were sedimented 6:1 whole blood to dextran sulphate (2% w/v; Sigma-Aldrich, Poole, UK) in saline (0.154 M; Sigma-Aldrich) for 30 minutes. Isotonic Percoll was made using 9:1 v/v Percoll (GE Healthcare) to sterile NaCl (1.54 M). This isotonic Percoll was further diluted with sterile NaCl (0.154 M) in a 4:1 ratio to make 80% or 1.27:1 to make 56%. A discontinuous density gradient was made in a 15 mL centrifuge tube by layering 2.5 mL of 80% Percoll underneath 5 mL of 56% Percoll. The remaining cellular fraction (buffy

coat) was then layered on top of the 56% Percoll (Figure 2.1) and centrifuged at 470 *g* with acceleration of 1 and no brake for 20 minutes. The top plasma and peripheral blood mononuclear cell (PBMC) layer were removed and discarded, and then the lower granulocyte band obtained and diluted to 15 mL in phosphate-buffered saline (PBS; Sigma-Aldrich) and centrifuged at 300 *g* for 5 minutes. The pellet was resuspended in RT double-plus media (RPMI 1640 containing 2 mM L-glutamine supplemented with Penicillin/Streptomycin (1% w/w; all Sigma-Aldrich)). Cells were counted with a haemocytometer and diluted to required concentration in double-plus media.

2.3.1 Assessment of neutrophil purity

Neutrophil purity was assessed by adding 100 µL of neutrophils at $1x10^6$ cells/mL to a cytospin chamber attached to a frosted glass slide (ThermoFisher Scientific, Loughborough, UK) and centrifuged in a cytospin (ThermoFisher Scientific) at 300 revolutions per minute (RPM) for 5 minutes (Figure 2.2). The glass slide was then removed and stained using a Quick-Diff staining kit (Reagena, Finland) by dipping in methanol for 10 seconds, solution I for 30 seconds and solution II for 30 seconds followed by rinsing in distilled water. Slides were imaged using a light microscope (Wilovert, Germany). The number of cells and neutrophils were counted in the field of view at x20 magnification, and the percentage of neutrophils calculated. A purity of >95% was typically observed.

2.4 Isolation of PBMCs from whole blood

Human blood was collected using the vacutainer system into 6 mL lithium heparin containing tubes. In a 15 mL centrifuge tube, 2.5 mL of Histopaque 1077 (Sigma-Aldrich) was layered on

top of 2.5 mL of Histopaque 1119 (Sigma-Aldrich) as previously described (Munir *et al.*, 2015). Whole blood was then layered on top and centrifuged at 800 *g* for 30 minutes with acceleration 1 and brake 0 at RT. The top layer of cells containing the PBMCs was then pipetted into a fresh 15 mL centrifuge tube and diluted in double-plus media. Cells were centrifuged at 300 *g* for 5 minutes with acceleration and brake of 9 at RT. The supernatant was discarded; the pellet was resuspended in double-plus media and counted with a haemocytometer before diluting further in double-plus media as required.

2.5 Collection of serum and plasma from blood

Human blood was collected using the vacutainer system (BD Biosciences) into either Potassium-Ethylenediaminetetraacetic acid (K2EDTA)-containing, for plasma, or silica clot activator serum clotting containing, for serum, tubes. The vacutainer was centrifuged at 1000 *g* for 10 minutes and the plasma or serum pipetted off the top into 500 µL aliquots that were frozen at -20^{o}C for up to two months before long-term storage at -80^{o}C.

2.5.1 Pooling of serum and plasma samples

Serum pools were made by thawing stored serum from randomly selected donors (healthy young (HY), COPD and AECOPD) or selected age-matched healthy elderly (HE) donors and combining 300 µL of each sample together before re-aliquoting into 110 µL aliquots and storing at -20^{o}C until required. In all cases, each pooled aliquot was thawed immediately before use and any remainder discarded.

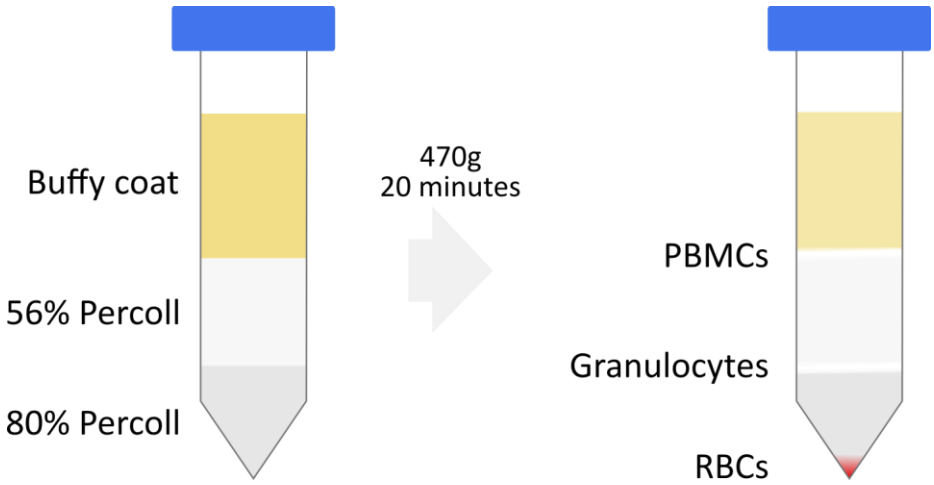

Figure 2.1: Diagram of the setup of the neutrophil isolation

Percoll and the buffy coat were layered as shown prior to centrifugation at 470 *g* with acceleration of 1 and no brake for 20 minutes. After centrifugation, remaining red blood cells (RBCs) pellet at the bottom, the granulocytes form a band at the interface between 80% and 56% Percoll, and the remaining peripheral blood mononuclear cells (PBMCs) form a layer on top of the 56% Percoll.

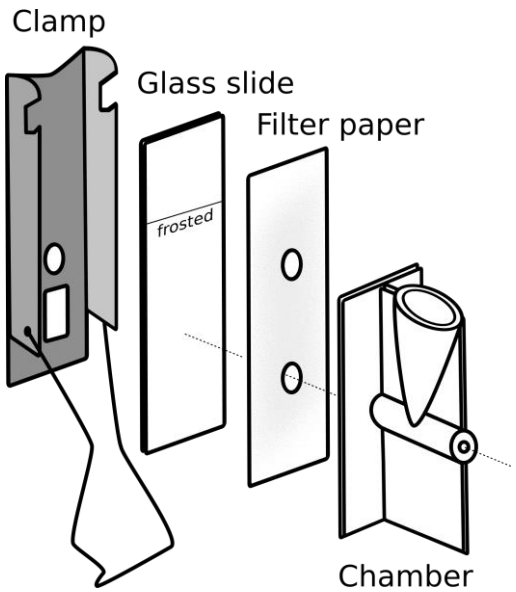

Figure 2.2: Diagram of the cytospin setup

All four sections were clamped together in the order shown prior to the addition of cells into the top of the chamber. This was then loaded into a cytospin centrifuge.

2.6 Phenotyping of isolated neutrophils by flow cytometry

The antibodies used for flow cytometry are summarised in Table 2.1. The stated dilutions were used in all experiments unless specifically expressed otherwise.

2.6.1 Validating antibodies for flow cytometry

Neutrophils at 1x10^6 cells/mL in double-plus media were added to 12x75 mm polystyrene tubes (BD Biosciences, USA; total of 1x10^5 cells) and incubated with 5 µL (1:20), 2.5 µL (1:40) or 1 µL (1:100) of the relevant antibody and corresponding concentration of the isotype control for 20 minutes on ice in the dark. Cells were washed twice with 100 µL of BSA (2% w/v in PBS; Sigma-Aldrich) and centrifugation at 300 *g* for 5 minutes. Samples were resuspended in 200 µL of 2% BSA in PBS and then analysed by flow cytometry using a 4-laser Fortessa X20 flow cytometer (BD Biosciences, UK) or a 2-laser Accuri C6 flow cytometer (BD Biosciences, UK) as described in Section 2.6.3. For each sample, a staining index was calculated using median fluorescence intensity (MFI) as previously described (Maecker *et al.*, 2004) using the following equation:

$$Staining\ Index = \frac{(MFI\ antibody - MFI\ isotype\ control)}{2 \times robust\ Standard\ Deviation\ of\ the\ isotype\ control}$$

In further experiments, the identified optimal antibody concentration was used during the incubation step (Table 2.1).

2.6.2 Sample preparation and staining

Neutrophils at 1x10^6 cells/mL in double-plus media at 100 µL/well were added to wells of a polyvinyl chloride 96-well U-bottomed plate (Costar, Loughborough, UK) and incubated with

relevant antibodies (Table 2.1) for 20 minutes on ice in the dark. Cells were washed with 100 µL of BSA (2% v/v in PBS), centrifuged at 300 *g* for 5 minutes with acceleration and brake of 9 at RT. Pellets were resuspended in 100 µL of annexin V Staining Buffer (BioLegend, London, UK) before centrifugation at 300 *g* for 5 minutes with acceleration and brake of 9 at RT before cell pellets were resuspended in either 100 µL of annexin V Staining Buffer (BioLegend) or 100 µL of PE-conjugated annexin V (1:40 in annexin V Staining Buffer; both BioLegend based on (Rieger *et al.*, 2011)). Cells were then incubated for 15 minutes at RT in the dark and then washed in 100 µL of annexin V Staining Buffer at 300 *g* for 5 minutes. Cell pellets were resuspended in either 100 µL annexin V Staining Buffer or 100 µL of 7-Aminoactinomycin D (7AAD; diluted 1:20 in annexin V Staining Buffer; all BioLegend, UK). Samples were transferred to 12x75mm polystyrene tubes (BD Biosciences, UK) containing 130 µL of annexin V Staining Buffer before running on a 4-laser Fortessa X20 flow cytometer (BD Biosciences, UK) or a 2-laser Accuri C6 flow cytometer (BD Biosciences, UK) as described in Section 2.6.3.

2.6.2.1 Addition of pooled serum and plasma

For experiments using pooled plasma, neutrophils at 1x10^6 cells/mL in double-plus media at 100 µL/well were added to wells of a polyvinyl chloride 96-well U-bottomed plate (Costar, Loughborough, UK) and incubated with 100 µL of either double-plus media, autologous plasma (AP), pooled plasma from HE donors or pooled plasma from stable COPD donors for 30 minutes at room temperature. Cells were centrifuged at 300 *g* for 5 minutes and washed twice with double-plus media prior to antibody staining as detailed in Section 2.6.2.

2.6.3 Analysis of flow cytometry data

Gates were set up in FACSDiva (Version 7; BD Biosciences, USA) or BD Accuri C6 Software (Version 1; BD Biosciences, USA) to exclude doublets, gate for granulocytes and, where viability dyes were included, live cells as shown in Figure 2.3. Samples were run at medium speed (default settings on both flow cytometers). Data were exported from FACSDiva or BD Accuri C6 Software as Flow Cytometry Standard (FCS) files and analysed using FlowJo (Version 10.6, BD Biosciences, USA). The standard gating strategy used is shown in Figure 2.3 to exclude doublets, gate for granulocytes using both forward (FSC) and side scatter (SSC) and neutrophil markers and, where viability dyes were included, live cells. For each channel, the raw median fluorescence intensity (MFI) and the percentage positive and negative was exported into a table. MFI values of the samples were adjusted by $MFI_{sample} - MFI_{isotype}$. For further analysis of neutrophil populations, only live neutrophils (indicated in Figure 2.3) were analysed to identify overactive senescent neutrophils (Figure 2.4a), segmentation status (Figure 2.4b) senescence (Figure 2.4c), or activation (Figure 2.4d).

Computational analysis of high-dimensional data was also used to analyse neutrophil phenotypes. Live neutrophils (Figure 2.4) were exported as FCS files for each sample and clustered using the Rphenograph clustering method (Levine *et al.*, 2015) and t-Distributed Stochastic Neighbor Embedding (t-SNE) visualisation (Van Der Maaten and Hinton, 2008) within the cytofkit package (H. Chen *et al.*, 2016) to identify neutrophil phenotypes without traditional manual gating strategies. The output of this analysis was used to generate heat-map profiles and investigate the expression profiles of each cluster according to the markers detailed in Table 2.1.

Table 2.1: Neutrophil phenotyping antibody panel.

Target	Alternative names	Clone	Conjugate	Manufacturer	LOT#	Concentration (µg/mL)	Dilution	Assay
CD16	FcγRIII	eBioCB16	FITC	eBiosciences	E13735-104	25	1:100	V
CD16	FcγRIII	3G8	AF700	BioLegend	B205739	500	1:100	V
CD16	FcγRIII	3G8	AF700	Invitrogen	1218921C	100	1:20	V
CD16	FcγRIII	B73.1	AF700	BioLegend	B201272	300	N/A	V
CD16	FcγRIII	VEP-13	FITC	Miltenyi Biotec	5130805659	N/A	1:100	V
CD10	Neprilysin, CALLA	HI10a	BV510	BioLegend	B253991 B248239	100	1:20	V, NP, PP
CD11b	Mac-1, CR3	ICRF44	BV786	BioLegend	B245198	100	1:40	V, NP, PP
CD11c	CR4	S-HCL-3	FITC	BioLegend	B231151	200	1:40	V, NP
CD16	FcγRIII	eBioCB16	AF700	eBiosciences	4348114	25	1:100	V, NP, PP
CD54	ICAM-1	HCD54	APC	BioLegend	B215798	200	1:100	V, NP
CD62L	L-selectin	DREG-56	BV605	BioLegend	B213455	100	1:100	V, NP, PP
CD66b	CEACAM8	G10F5	APC	BioLegend	B245473	200	1:100	V, NP, PP
CD66b	CEACAM8		AF700	BioLegend				V
CD182	CXCR2	5E8/CXCR2	FITC	BioLegend	B225522	500	1:40	V, NP, PP
CD184	CXCR4	12G5	BV421	BioLegend	B232492	100	1:20	V, NP
HLA-DR	MHCII	L243	BV421	BioLegend	B236256	100	1:40	V, NP
CD274	PD-L1, B7-H1	29E.2A3	BV605	BioLegend	B239150	100	1:100	V, NP
CD274	PD-L1, B7-H1	130021	APC	R&D Systems	ABKW0214101	10	1:100	V

Legend: The concentration is the stock concentration the antibody was supplied at and the dilution is the standard dilution the antibody was used in the staining procedure unless otherwise stated in the results section. The assays each antibody appears in are listed as V: validation experiments; NP: neutrophil phenotyping; PP: pooled plasma experiments.

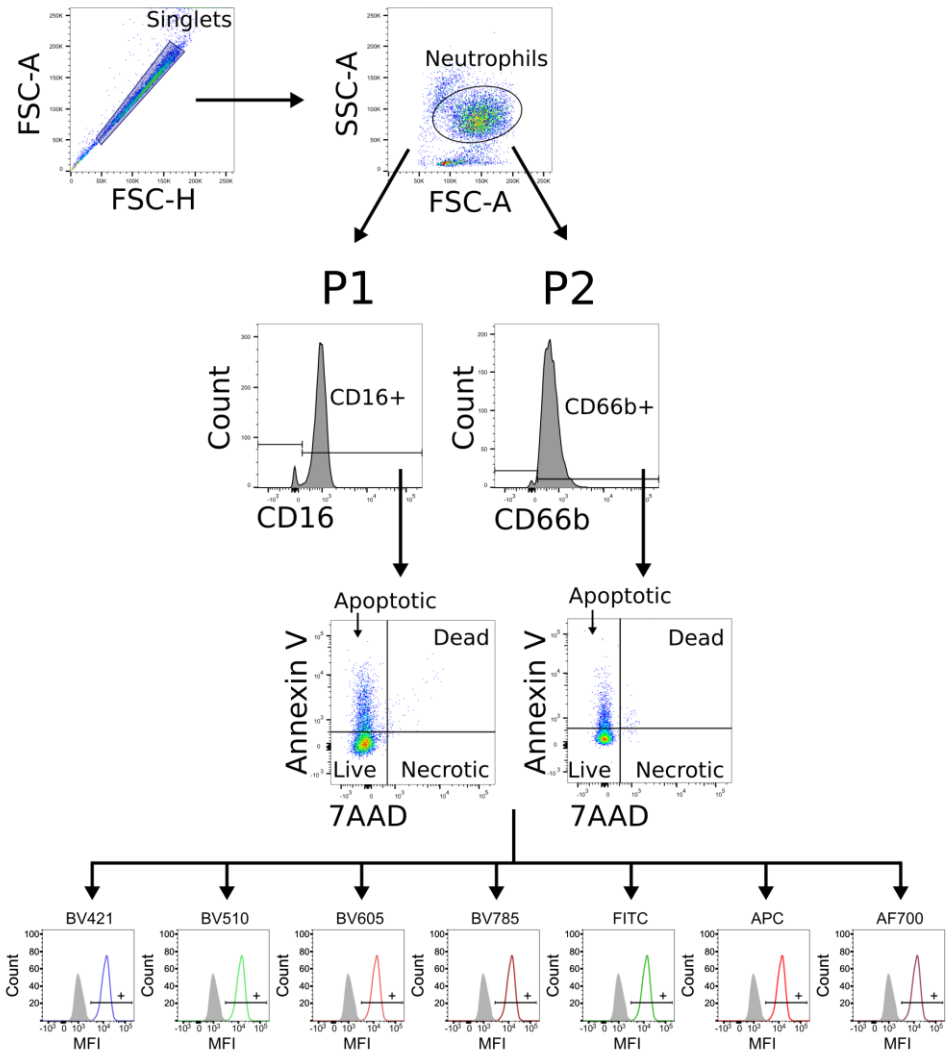

Figure 2.3: Initial flow cytometry gating strategy for neutrophil phenotyping experiments

Firstly, doublets were excluded based on cell circularity, followed by identification of granular cells (neutrophils). Cells positive for CD16 (Panel 1, P1) or CD66b (Panel 2, P2) were selected and viability assessed using 7AAD and Annexin V to identify live (double negative), apoptotic (Annexin V single positive), dead (double positive) or cells dead through a cellular process other than apoptosis, such as necrosis (7AAD single positive). Live and apoptotic cells were then assessed for the surface expression of each subsequent marker by median fluorescence intensity (MFI) and percentage positive (%+).

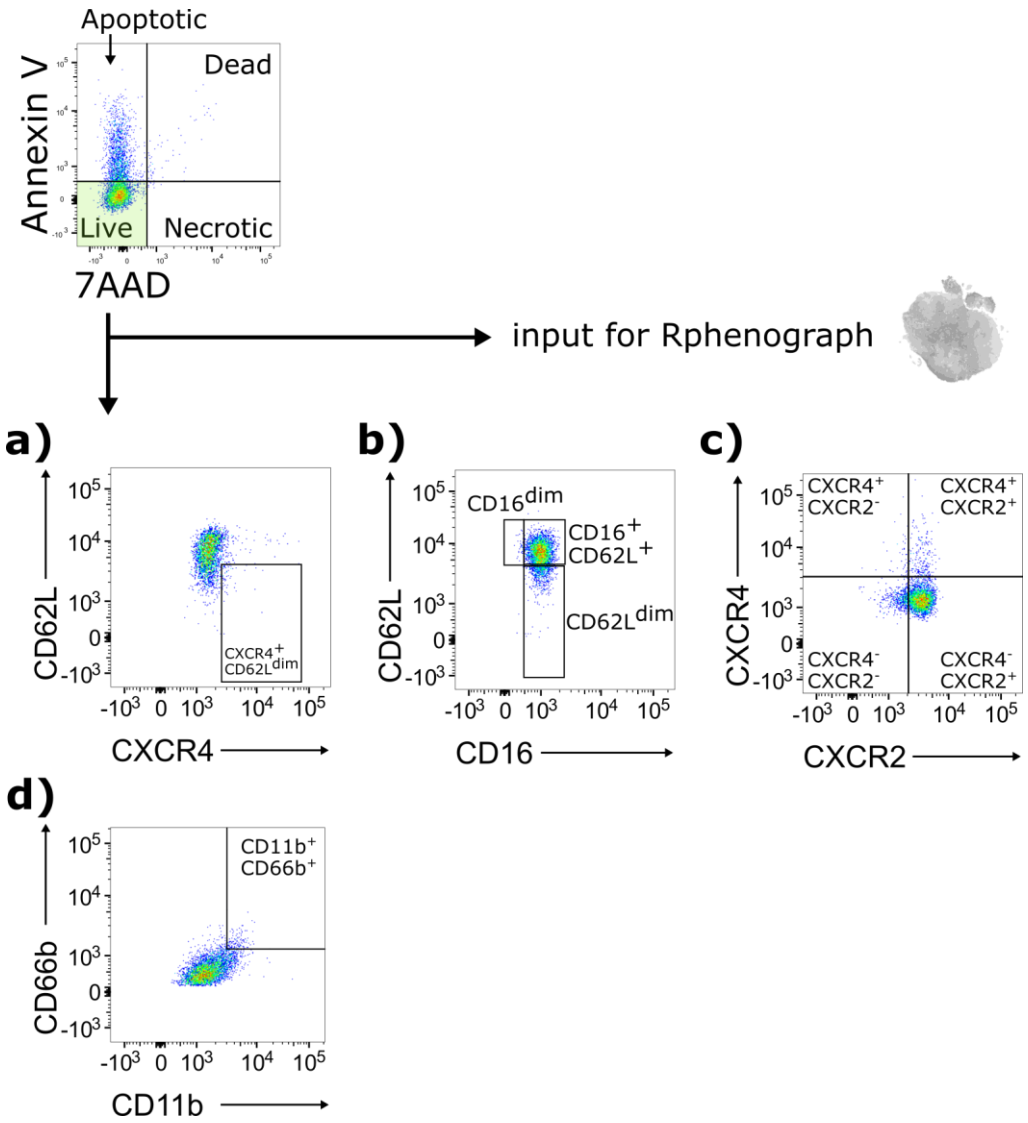

Figure 2.4: Extended flow cytometry gating strategy for neutrophil phenotyping experiments

Live neutrophils (as identified in Figure 2.3) were further analysed based on **a)** CD62L and CXCR4 expression, **b)** CD62L and CD16 expression, **c)** CXCR4 and CXCR2 expression or **d)** CD66b and CD11b expression. Live neutrophils were also used as the input for t-Stochastic Neighbor Embedding (t-SNE) analysis performed using Rphenograph.

2.7 Chemotaxis

Chemotaxis was assessed using Insall chambers (Weber Scientific, Teddington, UK) as previously described (Sapey *et al.*, 2014) and shown in Figure 2.5. BSA (1.125% v/v; Sigma-Aldrich) was added to neutrophils at $2x10^6$ cells/mL isolated as described in Section 2.3. Square 22x22 mm glass coverslips (Leica, UK) were sterilised in H_2SO_4 (0.4M; Sigma-Aldrich) and rinsed in double distilled (dd) H_2O before allowing to dry on filter paper under a petri dish lid (Corning, USA). Once dry, 400 µL of 7.5% BSA (Sigma-Aldrich) was added to the coverslip ensuring it covered the entire surface before tipping off and leaving to dry for 30 seconds under a petri dish lid. Then, 400 µL of neutrophils prepared as in Section 2.3 were then added to the coverslip and incubated at room temperature for 20 minutes. The Insall chamber was rinsed with double-plus media, filled level with double-plus media and then the coverslip inverted over the chamber once the excess neutrophils had been discarded. The remaining liquid in the chemoattractant well was then removed using filter paper and filled with 70 µL of one of the following chemoattractants: CXCL8 (100nM; Bio-techne), fMLP (10nM; Sigma-Aldrich), CXCL12 (1.25µM; Tonbo Biosciences), or fresh double-plus media. A gradient was allowed to form for 1 minute before imaging using a Leica DM 6000 with Leica Application Suite X software (Leica Microsystems, Version 3.3.0.16799) set to record a brightfield image at 20x magnification every 20 seconds for 12 minutes. The resulting data was exported as a combined Tagged Image File Format (TIFF) image for later analysis.

2.7.1 Inhibition of CD10

A CD10 inhibitor, phosphoramidon, was used to block CD10 prior to assessment of neutrophil chemotaxis. Isolated neutrophils were incubated with either double plus media, 0.5 µM, 1 µM,

10 µM or 50 µM of phosphoramidon, covering the concentration range of previously published data, for 30 minutes prior to chemotaxis (Hofman *et al.*, 1998; Stuardo *et al.*, 2004).

2.7.2 Analysis of neutrophil chemotaxis

Combined TIFF images were opened using ImageJ (Version 1.52i; National Institutes of Health, USA). Ten cells were randomly selected and then tracked using the included "Manual Tracking" plugin as previously described (Sapey *et al.*, 2014) and validated within the group where this sample size gave a representative result of the population. Cells were excluded if their path during the recording was substantially altered by collision with another cell. Analysis generated pixel positions for each frame for every cell tracked that was further analysed by calculating the following parameters to describe the movement of the cell divided into two types, per frame and whole time-lapse (Figure 2.6):

Per frame:

- **Speed:** the distance travelled by the cell divided by time, calculated as pixels per frame and converted to µm/minute using the frame length and distance per pixel;
- **Velocity:** the speed travelled towards (or away from) the chemoattractant, calculated from the movement in the y-direction only;
- **Chemotaxis index:** a measure of movement towards the chemoattractant, calculated from the sine transformation of the angle in radians between the cell movement and the x-axis (θ);
- **Persistence:** a measure of the change in the angle of movement of the cell, calculated by the equation $\frac{1}{2}\cos(\theta) + \frac{1}{2}$, where x is the change of the angle in radians.

Whole time-lapse:

- **Displacement:** the distance between the start point and endpoint of the track. This was independent of direction by removing the sign as follows $\sqrt{(x)^2}$;
- **Cumulative distance:** the total distance travelled by the cell during the track, calculated at each frame. This was independent of direction by removing the sign as follows $\sqrt{(x)^2}$;
- **Directness:** a measure of straightness of the path taken by the cell, calculated by displacement/cumulative distance;
- **Centre of mass (COM):** the coordinates of the endpoint of the cell measured from a starting position of 0,0.

Each of these parameters was then averaged to provide a result for each condition formed from ten individual cells.

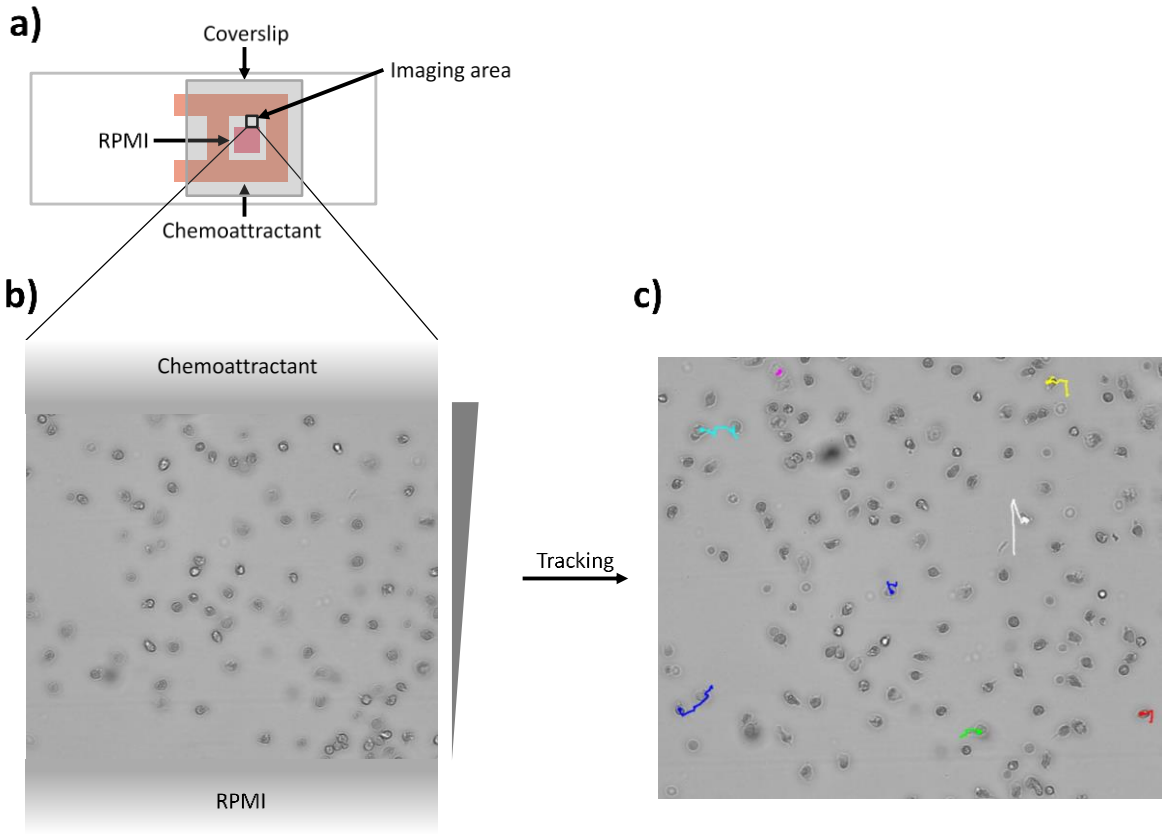

Figure 2.5: Schematic of an Insall chamber and tracking of neutrophils

a) Isolated neutrophils were adhered to a coverslip before inversion onto the Insall chamber. RPMI (as a negative control) or chemoattractant (such as CXCL8) was loaded into the outer well. **b)** a typical brightfield image of neutrophils adhered to the coverslip with **c)** an example of ten tracked cells using the ImageJ "manual tracking" plugin overlaid with the cell path.

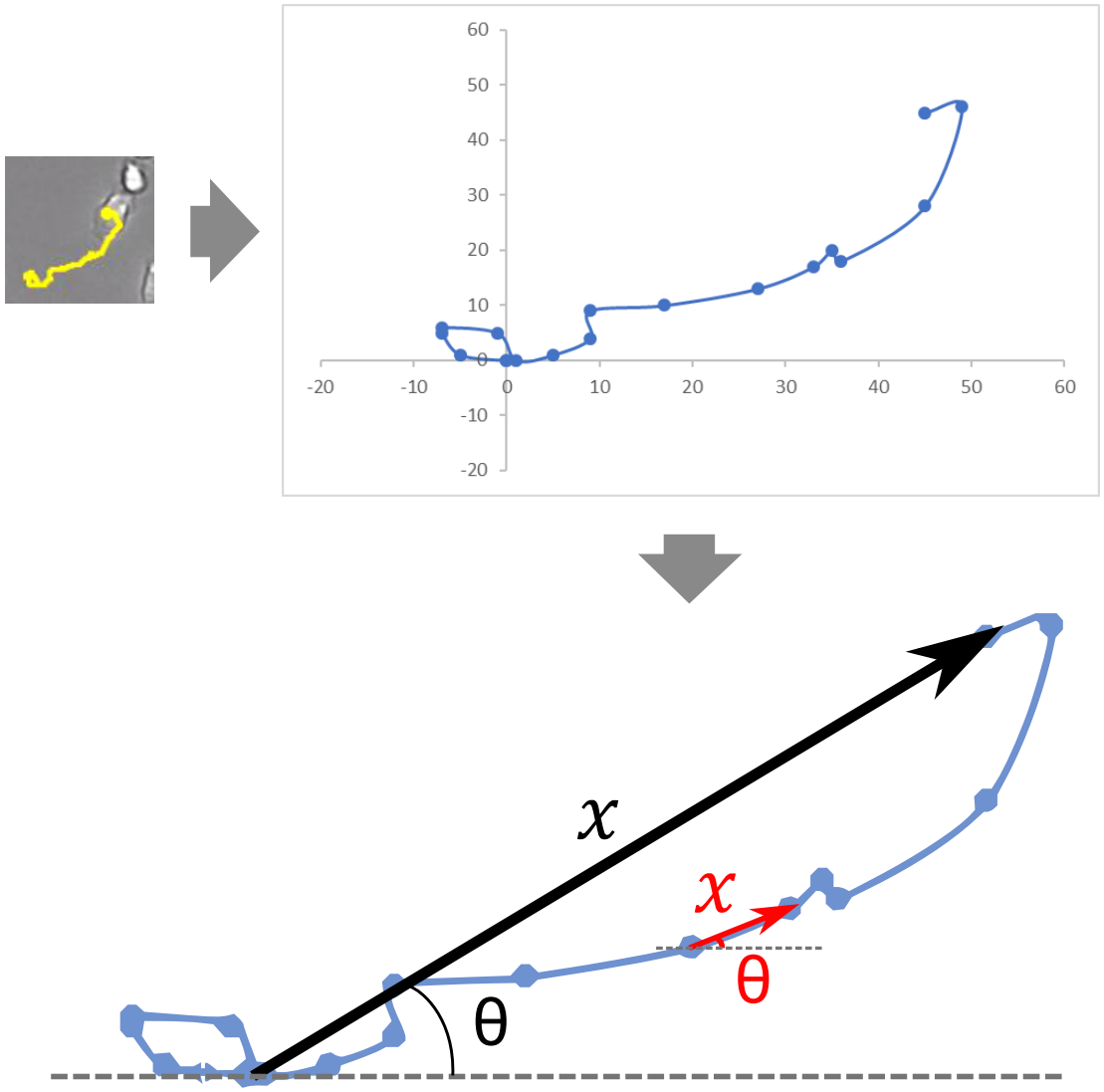

Figure 2.6: Schematic of neutrophil chemotaxis analysis

Conversion from light microscopy image and image trace to plotted points allowed the analysis of parameters for a single tracked cell. The black arrow, forming the angle (θ) and distance (x) show parameters calculated over the whole time-lapse. Parameters calculated per frame are indicated by the red arrow and symbols.

2.8 Quantification of CXCL12 using enzyme-linked immunosorbent assay (ELISA)

CXCL12 levels in platelet-poor human plasma samples were quantified using the Quantikine CXCL12 ELISA Kit (Bio-techne) according to manufacturer's guidelines. Briefly, all supplied reagents and frozen plasma samples were thawed to room temperature. Platelet removal was achieved by spinning plasma samples at 10000 *g* for 10 minutes at 4^{o}C in a microcentrifuge (Prism R; Labnet, NJ, USA). Lyophilised CXCL12 was reconstituted in distilled water and diluted to 10000 pg/mL in supplied diluent to obtain the top CXCL12 standard. A serial 1:2 dilution in the supplied diluent was subsequently performed down to a final concentration of 156 pg/mL, including a diluent blank as the 0 pg/mL control. To each well of the supplied capture-antibody coated 96-well plate, 100 µL of assay diluent was added, followed by 100 µL of each standard or sample in duplicate. The plate was incubated for 2 hours on an orbital plate shaker (ThermoFisher Scientific, UK) at 500 RPM. The plate was then washed 4 times using supplied wash buffer ensuring all liquid from the plate was removed by blotting on clean absorbent paper. To each well, 100 µL of supplied detection antibody was added and incubated for 2 hours on a shaker as previously described. Again, the plate was washed as described and 200 µL of a 1:1 solution of supplied reagent A and reagent B was added to each well. The plate was incubated for 30 minutes in the dark, followed by addition of 50 µL of supplied stop solution to each well. The plate was then read on an absorbance plate reader (BioTek Synergy HT; NorthStar Scientific, VT, USA) at 450 nm and 540 nm. The final absorbance value was obtained as follows:

$$Absorbance = Abs_{450} - Abs_{540} - Abs_{blank}$$

The concentration of CXCL12 was obtained from direct interpolation of the standard curve fitted using a 4-parameter sigmoidal least-squares fit. Assay range was 156 -10000 pg/mL with a sensitivity of 47 pg/mL.

2.9 Extraction of RNA

Cellular RNA was stabilised by pelleting isolated neutrophils (Section 2.3), discarding the supernatant and resuspending in 1 mL of RNALater (ThermoFisher Scientific, UK) and storing at -20^{o}C for up to 1 week before transferring to long-term storage at -80^{o}C. The total number of cells stored was recorded for each sample.

RNA was isolated using the RNEasy Mini Kit (QIAGEN, Manchester, UK) with QIAshredder and RNase-free DNase (both QIAGEN, UK) according to the manufacturer's guidelines. Briefly, neutrophils in RNALater were thawed on ice and up to 1x10^{7} neutrophils were removed into a fresh 1.5 mL microcentrifuge tube and diluted up to 1.5 mL in ice-cold PBS. Cells were centrifuged at 500 *g* for 5 minutes and the supernatant discarded. Cells were disrupted using 350 µL of supplied RLT buffer before transferring to a supplied QIAshredder tube. Tubes were centrifuged at 10000 *g* for 2 minutes and 350uL of 70% ethanol added to each collection tube. Samples were then transferred to a supplied RNeasy spin column and centrifuged at 9000 *g* for 30 seconds. The flow-through was discarded, 350 µL of supplied RW1 added to the column and centrifuged at 9000 *g* for 30 seconds. The flow through was discarded and 80 µL of DNase mix (1:7 mix of supplied DNase I stock to Buffer RDD) added to each column. Columns were incubated at RT for 15 minutes before adding 350 µL of RW1 and centrifuged at 9000 *g* for 30 seconds. The flow-through was discarded and 500 µL of supplied Buffer RPE working solution was added and centrifuged at 9000 *g* for 30 seconds. The flow-through was discarded and 500

µL of Buffer RPE was added and centrifuged at 9000 *g* for 2 minutes. Each column was placed in a fresh collection tube and centrifuged at 9000 *g* for 1 minute. Each column was then transferred to a supplied 1.5 mL collection tube and 30 µL of supplied nuclease-free water was added to the column and centrifuged at 9000 *g* for 1 minute. Samples were immediately placed on ice and tested using 1 µL of sample on a Nanodrop 2000 (ThermoFisher Scientific, UK) where ratios (260nm to 280nm and 260nm to 230nm) above 2.0 indicated good sample purity, between 2.0 and 1.8 indicated adequate purity and below 1.8 indicated poor purity. Further quality control was carried out by University of Birmingham Genomics Service (Birmingham, UK) including fluorescence quantification using Qubit (ThermoFisher Scientific) and RNA integrity score (RIN) using a Tapestation (Agilent, CA, USA) where a RIN score of 8 or higher is considered good, between 7 and 8 adequate and below 7 as poor. RNA was stored immediately at -80^{o}C.

2.10 RNA sequencing and analysis

Isolated RNA was thawed and diluted to 11.6 ng/mL in 30uL of nuclease-free water and placed into a skirted 96-well PCR plate and sealed with a PCR adhesive plate seal (both ThermoFisher Scientific, UK). Samples were shipped on dry ice to the Oxford Genomics Centre for sequencing: poly-A mRNA selection; cDNA generation; 150 base pair paired-end sequencing at 300 million reads per unit; sequence alignment to GRCh37.EBVB95-8wt.ERCC reference genome.

2.10.1 Analysis workflow

An overview of the analysis pipeline is shown in Figure 2.7. Sequence data in .fastq format was uploaded to the Galaxy web platform at usegalaxy.eu (Afgan *et al.*, 2016) using FileZilla (Tim Kosse; Version 3.46.0). The human genome GRCh38.p13.genome and annotations gencode.v33.annotation were obtained from GENCODE (Frankish *et al.*, 2019) and were also uploaded to Galaxy. Each paired read was aligned using RNA STAR (Dobin *et al.*, 2013), gene counts calculated with FeatureCounts (Liao, Smyth and Shi, 2014) and gene abundance calculated using StringTie2 (Kovaka *et al.*, 2019). Differential expression analysis was performed using DESeq2 (Love, Huber and Anders, 2014). The Galaxy server that was used for these calculations is in part funded by Collaborative Research Centre 992 Medical Epigenetics (DFG grant SFB 992/1 2012) and German Federal Ministry of Education and Research (BMBF grants 031 A538A/A538C RBC, 031L0101B/031L0101C de.NBI-epi, 031L0106 de.STAIR (de.NBI)). The full workflow can be seen in Figure 2.7 and freely accessible at https://usegalaxy.eu/u/mxh619/w/rnaseq-primary-analysis-mh; https://usegalaxy.eu/u/mxh619/w/downstream-rna-analysis.

Gene ontology (GO) analyses were performed using a combination of ShinyGO (Ge, Jung and Yao, 2019) and ClueGO (Bindea *et al.*, 2009). Heatmaps were generated using Shinyheatmap (Khomtchouk, Hennessy and Wahlestedt, 2017).

2.11 Figures and graphics

All graphs were generated using Graphpad Prism (Version 8.4.3 for Windows, GraphPad Software, San Diego, California USA). Where appropriate, figures and diagrams are original and generated using Inkscape (Version 0.92, Inkscape.org). Other third-party software is referenced in the specific sections where they were used (Sections 2.6.3 and 2.7.2).

2.12 Statistical analysis and power calculations

Data were assessed for a normal distribution using the Shapiro-Wilk test, where a p-value>0.05 was considered normally distributed. Pilot data from previous work indicated 14-20 samples gave an 80% power to detect a 20% difference in both chemotaxis and receptor marker expression.

For normally distributed data, parametric tests were used. A paired t-test was used for data between two treatment groups on the same participants. For non-related samples (e.g. healthy vs. COPD), an unpaired t-test was used. For analysis between more than two independent groups where all groups were normally distributed, a one-way ANOVA was performed. Multiple comparisons were then carried out using either Tukey's multiple comparisons (comparing all groups to each other) or Dunnett's multiple comparisons (comparing data to a control group). Pearson's correlation coefficients were used to determine associations between two variables. Analysis was performed by Graphpad Prism (Version 8.4.3 for Windows, GraphPad Software, San Diego, California USA).

For non-normally distributed data, or where at least one comparison group was non-normally distributed, non-parametric tests were used. Wilcoxon matched-paired signed-rank test was

used for analysing two treatment groups on the same participants. For non-related samples (e.g. healthy vs. COPD) a Mann-Whitney test was used. For analysis between more than two independent groups, Kruskal-Wallis analysis was performed. Multiple comparisons were then carried out using Dunn's multiple comparisons. Spearman's rank correlation coefficients were used to determine associations between two variables. Analysis was performed by Graphpad Prism (Version 8.4.3 for Windows, GraphPad Software, San Diego, California USA).

For demographic data where information was categorical, the Fishers Exact test was used. Analysis was performed using the fisher.test command within R (Version 4.0.0 for Windows, R Core Team, 2018)

In all cases, a p-value<0.05 was considered to be statistically significant.

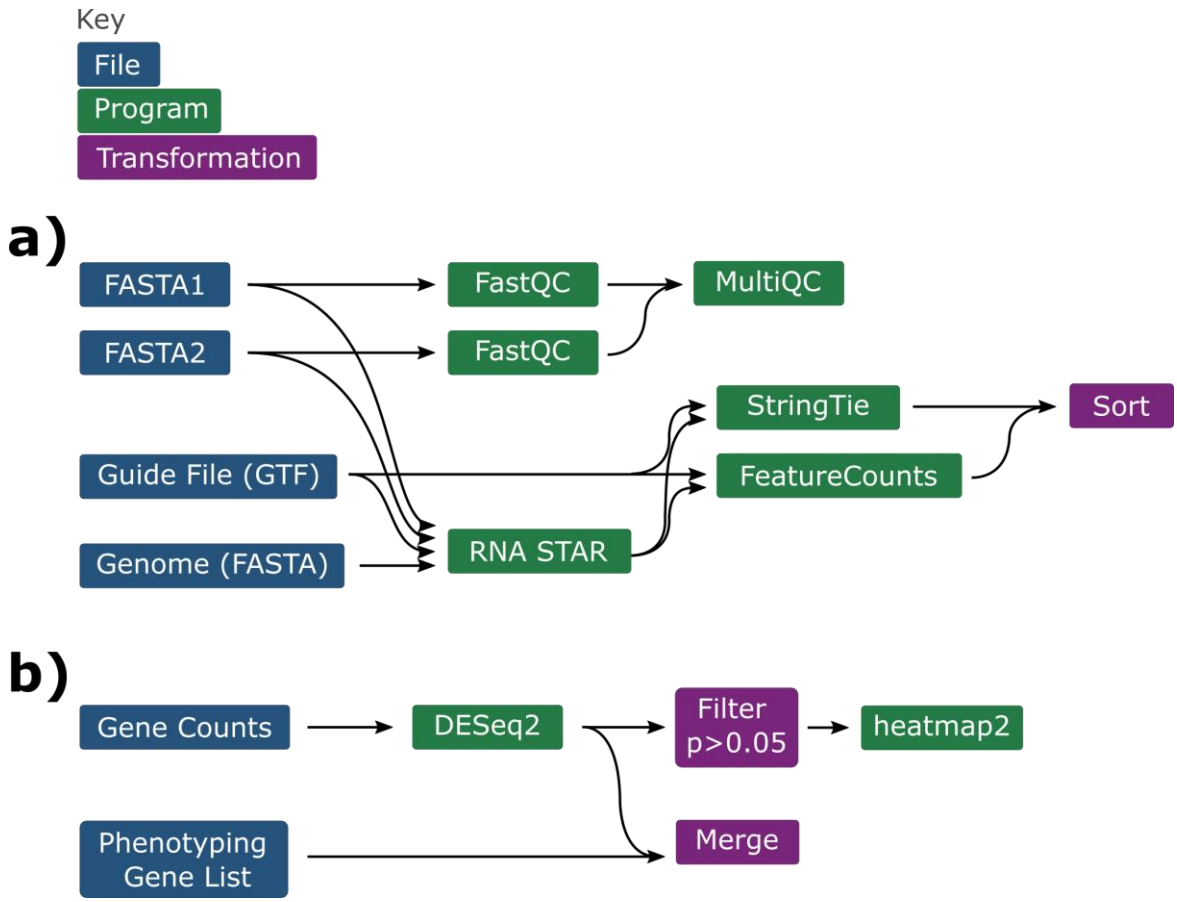

Figure 2.7: Simplified workflow for RNASeq analysis in Galaxy

a) Sequenced FASTA files (forward, 1; reverse, 2), including annotated gene information (guide file) and the complete human genome were uploaded to the Galaxy server. FastQC analysis was performed on each FASTA file and merged into a single file using MultiQC. Both FASTA files, the guide file and genome file were used to map the sequence to the genome. Gene expression and quantification was performed using StringTie and FeatureCounts before sorting based on total expression of identified genes. **b)** Normalised gene counts from FeatureCounts, grouped based on participant group, were used as the input for differential gene expression. The counts and p-value from this output was merged with genes of interest based on the flow cytometry phenotyping panel (Table 2.1), and all other genes with a p-value <0.05 were filtered before plotting on a heatmap of gene expression.

CHAPTER 3:

VALIDATING THE USE OF MULTI-COLOUR FLOW CYTOMETRY TO PHENOTYPE NEUTROPHILS

3.1 Brief introduction

Flow cytometry is a common technique used in an increasing array of applications including measuring effector functions (for example phagocytosis; Miksa *et al.*, 2009), cell viability and intracellular markers (Adan *et al.*, 2017). Whilst being a staple in both clinical (Bakke, 2000) and research settings, variations in reagents and flow cytometers leads to poorly comparable results between experiments and studies and, therefore, rigorous validation of reagents and standardisation of methodology is required (Maecker, McCoy and Nussenblatt, 2012).

The analysis of flow cytometry data also poses a challenge for the reproducibility of data. A variety of methods can be employed to analyse flow cytometry data (Bashashati and Brinkman, 2009), many of which require time and an in-depth understanding of the data to avoid erroneous placing of gates and reporting of incorrect or misleading statistics.

Here we establish two antibody panels to assess the phenotype of isolated human neutrophils from peripheral blood. Appropriate fluorophores and dyes that could be used as part of a multi-parameter flow cytometry panel to quantify surface expression of markers and cell viability were identified. A robust gating strategy was developed to identify neutrophil viability and phenotypes based on surface expression of key functional markers. Standardisation beads were validated for use to ensure cytometer performance between experiments based on EuroFlow guidelines (Kalina *et al.*, 2012).

3.2 Results

3.2.1 Target selection

In order to investigate the phenotype of neutrophils based on their surface expression, a flow cytometry panel was designed to target various of receptors and adhesion molecules linked to neutrophil activation, senescence, pro-inflammatory and anti-inflammatory phenotypes (as introduced in Section 1.6) and are summarised in Table 3.1. In total, 13 targets were identified and, given the limitation to simultaneously detect these markers using the flow cytometers available, split between two panels as indicated in Table 3.1.

3.2.2 Fluorophore selection

A spectral viewer (Figure 3.1a) was used to identify 8 fluorophores that had minimal spectral overlap, were readily commercially available as antibody conjugates and compatible with 7AAD - the chosen DNA dye for these experiments due to its low cost and previously validated use (Schmid *et al.*, 1992). Due to the spatial separation of the excitation lasers, each laser path could be considered individually (Figure 3.1b-e). Each fluorophore was then matched with a target depending on three characteristics: relative fluorophore brightness; predicted target abundance and antibody availability. Lower abundance targets were matched with a relatively brighter fluorophore to enable better detection and, conversely, high abundance targets with dimmer fluorophores to avoid saturation of the signal and increasing spectral overspill. These results are summarised in Table 3.2.

Table 3.1: Target selection, functional relevance and panel distribution

Target	Alternative names	Function	Panel	References
Activation				
CD11b	Mac-1, CR3	Found in combination with CD18 to form complement receptor 3 (CR3), it contributes to several neutrophil functions including adhesion, migration and degranulation. It is also rapidly recruited to the plasma membrane upon neutrophil activation.	1,2	(Arnaout MA, 1990; Van Spriel *et al.*, 2001)
CD66b	CEACAM8	CD66b a cellular adhesion molecule and is also exclusively expressed by neutrophils and can therefore be used to identify neutrophils in mixed cell populations. Expression of CD66b increases upon neutrophil stimulation.	2	(Lakschevitz *et al.*, 2016)
CD62L	L-selectin	The only selectin expressed on neutrophils and mediates rolling along the endothelium. L-selectin is also shed upon neutrophil activation and extravasation.	1	(Stadtmann *et al.*, 2013)
Senescence				
CD182	CXCR2	C-X-C receptor 2 is the chemokine receptor for IL-8 (CXCL8) and is expressed on mature neutrophils. It has been implicated in the neutrophil senescence, as aged neutrophils show reduced CXCR2 expression.	1	(Eash *et al.*, 2010; Stadtmann and Zarbock, 2012)
CD184	CXCR4	C-X-C receptor 4 is the chemokine receptor for stromal-derived factor 1 (SDF-1 or CXCL12). It is highly expressed during neutrophil maturation and surface expression is lost before release into the bloodstream. Re-expression of CXCR4 is thought to occur during senescence; allowing homing back to the bone marrow.	1	(Eash *et al.*, 2010)
Pro-inflammatory				
HLA-DR	MHC-II	A potential role has been identified for neutrophils to express HLA-DR and cross-present antigen to CD4+ T cells, thereby promoting T cell proliferation.	2	(Vono *et al.*, 2017)

Target	Alternative names	Function	Panel	References
Anti-inflammatory				
PD-L1	CD274	Programmed-death ligand 1 is a ligand for programmed-death receptor 1 (PD-1) and ligation is known to inhibit T cell activation. Some evidence suggests a role in cancer-associated neutrophils in immunosuppression.	2	(McNab *et al.*, 2011)
Viability				
Annexin V		Binds phosphatidylserine, displayed on the outer leaflet of the plasma membrane as cells undergo apoptosis.	1,2	(Fadok *et al.*, 1998; Schutte *et al.*, 1998)
7AAD		A DNA-binding dye that can only penetrate a permeabilised plasma membrane, a process that occurs during cell death.	1,2	(Schmid *et al.*, 1992)
Other				
CD54	ICAM-1	Reverse endothelial migration causes increased expression of CD54 on the surface of neutrophils.	1	(Buckley *et al.*, 2006)
CD11c	CR4	An integrin that forms part of complement receptor 4 and has been shown to be increased on neutrophils from sepsis patients, indicative of systemic inflammation and inhibit T cell responses.	2	(Pillay *et al.*, 2012; Lewis *et al.*, 2015)
CD10	Neprilysin, CALLA	An enzyme that hydrolysis fMLP, however, is not expressed on immature neutrophils and expressed on maturation and release into the bloodstream.	1,2	(Marini *et al.*, 2017)
CD16	FcγRIII	An Fc gamma receptor constitutively expressed by human neutrophils that is functionally involved in the detection of immune complexes.	1	(Fossati *et al.*, 2002a)

Legend: Each target and common alternative names listed with an overview of function alongside the panel the marker is included in.

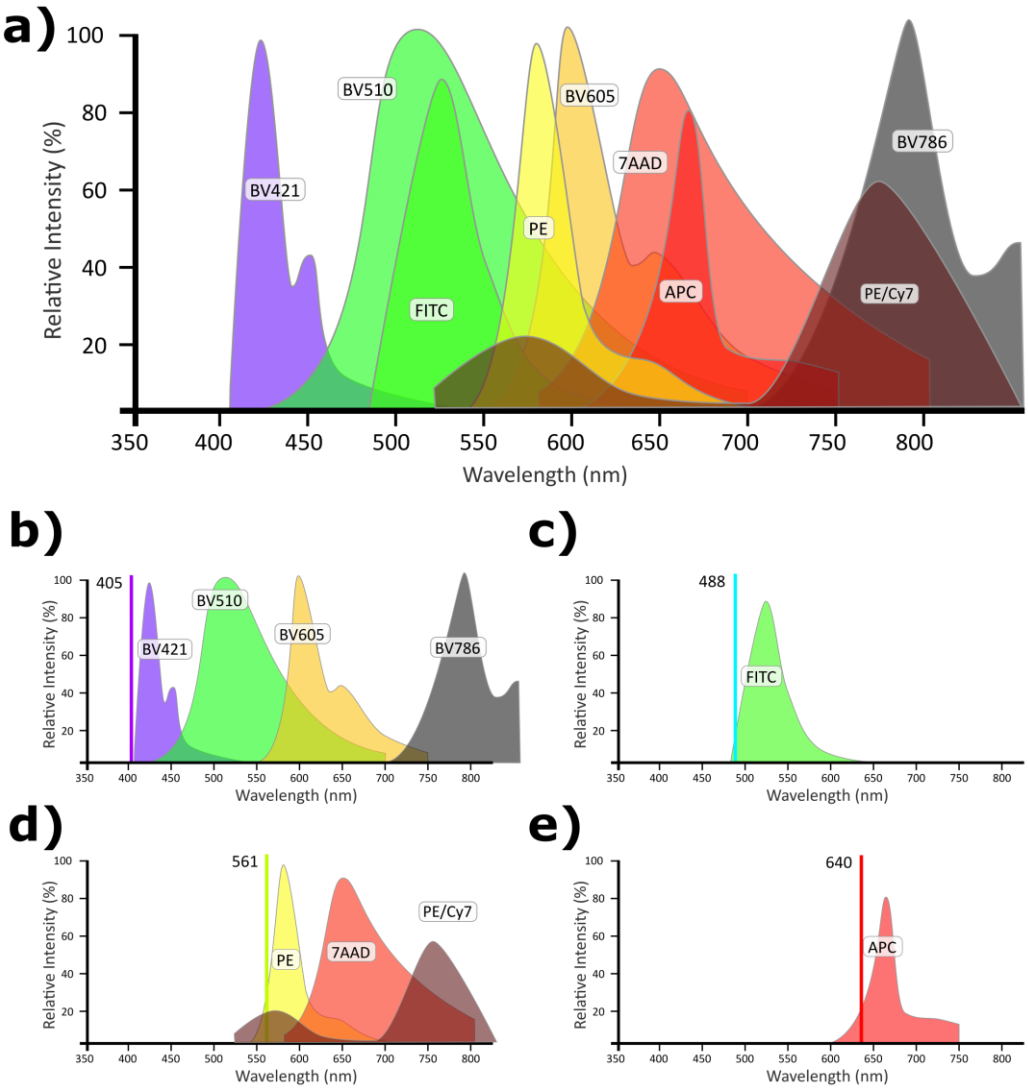

Figure 3.1: Emission spectra of conjugates for a 9-colour flow cytometry panel

Schematic representation of relative intensity (%) of **a)** each fluorophore combined and then separated by excitation laser: **b)** 405nm, **c)** 488nm, **d)** 561nm, **e)** 640nm as denoted by vertical line.

Table 3.2: Relative fluorophore brightness and target abundance.

Fluorophore	Relative brightness	Matched target	Predicted abundance
BV421	Very bright	CXCR4	Low
		HLA-DR	Low/unknown
PE	Very bright	Annexin V	Variable
PE/Cy7	Bright	CD16	High
BV605	Bright	PD-L1	Low/unknown
		CD62L	High
BV786	Bright	CD11b	High
APC	Bright	CD54	Low
		CD66b	Moderate
FITC	Moderate	CXCR2	High
		CD11c	Moderate
BV510	Moderate	CD10	Moderate

Legend: Relative brightness listed were obtained from BD Biosciences online supplement (BD Biosciences, 2014). Predicted target abundance determined from existing literature as discussed in Section 1.6.

3.2.3 Titration of antibodies

To obtain the optimal concentrations of each antibody, a titration was carried out using isolated neutrophils (Figure 3.2, Figure 3.3 and Figure 3.4) or, where expression by neutrophils was unknown, PBMCs (Figure 3.5 and Figure 3.6) from healthy volunteers incubated with the antibody (starting with the manufacturer recommended 1:20 dilution) or matched isotype control (Table 3.3). In each case, the median fluorescence intensity (MFI) was recorded, and a staining index calculated (Section 2.6.3) and summarised in Table 3.4.

Optimal antibody concentrations were determined by clear distinctions between the stained population and isotype control population on histograms and, where necessary, informed by the staining index. For clarity, these analyses have been split into activation markers (CD11b and CD66b), markers that are highly or moderately expressed on neutrophils (CD62L, CD11c, CD10, CD16 and CXCR2) and markers that have low expression on neutrophils (PD-L1, HLA-DR, CXCR4 and CD54). The staining index for all antibodies in Sections 3.2.3.1 - 3.2.3.3 and the selected antibody concentrations for all future experiments are summarised in Table 3.4.

3.2.3.1 CD11b and CD66b

Stimulation of isolated neutrophils with 10µM fMLP increased the MFI of CD11b (Figure 3.2a) and, to a lesser extent, CD66b (Figure 3.2b) compared with unstimulated neutrophils as expected (Abdel-Salam and Ebaid, 2014; Krogh Nielsen *et al.*, 2017). Reducing the concentration of anti-CD11b reduced the staining index between unstained and unstimulated neutrophils, however, increased the staining index between unstimulated and stimulated neutrophils (Table 3.4). Therefore, a concentration of 1:40 was selected to achieve the best balance between separating both activated and unactivated cells from CD11b negative cells.

In contrast, reducing the concentration of anti-CD66b had little effect on either staining indexes (Figure 3.2b and Table 3.4) and therefore the lowest concentration of 1:100 was selected for future experiments.

3.2.3.2 CD62L, CD11c, CD10, CD16 and CXCR2

For anti-CD62L (Figure 3.2c), CD10 (Figure 3.3b) and CD16 (Figure 3.3c), reducing the concentration of the antibody reduced the MFI and also reduced the staining index (Table 3.4). As both CD62L and CD16 are highly expressed and good separation was observed between positive and negative peaks (Figure 3.2c and Figure 3.3c respectively), the lowest antibody concentration was selected despite lower staining indexes.

Reducing the antibody concentration of anti-CD11c (Figure 3.3a) and anti-CXCR2 (Figure 3.4) reduced the MFI but also reduced staining by the isotype control, therefore increasing the staining index between 5uL and 2.5uL (Table 3.4). Based on this, a concentration of 1:40 was chosen for both antibodies to provide the best resolution between positive and negative peaks.

As there was poor separation between the unstained and anti-CD10 stained population (Figure 3.3b) and a reduction in the staining index with reducing antibody concentration, the highest concentration (1:20) was selected for anti-CD10.

3.2.3.3 PD-L1, HLA-DR, CXCR4 and CD54

For anti-PD-L1 (Figure 3.5), HLA-DR (Figure 3.6a), and CXCR4 (Figure 3.6b), reducing the concentration of the antibody reduced the MFI and also reduced the staining index (Table 3.4). However, good separation between the positive and negative peak was observed with anti-PD-L1 (Figure 3.5) and, therefore, the lowest antibody concentration (1:100) was

selected. Poor separation was observed with anti-CXCR4 and so the highest concentration (1:20) was selected.

In contrast to all other antibodies, reducing the antibody concentration of CD54 (Figure 3.6c) increased the staining index between 1:20 and 1:40, and again to 1:100 (Table 3.4). Good separation between peaks was also observed and therefore, the lowest concentration (1:20) was selected.

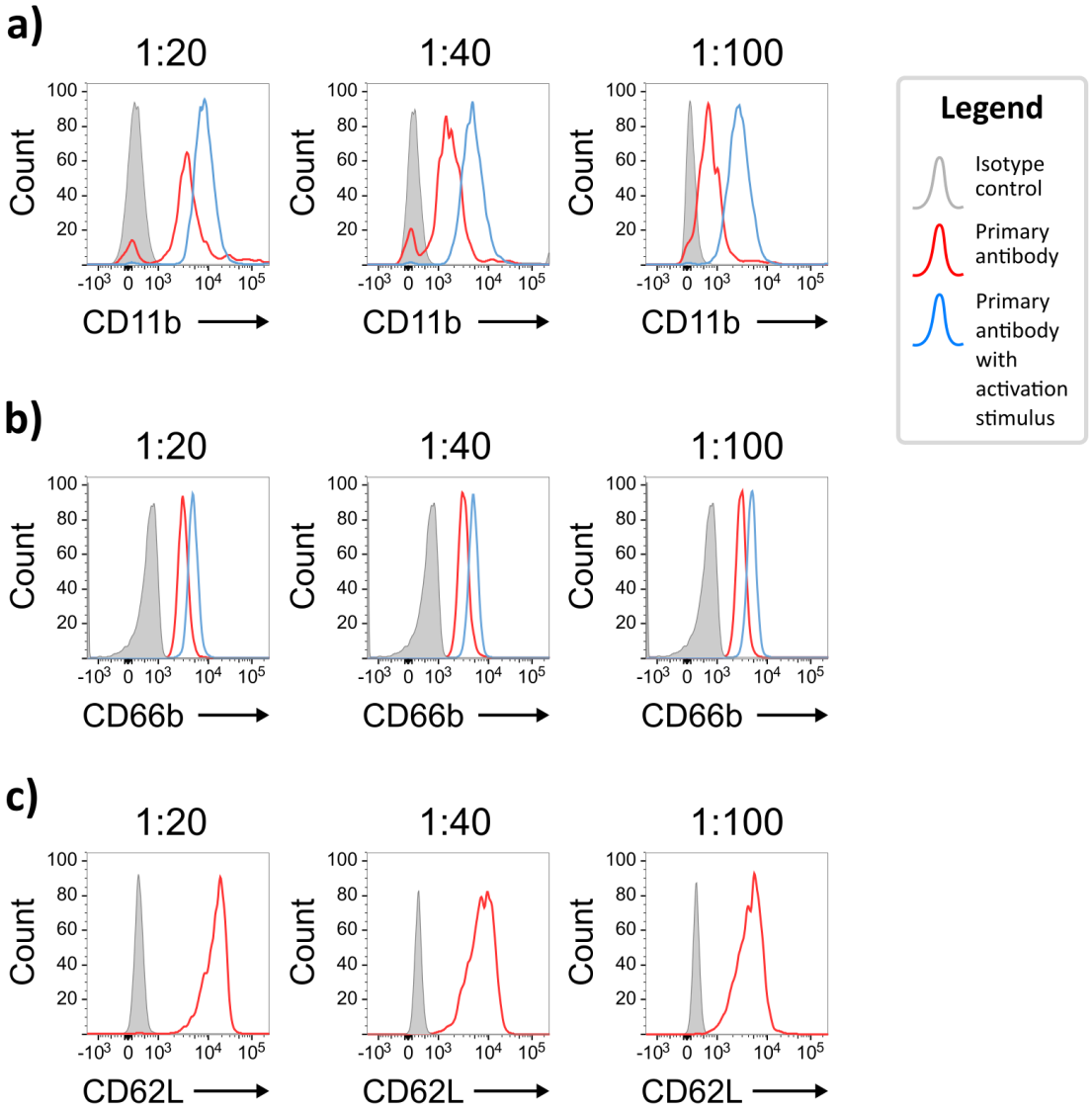

Figure 3.2: Titration of anti-CD11b, anti-CD66b and anti-CD62L

Isolated neutrophils from a healthy donor (n=1) were untreated (red trace) or treated with 10µM fMLP (blue trace) for 20 minutes. Neutrophils were then incubated with the concentration of antibody shown above each plot and the fluorescence intensity of **a)** anti-CD11b, **b)** anti-CD66b or **c)** anti-CD62L (red trace) or corresponding isotype control (grey trace) were measured by flow cytometry.

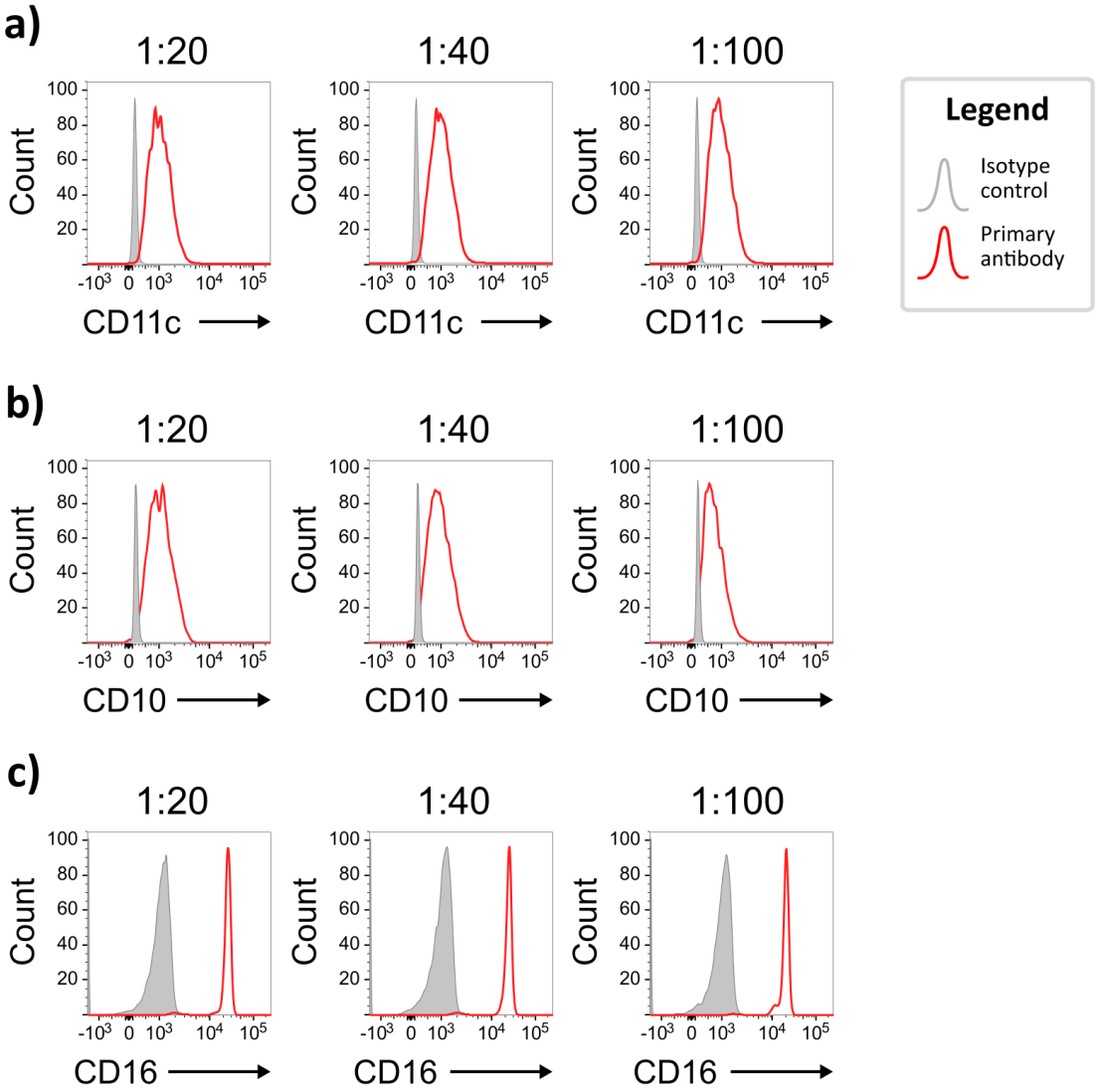

Figure 3.3: Titration of anti-CD11c, anti-CD10, anti-CD16

Isolated neutrophils from a healthy donor (n=1) were incubated with the concentration of antibody shown above each plot. The fluorescence intensity of **a)** anti-CD11c, **b)** anti-CD10 or **c)** anti-CD16 (red trace) or corresponding isotype control (grey trace) were measured by flow cytometry. Dilution denoted above each plot.

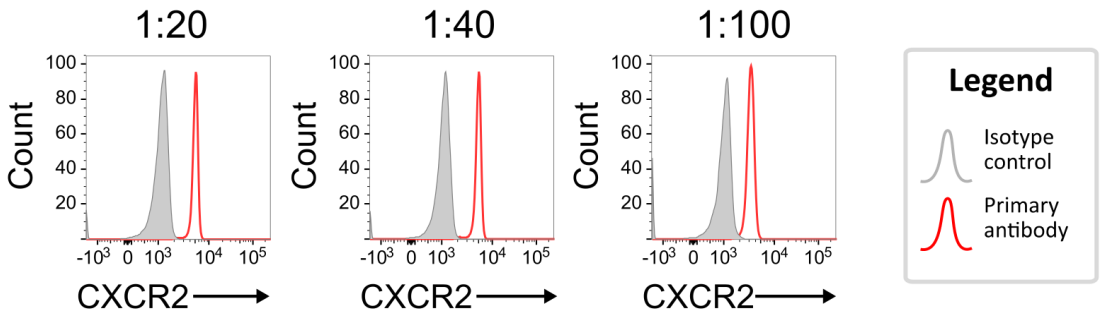

Figure 3.4: Titration of anti-CXCR2

Isolated neutrophils from a healthy donor (n=1) were incubated with the concentration of antibody shown above each plot. The fluorescence intensity of anti-CXCR2 (red trace) or corresponding isotype control (grey trace) were measured by flow cytometry.

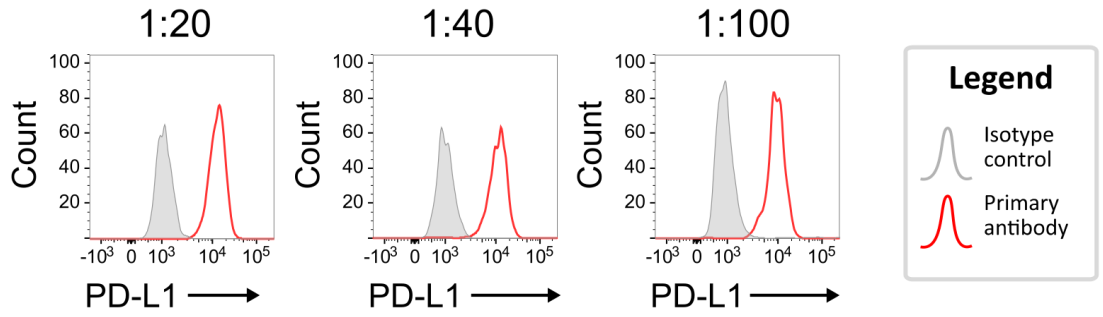

Figure 3.5: Titration of anti-PD-L1

Isolated PBMCs from a healthy donor (n=1) were incubated with 10ng/mL phytohaemagglutinin for 3 days prior to antibody incubation at the stated dilution above each plot. The fluorescence intensity of anti-PD-L1 (red trace) or corresponding isotype control (grey trace) measured by flow cytometry.

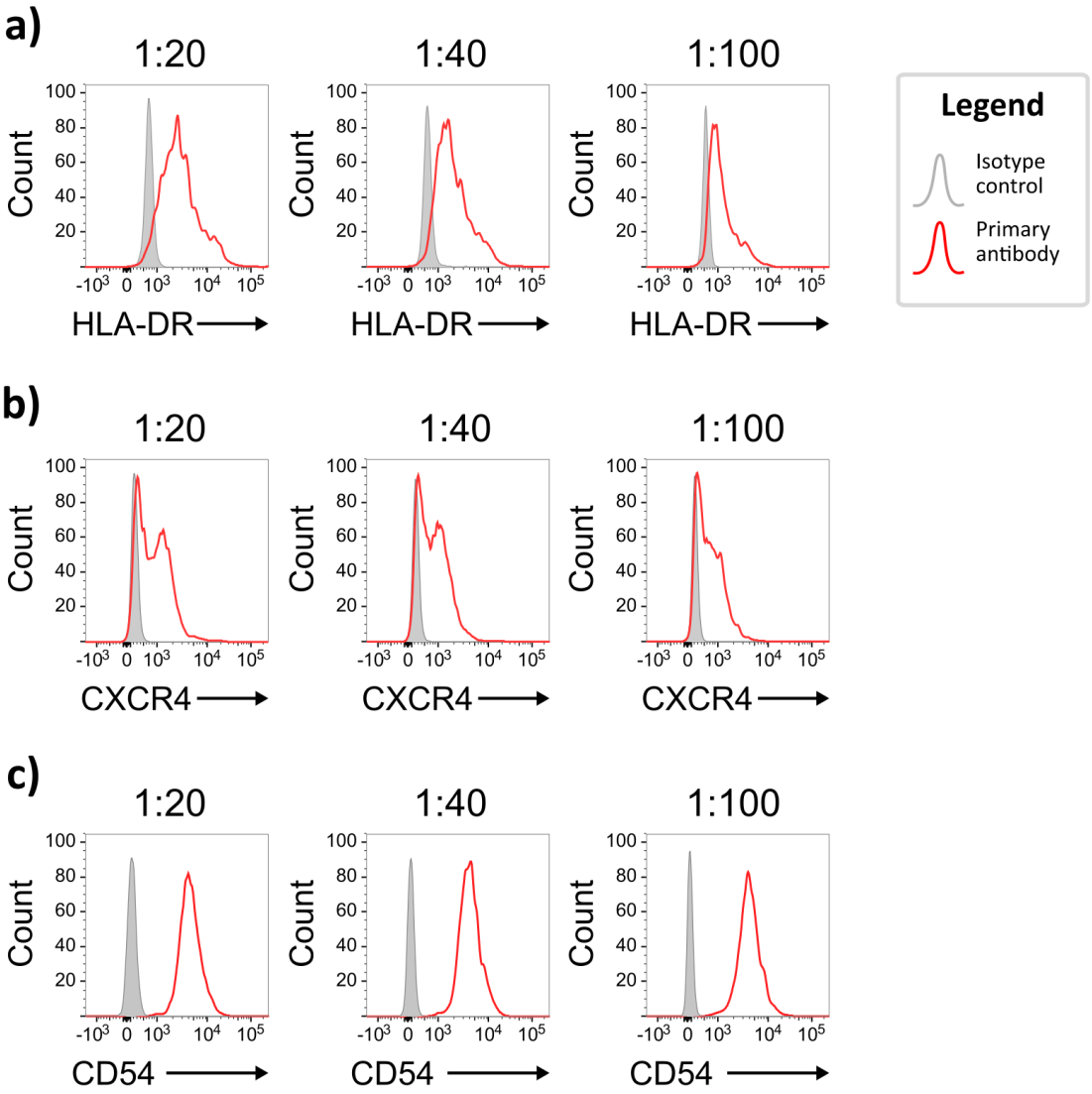

Figure 3.6: Titration of anti-HLA-DR, anti-CXCR4 and anti-CD54

Isolated PBMCs from a healthy donor (n=1) were incubated with the concentration of antibody shown above each plot. The fluorescence intensity of **a)** anti-HLA-DR, **b)** anti-CXCR4, **c)** anti-CD54 or corresponding isotype control (grey trace) were measured by flow cytometry.

Table 3.3: Primary antibodies and corresponding isotype controls with dilutions.

Primary antibody	Concentration (µg/mL)	Isoform	Isotype concentration (µg/mL)	Primary dilution	Isotype dilution
CD11b	100	IgG1, κ	100	1:20	1:20
CD66b	200	IgM, κ	200	1:20	1:20
CD62L	50	IgG1, κ	100	1:20	1:40
CD11c	100	IgG2b, κ	200	1:20	1:40
CD10	100	IgG1, κ	100	1:20	1:20
CD16	200	IgG1, k	200	1:20	1:20
CXCR2	400	IgG1, κ	500	1:20	1:25
PD-L1	150	IgG2b, κ	100	1:20	3:40
HLA-DR	50	IgG2a, κ	100	1:20	1:40
CXCR4	100	IgG2a, κ	100	1:20	1:20
CD54	100	IgG1, κ	200	1:20	1:40

Legend: Each primary antibody, concentration and immunoglobin (Ig) type is listed followed by the corresponding isotype control concentration and top dilution.

Table 3.4: Primary antibody titrations and concentration selection.

Primary antibody	Concentration (µg/mL)	Staining Index			Selected dilution
		1:20	1:40	1:100	
CD11b (unstimulated)	100	7.6	3.8	2.3	1:40
CD11b (stimulated)	100	0.8	1.7	2.4	
CD66b (unstimulated)	200	12.6	12.8	11.7	1:100
CD66b (stimulated)	200	1.6	1.6	1.8	
CD62L	50	60.2	34.4	22.8	1:100
CD11c	100	7.9	8.3	7.5	1:40
CD10	100	9.1	8.0	5.6	1:20
CD16	200	229.8	216.2	178.6	1:100
CXCR2	400	17.7	20.1	8.3	1:40
PD-L1	150	15.6	14.2	11.2	1:100
HLA-DR	50	6.6	4.2	2.5	1:40
CXCR4	100	6.5	6.0	5.0	1:20
CD54	100	17.2	21.8	27.0	1:100

Legend: Each primary antibody and stock concentration is listed followed by the calculated staining index for each dilution. Where applicable, stimulated designates pre-incubation with 10µM fMLP and the staining index calculated to compare between unstimulated and stimulated peaks.

3.2.4 Validation of combined antibody panel

After determining optimal concentrations, antibodies were combined in smaller groups to assess spectral compatibility and initially identify antibody cross-reactivity. Neutrophils from healthy young (<40 years old; HY) participants were stained with different antibody combinations with or without viability dyes and the MFI of each marker recorded (Table 3.5). In combinations P1 and P1a, 'arching' (where cell populations curve on a dot plot, indicative of uncompensated data or spectral overlap) was observed in the annexin V vs 7AAD plots compared to P1b (Figure 3.7a). The same observation was observed in combinations P2 and P2a compared to P1b (Figure 3.7b). Importantly, both P1a and P2a do not contain annexin V and, therefore, should have no annexin V positive events. In the absence of both annexin V and 7AAD, but with all other antibodies (viability fluorescence minus one, FMO), annexin V positive events were recorded (Figure 3.7a). From these data, it was likely another fluorophore was spilling over into the annexin V detection channel. Quantification of these data highlight the increased detection of apoptotic and dead cell events in P1, P1a, P2, P2a when compared to conditions P1b and P2b (Figure 3.7c). These combinations were used to identify a potential antibody that could spill into the annexin V channel.

Based on the above analysis, and the PE-Cy7 conjugate, anti-CD16 was identified as a potential candidate for spectral overlap. In order to investigate the role of anti-CD16 in producing annexin V positive events, neutrophils from an elderly participant without COPD (HE, Figure 3.8a) and with COPD (Figure 3.8b) were stained with all P1 or P2 antibodies with and without anti-CD16 (conjugated to PE/Cy7). Neutrophils from either donor demonstrated an increase in the proportion of detected apoptotic cells with anti-CD16 compared to without anti-CD16

staining (Figure 3.8). Furthermore, neutrophils stained with all P1 or P2 antibodies without anti-CD16 or viability dyes (Sub-panel c, Table 3.5) did not show any annexin V or 7AAD positive events as expected (Figure 3.9), suggesting PE/Cy7-conjugated anti-CD16 was altering the detection of annexin V positive events. Therefore, an alternative fluorophore of anti-CD16 was obtained to mitigate this effect.

AlexaFluor™700 (AF700), was identified as a suitable fit in the existing panel and commercially available conjugate for anti-CD16 and is reflected in the spectral analysis (Figure 3.10). To determine the optimal concentration, neutrophils from a HY volunteer were stained with different concentrations of AF700-conjugated anti-CD16 (BioLegend, clone 3G8, Figure 3.11). A staining index could not be calculated for this sample as the isotype control had a reported fluorescence of zero, however, a clear separation was observed between peaks (Figure 3.11) and, therefore, the lowest concentration (1:100) was selected. Further validation of anti-CD16 within both antibody panels is described separately in Chapter 4.

Table 3.5: Antibody combinations used in initial panel validation.

Fluorophore	Target	P1	P1a	P1b	P1c	AnV / 7AAD FMO	Target	P2	P2a	P2b	P2c
BV421	CXCR4	■	■		■	■	HLA-DR	■	■		■
BV510	CD10	■		■	■	■	CD10	■		■	■
BV605	CD62L	■	■		■	■	PD-L1	■	■		■
BV785	CD11b	■		■	■	■	CD11b	■		■	■
FITC	CXCR2	■	■		■	■	CD11c	■	■		■
PE	AnV	■		■			AnV	■		■	
PE/Cy7	CD16	■	■			■	CD16	■	■		
APC	CD54	■		■	■	■	CD66b	■		■	■
7AAD	DNA	■	■				DNA	■	■		

Legend: Each fluorophore and corresponding target for Panel 1 (P1) and Panel 2 (P2) are shown. Grey highlighted cells indicate the inclusion of that antibody in the group. Viability dyes [Annexin V (AnV)/7-aminoactinomycin (7AAD)] were excluded from P1 using a fluorescence minus one (FMO) group as shown.

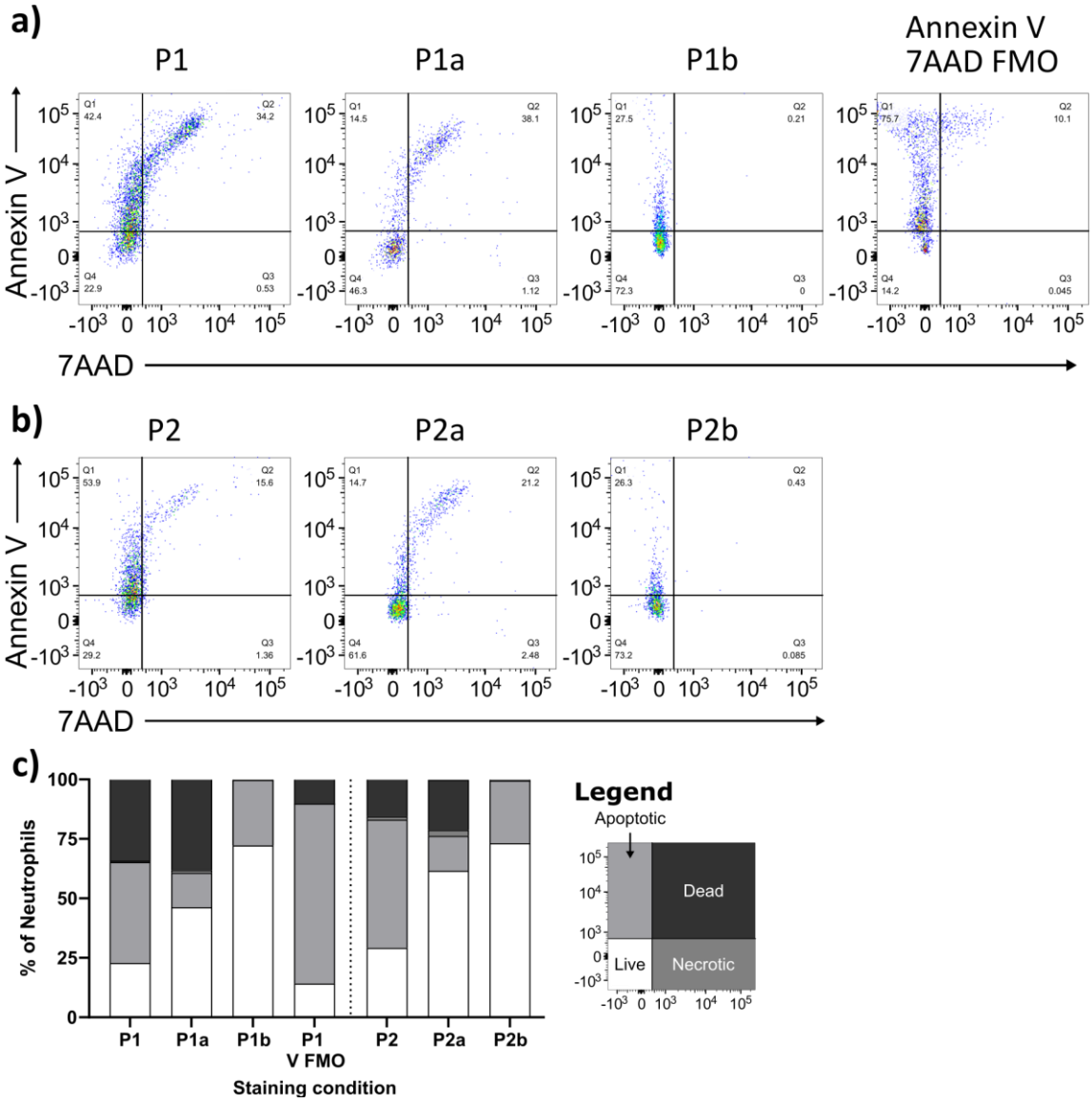

Figure 3.7: Viability flow plots using different antibody combinations

Neutrophils from a healthy young participant (n=1) were isolated and stained with different antibody combinations (Table 3.5) and viability was assessed using annexin V and 7AAD. **a,b)** Flow cytometry plots show 7AAD and annexin V fluorescence intensity. **c)** Quantification of each condition in (a) and (b) in stacked histograms, divided into the percentage of neutrophils that were live (white), apoptotic (light grey), necrotic (dark grey) or dead (black). Data are for n=1 donor.

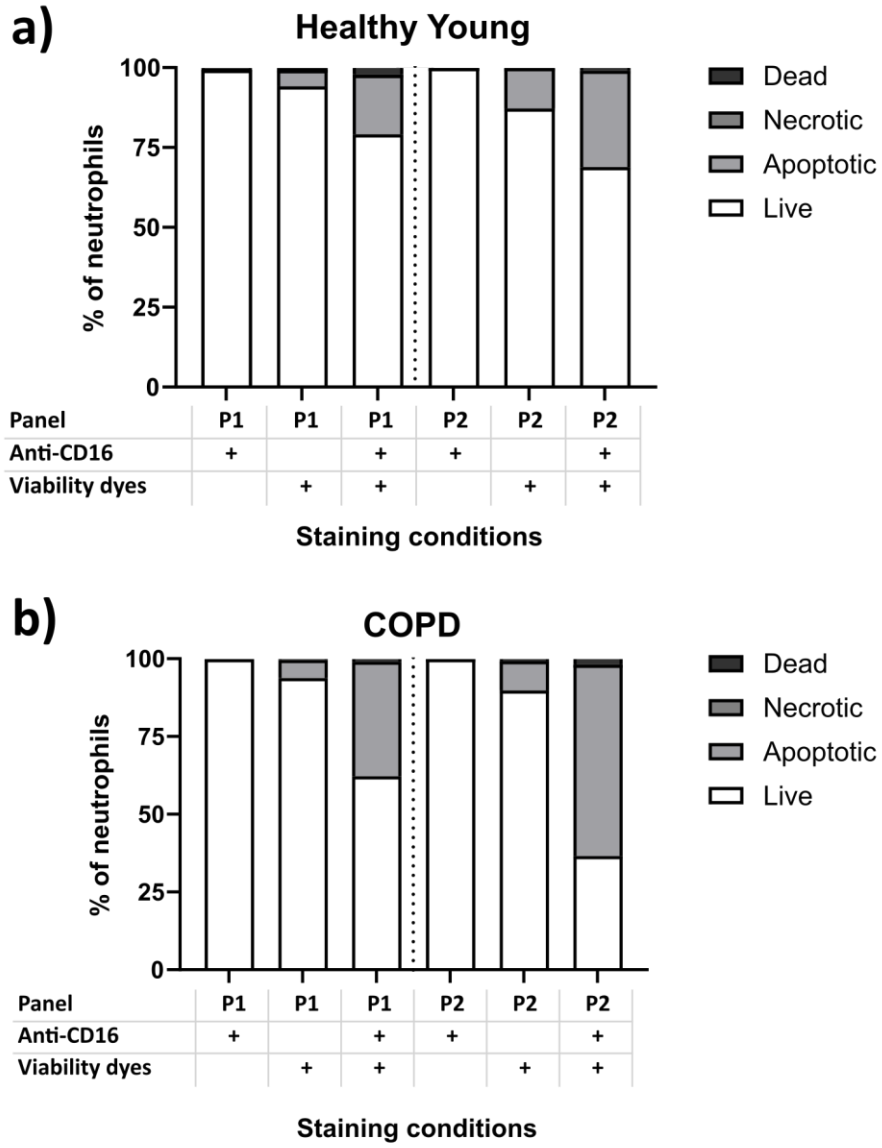

Figure 3.8: Viability of neutrophils stained with and without PE/Cy7-conjugated anti-CD16

Neutrophils from **a)** healthy young (n=1) and **b)** a patient with COPD (n=1) were isolated and treated as shown (full panel shown in Table 3.5). Stacked histograms show the percentage of total neutrophils that are live (empty), apoptotic (light grey), necrotic (dark grey) or dead (black) for each staining condition.

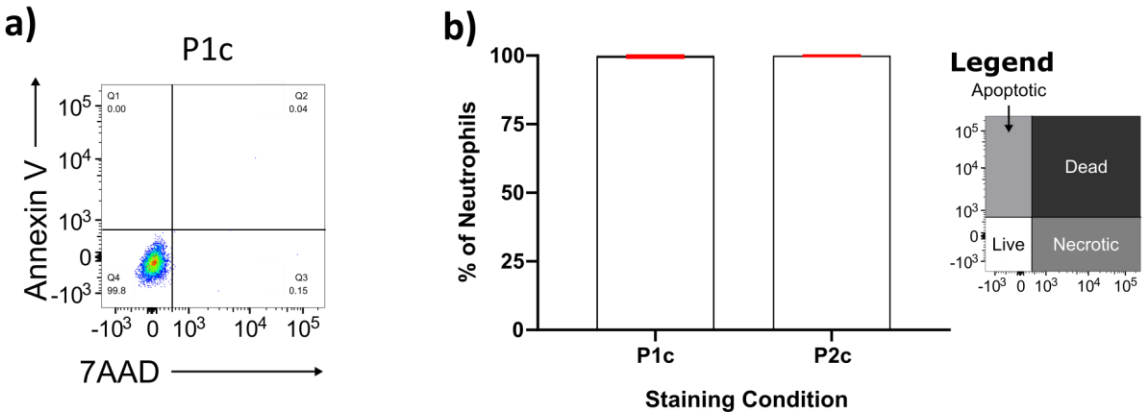

Figure 3.9: Viability of neutrophils stained without PE/Cy7-conjugated anti-CD16 or viability dyes

Neutrophils from healthy young participants (n=2) were isolated and stained with panel P1c or P2c (Table 3.5). **a)** Representative viability flow cytometry plot of isolated neutrophils stained with panel P1c. **b)** Stacked histograms show median percentage of neutrophils that are live (empty), apoptotic (light grey), necrotic (dark grey) or dead (black) for each staining condition. Error bars (red) indicate the range for n=2 participants.

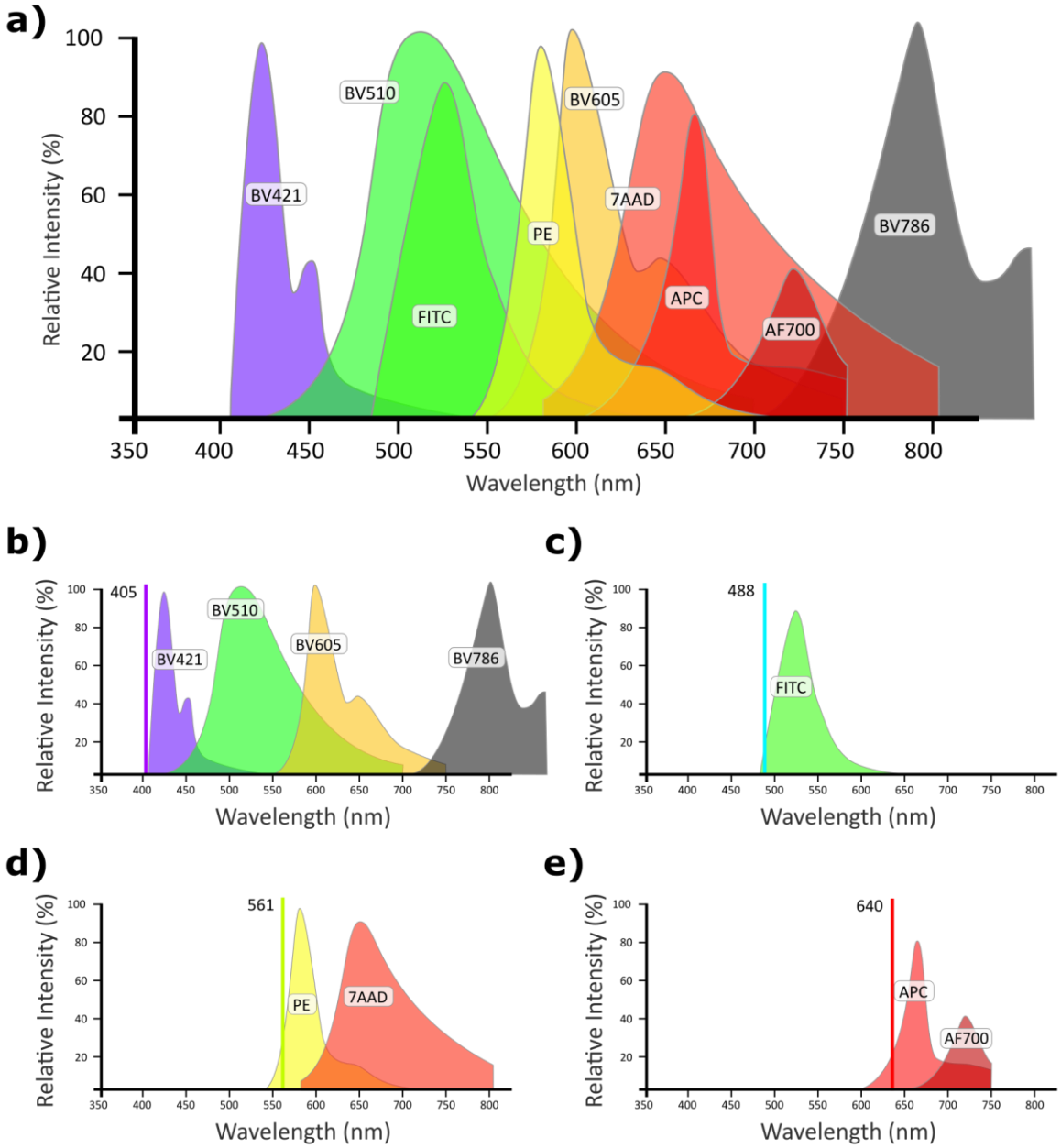

Figure 3.10: Spectral view of conjugates for a 9-colour flow cytometry panel replacing PE/Cy7 for AF700

Schematic representation of relative intensity (%) of **a)** each fluorophore combined and then separated by excitation laser: **b)** 405nm, **c)** 488nm, **d)** 561nm, **e)** 640nm as denoted by vertical line.

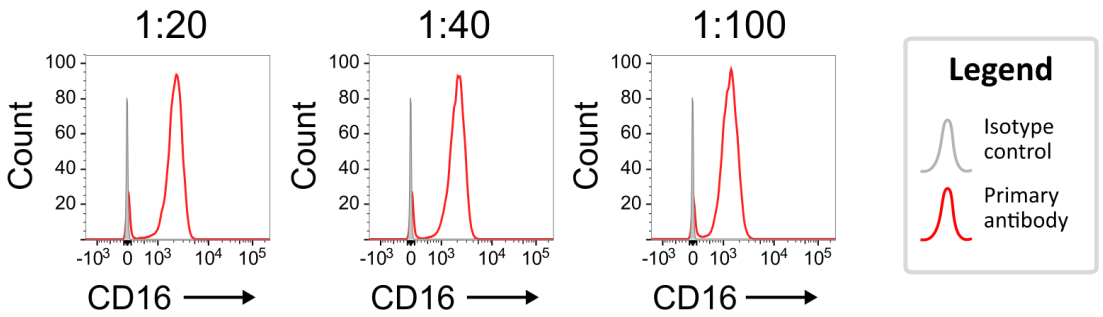

Figure 3.11: Titration of AF700-conjugated anti-CD16

Isolated neutrophils from a healthy donor (n=1) were incubated with anti-CD16 at the dilutions indicated above each plot. The fluorescence intensity of anti-CD16 (red trace) or corresponding isotype control (grey trace) were measured by flow cytometry.

3.2.5 Single-stain analysis

In order to determine the impact of spectral overlap on the entire panel, neutrophils from HY participants were stained individually with each antibody and compared to the entire panel. Changes between the single stain controls and the entire panel were then used to determine if the detection of the given marker was influenced by other antibodies in the panel.

An increase in the MFI between unstained cells and single-stained samples was observed for anti-CD16, CD62L, CXCR2 and CD11b (Figure 3.12) and anti-CD66b, CD10, CD11c and CD54 (Figure 3.13), indicating basal expression of these markers. Little or no difference was observed between unstained and single-stained samples for anti-CXCR4, HLA-DR and PD-L1, indicating little or no basal expression of these markers.

For anti-CD16, CD62L, CXCR2 and CD11b antibodies (Figure 3.12), a decrease in the MFI was observed between the single stain control and the whole panel, and thus indicated a reduction in the sensitivity of these channels.

In contrast, anti-CD66b, CD10, CD11c and CD54 (Figure 3.13) and anti-CXCR4, HLA-DR and PD-L1 (Figure 3.14) showed no change in the MFI was observed between the single stain control and the whole panel. These data indicated no reduction in sensitivity in these channels.

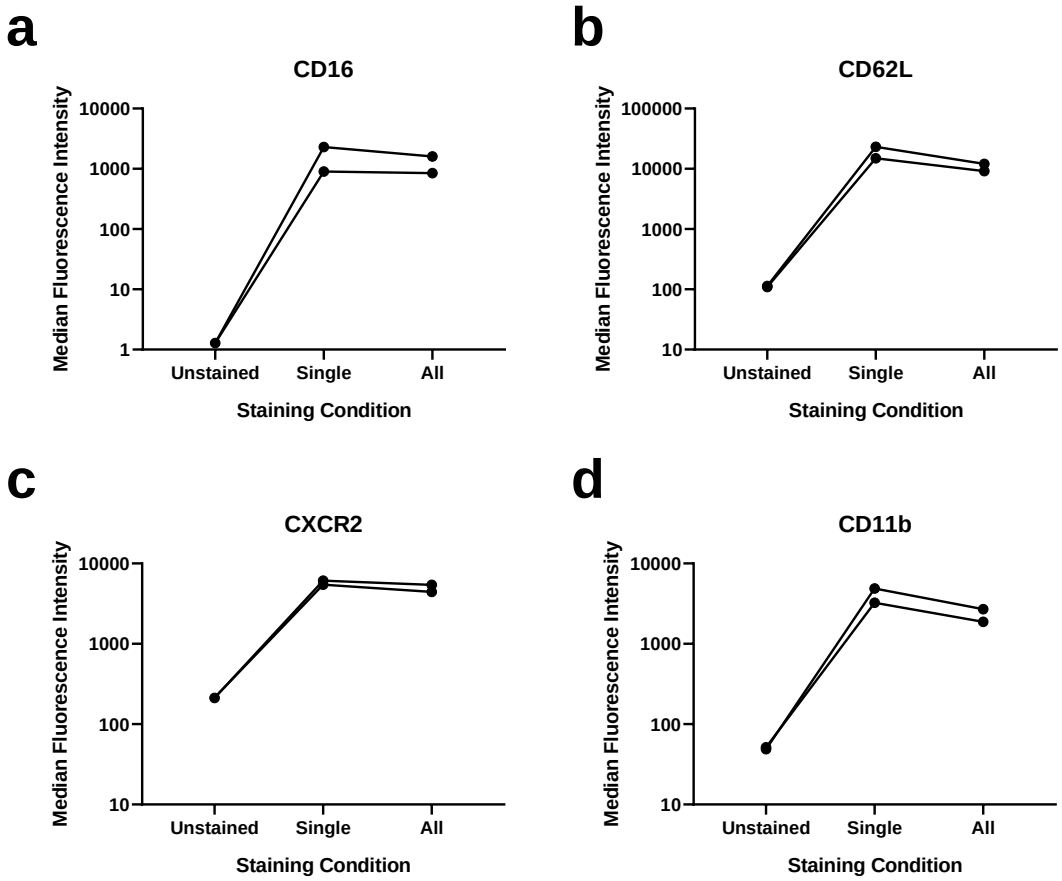

Figure 3.12: Median fluorescence intensity of neutrophils stained individually with anti-CD16, anti-CD62L, anti-CXCR2, anti-CD11b or in combination with all panel antibodies

Neutrophils from healthy young participants (n=2) were isolated and left unstained or stained individually with **a)** anti-CD16, **b)** CD62L, **c)** CXCR2, **d)** CD11b or with all relevant antibodies (All, as shown in AnV / 7AAD FMO in Table 3.5) and the median fluorescence intensity (MFI) recorded. The MFI is plotted for each participant.

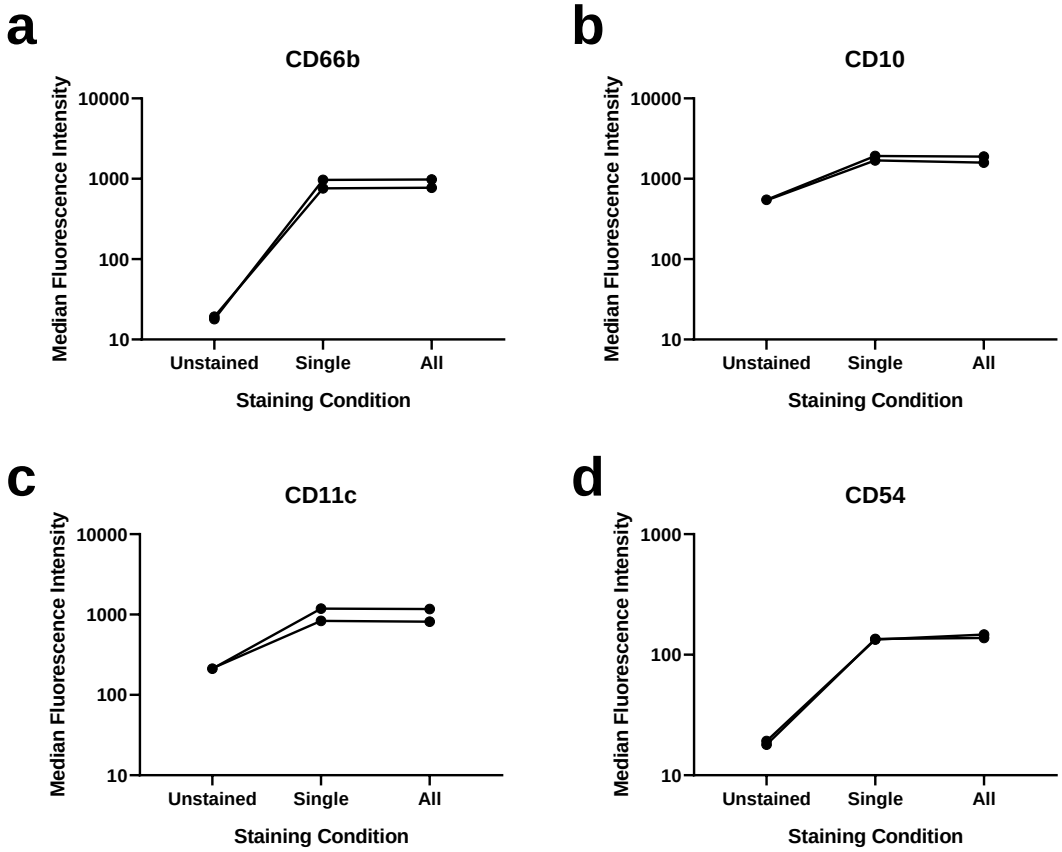

Figure 3.13: Median fluorescence intensity of neutrophils stained individually with anti-CD66b. anti-CD10, anti-CD11c, anti-CD54 or in combination with all panel antibodies

Neutrophils from healthy young participants (n=2) were isolated and stained individually with **a)** anti-CD66b, **b)** CD10L, **c)** CD11c, **d)** CD54 or with all relevant antibodies (All, as shown in AnV / 7AAD FMO in Table 3.5) and the median fluorescence intensity (MFI) recorded. The MFI is plotted for each participant.

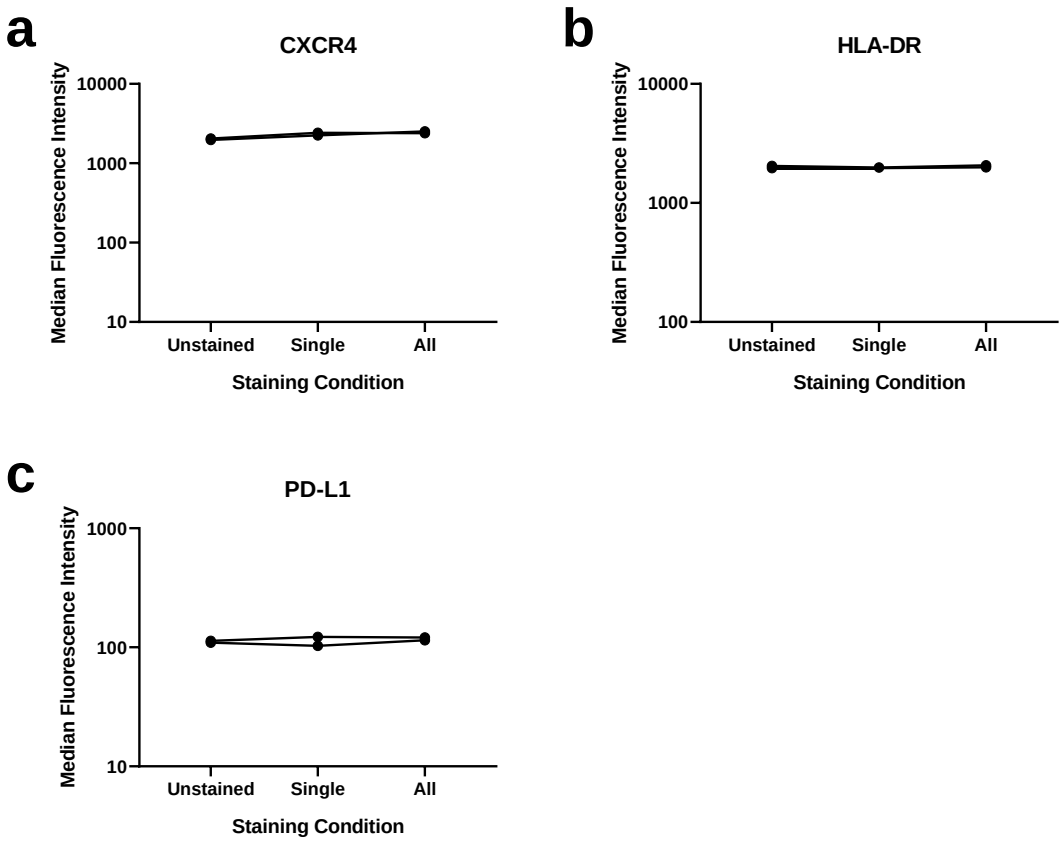

Figure 3.14: Median fluorescence intensity of neutrophils stained individually with anti-CXCR4, anti-HLA-DR, anti-PD-L1 or in combination with all panel antibodies

Neutrophils from healthy young participants (n=2) were isolated and stained individually with **a)** anti-CXCR4, **b)** HLA-DR, **c)** PD-L1 or with all relevant antibodies (All, as shown in AnV / 7AAD FMO in Table 3.5) and the median fluorescence intensity (MFI) recorded. The MFI is plotted for each participant.

3.2.6 Use of fluorescence minus one controls

In order to assist with setting gates, FMO controls were used. Based on the initial validation information, it was identified that CXCR4, HLA-DR and viability markers most required these controls to accurately discriminate between positive and negative cells. Therefore, controls that contained staining for every other marker in the panel excluding each of these were used to inform the gate placement (Figure 3.15). As shown, gates were drawn around the FMO population to allow accurate gating of any positive events where it was not possible to clearly distinguish the positive and negative populations. In all other cases, isotype controls were used to perform a similar function where the positive population was separated from the negative population.

3.2.7 Neutrophil dim and bright gating calculations

Identifying bright and dim populations using flow cytometry is usually poorly described or based only on the visual appearance of the flow cytometry plot. To ensure a more robust gating strategy, neutrophils from HY participants were isolated and stained with panel 1 or panel 2 antibodies (Table 3.5). The fluorescence intensity value at the 10th percentile, for determining dim populations, or 90th percentile for bright populations was determined for each sample and the median value used as the threshold (Figure 3.16a-d). The exact values calculated are shown in Table 3.6.

In order to ensure this method enabled identification of activated neutrophils, neutrophils from HY participants were incubated with 10 µM fMLP and expression of anti-CD11b and CD66b measured. Addition of fMLP resulted in an increase in both CD11b and CD66b and a respective increase in the percentage of $CD11b^{bright}CD66b^{bright}$ neutrophils (Figure 3.16e).

3.2.8 Use of Sphero Calibration Particles to maintain comparable laser performance

As this project relies heavily on comparing the expression of surface proteins using the MFI of each sample, a method of ensuring accurate reporting of fluorescence between samples was required. Sphero Calibration Particles (or SpheroBeads; Fisher Scientific) were used to generate fluorescence intensity values that would act as target values for each individual sample. Eight fluorescent peaks are detected in each channel and a peak in the upper range of the detector without being saturated was selected to provide accurate target values (Figure 3.17). The first run generated the target values shown in Table 3.7 and a 10% tolerance was set for subsequent experiments. Data were only collected once the flow cytometer was within these target ranges.

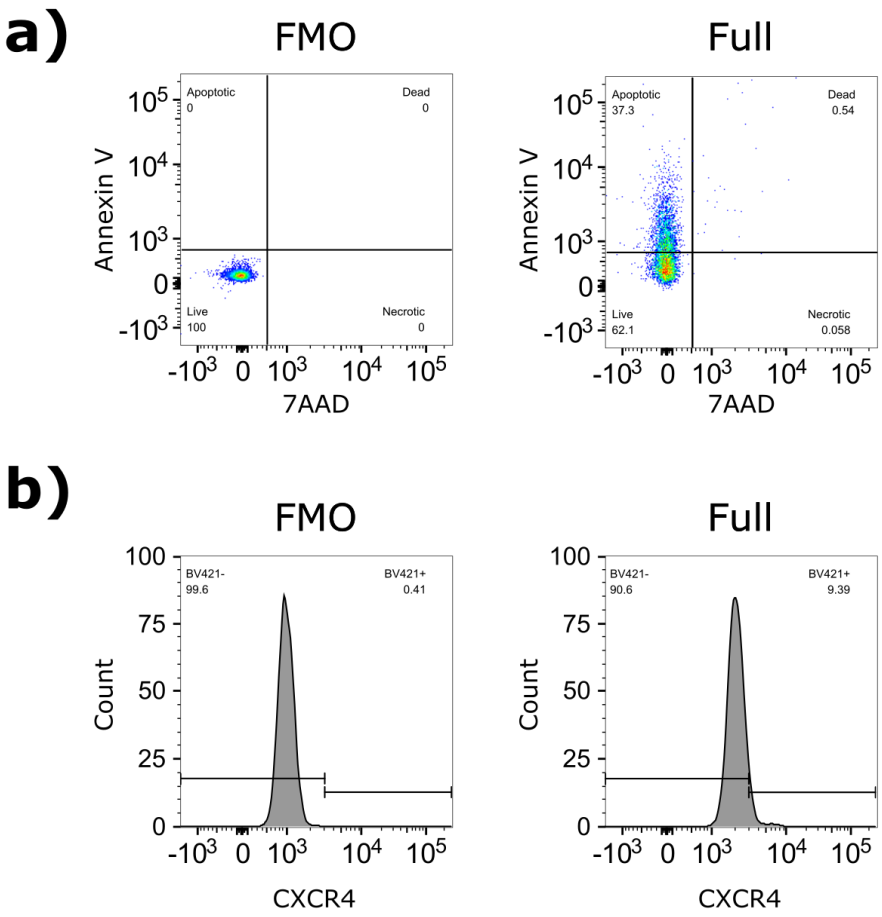

Figure 3.15: Fluorescence intensity of neutrophils stained with all panel antibodies or in fluorescent minus one controls

Healthy young neutrophils (n=1) were stained with all P1 antibodies (Full, as shown in Table 3.5) or without **a)** viability dyes or **b)** anti-CD184 (fluorescence minus one, FMO).

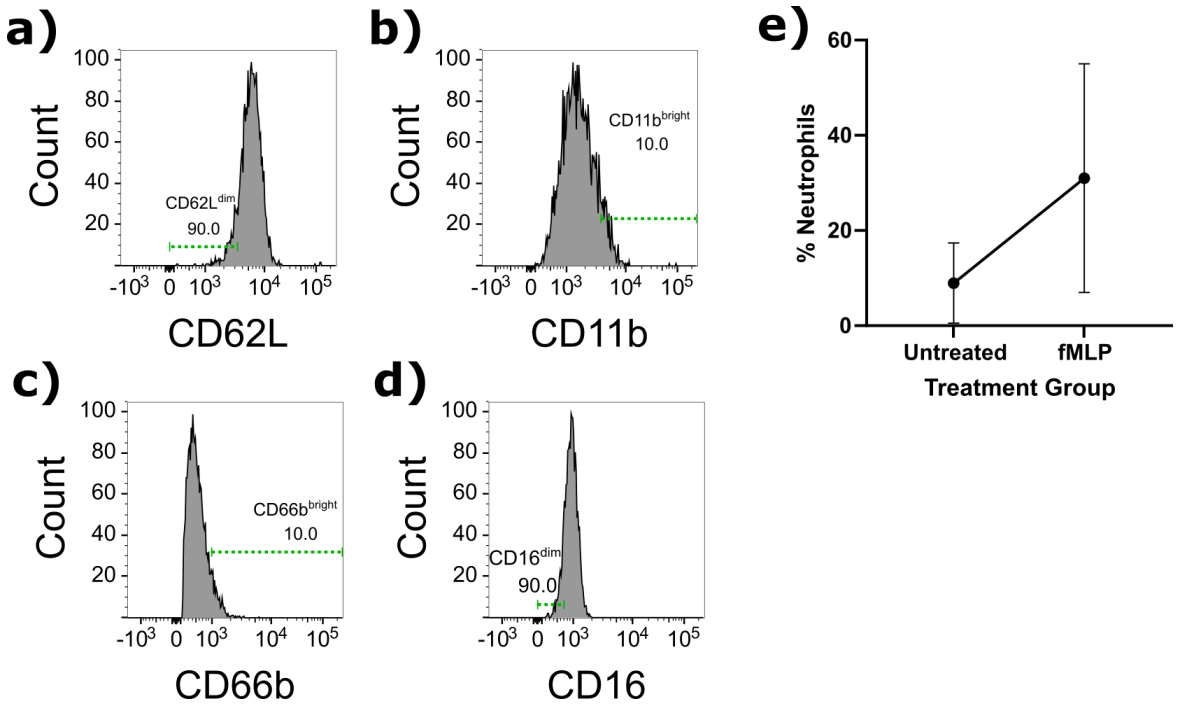

Figure 3.16: Mathematical calculation of gate cut-off values and activation marker validation

Neutrophils were isolated from healthy young donors (n=15) and stained with all panel 1 or panel 2 antibodies (Table 3.5). The fluorescence intensity for **a)** CD62L, **b)** CD11, **c)** CD66b, and **d)** CD16 were measured and the fluorescence value at either the 10th percentile (a and d) or 90th percentile (b and c) was recorded. The mean value across all samples was then used to determine 'dim' or 'bright' respectively. **e)** Neutrophils from healthy young individuals (n=2) were incubated with either RPMI (untreated) or 10 µM N-Formylmethionyl-leucyl-phenylalanine (fMLP) for 20 minutes prior to antibody staining. The percentage of neutrophils above the 10% threshold for both CD11b and CD66b (section 2.x) were recorded, where error bars show interquartile range.

Table 3.6: Fluorecence thresholds for dim and bright gates

Gate	Channel	MFI
$CD11b^{bright}$	BV768	3021
$CD66b^{bright}$	APC	1164
$CD16^{dim}$	AF700	831
$CD62L^{dim}$	BV605	4490

Legend: Gate threshold values for determining dim and bright populations

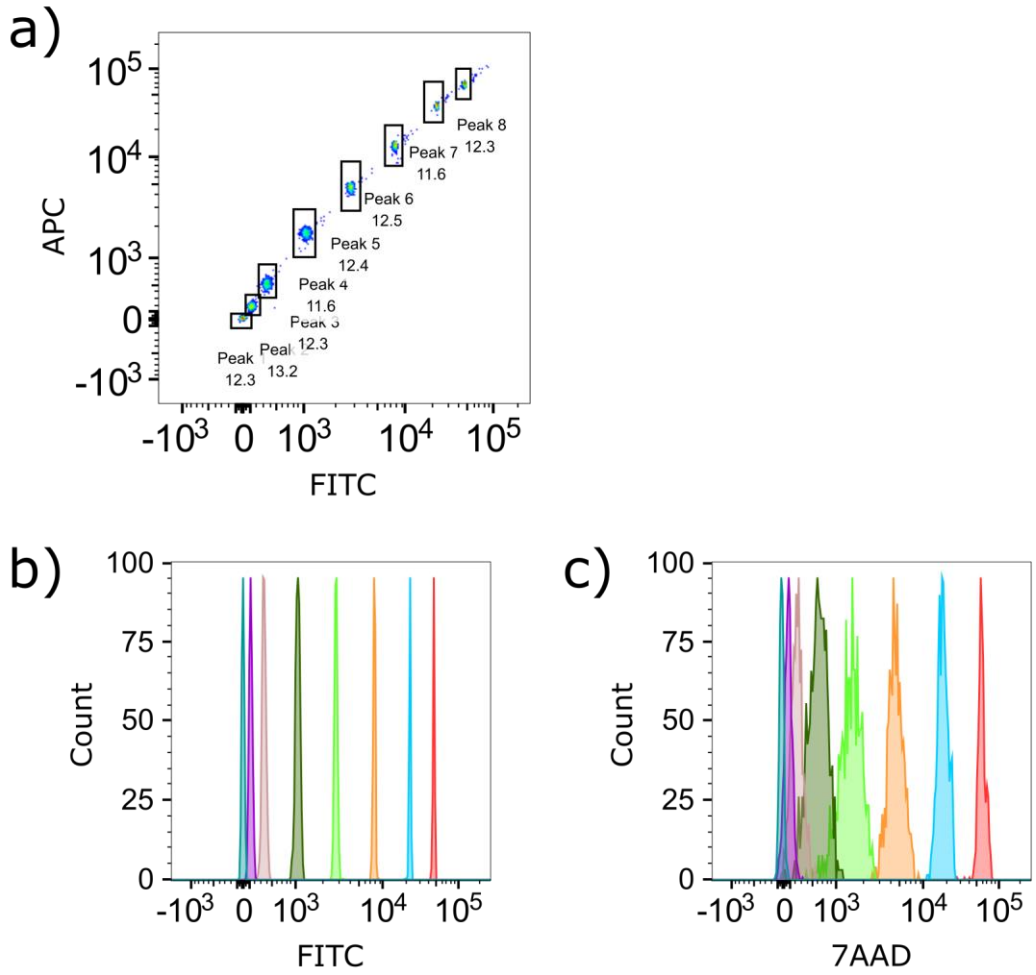

Figure 3.17: SpheroBeads shown on FITC and APC channels

One drop of SpheroBeads was added to 500uL of water and 4000 bead events collected. **a)** Beads were gated using FITC and APC as 8 clear populations can be visualized. Each of the 8 peaks were plotted for each channel and **b)** FITC and **c)** 7AAD are represented.

Table 3.7: Target MFI values from SpheroBeads.

Fluorophore	Bead	Target MFI	Upper limit	Lower limit
BV421	6	22500	24750	20250
BV510	7	36020	39622	32418
BV605	7	3270	3597	2943
BV785	7	113674	125041	102307
FITC	7	73805	81186	66425
PE	7	47530	52283	42777
AF700	7	3300	3630	2970
APC	7	51876	57064	46688
7AAD	7	61000	67100	54900

Legend: Each fluorophore and corresponding target value is shown, along with upper and lower limits for a 10% tolerance.

3.3 Discussion

Flow cytometry is a widely used technology routinely used in both research and diagnostic capacities (Davidson *et al.*, 2012). At the time of writing, BioLegend (a major supplier of antibodies for use in flow cytometry) listed over 15000 primary antibodies designed for use in flow cytometry (BioLegend, 2021). There are now many commercially available fluorophores and advances in synthetic biology have led to many different options available to researchers (Martynov *et al.*, 2016). These advances provide the opportunity to collect large amounts of information on an individual cell by using multiple antibodies conjugated to different fluorophores, but also increase the complexity in deciding what fluorophores to use. A report highlighted the lack of standardisation in antibody production and use (Bradbury and Plückthun, 2015), combined with only around half of 6000 tested antibodies recognising only their reported target (Berglund *et al.*, 2008). Whilst many antibody suppliers have robust validation procedures, they cannot account for each individual experiment and antibody combination and, therefore, researchers must carry out their own validation. In this chapter, each antibody was individually titrated and optimised for use in two multiparameter panels with PE/Cy7-conjugated anti-CD16 being identified as incompatible and was replaced with an AF700-conjugated anti-CD16 antibody.

3.3.1 Importance of panel design

The selection of antibody conjugates is the first step in designing a successful multiparameter flow cytometry panel. The fluorophores in this panel were chosen to minimise the spectral spill-over from one channel to another. This ensured that individual antibodies were detected and is considered a fundamental principle underpinning multiparameter flow cytometry

(Nguyen *et al.*, 2013). Another important reason for minimising overlap in fluorophore selection was to reduce the compensation required as compensation negatively impacts the sensitivity of detection and limits the range of the detector (Maecker *et al.*, 2004). Compensation is, however, necessary in all multi-colour flow cytometry experiments and involves the subtraction of any fluorescence originating from a different fluorophore to the one being detected (Bagwell and Adams, 1993). The importance of correct compensation has also been highlighted in phenotyping panels and can be predicted from the fluorophores used (Njemini *et al.*, 2014). Using well-separated fluorophores in this panel, therefore, increased the ability to accurately detect changes in surface protein expression. With larger panels, a reduction in sensitivity is inevitable due to the broad emission spectra seen with all fluorophores. To improve detection between antibodies, each fluorophore was matched to a target based on the fluorophore brightness and target abundance. Fluorophore brightness indexes are produced by multiple manufacturers and are readily available online (BioLegend, no date). Targets highly expressed on neutrophils, such as CXCR2, were matched with relatively dimmer fluorophores and low expressed targets, such as CXCR4, were matched with relatively bright fluorophores. The loss of sensitivity observed in 4 markers reduced the ability to detect small differences in these markers, but the validation steps carried out minimises the impact of this on downstream data analysis.

3.3.2 Validation of specificity and antibody concentration

Nearly all commercially available antibodies are already validated for their specificity for a given target and multiple processes have been described for achieving this (Marcon *et al.*, 2015). In this panel, the optimal concentration of each antibody was determined using a

titration on a cell type known to express the target. Whilst not a direct measure of antibody specificity, it was able to show that the target could be visualised using flow cytometry and enabled calculation of a staining index. The staining index is a relative measure of the ability to discriminate a positive population and a negative population in flow cytometry and can aid in selecting appropriate dilutions of antibody (Maecker *et al.*, 2004) and a basic version using a three-step titration was implemented here. For the majority of antibodies tested in this chapter, the staining index reduced as the concentration of antibody was decreased. However, reduction in the spread of non-specific binding (measured using an isotype-control) increased this index in some cases and informed selection of a lower antibody concentration. As demonstrated by the data presented in this chapter, a higher concentration of antibody does not always increase the separation between the positive and negative population as higher antibody concentrations can increase non-specific binding. However, lower staining indexes don't necessarily mean poor separation as they are only one tool used, and where clear separation is observed, lower antibody concentrations that provide a cost benefit are preferred. Titration of antibodies is common practice in flow cytometry experiments and is usually described in guides or blogs (UCFlow, 2009), but is very poorly reported in the literature, with many papers simply stating that it was carried out (e.g. Tanaka *et al.*, 2004) or not reporting at all (e.g. Schlub *et al.*, 2010). This may, however, be as a result of publishers not requiring or allowing the publication of validation data and instead assume it has been carried out correctly.

So far, these two sections deal with each specific antibody and how, in theory, they will interact together. It is important however to experimentally validate the compatibility of every antibody in the panel together.

3.3.3 Validation of spectral overlap and antibody compatibility

Validation of each antibody has been carried out to ensure reliable results from surface expression on human neutrophils is obtained. Initially, spectral overlap was suspected between the PE/Cy7 conjugated anti-CD16 and the detection of PE-conjugated annexin V due to a high percentage of annexin V positive events in a sample containing PE/Cy7-conjugated anti-CD16 but no annexin V. Whilst PE and PE/Cy7 fluorophores have been used together in many flow cytometry panels, it has been shown that PE/Cy7 shows one of the highest degradation rates of PE tandem dyes (Hulspas, Dombkowski, *et al.*, 2009) and substantial spill over into the PE channel is reported for some antibody manufacturers (Johansson and Macey, 2014). Particular caution with PE/Cy conjugates was also recommended from a published workshop on multiparameter flow cytometry as the decay time after illumination may lead to overlap between lasers (Bayer *et al.*, 2007). Spectral overlap, or poorly compensated data, presents with a characteristic 'arching' of the cell population when viewed on a two-dimensional plot (Roederer, 2001). This was observed in the PE channel in samples containing the PE/Cy7 tandem conjugated anti-CD16 antibody and was absent when anti-CD16 was excluded. Therefore, an alternative fluorophore was sought to negate spectral overlap between the PE fluorophore and the PE/Cy7 tandem.

After changing PE/Cy7 for AF700-conjugated anti-CD16, further spectral compatibility was assessed using single-stained samples compared to staining with the whole antibody panel. Any increases in MFI between the single-stain and whole panel could indicate further spectral overlap, however, this was not observed. This step also tests the compatibility of each antibody in the whole panel – highlighting any potential antibody-antibody interactions.

Antibody cross-reactivity usually refers to the ability of an antibody to bind a specific antigen across different species (Moreira *et al.*, 2015) and very little attention is given to the ability of antibodies to interact with each other. Apart from secondary antibodies that are specifically designed to bind the Fc portion of antibodies (Min *et al.*, 2016), the literature does not report any antibody-antibody interactions. The European Journal for Immunology extensively reviewed flow cytometry practice and ensuring antibody-antibody did not exist was not mentioned and may also indicate how rare these events may be (Cossarizza *et al.*, 2017). Chapter 4 of this thesis details the potential pitfalls if this step is omitted and therefore comparisons of single-stained cells against the whole panel as shown in this chapter provides two key benefits: recording the expected baseline expression of that particular marker and detecting any antibody-antibody incompatibilities.

3.3.4 Importance of correct controls

There has been much debate on the number and type of controls that flow cytometry experiments should include. As part of this validation work, isotype controls were used for each antibody as a means of correcting for background staining and to match many published papers that still report using isotype-corrected data as a relatively low-cost control. Isotype controls are widely used and commonly reported (Hulspas, O'Gorman, *et al.*, 2009), but it is important the correct isotype is used as they can vary considerably (Maecker and Trotter, 2006). They also have limitations and do not specifically aid the placing of gates during analysis. There have been many calls to move away from reliance on isotype controls, instead using a cell population that does not express the marker of interest (O'Gorman and Thomas,

1999). This is a challenge in some experimental models and may require the use of cell lines if there are no similar primary cells that fit this criterion.

The move towards using FMO controls is generally a positive move, however, FMO controls do not serve the same purpose as isotype controls and instead aid placement of gates (Baumgarth and Roederer, 2000; Bayer *et al.*, 2007). FMO controls do not provide any information on background staining from a particular antibody (Hulspas, O'Gorman, *et al.*, 2009). It is also important to distinguish between two key questions: the level of expression on a particular cell, determined by MFI and the percentage of cells expressing a given marker, identified by either a 'positive' or 'negative' cell. Isotype controls will not help discriminate positivity (Bayer *et al.*, 2007) and will require an FMO control. However, distinct bi-modal expression as observed in CD4+ and CD8+ T cells are unlikely to require an FMO control as clear distinctions will exist. In a 9-colour panel, to have an FMO for each marker would require 9 separate controls, resulting in each antibody being used 9 times – once each in other FMO controls, absent from its own FMO but included in the full panel of antibodies. Clearly, there is a large cost implication to using an FMO for each marker and therefore based on the single-stain validation data, CXCR4 and HLA-DR, along with the viability dyes would have an FMO to aid with gating for positive samples. Unlike isotype controls, there is little report of them being used in published papers, yet are vital to ensure accurate discrimination between positive and negative events (Cossarizza *et al.*, 2017).

Again, journals may not require researchers to disclose the controls that were used in the experiments and papers where FMO controls are vital, for example measuring the change in

receptor expression in different populations (Zhang *et al.*, 2015), do not mention the use of these controls.

In this thesis, SpheroBeads are used to ensure the fluorescence intensity recorded by the flow cytometer between each experiment is consistent. This approach was developed by EuroFlow (a consortium comprised of 20 diagnostic research groups) that uses this technique to allow multi-centre flow cytometry of leukocytes to generate directly comparable data (Theunissen *et al.*, 2017). Generating tables of MFI values, as has been done in this thesis, allows the replication of data on a comparable machine as each laser can be configured to give the same fluorescence output. Publication of these data for any flow cytometry experiment would greatly improve transparency and reproducibility in published work.

3.3.5 Determining phenotypes from surface expression

As neutrophils constitutively express markers such as CD16, CD11b and CD66b, it is not accurate to describe changes in these markers by the percentage of positive cells as all neutrophils will inherently be 'positive' for these markers, but instead by measuring the absolute fluorescence intensity. Exceptions do exist in specific genetic diseases such as leukocyte adhesion deficiency (LAD), where leukocytes lack surface expression of CD11 and CD18 (Hogg *et al.*, 1999; Jasani, Nanavati and Kabra, 2014), but generally measuring changes in constitutively expressed markers must be done by fluorescence intensity. This does have issues, especially as the fluorescence intensity can vary with cytometer laser voltage and between instruments (Ashhurst and Smith, no date), putting importance on being able to ensure this is comparable between runs as described in the use of SpheroBeads. Ensuring a

stringent control on the output of the cytometer collecting the data greatly reduces sample-to-sample variation due to technical setup.

Some previous studies and groups have attempted to identify cell phenotypes based on either bright or dim expression for particular markers, including categorisation of activated neutrophils as being 'bright' for CD11b, CD66b and 'dim' for CD62L (Fortunati *et al.*, 2009), or hypersegmented neutrophils as being $CD16^{bright}$ and $CD62L^{dim}$ (Tak *et al.*, 2017). Highlighting these populations allows the splitting of potentially functionally distinct neutrophil populations, however, relies on manual selection of the population that does not usually have a distinct separation. Considerable ambiguity can occur with this gating method and many publications do not show how these gates were determined, but also have different gating positions even within the same dataset (Zhang *et al.*, 2015). Here, the use of MFI allows for determination of surface expression, whilst gating for subpopulations is performed in line with previously published studies, where appropriate, to supplement these data.

3.3.6 Conclusion

This chapter provides a detailed analysis of the construction and validation of two multi-parameter flow cytometry panels to enable immunophenotyping of human neutrophil populations. Using an evidence-based and methodical approach, these experiments ensure that these panels have good sensitivity to detect the selected markers on the cell surface, the correct controls for downstream data analysis and robust measures to ensure quality control between samples. It also highlights potential pitfalls that could be made if this process is not carried out, and the shortcomings in published data that prevent transparency and reproducibility of data.

CHAPTER 4:

EXTENDED VALIDATION: ANTI-PD-L1 ANTIBODY BINDS THE ALEXAFLUOR™ 700 FLUOROCHROME

4.1 Brief introduction

Programmed Death receptor protein 1 (PD-1) has been the focus of multiple cancer immunotherapies (Rotte, 2019) and is recognised as an important immune checkpoint (Dermani *et al.*, 2019). PD-1 is predominantly expressed on T cells and engagement of PD-1 inhibits T cell proliferation and activation, maintaining immune tolerance in health (Keir *et al.*, 2008; Wei *et al.*, 2013), and has been implicated in evasion of immune-mediated clearance of cancer cells (Dong, Sun and Zhang, 2015). Programmed death receptor ligand 1 (PD-L1) is the major ligand of PD-1 and is expressed on multiple cell types, including dendritic cells (Karwacz *et al.*, 2011) and macrophages (Liu *et al.*, 2019). PD-L1 is also expressed by certain cancers, where it inhibits T cell-mediated killing of the tumour cells (J. Chen *et al.*, 2016). There is an evolving clinical programme to block the action of PD-L1 on T cell inhibition as a therapeutic in cancer therapy (Huang *et al.*, 2015; Hahn *et al.*, 2017). It is clear that the PD-1 axis has a role in the suppression of T cell function and is involved in the progression of some cancers, but the importance of this axis may extend to other disease areas and cell types.

Far less is known about the expression of PD-L1 on neutrophils and its importance in neutrophil function in health and disease. Neutrophil PD-L1 may be of interest in inflammatory diseases, such as COPD, where neutrophils are abundant, but bacterial clearance appears reduced (Hodge and Reynolds, 2012). Moreover, it has been postulated that PD-L1 expression is reduced on macrophage and dendritic cells in COPD, rather than increased as in cancer (Stoll, Virchow and Lommatzsch, 2016). Neutrophil PD-L1 expression has also been implicated in other inflammatory conditions: for example, an increased proportion of PD-L1-expressing neutrophils have been detected in the blood of patients with SLE, and this increase positively

correlated with disease severity (Luo *et al.*, 2016). Moreover, human neutrophils up-regulated PD-L1 expression when exposed to conditioned media from cancer-associated fibroblasts, leading to a reduction in T cell proliferation and increased neutrophil survival *in vitro* (Cheng *et al.*, 2018). Furthermore, isolated blood neutrophils from patients with active tuberculosis infection have been reported to have elevated levels of PD-L1 surface expression compared to neutrophils isolated from healthy volunteers (McNab *et al.*, 2011). Thus, current evidence suggests that neutrophils can express PD-L1 during infection and in chronic inflammatory diseases and that this heightened expression may play a role in disease pathogenesis. Importantly, the impact of altering the PD-1/PD-L1 axis in chronic inflammatory lung disease is not yet fully understood. Due to the role of PD-L1 in immunosuppression, it is possible that increased expression of PD-L1 on neutrophils from patients with COPD may contribute to reduced efficiency of bacterial clearance, or be a beneficial compensatory response to inflammation in these patients. However, expression by neutrophils from healthy volunteers and patients with COPD is largely unknown.

4.1.1 Hypothesis and aims

We hypothesised that PD-L1 expression could be measured in combination with other antibodies using flow cytometry without any changes occurring due to this procedure and that expression would be raised in patients with COPD. We aimed to investigate the expression profile of PD-L1 on neutrophils from these groups and ensure the compatibility of anti-PD-L1 antibodies with other antibodies widely used to identify the phenotype of human neutrophils.

4.2 Results

As discussed in Section 3.2.4, due to incompatibilities with PE/Cy7-conjugated anti-CD16 and other fluorophores in the antibody panels, identification of a suitable alternative fluorophore was carried out theoretically from spectral analysis. AlexaFluor™700 (AF700) was identified as a suitable and commercially available conjugate for anti-CD16. The AF700-conjugated anti-CD16 (BioLegend, clone 3G8) was titrated as shown in Figure 3.11 and a dilution of 1:100 was selected as an appropriate concentration for antibody staining.

4.2.1 Validation of AF700-conjugated anti-CD16

In all experiments, the gating strategy shown in Figure 4.1 was used to identify neutrophils and assess viability. To validate AF700-conjugated anti-CD16 in the existing antibody panels, neutrophils from an elderly participant with or without COPD (see Section 2.2 for cohort definitions) was stained with two different but overlapping antibody panels (P1 and P2; Table 4.1) and viability dyes in the presence or absence of the AF700-conjugated anti-CD16 antibody. All neutrophils were detected as live when viability dyes were absent (Figure 4.2). A reduction in the proportion of live cells was observed in both participants when neutrophils were stained with AF700-conjugated anti-CD16 in addition to P1 and P2 antibody panels compared to neutrophils not stained with AF700-conjugated anti-CD16 (Figure 4.2). Therefore, anti-CD16 antibody may be compromising the viability of isolated neutrophils in these donors.

4.2.2 Investigating neutrophil viability with anti-CD16

In order to further investigate neutrophil survival when incubated with AF700-conjugated anti-CD16 antibody, neutrophils were isolated from HY donors and incubated with viability dyes with or without anti-CD16 (Figure 4.3a). There was no significant reduction in the percentage of live neutrophils in the presence of the viability dye and anti-CD16 antibody compared to those stained with the viability dyes alone. In order to mitigate an age or disease bias, neutrophils from HE participants (Figure 4.3b) and patients with COPD (Figure 4.3c) were isolated and, as with the young cohort, incubated with viability dyes with or without anti-CD16. Again, no significant differences in the percentage of live neutrophils were observed, indicating antibody incubation did not significantly impact on cell viability.

4.2.3 Investigating changes in BV605 fluorescence with anti-CD16

Whilst no impact on cell viability was seen, it was important to ensure there were no changes in marker surface expression within the panel. Isolated neutrophils from an elderly individual without COPD (HE, Figure 4.4a) and with COPD (Figure 4.4b) incubated with AF700-conjugated anti-CD16 antibody led to an increase in the detected level of PD-L1 expression (BV605-conjugated PD-L1 in P2, Figure 4.4). However, incubation with anti-CD16 did not alter the detected expression of CD62L (BV605-conjugated anti-CD62L in P1, Figure 4.4). In order to investigate whether these findings were due to spectral overlap between AF700 and BV605, isolated neutrophils from HY individuals were incubated with either AF700-conjugated anti-CD16 or BV605-conjugated anti-PD-L1 antibodies alone, and the fluorescence detected in the AF700 and BV605 channel measured. As expected, an increase in the MFI of AF700 was detected when neutrophils were incubated with anti-CD16, but there was no increase in the

MFI of BV605 (Figure 4.5a). There was also no increase in the MFI of AF700 or BV605 when neutrophils were incubated with anti-PD-L1 (Figure 4.5b). These data indicated that AF700-conjugated anti-CD16 was not spilling into the BV605 detector and that under these conditions, isolated neutrophils do not express detectable levels of PD-L1.

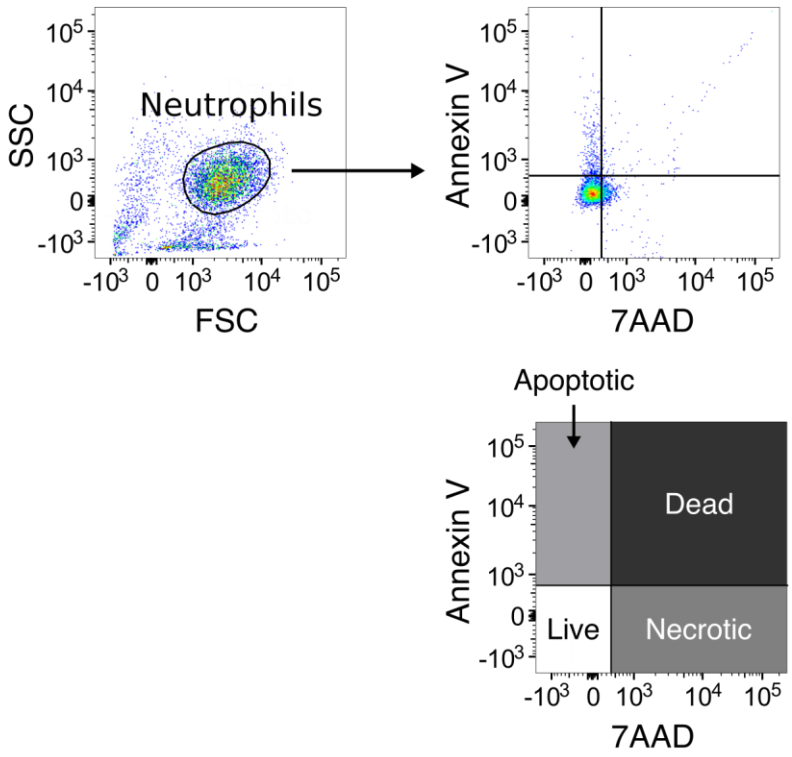

Figure 4.1: Representative flow cytometry plots showing gating of neutrophils and classification of live, apoptotic, necrotic and dead cells

Isolated neutrophils are gated based on forward scatter (FSC) and side scatter (SSC) to remove any remaining contaminating cells. Neutrophil viability was assessed by staining for annexin V and 7AAD and reported as live (double negative), apoptotic (annexin V positive), necrotic (7AAD positive) or dead (double positive).

Table 4.1: Antibodies and dyes included in each panel for validation of anti-CD16

Target	Fluorophore	Panel
CXCR4	BV421	1
CD62L	BV605	1
CXCR2	FITC	1
CD54	APC	1
CD10	BV510	1,2
CD11b	BV786	1,2
AnV	PE	1,2
DNA	7AAD	1,2
HLA-DR	BV421	2
PD-L1	BV605	2
CD11c	FITC	2
CD66b	APC	2

Legend: Each antibody or dye target is listed alongside the respective fluorophore and which panel it is included in as validated in Chapter 3.

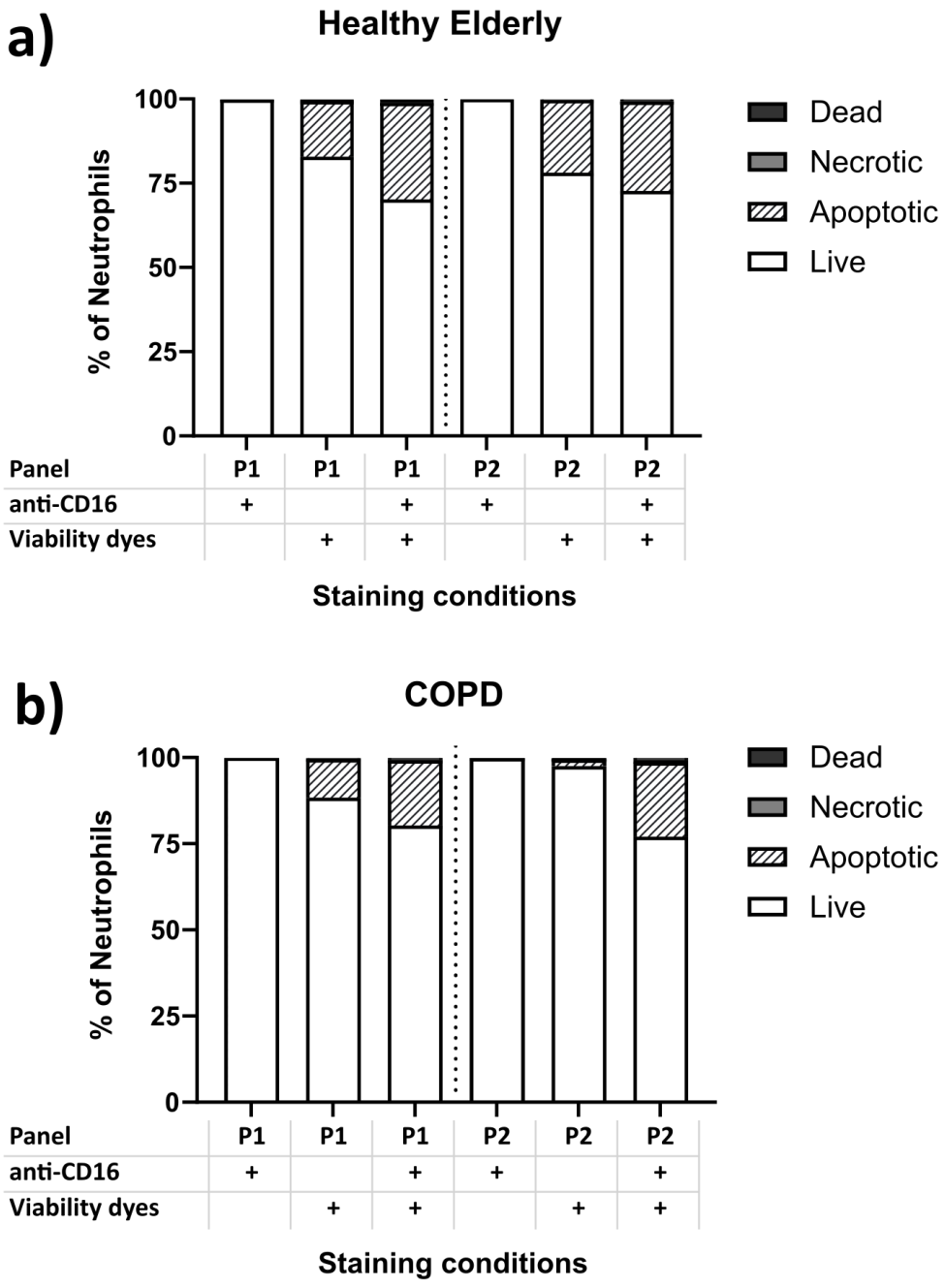

Figure 4.2: Viability of neutrophils with and without AF700-conjugated anti-CD16

Isolated neutrophils from **a)** a healthy elderly (HE, n=1) and **b)** a patient with COPD (n=1) were stained with either panel 1 (P1) or panel 2 (P2) antibodies (Table 4.1) with or without AF700-conjugated anti-CD16 antibody and viability dyes. Stacked histograms show percentage of total neutrophils that are live (annexin V and 7AAD negative), apoptotic (annexin V positive), necrotic (7AAD positive) or dead (annexin V and 7AAD positive).

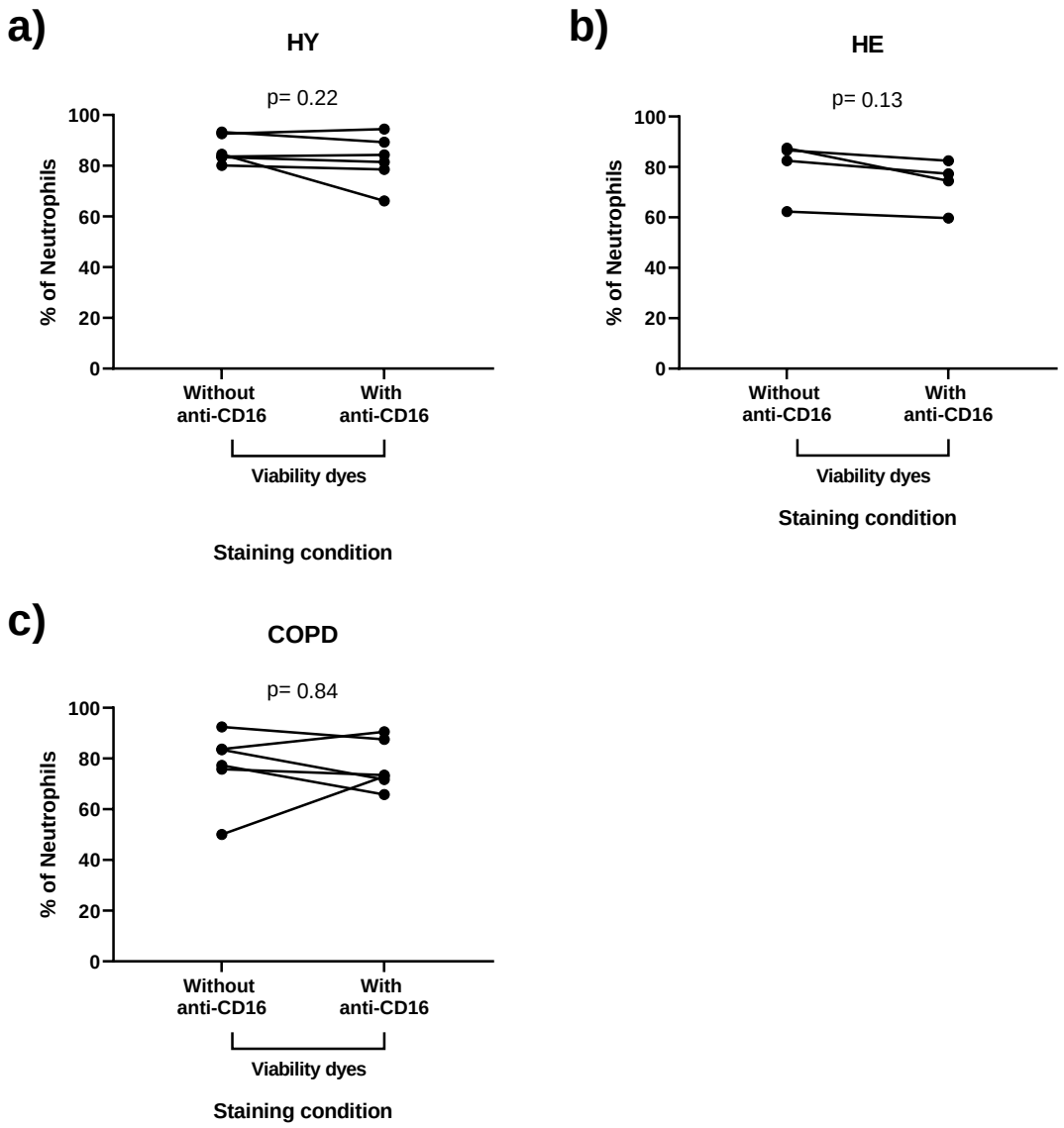

Figure 4.3: Percentage of live neutrophils from healthy young donors following staining with and without AF700-conjugated anti-CD16 antibody

Neutrophils were isolated from **a)** healthy young (HY, n=6), **b)** healthy elderly participants without COPD (HE, n=4) and **c)** participants with COPD (n=6) and stained with viability dyes with or without AF700-conjugated anti-CD16. Paired data are shown for the percentage of live neutrophils (negative for both annexin V and 7AAD). Statistical analysis performed using Wilcoxon matched-pairs signed rank test.

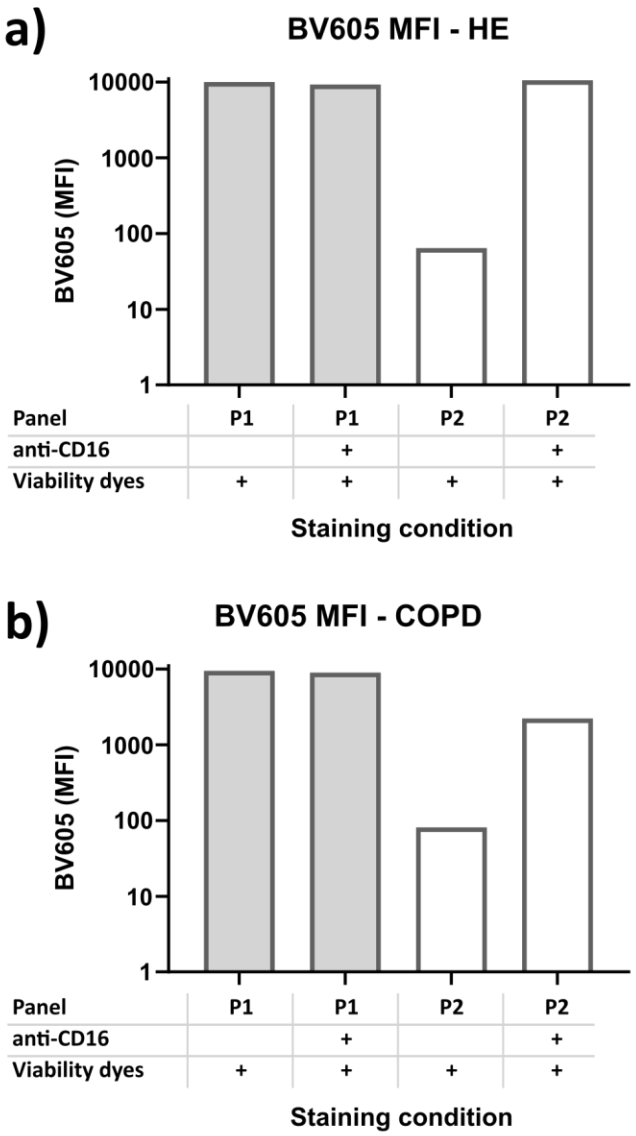

Figure 4.4: BV605 median fluorescence intensity detected on neutrophils from elderly donors with and without COPD following incubation with and without AF700-conjugated anti-CD16 antibody

Isolated neutrophils from **a)** elderly participants without COPD (HE, n=1) and **b)** participants with COPD (n=1) stained with panel 1 (P1) or panel 2 (P2) antibodies (Table 4.1) with or without AF700-conjugated anti-CD16 and viability dyes. Median fluorescence intensity (MFI) shown for 5000 neutrophil events on a log(10) scale.

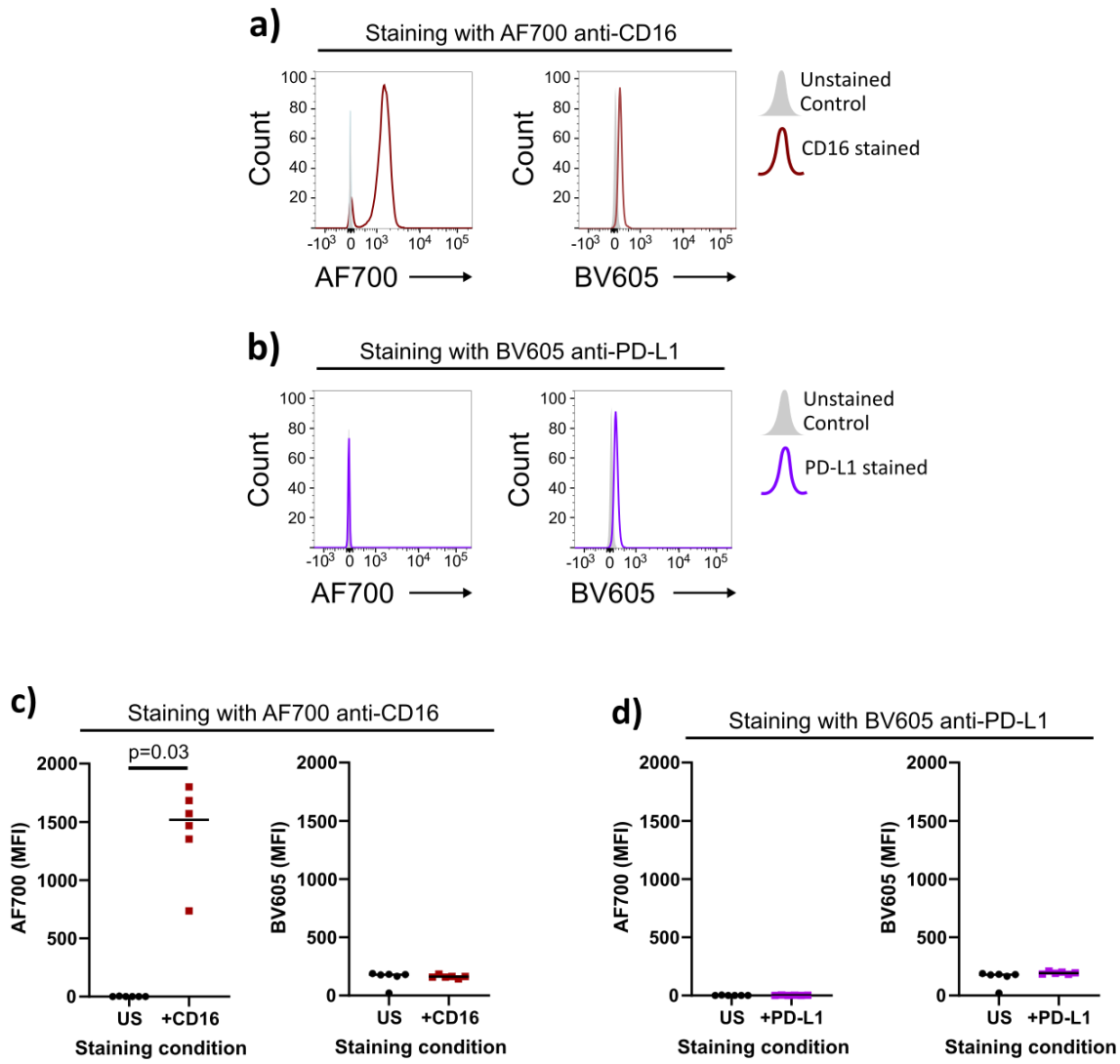

Figure 4.5: Median fluorescence intensity of AF700 and BV605 of isolated neutrophils stained with AF700-conjugated anti-CD16 or BV605-conjugated anti-PD-L1

a and b) Representative histogram plots of (left) AF700 and (right) BV605 fluorescence of isolated neutrophils from healthy young (HY, n=6) unstained (US) or stained with either a) AF700-conjugated anti-CD16 or b) BV605-conjugated anti-PD-L1. **c and d)** Quantification of the median fluorescence intensity (MFI) of AF700 and BV605 for c) anti-CD16 or d) anti-PD-L1. Each point represents one independent donor (total n=6 paired results) with the median shown. Statistical analysis performed using Wilcoxon signed-rank paired test.

4.2.4 Changes detected PD-L1 expression are due to anti-CD16

In order to assess if PD-L1 expression was influenced by anti-CD16, neutrophils from HY donors were incubated with AF700-conjugated anti-CD16 or BV605-conjugated anti-PD-L1 alone or in combination and the fluorescent signal from both detection channels measured (Figure 4.6). Co-incubation of neutrophils with these two antibodies led to an increase in the MFI of AF700 (Figure 4.6b), indicating CD16 staining. Under these conditions, the MFI of BV605 was significantly higher compared to staining with anti-PD-L1 alone (Figure 4.6c: without anti-CD16 median MFI=193, with median MFI=4888, p=0.03). Therefore, the detected expression of PD-L1 was dependant on incubation with AF700-anti-CD16 antibody.

4.2.5 Identification of alternative anti-CD16 antibodies

Given that AF700-conjugated anti-CD16 altered the amount of PD-L1 detected on the surface of neutrophils, alternative clones and conjugates of commercially available anti-CD16 antibodies (Table 4.2) were used to determine if antibody incubation altered cell viability and PD-L1 expression. Neutrophils from HY participants were incubated with each anti-CD16 antibody in combination with BV605-conjugated anti-PD-L1. There was no significant difference in the viability of neutrophils following staining with any of the anti-CD16 antibodies tested (Figure 4.7).

Antibodies from eBiosciences (clone eBio-CB16, Figure 4.8a) and Invitrogen (clone 3G8, Figure 4.8b) showed a similar increase in the detected expression of PD-L1 as with BioLegend's anti-CD16 (clone 3G8). In contrast, the anti-CD16 antibody from Miltenyi (clone VEP-13) did not demonstrate an increase in the MFI of PD-L1 (Figure 4.8c). Therefore, not all anti-CD16

antibodies resulted in an increase in the detected expression of PD-L1 and the underlying cause required further investigation.

4.2.6 Changes in PD-L1 expression are conserved in COPD

In order to investigate if the changes in PD-L1 expression were recapitulated by neutrophils from patients with COPD, isolated neutrophils from both elderly participants with and without COPD were incubated with different anti-CD16 antibodies as described above and the BV605-conjugated anti-PD-L1 antibody (Figure 4.9). Similar to the healthy participant group, an increase in detectable expression of PD-L1 was observed when neutrophils were stained with the anti-CD16 antibodies from with BioLegend 3G8, eBiosciences eBioCB16 and Invitrogen 3G8 (Figure 4.9). Whilst the same trend was observed with HE participants without COPD (Figure 4.9a-c), the sample number prevented this reaching statistical significance. Once again, there was no increase in detectable PD-L1 expression when incubated with Miltenyi VEP-13 anti-CD16 in either participant group (Figure 4.9d,h). These data point to the possibility that the increase in detected PD-L1 expression was age and antibody clone independent, but fluorophore dependent.

4.2.7 Changes in PD-L1 expression depend on the AF700 fluorophore

In order to identify if the detected increase in PD-L1 was dependent on AF700, a direct comparison of anti-CD16 (clone eBio-CB16) conjugated to either AF700 or FITC was carried out. A significant increase in the detectable PD-L1 expression was observed on neutrophils isolated from HY donors following incubation with AF700 conjugated (Figure 4.10a), but not FITC-conjugated anti-CD16 antibody (Figure 4.10b), suggesting that AF700 was a key factor in this phenomenon.

4.2.8 Neutrophils do not directly engage with AF700

To further investigate if neutrophils directly interact with AF700, neutrophils from HY participants were incubated with a non-specific mouse anti-human IgG1 (isotype control) conjugated to AF700. Incubation with anti-IgG1 demonstrated a comparable MFI of PD-L1 (Figure 4.11 median MFI [IQR] = 178.7 [95.5-202.8]) to previous incubation with anti-PD-L1 alone (Figure 4.6 median MFI [IQR] = 193 [182.9-203.8]). However, incubation with AF700-conjugated anti-CD16 still resulted in an increase in the MFI of PD-L1 (Figure 4.11). These data highlight that AF700 alone was not responsible for the increase in PD-L1 expression and that neutrophils are not directly binding AF700 to give a false-positive signal.

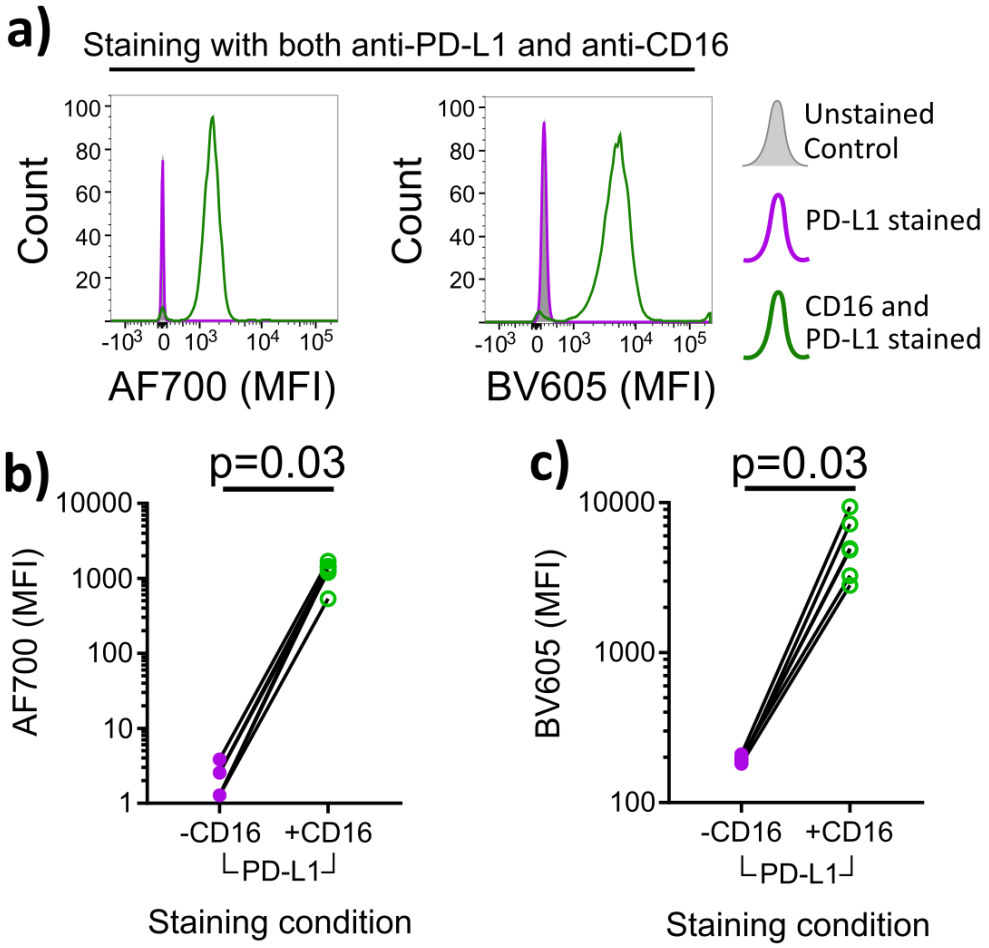

Figure 4.6: Median fluorescence intensity of AF700 and BV605 of isolated neutrophils stained with BV605-conjugated anti-PD-L1 with or without AF700-conjugated anti-CD16

a) Representative histogram plots of (left) AF700 and (right) BV605 fluorescence of isolated neutrophils from healthy young (HY, n=6) unstained (grey), stained with BV605-conjugated anti-PD-L1 alone (purple trace) or in combination with AF700-conjugated anti-CD16 (green trace). **b and c)** Quantification of the median fluorescence intensity (MFI) of (b) AF700 and (c) BV605 on a log(10) scale. Each point represents one independent donor (total n=6 paired results). Statistical analysis performed using Wilcoxon signed-rank paired test.

Table 4.2: Alternative anti-CD16 antibodies

Manufacturer	Clone	Fluorophore
eBiosciences	eBioCB16	AF700
Invitrogen	3G8	AF700
Miltenyi	VEP-13	FITC

Legend: Each anti-CD16 antibody is listed by manufacturer alongside the respective clone and fluorophore.

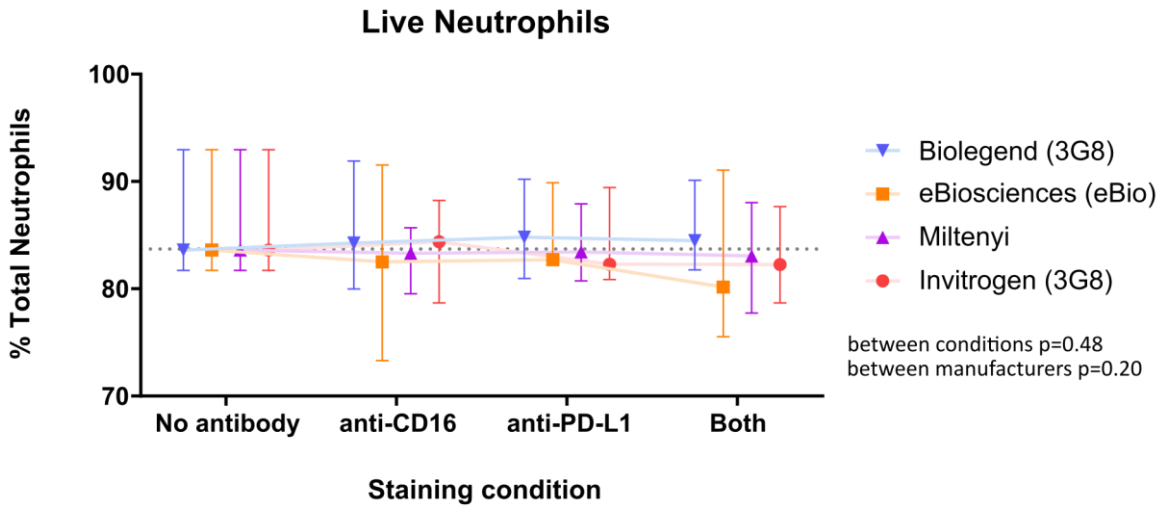

Figure 4.7: Viability of neutrophils incubated with different clones and suppliers of anti-CD16

Isolated neutrophils from healthy young participants (n=6) were assessed for viability using annexin V and 7AAD following antibody incubation. Median percentage live neutrophils without antibody incubation indicated by dotted grey line. Data are shown as median with interquartile range for n=6 individual donors. ANOVA analysis showed no significant effect of staining condition or manufacturer on neutrophil viability.

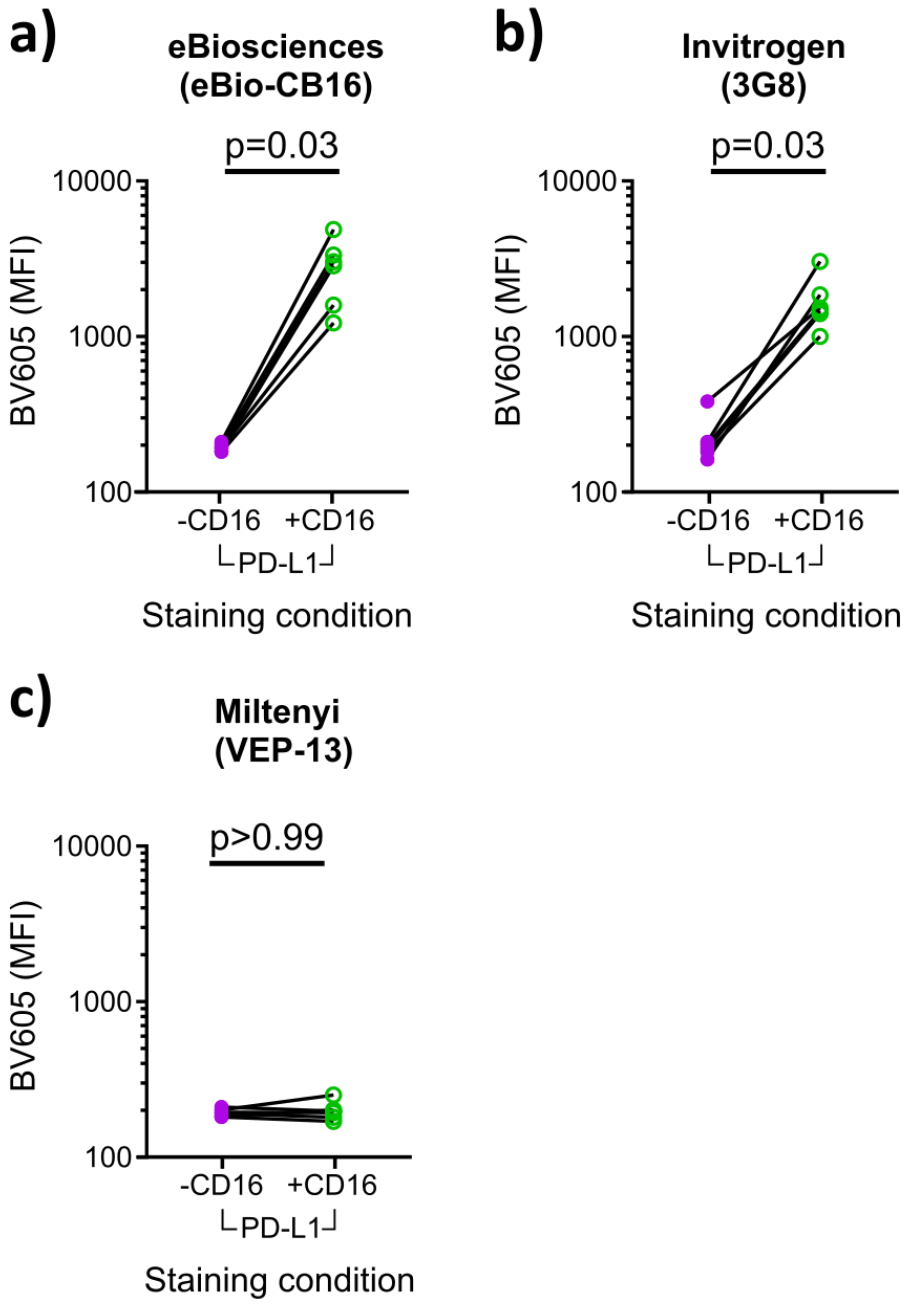

Figure 4.8: Median fluorescence intensity of BV605 with neutrophils from healthy young incubated with both BV605-conjugated anti-PD-L1 and anti-CD16

Isolated neutrophils from healthy young participants (n=6) were incubated with anti-PD-L1 with or without **a)** eBiosciences eBio-CB16, **b)** Invitrogen 3G8 or **c)** Miltenyi VEP-13 anti-CD16. Median fluorescence intensity (MFI) of BV605 shown on a log(10) scale. * = P<0.05 determined using Wilcoxon signed-rank paired test.

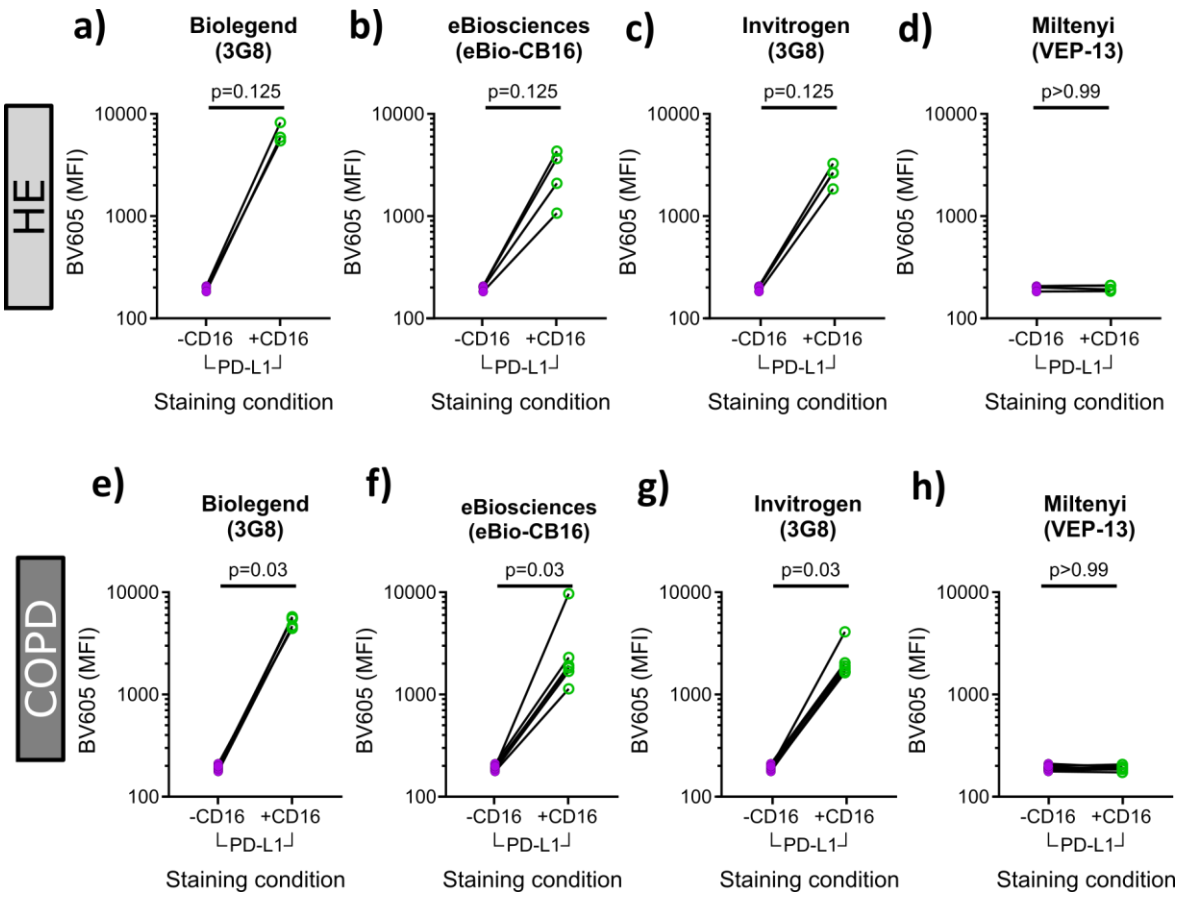

Figure 4.9: Median fluorescence intensity of BV605 with neutrophils from healthy elderly and COPD participants incubated with both BV605-conjugated anti-PD-L1 and anti-CD16

Isolated neutrophils from **a-d)** elderly participants without COPD (n=5) or **e-h)** with COPD (n=6) were incubated with anti-PD-L1 with or without **a,e)** BioLegend 3G8, **b,f)** eBiosciences eBio-CB16, **c,g)** Invitrogen 3G8 or **d,h)** Miltenyi VEP-13 anti-CD16. Median fluorescence intensity (MFI) of BV605 shown on a log scale. Statistical analysis performed using Wilcoxon signed-rank paired.

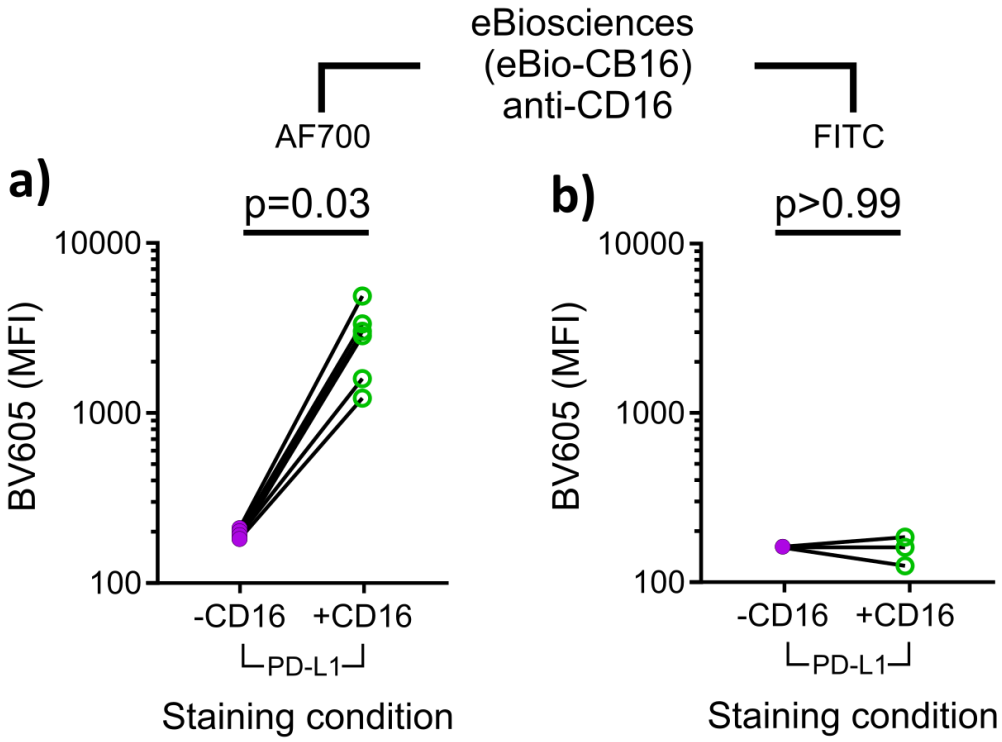

Figure 4.10: Median fluorescence intensity of BV605 with neutrophils from healthy young participants incubated with anti-PD-L1 and anti-CD16

Isolated neutrophils were incubated with BV605-conjugated anti-PD-L1 and either **a)** AF700-conjugated (as shown in Figure 4.8a) or **b)** FITC-conjugated eBiosciences eBio-CB16 anti-CD16. Median fluorescence intensity (MFI) of BV605 shown on a log(10) scale. Each point represents one independent donor (a: n=6 paired results; b: n=3 paired results). Statistical analysis performed using Wilcoxon signed-rank paired test.

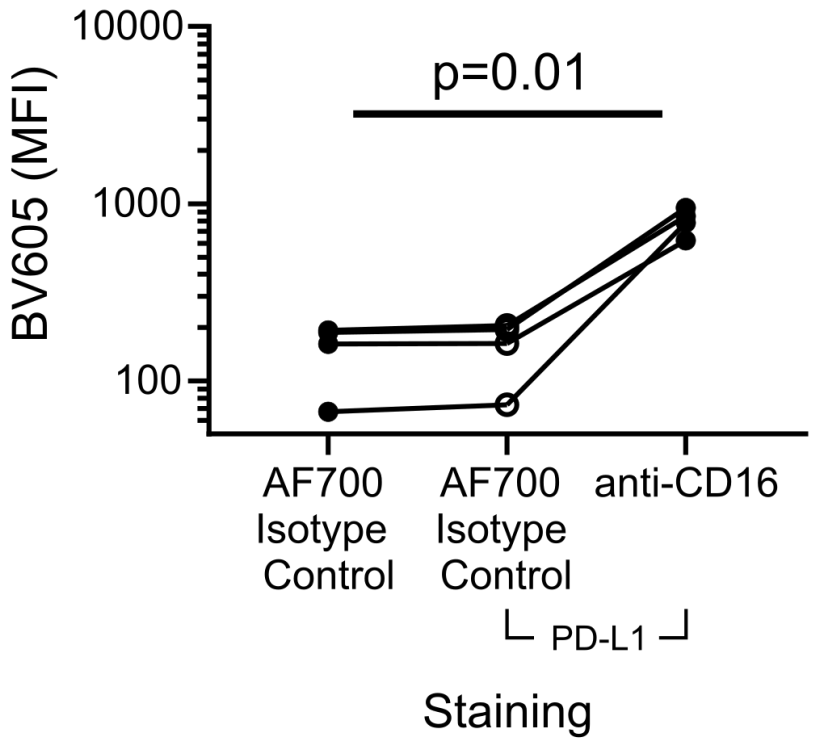

Figure 4.11: Median fluorescence intensity of BV605 of neutrophils from healthy young participants incubated with anti-PD-L1 and either AF700-conjugated anti-IgG1 or anti-CD16

Isolated neutrophils from healthy young participants (n=4) were incubated with a combination of AF700-conjugated IgG1, BV605-conjugated anti-PD-L1 and AF700-conjugated anti-CD16. The median fluorescence intensity (MFI) of BV605 is shown on a log(10) scale. Statistical analysis performed using a Friedman test (shown) with Dunn's multiple comparison.

4.2.9 Incubation of neutrophils with different clones of anti-PD-L1

As the increase in the MFI of PD-L1 occurred with the AF700 fluorophore, an alternative clone of anti-PD-L1 was obtained to investigate direct binding of anti-PD-L1 to the AF700 fluorophore. Neutrophils from a HY participant were incubated with AF700-conjugated anti-CD16 (clone eBio-CB16) and either clone 29E.2A3 (BioLegend, used in all previous experiments conjugated to BV605) or clone 130021 (R&D Systems, conjugated to APC) of anti-PD-L1.

As previously discussed, incubation of neutrophils with anti-PD-L1 (clone 29E.2A3) and anti-CD16 resulted in the increase of the MFI of PD-L1 (Figure 4.12a, purple trace). When neutrophils were incubated with the alternative anti-PD-L1 antibody (clone 130021) and anti-CD16, only an increase in the MFI of CD16 was observed (Figure 4.12b, dark red trace). The absence of increased PD-L1 signal suggested that anti-CD16 successfully and specifically binds CD16 on the neutrophil surface followed by binding of anti-PD-L1 (clone 29E.2A3) to the AF700 fluorophore – an interaction that was not recapitulated with an alternative anti-PD-L1 clone.

4.2.10 Direct binding of anti-PD-L1 clone 29E.2A3 to AF700 fluorophore

To further investigate direct binding of clone 29E.2A3 of anti-PD-L1 to AF700, further analysis of previous data was performed. When isolated neutrophils from HY participants were incubated with anti-CD16 (BioLegend, clone 3G8) and anti-PD-L1 (BioLegend clone 29E.2A3), the MFI of CD16 decreased compared to incubation with anti-CD16 alone (Figure 4.13, median MFI [IQR] without anti PD-L1 = 1519 [1199-1713] compared with anti-PD-L1 = 1358 [1031-1515], p=0.03). These data indicate disruption of the AF700 fluorescence signal.

In order to confirm the requirement of AF700 but negate the requirement of anti-CD16 in the binding of anti-PD-L1 (clone 29E.2A3), an AF700-conjugated anti-CD66b antibody was obtained. Neutrophils from HY volunteers were incubated with anti-PD-L1 and anti-CD66b either separately or together as previously for anti-CD16 antibodies. An increase in the detected PD-L1 expression was observed on neutrophils incubated with both antibodies (Figure 4.14a). This increase in MFI was less than previously observed with anti-CD16 incubation (Figure 4.6c), however, expression of CD66b appeared lower than CD16 on neutrophils (Figure 4.14b compared with Figure 4.6b). Incubation of neutrophils with this clone of anti-PD-L1 also resulted in a reduction in the MFI from CD66b compared with anti-CD66b alone (Figure 4.14c), supporting disruption of the AF700 signal. Furthermore, sequential staining with anti-PD-L1 and then anti-CD16 separated with a wash step failed to recapitulate the increase in the expression of PD-L1 (Figure 4.14d). Together, these results support the direct binding of anti-PD-L1 (clone 39E.2A3) to the AF700 fluorophore.

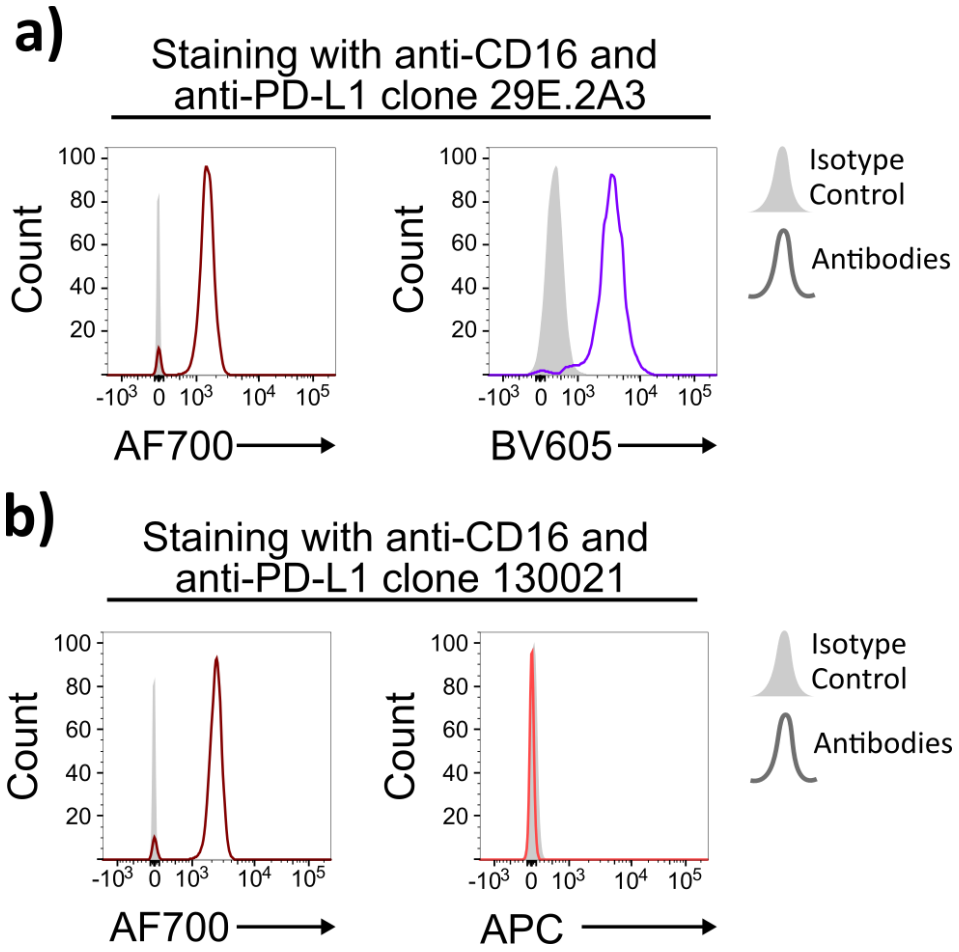

Figure 4.12: Fluorescence intensity plots of both CD16 and PD-L1 of neutrophils from a healthy young participant incubated with anti-CD16 and two clones of anti-PD-L1

Isolated neutrophils from a healthy young participant (n=1) were stained with anti-CD16 and either anti-PD-L1 **a)** BioLegend clone 29E.2A3 or **b)** R&D Systems clone 130021. The flow cytometry plots of fluorescent intensity of both AF700 (CD16) and either BV605 (PD-L1 clone 29E.2A3) or APC (PD-L1 clone 130021) are shown. Each panel shows 5000 neutrophils from n=1 donor.

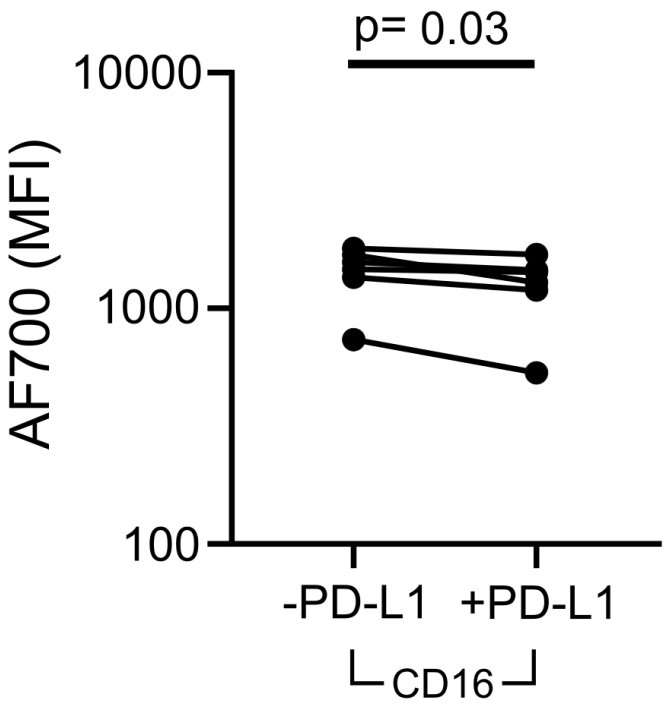

Figure 4.13: Median fluorescence intensity of CD16 on neutrophils from healthy young participants incubated with anti-PD-L1 clone 29E.2A3 and AF700-conjugated anti-CD16 clone 3G8 antibodies

Isolated neutrophils from healthy young participants (n=6) were incubated with anti-CD16 in the presence or absence of anti-PD-L1 antibody. The median fluorescence intensity (MFI) of AF700 for each sample is shown on a log(10) scale. Statistical analysis performed using Wilcoxon signed-rank paired test.

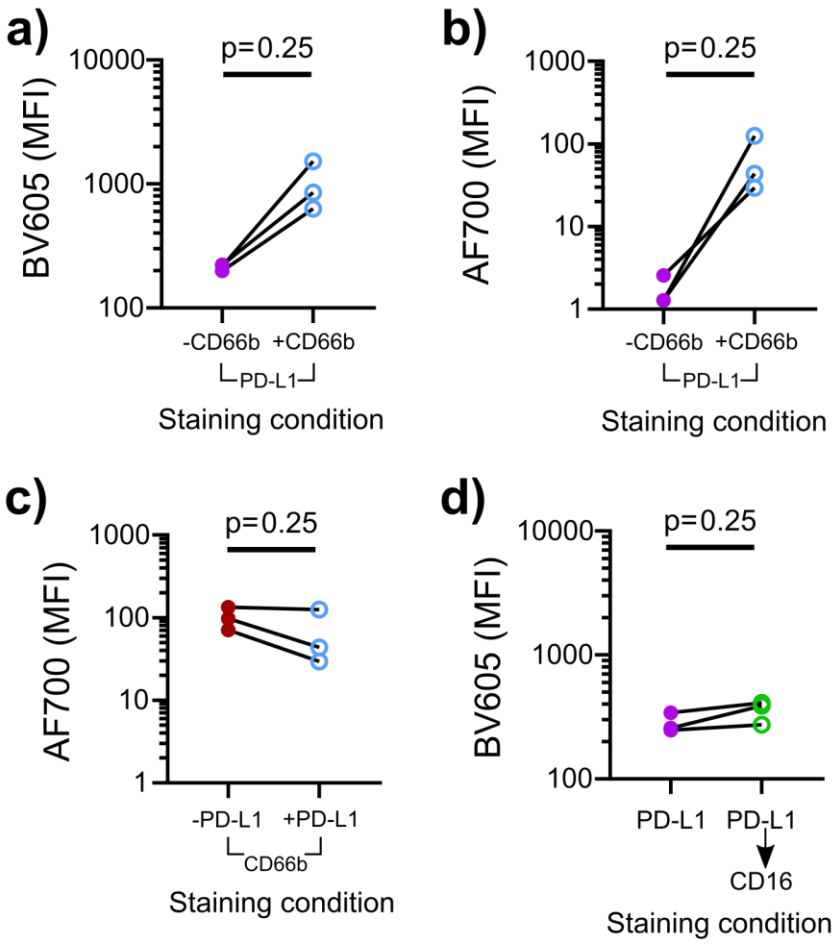

Figure 4.14: Median fluorescence intensity of PD-L1 clone 29E.2A3, CD66b and CD16 of neutrophils from healthy young participants incubated with anti-PD-L1 and AF700-conjugated anti-CD66b or anti-CD16

Isolated neutrophils from healthy young participants (n=3) were incubated with combinations of anti-PD-L1 (BV605) and anti-CD66 (AF700) and the median fluorescence intensity (MFI) of **a)** BV605 or **b)** AF700 measured. **c)** MFI of AF700 in the presence or absence of anti-PD-L1 antibody. **d)** MFI of BV605 on neutrophils incubated with anti-PD-L1 and washed prior to incubation with anti-CD16 (n=2). The median fluorescence intensity is shown on a log(10) scale for all plots. Statistical analysis performed using Wilcoxon signed-rank paired test.

4.3 Discussion

PD-L1 expression by circulating immune cells has been implicated in cancer (Bocanegra *et al.*, 2019), and the potential role of PD-L1 in inflammatory diseases, such as COPD, required measurement of PD-L1 surface expression on isolated neutrophils. Flow cytometry is a widely used and accepted technology for addressing this question, however, it relies heavily on fluorescent target-specific monoclonal antibodies (Maecker and Trotter, 2006). With an array of different clones and fluorophores commercially available, it is generally assumed that these antibodies are already well-validated and ready to use in complex antibody panels without further end-user validation. These series of experiments were carried out to ensure that accurate measurement of PD-L1 expression could be determined using flow cytometry in a multi-parametric panel.

As demonstrated, initially it appeared that PD-L1 expression could be induced by the binding of CD16 antibodies to neutrophils, raising both an issue for the measurement of baseline expression of PD-L1 in different patient groups, but also a potential novel signalling pathway in neutrophils. However, careful validation identified that the detected PD-L1 expression was due to a previously undescribed interaction between a specific clone of anti-PD-L1 antibody and the AF700 fluorophore of the CD16 antibody. These results demonstrate that failure to correctly validate this antibody would have led to the incorrect reporting of PD-L1 expression by neutrophils, undermining the accuracy of the results. Other such interactions likely exist given the number of commercial and specialist antibodies that are produced for flow cytometry.

4.3.1 Evidence that anti-CD16 may have caused a functional effect

AF700 was identified as a suitable fluorophore despite having a 'dim' relative brightness (BD Biosciences, 2014), as CD16 is highly expressed on neutrophils. Introduction of AF700-conjugated anti-CD16 antibody into the panel appeared to slightly reduce neutrophil viability and increase PD-L1 expression. It has previously been shown that cross-linking of the CD16 molecule on neutrophils can initiate calcium signalling (Kimberly *et al.*, 1990) and, therefore, potentially lead to PI3K activation (Chuang *et al.*, 2000). PI3K signalling in neutrophils is linked with both activation of neutrophil functions and the inhibition of apoptosis (Klein *et al.*, 2001) – in contrast to the slight increase in apoptosis observed in these data. Further repeats revealed that viability was not significantly altered, however, apparent robust induction of PD-L1 remained. Possible links to the PI3K signalling pathway may explain this effect as PD-L1 expression in epithelial cells has been linked to PI3K signalling (Ritprajak and Azuma, 2015). Therefore, it was feasible that ligation using this anti-CD16 antibody was causing functional changes in these isolated neutrophils and raised concerns about the use of this anti-CD16 antibody for subsequent analysis given the aim of our study was to measure PD-L1 expression without manipulation of isolated neutrophils.

4.3.2 Are changes in PD-L1 due to fluorescence artefacts?

Due to the chemistry of fluorophore and fluorescent molecules, spectral overlap is an important consideration in panel design. Given the separation of AF700 and BV605 in both excitation laser and emission wavelength (AF700: excitation laser 640nm, emission peak 719nm; BV605: excitation laser 405nm, emission peak 605nm) spectral overlap between AF700 and BV605 was highly unlikely. Assessing each antibody in turn is the best way to ensure

signals are not giving false-positive results and is in line with published guidance (Maecker and Trotter, 2006). Following this, no evidence of spectral overlap was detected. Although this important step in the validation of multiparametric flow cytometry is highlighted in publications and technical bulletins from manufacturers (Roederer, 2001; BD Biosciences, 2009), it is not usually included within publications or supplemental documents. Given spectral overlap did not explain the rise in detected PD-L1 expression and this epitope of anti-CD16 may be functional, other manufacturers and clones of anti-CD16 were sought.

4.3.3 Reproducibility of PD-L1 induction with participants and anti-CD16 clones

It is known that neutrophils change with both age (Whyte *et al.*, 1993; Sapey *et al.*, 2014) and the presence of COPD (Yoshikawa *et al.*, 2007; Hughes, Sapey and Stockley, 2019). Therefore, it was desirable to understand if the expression of PD-L1 in response to anti-CD16 antibody altered in these participant groups as differences may be indicative of alterations in activation pathways and protein expression. Presently, no investigations have specifically addressed age-related changes in PD-L1 expression on any cell type, however, a meta-analysis of the efficacy of current PD-1 and PD-L1 inhibitors showed no difference with age (Elias *et al.*, 2018). Based on the data presented here, there was a conserved ability of several anti-CD16 antibodies to increase the MFI of PD-L1 across these groups that suggested a fundamental response of neutrophils that was not altered with age or the presence of COPD.

Furthermore, several different clones and manufacturers of anti-CD16 antibodies were also able to reproduce the increase in PD-L1. Patent law does not require antibody manufacturers to disclose or know the binding epitope of the antibody (Domeij, 2000) and, therefore, it was not possible to directly implicate the binding location of any anti-CD16 antibody to CD16 with

the increase in PD-L1. Functional antibodies do exist and are used in a variety of applications including activating T cells through CD3 engagement by anti-CD3 (Lécureuil *et al.*, 2007). Functional antibodies are epitope-dependant (Domina *et al.*, 2016), a notion supported by affinity-maturation of antibody responses in the adaptive immune system (Mishra and Mariuzza, 2018). Together, these provide support that these anti-CD16 antibodies may be giving a stimulatory signal to neutrophils. However, as the only antibody tested that did not produce this response was conjugated to a different fluorophore (FITC), another avenue arose that the effect seen was fluorophore-dependant rather than epitope-dependant.

Of note, one study found that neutrophils phagocytosed FITC-conjugated bacteria more readily than green-fluorescence protein (GFP)-tagged bacteria, suggesting that fluorophores could influence neutrophil behaviour. Neutrophils also express an array of surface receptors (Futosi, Fodor and Mócsai, 2013) and it was, therefore, poignant to investigate any impact of the AF700 fluorophore. Mouse IgG and IgM antibodies are commonly used as isotype controls to measure non-specific binding (Hulspas, O'Gorman, *et al.*, 2009) and here could be used to identify any functional changes due neutrophils binding the AF700 fluorophore rather than the antibody binding the neutrophil. However, this resulted in no detected changes due to AF700, prompting an investigation of alternative explanations to the increase in detected PD-L1 expression.

4.3.4 A novel and specific antibody binding of a fluorophore

Previous work has identified that the conjugation of monoclonal antibodies to fluorophores can change their binding affinity (Szabó *et al.*, 2018), but there does not appear to be any evidence suggesting monoclonal antibodies bind to both a specific antigen and a fluorophore.

As demonstrated, a direct head-to-head comparison of anti-CD16 fluorophore revealed fluorophore-dependence and that the binding of CD16 was not eliciting a change in PD-L1 expression. Attention then turned to the anti-PD-L1 antibody and, surprisingly, revealed that a different clone of anti-PD-L1 did not recapitulate the increase in detected PD-L1 expression. Use of another AF700-conjugated antibody, CD66b, and separating anti-PD-L1 incubation with wash steps before AF700-conjugated anti-CD16 staining further supported the interaction between the 29E.2A3 clone of anti-PD-L1 and AF700. The reduction in AF700 fluorescence when combined with anti-PD-L1 further suggested obstruction of the AF700 fluorophore, however, no mention of this effect exists in the literature. Together, these data support a novel and unreported interaction between a target-specific monoclonal antibody, clone 29E.2A3 anti-PD-L1 and a fluorophore, AF700.

4.3.5 Greater validation and transparency are required in all flow cytometry experiments

Flow cytometry is a fundamental tool to investigate the surface expression of receptors and ligands in immunobiology. The reliability of these data relies on antibodies that bind their target with specificity; that do not influence the binding of other antibodies; and are used in conjunction with fluorophores that can be detected separately. Despite the wide array of guidelines from manufacturers that highlight the importance of panel design (Edinger, no date; Thermo Fisher Scientific, 2017), there is no published standard for designing and testing custom panels. There have been improvements in tools available to researchers to improve the theoretical aspect of panel design (Bashratyan *et al.*, 2017), but each individual panel requires real-world validation, especially as antibodies can have functional effects on the cell of interest, modifying signalling pathways and altering cellular phenotype (Melidoni, Dyson

and McCafferty, 2015). In addition, failure to account for non-specific binding, such as through surface Fc receptors on the cells of interest, can lead to false-positive results (Andersen *et al.*, 2016). The data presented in this chapter highlight this, as the use of AF700-conjugated antibodies in combination with anti-PD-L1 (clone 29E.2A3) without validation would lead to the incorrect reporting of PD-L1 expression. Importantly, this type of interaction between a commercial monoclonal antibody and a commonly available fluorophore has not been reported in the literature previously.

Several papers and articles do describe the need for careful validation and how to use appropriate controls to assist in data interpretation (Maecker and Trotter, 2006; Bordeaux *et al.*, 2010). Using data presented in this chapter and building upon these publications, there should, however, be two distinct validation steps. Firstly, each antibody should be titrated to determine the optimum concentration using a known positive, such as a cell known to express the marker of interest. Secondly, the baseline expression and fluorescence intensity for each marker should be established on the cell of interest in isolation. These data can then be used to compare any potential changes when the antibodies are combined. The benefits are threefold: identification of spectral overlap, antibody-antibody interactions and functional antibodies.

Given the challenges of good panel design and knowledge of the consequences, clear strategies need to be employed to ensure accurate and reliable data are produced. There is a serious gap in this information provided by publications of flow cytometry data, with many only providing the target and fluorophore conjugate of the antibodies used, neglecting to identify the clone used and how it was validated.

4.3.6 Summary and study limitations

As highlighted, validation of antibodies in multi-parametric flow cytometry is required for reliable expression data to be obtained. Here it has been shown that incubation of isolated human neutrophils with both AF700-conjugated anti-CD16 and anti-PD-L1 resulted in an observed increase in PD-L1 expression. Through careful validation, the increase in MFI of PD-L1 was not due to increased expression but caused by an artefact of a specific clone of anti-PD-L1 that was capable of specifically binding the AF700 fluorophore, resulting in false-positive detection of PD-L1 expression. However, this study does not provide direct evidence of this binding interaction. This could be achieved through a technique such as surface plasmon resonance. (Hearty, Leonard and O'Kennedy, 2012). These data presented in this chapter describe a novel antibody-fluorophore cross-reactivity and further highlight the importance of rigorous validation of antibodies used in flow cytometry to prevent false-positive results.

The phenotyping of neutrophils performed in this thesis needed to commence prior to the completion of this entire validation and, therefore, anti-CD16 was not used in conjunction with anti-PD-L1 in subsequent experiments.

CHAPTER 5:

PHENOTYPING OF PERIPHERAL BLOOD NEUTROPHILS IN HEALTH AND STABLE COPD

5.1 Brief introduction

There has been much debate regarding neutrophil phenotypes and subtypes, and if different expression of surface receptors and ligands corresponds to different functional states or indeed separate and unique cell subtypes, similar to those observed in other immune cells such as T cells (Sagiv *et al.*, 2015; Brandau and Hartl, 2017; Christoffersson and Phillipson, 2018; Hellebrekers, Vrisekoop and Koenderman, 2018; Rosales, 2018; Silvestre-Roig *et al.*, 2019). The debate is not simply about how we define neutrophils, but has wider implications in a disease setting to guide therapies: is a 'subtype' of neutrophils detrimental and can it be modified, or does the environment the neutrophils encounter require alteration?

There is also a growing appreciation of the heterogeneity, both biological and clinical, of COPD, resulting in patients with varying degrees of lung function decline and responsiveness to treatment (Roy *et al.*, 2009; Miravitlles *et al.*, 2013). However, many studies (including clinical trials) do not separate patients into different subgroups based on biological differences (Calverley and Rennard, 2007): this may be, in part, due to the lack of understanding and ineffective tools to easily identify these differences. Stratification tends to be limited to clinical parameters, such as lung function or exacerbation frequency, that may poorly reflect the underlying molecular and cellular pathogenesis (Calverley and Rennard, 2007). The lack of stratification poses limitations when addressing the effectiveness of therapy, as patients may not respond to the same degree to a given drug or treatment regime due to differences in the underlying biology of COPD.

Many patients with COPD commonly have other chronic inflammatory conditions, such as CVD and T2D, that increase the inflammatory burden in these patients (Grosdidier *et al.*, 2014).

The neutrophil is one of the immune cells implicated in COPD, CVD and T2D, and alterations in neutrophil function within these diseases is a potential source of heterogeneity in patients with COPD (Hughes, McGettrick and Sapey, 2020b). Understanding the difference these co-morbidities have on neutrophil function and phenotype could determine the potential benefit different therapeutic interventions have on different patient populations.

To explore differences in neutrophil function, neutrophil phenotypes can be investigated by measuring the surface expression of receptors and ligands by neutrophils isolated from whole blood – a relatively well-established technique in both mice (Takashima and Yao, 2015) and humans (Millrud *et al.*, 2017). In many cases, a specific attribute (such as activation) is investigated in isolation (Noguera *et al.*, 2001). However, multiple phenotypes have been previously described such as immature, senescent and pro-inflammatory and can be investigated simultaneously using multi-parametric flow cytometry.

This study aimed to assess multiple neutrophil phenotypes using a combination of markers linked to neutrophil function that may be altered in healthy ageing and COPD. This was achieved using two well-validated flow cytometry panels. Neutrophils were obtained from a cohort of well-characterised patients with stable COPD, age-matched volunteers without COPD (healthy age-matched controls) and HY volunteers. Patients with COPD also had the presence of CVD or T2D recorded to allow stratification based on these co-morbidities. It was hypothesised that fundamental differences in neutrophil phenotypes exist between healthy people and those with COPD, especially regarding activation status and senescence. These differences would be exaggerated if multiple chronic inflammatory conditions were present in a given individual.

5.2 Results

5.2.1 Sample quality control

To ensure robust data collection, various quality control (QC) steps were performed for each experiment. These criteria were used to exclude any sample that did not meet these standards from further data analysis.

5.2.1.1 Controlling expression measurement with SpheroBeads

Consistent fluorescence detection from the flow cytometer was vital to record comparable surface expression levels across every patient sample using fluorescence intensity. To achieve this, SpheroBeads were used in each experiment to calibrate the laser power as established in Section 3.2.8, with target values for each detector determined with a tolerance of ±10% in line with EuroFlow guidelines (Kalina *et al.*, 2012). Every SpheroBead preparation collected prior to the neutrophil sample was within the 10% tolerance for channels AF700, BV421, BV510, BV605, FITC and 7AAD (Figure 5.1a). In the APC channel, run NP030 exceeded the upper 10% limit, as did NP042 in BV605 and BV786; NP047 in BV786 and NP004 in PE (Figure 5.1a). Samples that exceeded the tolerance in any channel were excluded from further analysis as the fluorescence intensity recorded was not comparable to other samples.

5.2.1.2 Exclusion of apoptotic samples

All samples were measured for viability and phenotype analysis was performed only on live cells. However, samples that contained below 25% viable neutrophils in either panel were excluded completely from analysis due to both low cell numbers for analysis and variations in the fluorescence intensity readings. Using this criterion, a total of 6 samples (7%) were

excluded (grey bars) from further analysis (Figure 5.1b). Remaining samples were deemed to have accurate fluorescence readings from a large enough sample of live neutrophils for phenotyping.

5.2.2 Gating strategy

Flow cytometry gating enabled the identification of specific neutrophil populations (Figure 5.2a). Doublets were excluded by selecting singlets using forward-scatter (FSC) height (-H) vs width (-W) and then selecting the granulocyte population by FSC area (FSC-A) vs. side-scatter area (SSC-A). Neutrophils were identified as positive for either CD16 (panel 1, see Figure 2.3) or CD66b (panel 2, see Figure 2.3). Neutrophil viability was assessed using annexin V and 7-aminoactinomycin (7AAD), where live cells were negative for both markers, apoptotic cells were identified as annexin V positive, necrotic (or alternative cell death) were identified as 7AAD positive and dead cells defined as positive for both markers. This strategy was followed for all neutrophil flow cytometry experiments unless otherwise stated. Additional neutrophil phenotypes were identified by gating live neutrophil events from Figure 5.2a as shown (Figure 5.2b-e). For individual markers, expression is shown with median fluorescence intensity as either nearly 100% of neutrophils were positive for a given marker (e.g. CD16) or showed the same outcome between the percentage positive events and MFI.

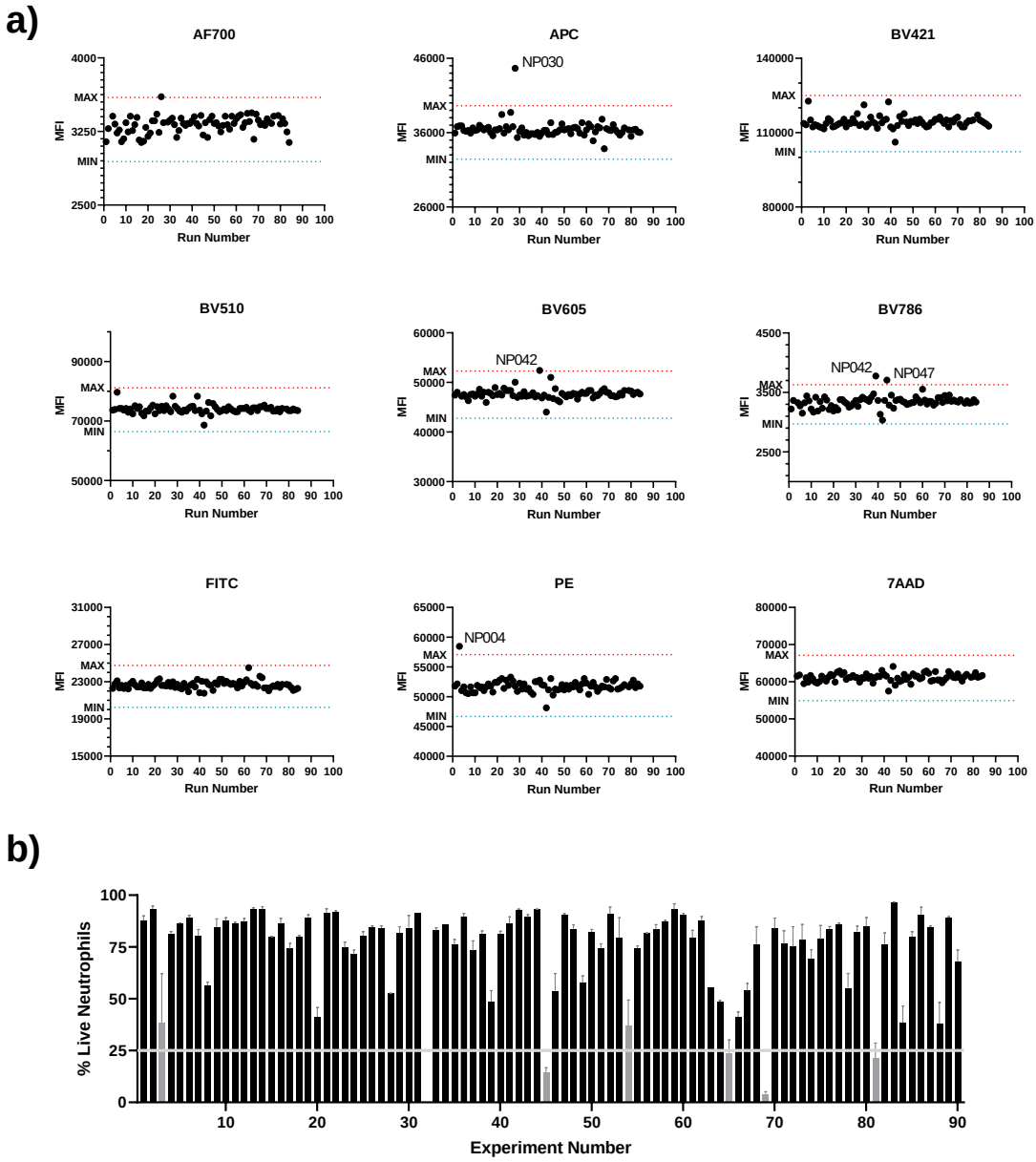

Figure 5.1: Sample quality control using SpheroBeads and cell viability

a) Median Fluorescent Intensity (MFI) was recorded for 4000 events in each fluorescent channel. Each point represents a separate run (n=100). A range of ±10% from the target value (shown in Table 3.7) is shown by the red (+10%) and blue (-10%) dotted lines, where any run outside this range was labelled with the run number (NP). **b)** Neutrophils from study participants were isolated and viability measured using annexin V and 7ADD staining. Cells negative for both markers were considered live. Bars show mean percentage of live cells for n=1 participant, averaged from samples in panel 1 and panel 2, with standard deviation. Horizontal line indicates the 25% cut-off value for exclusion, where if either value was <25%, the whole sample was excluded (grey bars).

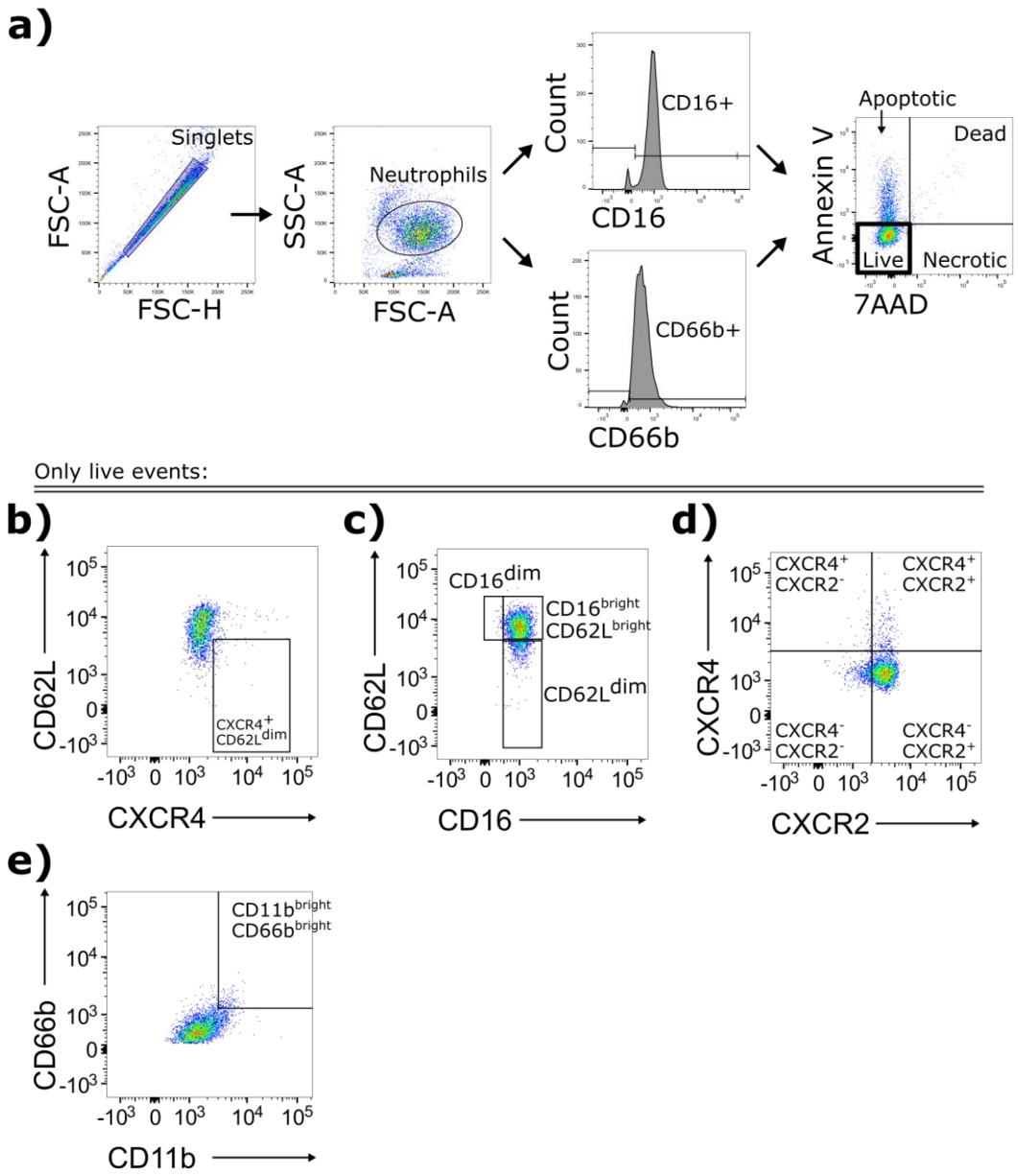

Figure 5.2: Gating strategy for neutrophil phenotyping

Neutrophils from whole blood were isolated, stained with antibodies and **a)** gated for single cells (based on forward scatter [FSC] area [A] and height [H]), neutrophils were further gated based on FSC-A and side scatter (SSC) and then either CD16+ (for panel 1) or CD66b+ (for panel 2) events to specifically identify neutrophils. Viability was assessed using annexin V and 7AAD and live cells identified as double negative events. Live events were then gated for **b)** CXCR4 and CD62L, **c)** CD16 and CD62L, **d)** CXCR2 and CXCR4, and **e)** CD66b and CD11b expression. Thresholds for gates were determined as detailed in section 3.2.7.

5.2.3 Changes in neutrophil phenotype with ageing and COPD

The expression of 11 surface markers on human neutrophils were measured across two flow cytometry panels (P1 and P2, as shown in Table 2.1). These markers were split into groups for analysis to assess particular neutrophil functions: activation status (CD11b, CD66b and CD62L); senescence (CXCR2 and CXCR4); inflammatory status (HLA-DR, PD-L1 and CD11c); reverse transmigration (CD54) and neutrophil maturity (CD10 and CD16).

5.2.3.1 Patient demographics

A total of 30 healthy volunteers and 41 patients with COPD were recruited in order to measure the peripheral blood neutrophil phenotype. The demographics of these participants can be seen in Table 5.1. There was no significant difference in age or sex between the healthy age-matched (HE) participants and patients with COPD (Table 5.1). Lung function was not carried out on healthy participants, but all had a complete absence of respiratory symptoms. A significant difference in smoking status was identified between HY and HE participants, with all HY participants being never-smokers (Table 5.1). A significant difference was also observed in smoking status and smoking history (measured as pack-years) between HE participants and patients with COPD. There was also a significant difference between the number of years HE participants had been ex-smokers compared to patients with COPD, with ex-smokers in the HE group quitting over twice as long ago. Notably, there were no current smokers in either healthy groups compared to 11 current smokers in the COPD group, measured from self-reported smoking status.

Table 5.1: Basic demographics for healthy and unstratified stable COPD study participants

	HY	HE	Stable COPD	p-value (HY vs HE)	p-value (HE vs COPD)
Number	15	15	41		
Age, median (IQR)	27 (23.5-31)	72 (70.75-74.25)	72 (68-73)	<0.01[k]	0.65[k]
Sex, M:F	8:7	10:5	25:16	0.71[f]	0.76[f]
Lung function					
FEV_1 % predicted, median (IQR)	N/A*	N/A*	57 (45-67)[1]	-	-
FVC % predicted, median (IQR)	N/A*	N/A*	79 (66-98.5)[1]	-	-
GOLD Grade (1:2:3:4)	N/A*	N/A*	5:22:9:4[1]	-	-
Smoking					
Smoking status (NS:Ex:C)	15:0:0	7:7:0[2]	0:30:11	<0.01[f]	<0.01[f]
Pack year history, median (IQR)	0 (0-0)	0.35 (0-9.0)[2]	49 (34.4-77.6)	-	<0.01[k]
Years ex-smoker, median (IQR)	N/A	31.5 (14.0-48.0)[2]	15 (6.5-17.5)	-	0.03[k]

Legend: Study participants split into healthy young controls without COPD (HY), healthy age-matched controls without COPD (HE) and patients with stable COPD (COPD). Median with interquartile range (IQR) to one decimal place are shown where appropriate. **Sex** shown as a ratio of male (M) to female (F). **GOLD Grade** displayed as a ratio of stage 1:2:3:4. **Smoking status** shown as ratio of never(NS):ex(Ex):current(C). Statistical analysis carried out using Kruskal-Wallis test (k) for continuous data and Fishers Exact test (f) for categorical data. FEV_1, forced expiratory volume in one second; FVC, forced vital capacity. *Spirometry not performed; [1] One patient was unable to perform spirometry. [2]Data unavailable for one participant.

5.2.3.2 Neutrophil activation

Contrary to the hypothesis, there was no difference in the expression of CD11b, CD66b or CD62L by neutrophils isolated from HY or HE participants, or from HE and those with COPD (Figure 5.3). However, a proportion of patients with COPD did demonstrate higher levels of CD11b and CD66b, indicated by a greater spread in the data (Figure 5.3a and b).

Measuring the proportion of $CD11b^{bright}CD66b^{bright}$ neutrophils, using the gating strategy shown in Figure 5.2, a similar pattern was observed to the expression of each individual marker (Figure 5.3a and b), with no difference between neutrophils isolated from HY or HE participants or from HE and those with COPD (Figure 5.4). Again, a small proportion of patients with COPD show a higher percentage of $CD11b^{bright}CD66b^{bright}$ neutrophils compared to both healthy groups (Figure 5.4). These data do not support the hypothesis that peripheral neutrophils from patients with COPD have an activated phenotype.

To assess the relationship between these activation markers, linear regression analysis was performed between CD11b and CD66b (Figure 5.5a). A significant positive correlation between CD11b and CD66b was observed (Figure 5.5a), supporting the use of CD11b and CD66b double-positive events to identify neutrophil activation.

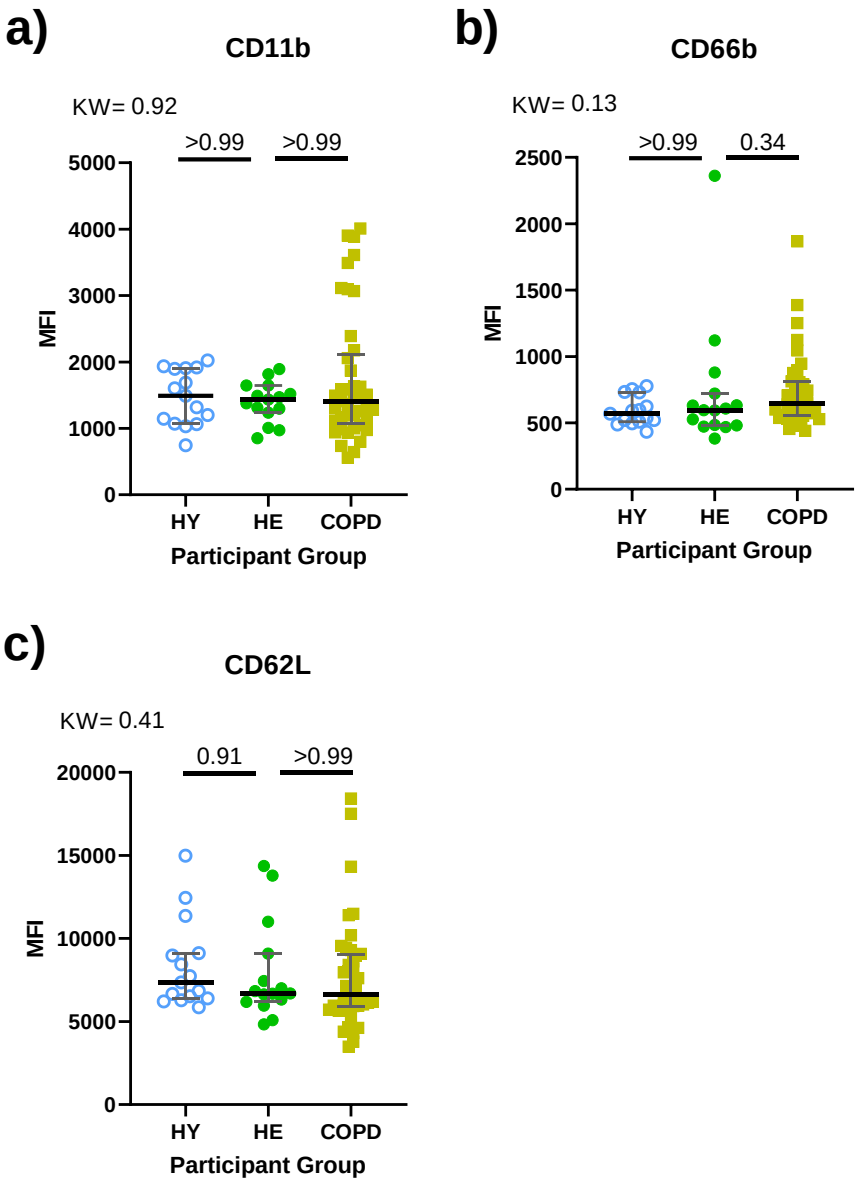

Figure 5.3: Median fluorescence intensity of activation markers of isolated neutrophils from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** CD11b, **b)** CD66b or **c)** CD62L for each participant group (HY n=15; HE n=15, COPD n=41). In each case, the horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups).

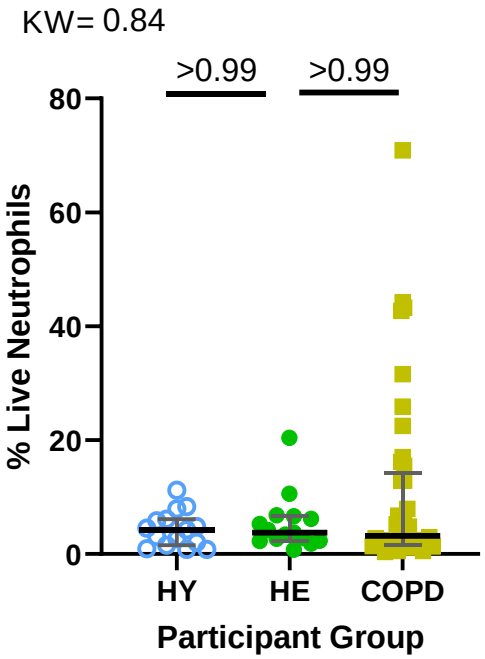

Figure 5.4: Percentage of CD11b^{bright}CD66b^{bright} live neutrophils isolated from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the percentage of CD11b^{bright}CD66b^{bright} live neutrophils (see Figure 5.2) recorded for each participant group (HY n=15; HE n=15, COPD n=41). In each case, the horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups).

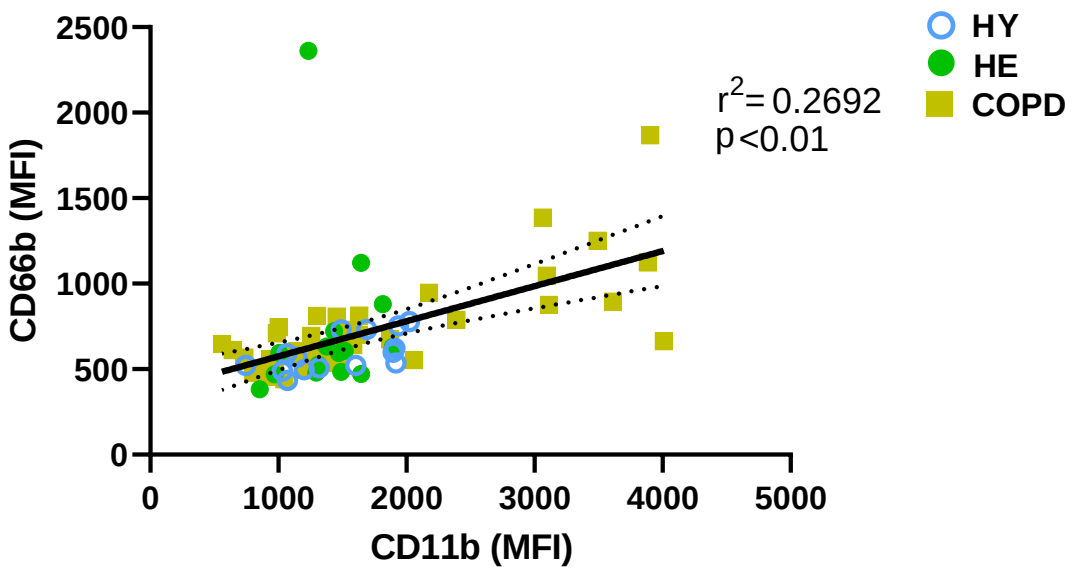

Figure 5.5: Correlation of activation markers on neutrophils from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the median fluorescence intensity (MFI) of CD11b and CD66b recorded for each participant group (HY n=15; HE n=15, COPD n=41). The correlation between CD11b expression and CD66b is shown. Linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for the linear regression.

5.2.3.3 Neutrophil senescence

Other studies indicate neutrophil senescence can be identified by a loss of CXCR2 and rise in CXCR4 levels. No difference in CXCR2 expression was observed between neutrophils isolated from HY or HE (Figure 5.6a). In contrast, neutrophils from patients with COPD showed, on average, a 12% reduction in CXCR2 expression (Figure 5.6b) compared with neutrophils from HE participants that negatively correlated with CD11b expression (Figure 5.6c). This change was not accompanied by an increase in CXCR4 expression, as comparable expression of CXCR4 was observed on neutrophils from all groups. These data indicate an altered neutrophil phenotype in patients with COPD, due to altered CXCR2 expression that correlated with activation, but not one consistent with senescence.

5.2.3.4 Neutrophil overactive senescence

A study of patients with ischemic stroke identified an increase in a population of neutrophils with CXCR4 expression and low CD62L expression, termed overactive senescent neutrophils (Weisenburger-Lile *et al.*, 2019). Neutrophils that expressed CXCR4 combined with low levels of CD62L were gated (CXCR4+CD62L^{dim}, Figure 5.2b) and the percentage of CXCR4+CD62L^{dim} events measured (Figure 5.7). No significant differences were identified between neutrophils isolated from HY and HE participants, or between HE and patients with COPD. However, there was a significant change across the groups as a whole, suggesting overactive senescent neutrophils may play a role in ageing and COPD, but further work is needed.

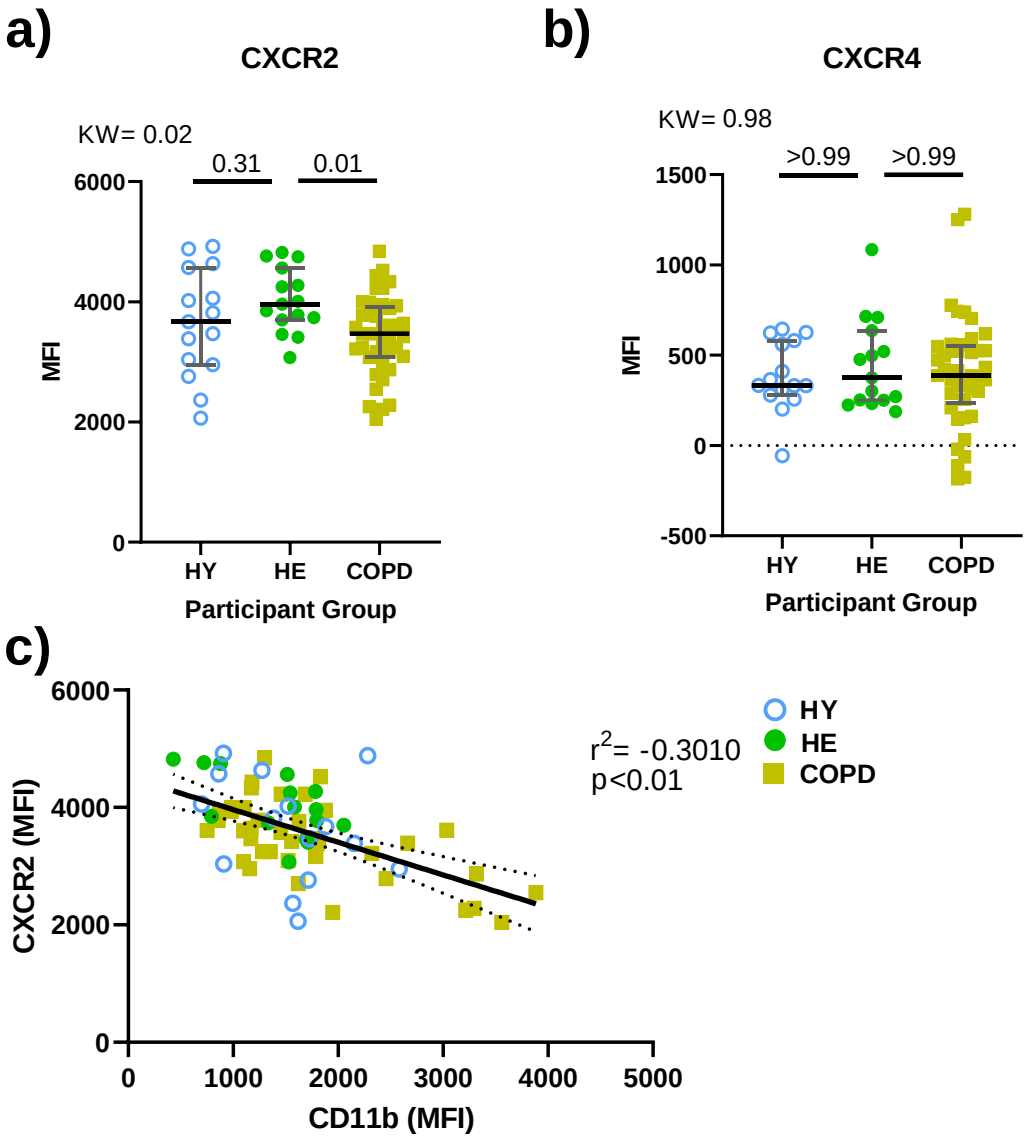

Figure 5.6: Median Fluorescence Intensity of senescence markers of isolated neutrophils from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** CXCR2 or **b)** CXCR4 for each participant group (HY n=15; HE n=15, COPD n=41). In each case, the horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups). **c)** The correlation between CD11b and CXCR2 expression. Linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for the linear regression.

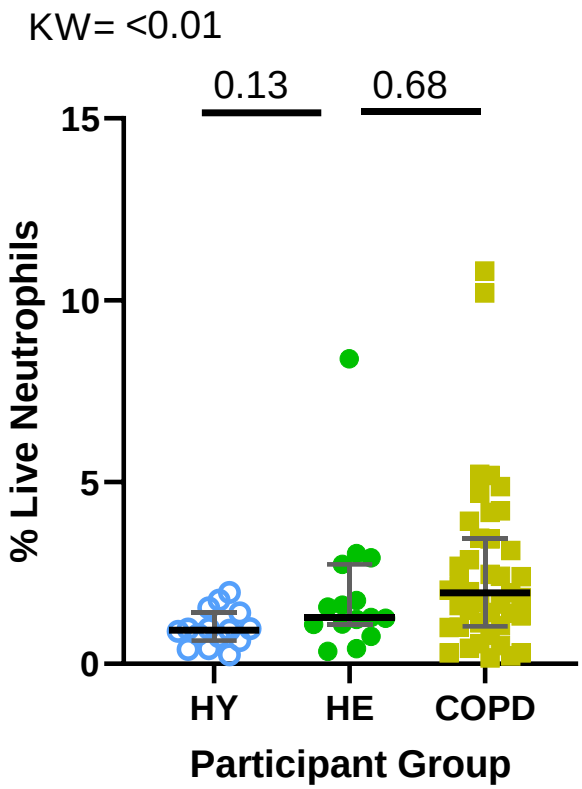

Figure 5.7: Percentage of CXCR4^{+}CD62L^{dim} live neutrophils isolated from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the percentage of CD16^{+}CD62L^{dim} live neutrophils (see Figure 5.2) recorded for each participant group (HY n=15; HE n=15, COPD n=41). The horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups).

5.2.3.5 Neutrophil inflammatory status

In order to assess neutrophil inflammatory status, the expression of HLA-DR, PD-L1 and CD11c were measured. In all three markers, no difference in expression was observed between neutrophils isolated from HY, HE or patients with COPD (Figure 5.8), with a comparable spread in all groups and markers. These data suggest that neutrophils do not change their anti-inflammatory or pro-inflammatory status in either healthy ageing or COPD.

5.2.3.6 Neutrophil reverse transmigration

In order to assess neutrophil reverse transmigration, the expression of CD54 was measured. Again, no difference in expression was observed between neutrophils isolated from HY, HE or patients with COPD (Figure 5.9), with a comparable spread in all groups. The absence of a change in CD54 expression suggests there is not an increase in reverse transmigrated neutrophils in healthy ageing or patients with COPD.

5.2.3.7 Neutrophil maturity

In order to assess neutrophil maturity, the expression of CD10 and CD16 was measured. No difference in CD10 expression was observed between neutrophils isolated from HY, HE or patients with COPD (Figure 5.10a). A small proportion of neutrophils from patients with COPD showed around an 80% increase in CD10 expression compared to the median expression of other patients (Figure 5.10a). A small, but statistically insignificant, increase in CD16 expression was observed between HY and HE participants (Figure 5.10b). However, a significant decrease in CD16 expression, on average by 16%, in neutrophils from patients with COPD compared to HE participants (Figure 5.10b). Together, these data suggest there is no difference in neutrophil maturity between HY and HE participants, but there may be a small

increase in circulating immature neutrophils in patients with COPD compared to HE volunteers, but there is large heterogeneity in this patient population. The change in CD16 in patients with COPD does not follow the same trend as between HY and HE participants, suggesting the difference may not reflect a pathogenic or accelerated ageing change.

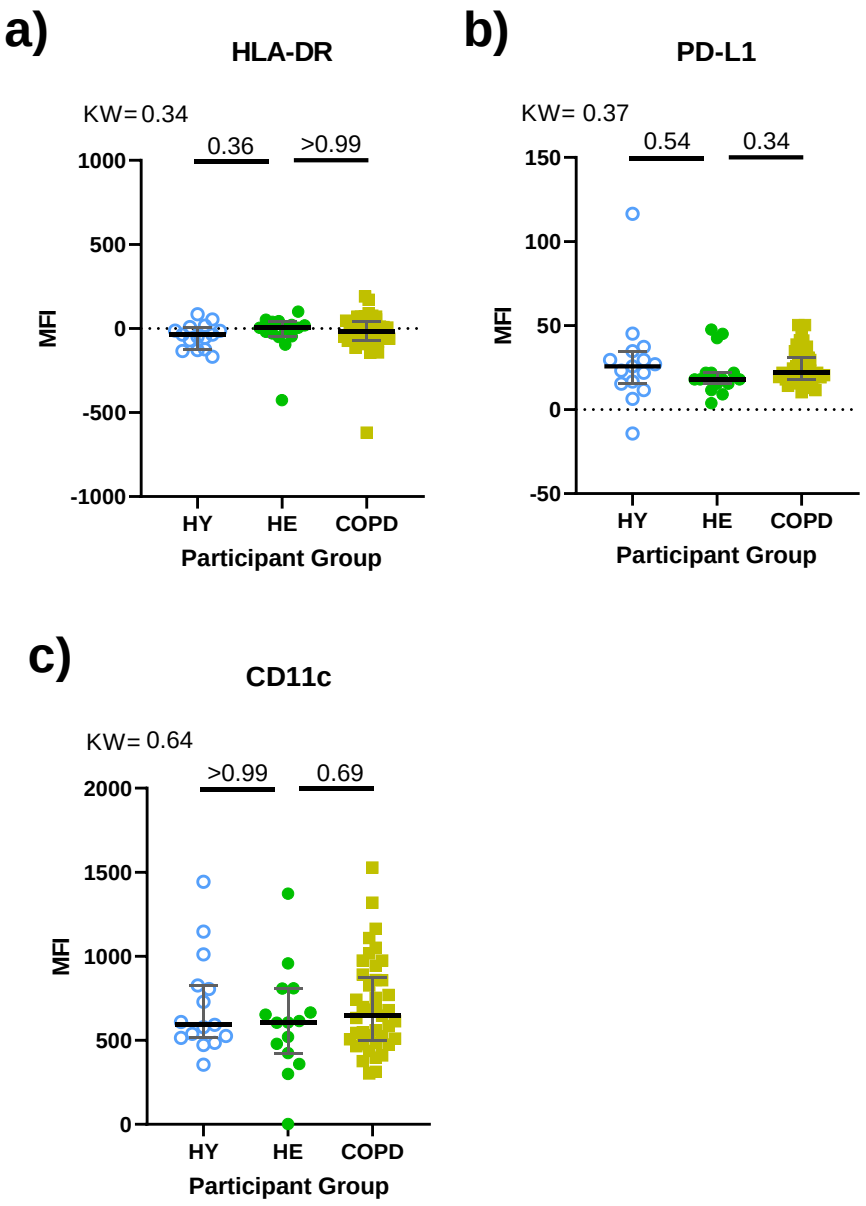

Figure 5.8: Median Fluorescence Intensity of inflammatory markers of isolated neutrophils from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** HLA-DR, **b)** PD-L1 or **c)** CD11c for each participant group (HY n=15; HE n=15, COPD n=41). In each case, the horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups).

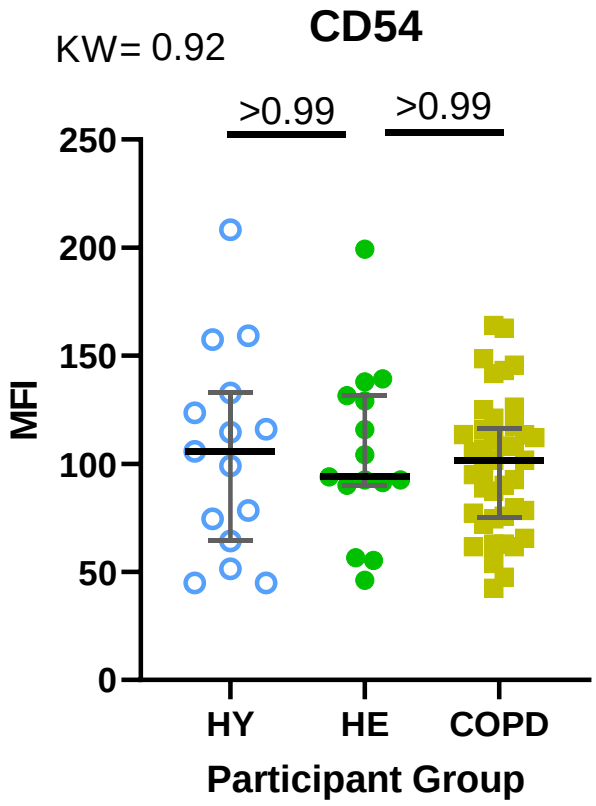

Figure 5.9: Median Fluorescence Intensity of CD54 of isolated neutrophils from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the median fluorescence intensity measured for CD54 for each participant group (HY n=15; HE n=15, COPD n=41). The horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups).

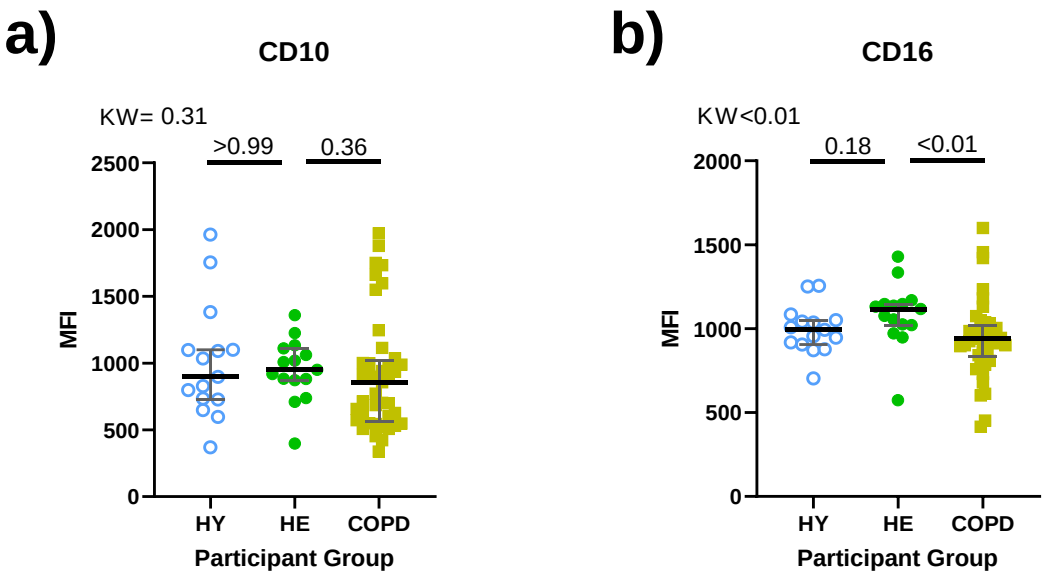

Figure 5.10: Median Fluorescence Intensity of CD10 and CD16 of isolated neutrophils from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** CD10 or **b)** CD16 for each participant group (HY n=15; HE n=15, COPD n=41). In each case, the horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups).

5.2.3.8 Hypersegmentation

Previous studies have identified hypersegmented neutrophils by CD16+CD62L^{dim} expression, with potential functional defects in this population (Pillay *et al.*, 2012). Neutrophils that expressed CD16 and low levels of CD62L were gated (Figure 5.2c) and the percentage of CD16+CD62L^{dim} events measured (Figure 5.11). A trend of increasing hypersegmented neutrophils was observed, with lowest levels in HY participants, increasing with age (HE) and further with the presence of COPD (Figure 5.11), however, this was not statistically significant. Sample heterogeneity may mask this change, but suggests that neutrophil hypersegmentation may be increased with age, and further in COPD, linking with the overall hypothesis of accelerated ageing.

5.2.3.9 Key correlations with clinical parameters

Clinically relevant parameters are important in guiding patient care and help identify the severity of COPD. Using CD11b to investigate correlations with activation status, there was no correlation between smoking history and neutrophil CD11b expression or CXCR2 expression (Figure 5.12a and c). However, there was a weak significant correlation between FEV_1 and CD11b expression, suggesting that lower lung function was associated with lower levels of peripheral neutrophil activation – although further investigation would be necessary to confirm and understand this finding. No correlation was seen with lung function and CXCR2 expression (Figure 5.12d). There was no difference in CD11b expression on neutrophils from patients who were current or ex-smokers (Figure 5.13a), or between GOLD Grades (Figure 5.13b).

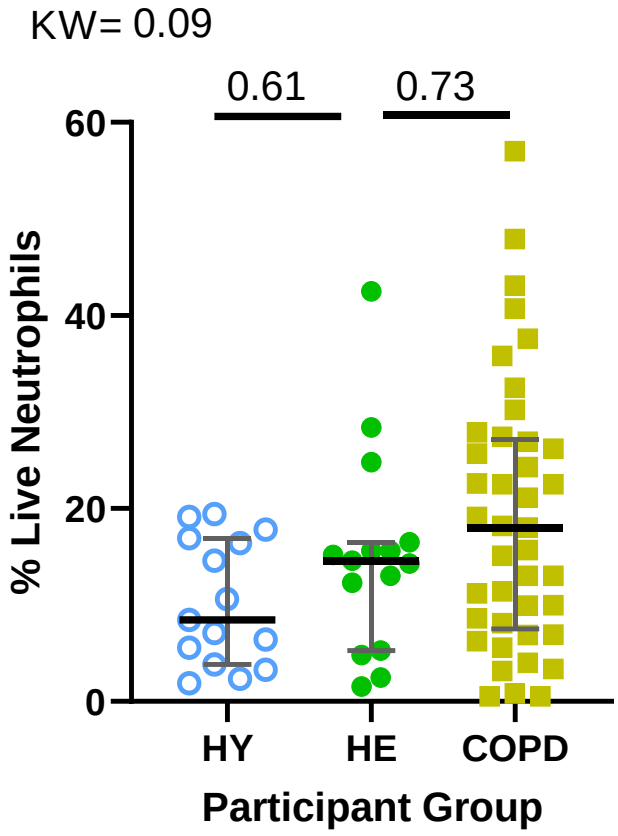

Figure 5.11: Percentage of CD16^{+}CD62L^{dim} live neutrophils isolated from healthy young (HY), elderly (HE) and patients with COPD

Neutrophils from whole blood were isolated, stained with antibodies and the percentage of CD16^{+}CD62L^{dim} live neutrophils (see Figure 5.2) recorded for each participant group (HY n=15; HE n=15, COPD n=41). The horizontal line indicates the median value. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison (indicated between groups).

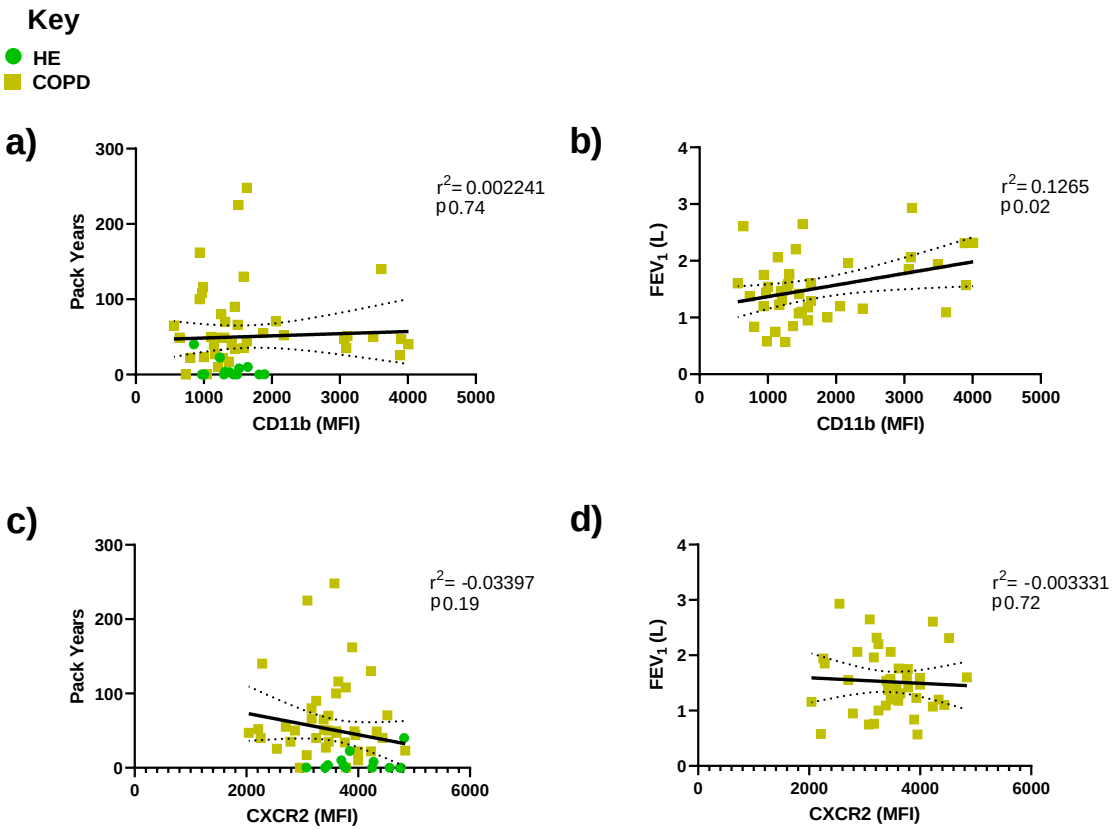

Figure 5.12: Correlation of CD11b and CXCR2 expression with lung function and smoking history

Neutrophils from HE (n=15) and COPD (n=41) whole blood were isolated, stained with antibodies and the fluorescence intensity of CD11b and CXCR2 measured. The correlation between CD11b median fluorescence intensity (MFI) and **a)** pack years and **b)** forced expiratory volume in one second (FEV_1) in litres (L) or CXCR2 MFI with **c)** pack years and **d)** smoking history are shown. In each case, linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for each linear regression.

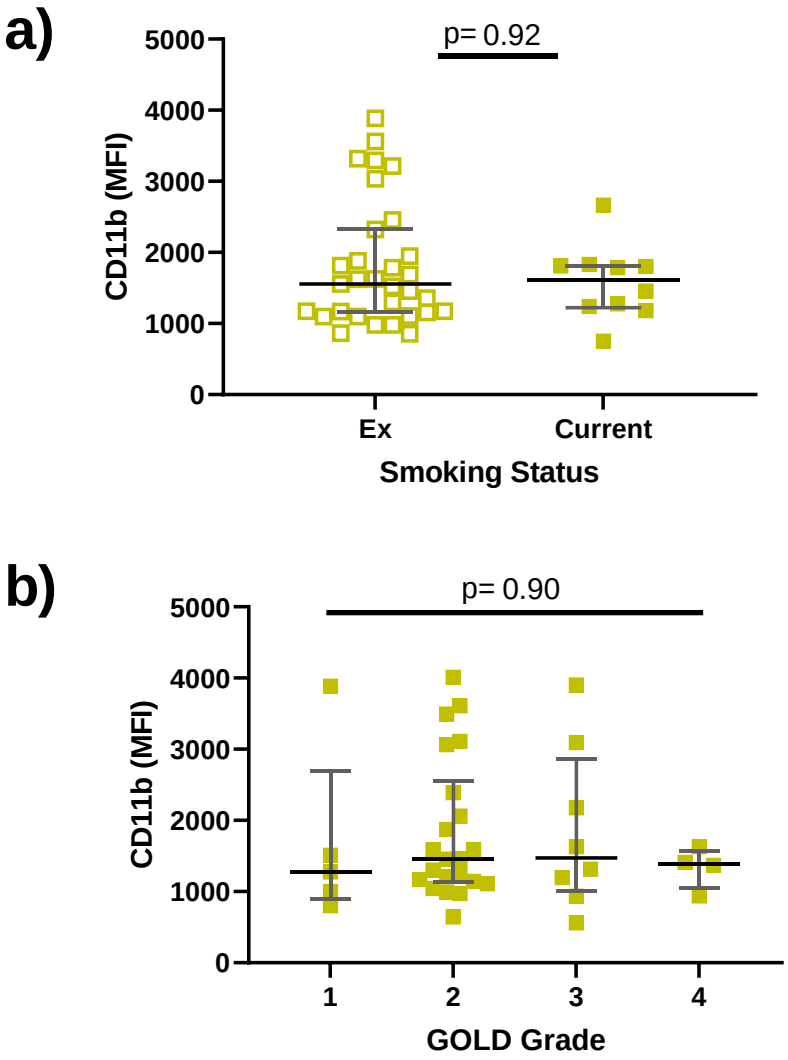

Figure 5.13: Comparisons of CD11b expression with smoking status and GOLD Grade

Neutrophils from patients with COPD (n=41) whole blood were isolated, stained with antibodies and the median fluorescence intensity (MFI) of CD11b measured. The expression of CD11b was grouped by **a)** smoking status and **b)** GOLD Grade. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using a) unpaired t-test or b) Kruskal-Wallis test.

5.2.4 Changes in neutrophil phenotype in multimorbidity

As highlighted in the introduction, heterogeneity exists between patients with COPD. A potential source of this heterogeneity is the presence of multiple chronic inflammatory diseases in a single patient – often referred to as multimorbidity. To investigate how neutrophil phenotypes may be altered in multimorbid patients, the cohort of patients with COPD was sub-divided into patients with known and clinically diagnosed T2D and CVD. Stratification of patients with COPD in this way may identify potential reasons for the heterogeneity seen in COPD. As before, neutrophil phenotypes were identified by surface expression: activation status (CD11b, CD66b and CD62L); senescence (CXCR2 and CXCR4); inflammatory status (HLA-DR, PD-L1 and CD11c); reverse transmigration (CD54) and neutrophil maturity (CD10 and CD16).

5.2.4.1 Participant demographics

The demographics for healthy age-matched participants and patients with COPD are shown in Table 5.2 – note that these patients with COPD are the same patients presented as a group in Section 5.2.3. There was no significant difference in age, or sex between any participant group (Table 5.2). There was a significant change in both FVC % predicted and GOLD Grade in patients with COPD, with those patients with all conditions (COPD, CVD and T2D) having a reduced FVC and more patients in GOLD Grade 3 (Table 5.2). Patients with all conditions also had a greater smoking history. There was no difference in the ex-smoker years (Table 5.2).

Table 5.2: Basic demographics for study participants, divided by co-morbidity for patients with stable COPD

	HE	Stable COPD	Stable COPD +CVD	Stable COPD +T2D	Stable COPD +CVD+T2D	p-value
Number	15	15	12	8	6	-
Age, median (IQR)	72 (70.75-74.25)	69 (66.0-72.0)	72 (70.3-74.3)	73.5 (67.5-76.0)	72 (67.3-79.5)	0.42[k]
Sex, M:F	10:5	9:6	7:5	4:4	5:1	0.79[f]
Lung function						
FEV_1 % predicted, median (IQR)	N/A	59.5 (48.8-65.5)[1]	66.5 (47.8-93.3)	56.0 (43.5-71.5)	39.5 (31.8-53.8)	0.15[k]
FVC % predicted, median (IQR)	N/A	94.0 (71.0-107)[1]	93.5 (74.0-109)	82.5 (76.5-94.3)	60.5 (39.0-67.5)	0.01[k]
GOLD Grade	N/A	0:11:2:1[1]	4:5:2:1	1:5:1:1	0:1:4:1	0.04[f]
Smoking						
Smoking status	7:7:0[2]	0:10:5	0:11:1	0:5:3	0:5:1	<0.01[f]
Pack year history, median (IQR)	0 (0-6.75)[2]	48.9 (22.8-89.0)	40 (22.0-65.0)	47 (35.0-55.0)	80 (47.8-116)	<0.01[k]
Years ex-smoker, median (IQR)	31.5 (14.0-48.0)[2]	17.0 (8.0-30.5)	15.5 (8.5-21.0)	7.0 (6.0-9.0)	15.0 (8.5-18.5)	0.13[k]

Legend: Study participants split into healthy age-matched controls without COPD (HE), patients with only stable COPD (COPD) and those also with a cardiovascular disease (+CVD), type-2 diabetes (+T2D) or both (+CVD+T2D). Median with interquartile range (IQR) to one decimal place are shown where appropriate. **Sex** shown as a ratio of male (M) to female (F). **GOLD Grade** displayed as a ratio of stage 1:2:3:4. **Smoking status** shown as ratio of never(NS):ex(Ex):current(C). Statistical analysis carried out using Kruskal-Wallis test (k) for continuous data and Fishers Exact test (f) for categorical data. FEV_1, forced expiratory volume in one second; FVC, forced vital capacity. [1]1 patient was not able to perform spirometry. [2]Data unavailable for one participant.

5.2.4.2 Neutrophil activation in multimorbidity

In order to assess activation status, the expression of CD11b, CD66b and CD62L were measured. There was no difference in the expression of CD11b, CD66b or CD62L by neutrophils between HE participants or COPD patients with any co-morbidity (Figure 5.14). A few patients in each of the COPD groups showed higher CD11b expression compared to the HE group (Figure 5.14a), however, this trend was not reproduced for CD66b or CD62L (Figure 5.14b and c). Investigating the proportion of cells displaying a $CD11b^{bright}CD66b^{bright}$ phenotype, no significant difference was observed between HE participants and any of the COPD groups (Figure 5.15). All groups tested showed a low percentage of $CD11b^{bright}CD66b^{bright}$ neutrophils (Figure 5.15), suggesting that systemic activation is comparable across all groups tested, but this observation may be hindered by the low sample number within the most multimorbid group (COPD with CVD and T2D).

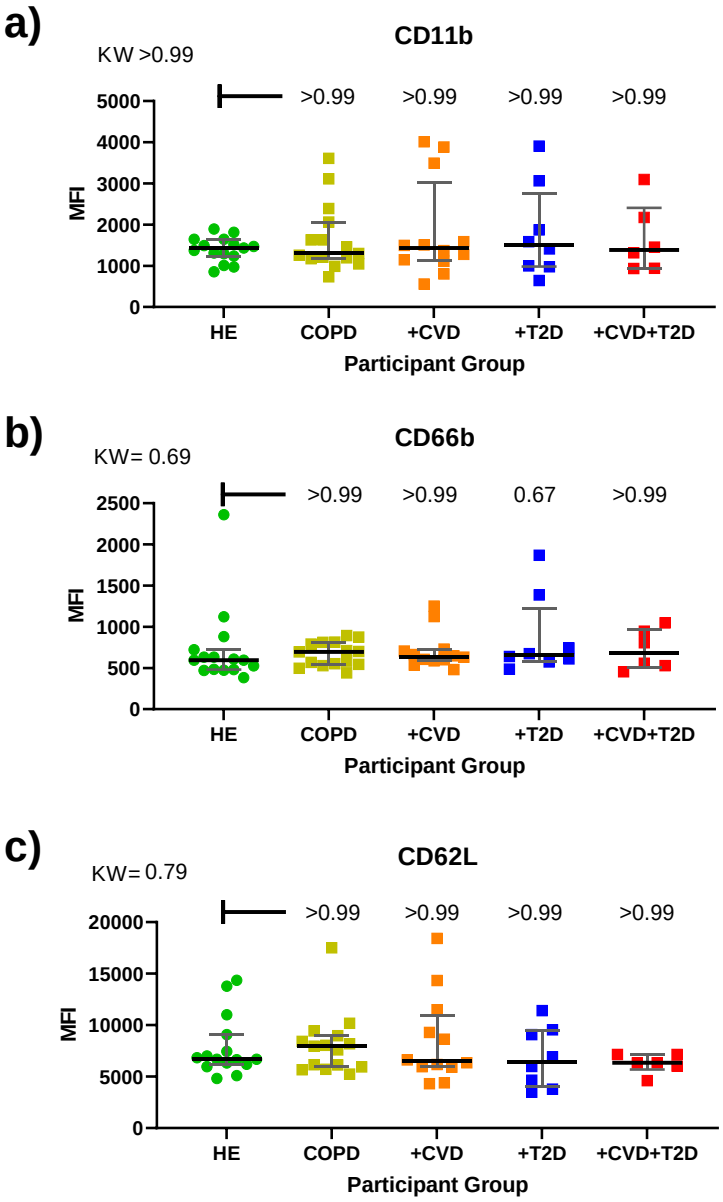

Figure 5.14: Median fluorescence intensity of activation markers on isolated neutrophils from healthy elderly (HE) and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12)), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Neutrophils were stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** CD11b, **b)** CD66b or **c)** CD62L. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

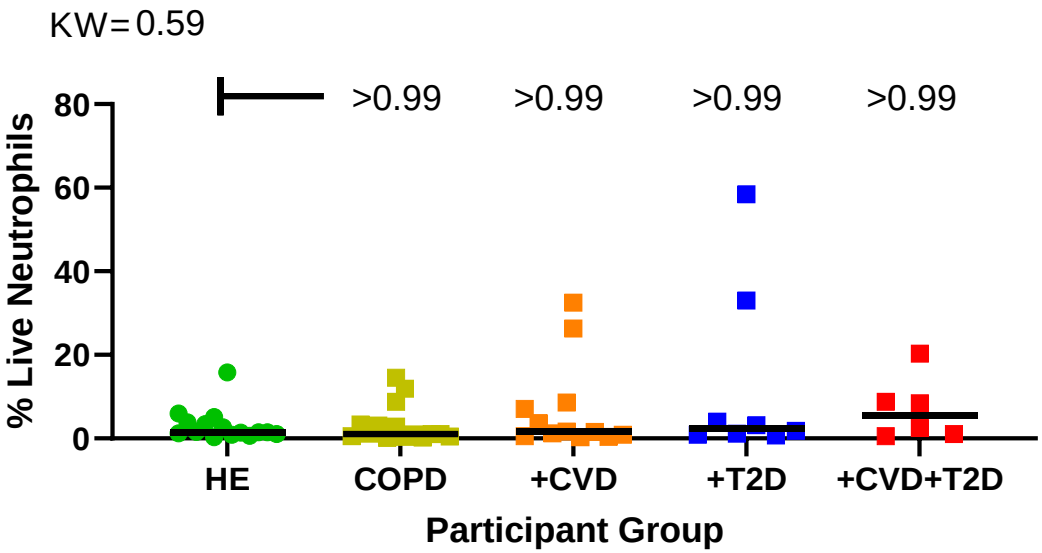

Figure 5.15: Percentage of CD11b^{bright}CD66b^{bright} live neutrophils isolated from healthy elderly (HE) and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12)), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Live neutrophils were gated based on CD11b and CD66b expression to determine the percentage of high-expressing neutrophils (see Figure 5.2). In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

5.2.4.3 Neutrophil senescence

Patients with COPD were stratified based on their co-morbidities to identify if the reduction in CXCR2 expression was altered by multimorbidity. No significant decrease in CXCR2 expression was observed between HE participants and patients with COPD and no known co-morbidities, or with those with COPD and T2D (Figure 5.16a). However, a decrease in CXCR2 expression was observed between HE participants and patients with COPD and CVD, and patients with COPD, CVD and T2D (Figure 5.16a). This change was not accompanied by an increase in CXCR4 expression, as comparable expression of CXCR4 was observed on neutrophils from all groups (Figure 5.16b). Of note, a statistically significant decrease in CXCR2 was still observed when analysing all the COPD groups collectively (Figure 5.16a). These data highlight that not all patients with COPD show an equal reduction in CXCR2 expression, indicating potential biological differences between these patient groups.

5.2.4.4 Overactive senescent neutrophils

In order to assess neutrophils that may display an overactive senescent phenotype, the expression of CXCR4 and CD62L was measured. Neutrophils that expressed CXCR4 combined with low levels of CD62L were gated (Figure 5.2b) and the percentage of CXCR4+CD62L^{dim} events measured (Figure 5.17). No significant differences were identified between HE participants and any of the patient populations with COPD. However, two patients with both COPD and T2D had more than 10% of neutrophils identified with this phenotype. Overall, these data support that the proportion of overactive senescent neutrophils is not altered by multimorbidity, but a small subset of patients may display this phenotype and could warrant further investigation.

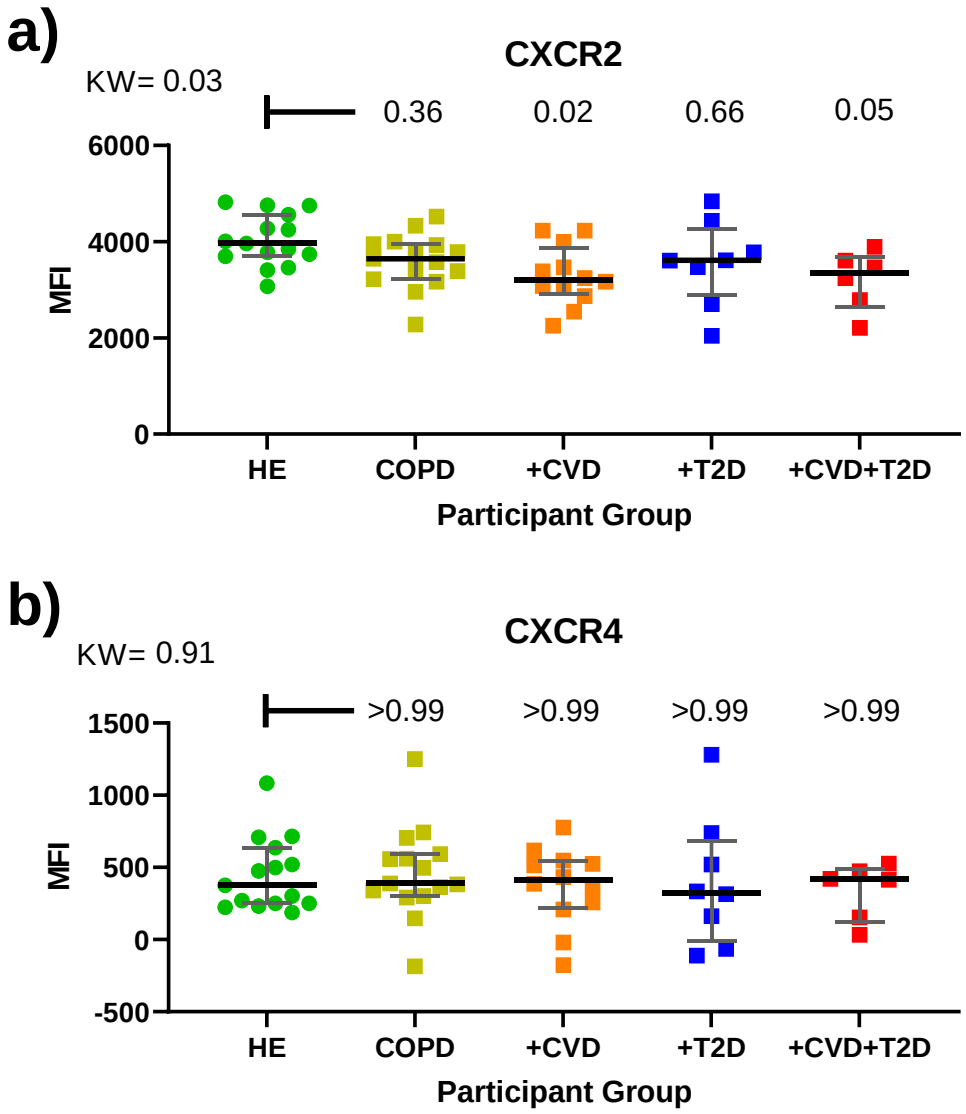

Figure 5.16: Median fluorescence intensity of senescence markers on isolated neutrophils from healthy elderly (HE) participants and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12)), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Neutrophils were stained with antibodies and the median fluorescence intensity measured for **a)** CXCR2 or **b)** CXCR4. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

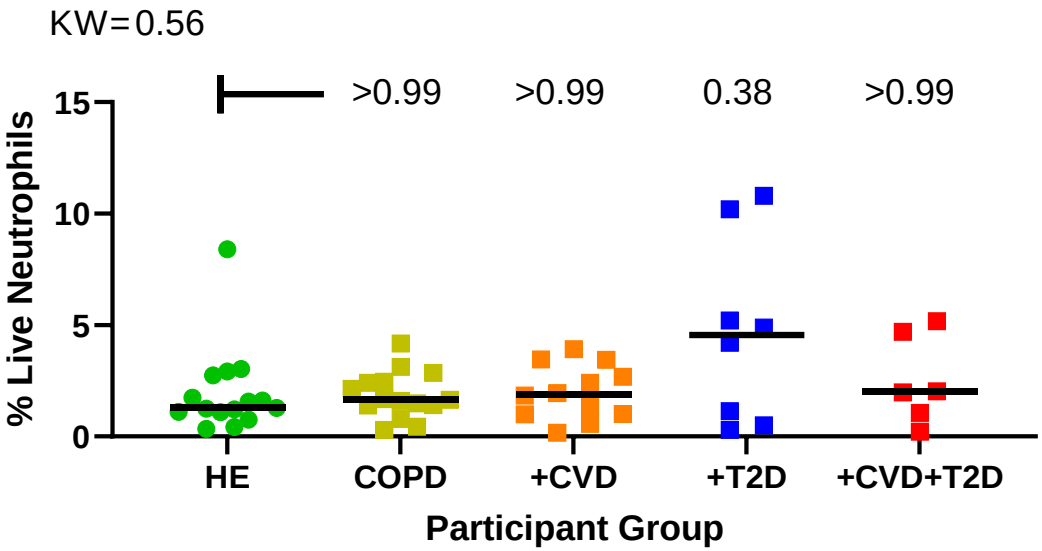

Figure 5.17: Percentage of CXCR4^{+}CD62L^{dim} live neutrophils isolated from healthy elderly (HE) participants and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12)), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Neutrophils were stained with antibodies and the percentage of CD16^{+}CD62L^{dim} live neutrophils recorded (see Figure 5.2). In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

5.2.4.5 Neutrophil inflammatory status

In order to assess neutrophil inflammatory status, the expression of HLA-DR, PD-L1 and CD11c were measured. In all three markers, no difference in expression was observed between neutrophils isolated from HE participants and any of the COPD groups (Figure 5.18).

5.2.4.6 Neutrophil reverse transmigration

In order to assess neutrophil reverse transmigration, the expression of CD54 was measured. Again, similar levels of CD54 expression was observed between neutrophils isolated from HE participants or patients with COPD irrespective of other diseases (Figure 5.19).

5.2.4.7 Neutrophil maturity

Neutrophil maturity was investigated by measuring the expression of CD10 and CD16. No difference in CD10 expression was observed between neutrophils isolated from HE participants or patients with COPD alone or with co-morbidities (Figure 5.20a). As seen in Figure 5.10, a proportion of neutrophils from patients with COPD showed lower CD10 expression, and whilst statistically insignificant here, patients with COPD and T2D (with or without CVD) showed relatively lower levels of CD10 (Figure 5.20a). The lack of statistical significance may be due to number of patients recruited but suggests there may be increased numbers of circulating immature neutrophils in these patient groups.

A small and statistically significant decrease was observed in CD16 expression between HE participants and patients with COPD alone ($p=0.03$) and patients with COPD and T2D (<0.01, Figure 5.20b). CD16 expression was reduced, on average, by 18% in neutrophils from patients with COPD and 31% in patients with COPD and T2D compared to HE participants (Figure

5.20b). In contrast, patients with COPD and CVD or both CVD and T2D did not show the same decrease, highlighting differences in CD16 expression for particular multimorbid groups.

5.2.4.8 Hypersegmentation

In order to assess neutrophil hypersegmentation, the expression of CD16 and CD62L was measured. Neutrophils that expressed CD16 and low levels of CD62L were gated (Figure 5.2c) and the percentage of CD16+CD62L^{dim} events measured (Figure 5.21). Whilst no significant differences were identified, the percentage of CD16+CD62L^{dim} neutrophils varied between the different populations tested, with the lowest levels observed in patients with COPD and CVD and the highest in patients with COPD and T2D. These data suggest hypersegmented neutrophils may be absent in patients with COPD and CVD, but variation makes this observation unclear.

5.2.4.9 Key correlations with clinical parameters

Neutrophil phenotypes that correlate with clinical parameters could indicate a functional link for further investigation, as the study carried out here is underpowered to draw strong conclusions. Lung function is one such important clinical parameter and may link with neutrophil functions leading to lung damage. Using CD11b as a marker of activation, there was no statistically significant correlation between neutrophil CD11b expression and smoking history (Figure 5.22a) or FEV_1 (Figure 5.22b) in any patient population. However, there was a weak negative correlation between smoking history and CD11b expression for patients with COPD, CVD and T2D, suggesting a higher pack year history may be related to lower neutrophil activation, albeit with very low numbers in this group, yet highlighting differences between patients with COPD based on multimorbidity.

Again, there were no significant correlations with FEV_1 and CXCR2 (Figure 5.23a) or CD16 (Figure 5.23b) expression. Of note, the most multimorbid group (COPD with CVD and T2D) seemed to show the opposite correlation to the group with only COPD. In addition, both patients with COPD alone and COPD with T2D showed lower CD16 expression compared to HE participants (Figure 5.20b) and patients with both COPD and T2D showed a weak negative correlation between CD16 expression and FEV_1 (Figure 5.23b). These data suggest that higher CD16 expression is associated with reduced lung function in patients without CVD, although further investigation is necessary.

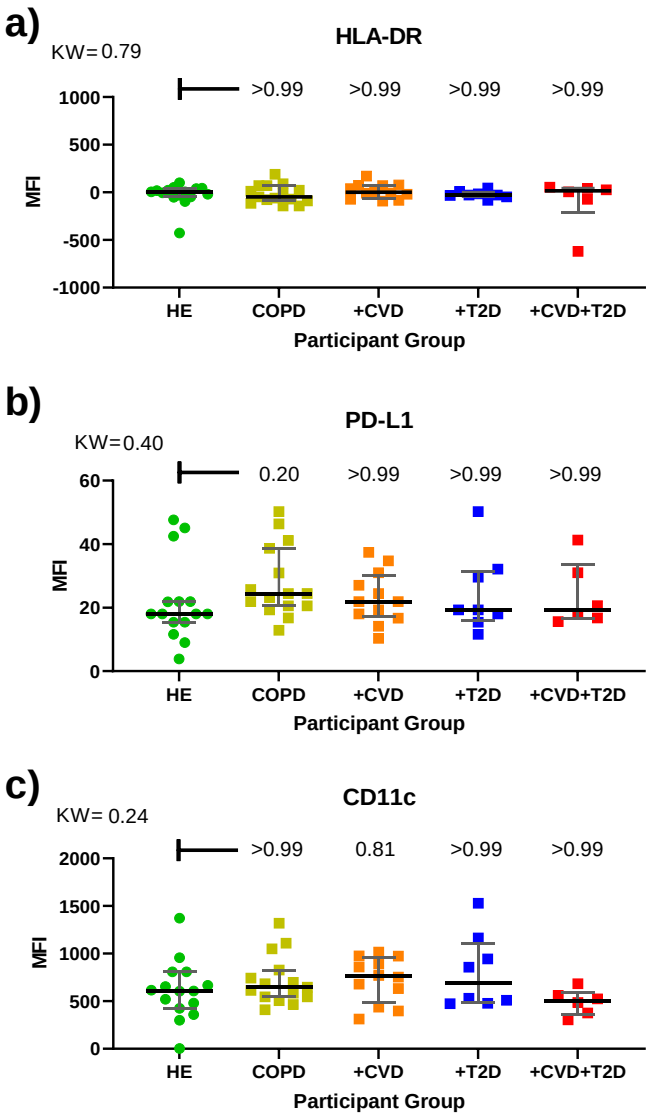

Figure 5.18: Median fluorescence intensity of inflammatory markers on isolated neutrophils from healthy elderly (HE) and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12)), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Neutrophils were stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** HLA-DR, **b)** PD-L1 or **c)** CD11c. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

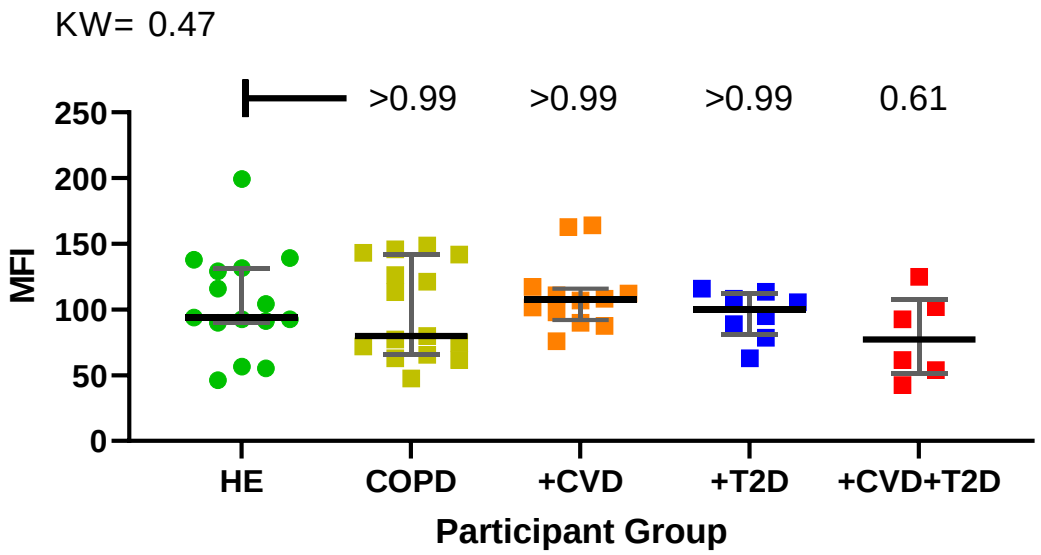

Figure 5.19: Median fluorescence intensity of CD54 on isolated neutrophils from healthy elderly (HE) and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12)), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Neutrophils were stained with antibodies and the median fluorescence intensity (MFI) measured for CD54. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

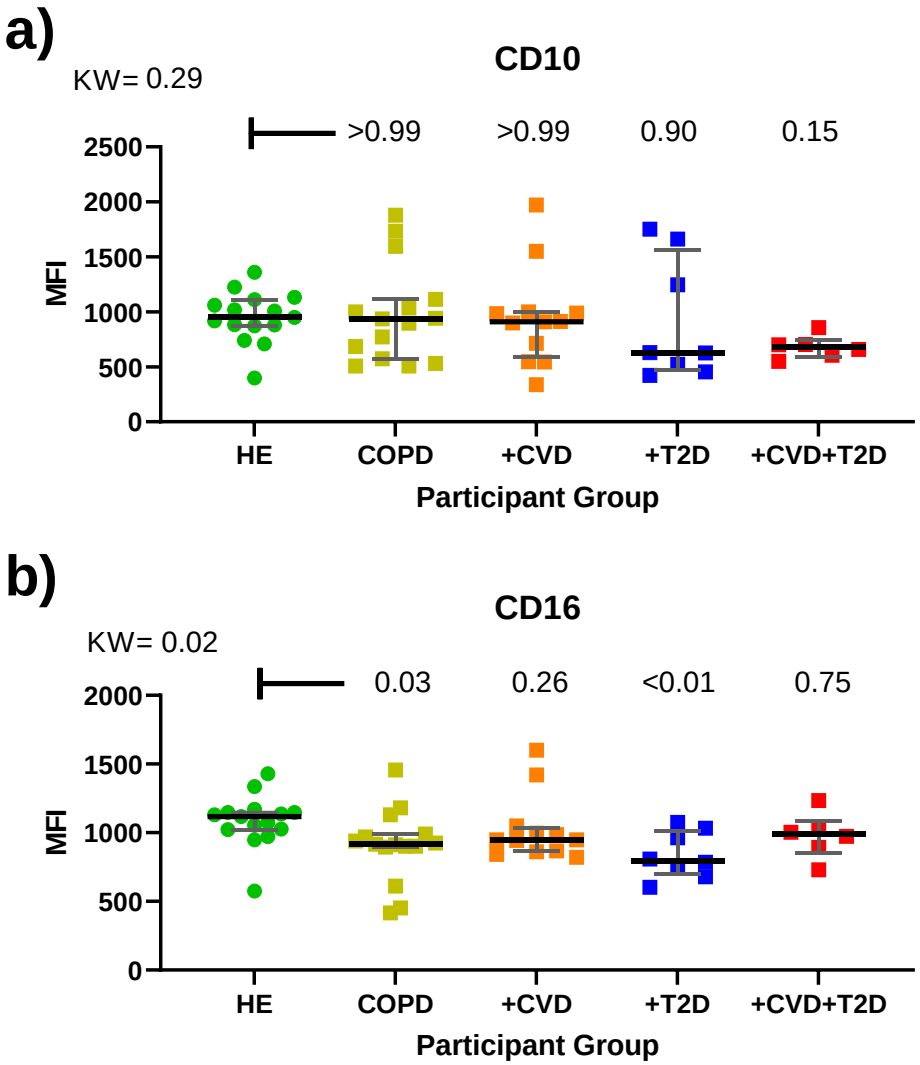

Figure 5.20: Median fluorescence intensity of maturity markers on isolated neutrophils from healthy elderly (HE) and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12)), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Neutrophils were stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** CD10 or **b)** CD16. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

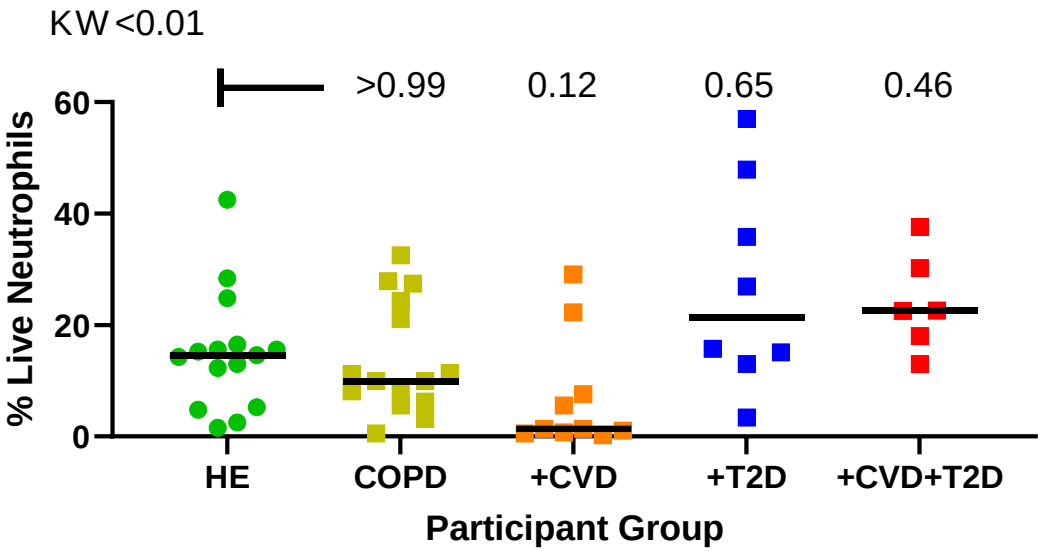

Figure 5.21: Percentage of $CD16^{+}CD62L^{dim}$ live neutrophils isolated from healthy elderly (HE) participants and patients with COPD alone or with co-morbidities

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). Neutrophils were stained with antibodies and the percentage of $CD16^{+}CD62L^{dim}$ live neutrophils measured. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using Kruskal-Wallis (KW) with Dunn's multiple comparison, all compared to HE.

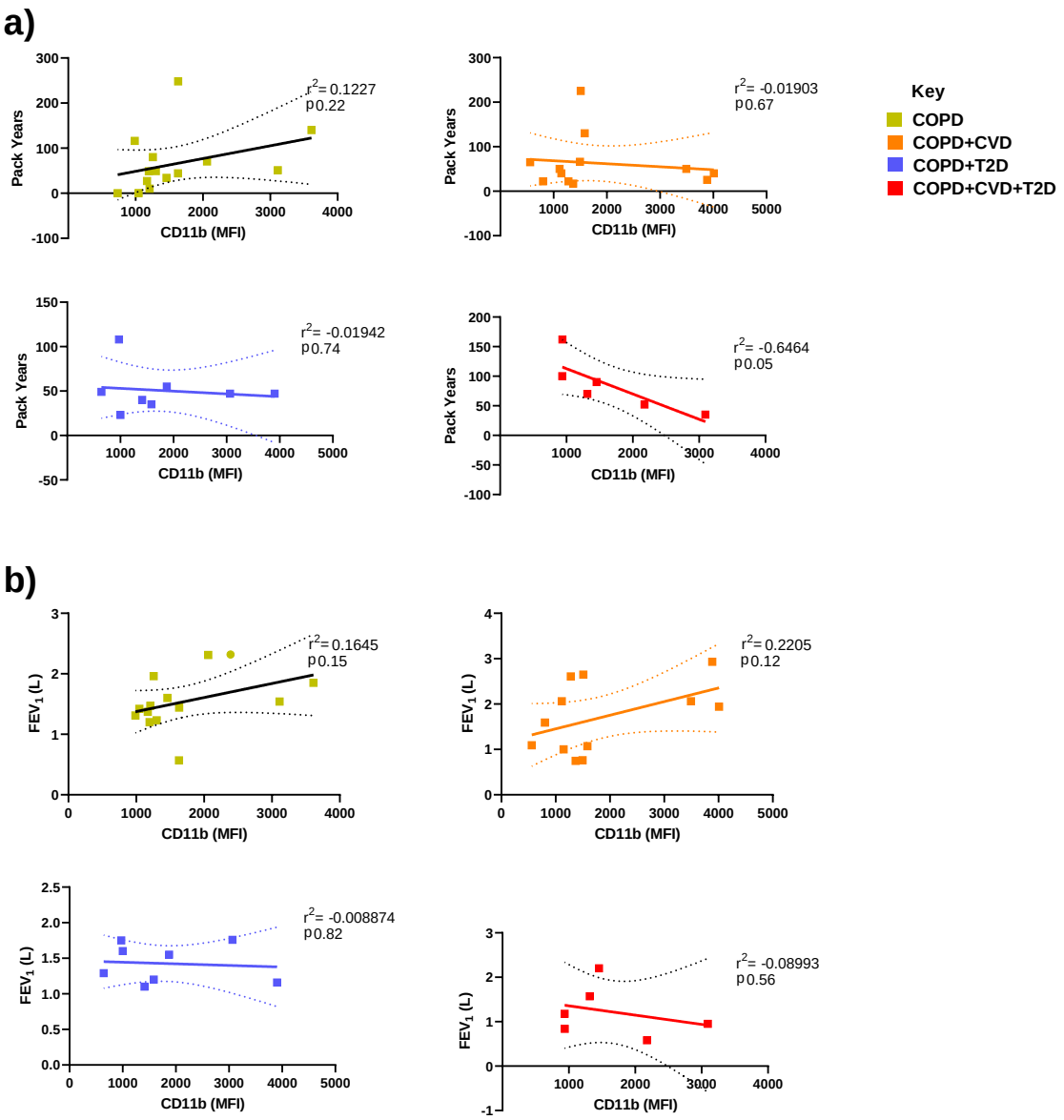

Figure 5.22: Preliminary assessment of CD11b expression on neutrophils from patients with COPD, stratified by co-morbidities, with lung function and smoking history

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). The correlation between CD11b expression and **a)** pack years and **b)** forced expiratory volume in one second (FEV_1) in litres (L) are shown. In each case, linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for each linear regression.

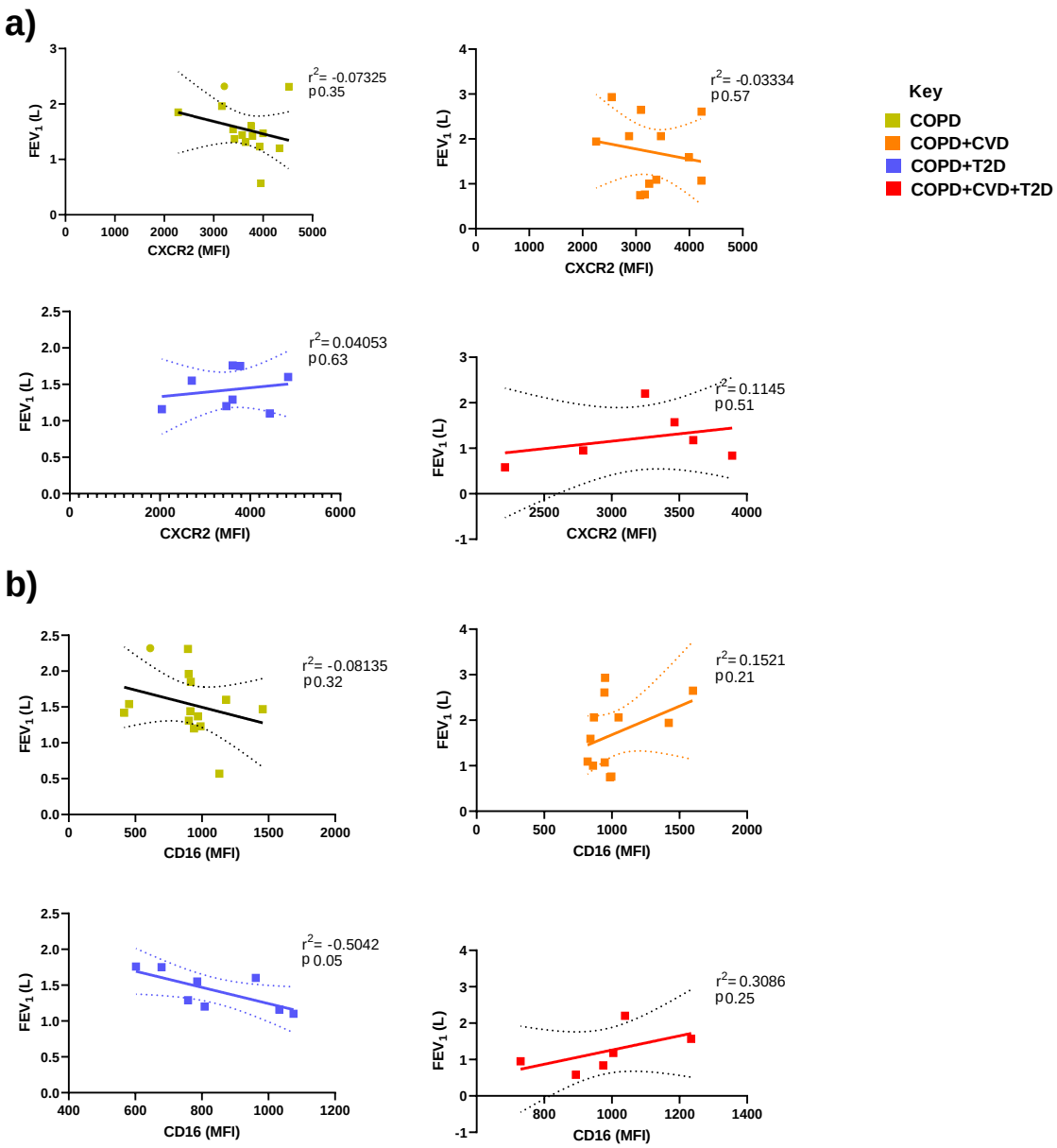

Figure 5.23: Preliminary assessment of CXCR2 and CD16 expression on neutrophils from patients with COPD, stratified by co-morbidities, with lung function

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) participants or patients with COPD (split into those with COPD alone (n=15), COPD with cardiovascular disease (+CVD; n=12), type-2 diabetes (+T2D; n=8), or both conditions (+CVD +T2D; n=6). The correlation between forced expiratory volume in one second (FEV_1) and a) CXCR2 or b) CD16 expression. In each case, linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for each linear regression.

5.2.5 Unassisted analysis using t-Distributed Stochastic Neighbor Embedding (t-SNE) and Rphenograph clustering

Investigating surface expression using MFI and manual gating strategies provide useful knowledge-driven analyses, however, there are pitfalls to these approaches with complex data sets. Primarily, it is not possible to investigate every possible expression profile, nor would this be a robust analysis approach. Dimension-reduction algorithms have been developed that enable multi-parameter datasets, such as those where there is expression of multiple markers on an induvial cell, to be clustered incorporating all these dimensions and visualised in a 2D plot.

Here, t-SNE visualisation with a clustering algorithm called Rphenograph was utilised to perform multi-dimension analysis of gated live neutrophils (Section 2.6.3) in the same combinations as presented in this chapter: HY, HE and COPD participants; HE and COPD participants stratified based on co-morbidities. These analyses provided a t-SNE plot, showing the distribution cells based on the similarities of the clusters (the closer together, the more similar the clusters), and quantification of the percentage of cells from each sample within each cluster. The expression of each marker for each cluster can also be quantified.

Broadly speaking, limited differences were identified using these analyses for any of the groups, owing to small absolute differences between the populations studied (HY, HE and COPD; HE compared with COPD stratified based on multimorbidity), despite statistical significance. However, these data still provide an interesting insight into the heterogeneity of neutrophils in these different participant groups and will now be described in turn with the aforementioned caveats.

5.2.5.1 Rphenograph analysis of healthy participants and all patients with COPD

Analysis of neutrophils from HY and elderly participants alongside patients with COPD a total of 25 clusters for panel 1 (Figure 5.24a). There was no clear separation between any of these clusters, with small regions with a higher expression for CXCR2, CD11b, CD62L, CD52 and CD10 (Figure 5.24b). Of note, regions with higher CD11b expression overlay regions with greater CD10 expression and lower CD62L expression (Figure 5.24b). There was a homogenous distribution of CXCR2 and CD16 in these plots (Figure 5.24b). These indicate a correlation between CD11b expression and CD10 expression, but generally a uniform expression profile between all neutrophils.

For panel 2, a total of 28 clusters were identified (Figure 5.25a), again very closely related with only clear regions of very low PD-L1 expression separating from neighbouring clusters (Figure 5.25b). Similarly to the analysis of panel 1, regions of higher CD11b expression appear to overlay with higher CD10 expression, CD11c expression and to a lesser extent CD66b expression (Figure 5.25b). These data support a correlation between CD11b and CD66b expression, but also that CD11c and CD10 expression may also associate with these changes on individual cells.

Taking these clusters, it is possible to separate out the participant groups to see if neutrophils within any given cluster comprise a greater or lesser amount of the total neutrophil population. Complete cluster percentages for panel 1 (Figure 5.26a) and panel 2 (Figure 5.26b) show very similar proportions, but statistically significant differences are highlighted in Figure 5.26c and d. As shown, patients with COPD had a higher proportion of neutrophils in cluster 4 and a lower proportion in cluster 21 compared to HE participants from panel 1 (Figure 5.26c)

– but with a median percentage change of <1% and 2% respectively. Within panel 2, neutrophils from patients with COPD had a greater proportion of neutrophils in cluster 4 and a lower proportion in cluster 9 and 15 (Figure 5.26d), with median percentage changes of 2%, <1% and <1% respectively. Overall, whilst statistically significant changes were identified, the absolute change between groups was very small and comparisons between these expression profiles within these clusters are not shown.

5.2.5.2 Rphenograph analysis of healthy elderly participants and patients with COPD stratified based on co-morbidities

The same analysis was performed as above (Section 5.2.5.1) for neutrophils from HE participants and those with COPD, this time stratified post-analysis based on their multimorbidity status. A total of 25 clusters were identified from panel 1 (Figure 5.27a). Again, regions with higher CD11b expression overlayed regions with greater CD10 expression (Figure 5.27b). There was a homogenous distribution of CXCR2 and CD16 in these plots (Figure 5.27b). These indicate a correlation between CD11b expression and CD10 expression, with only subtle differences between each cluster.

For panel 2, a total of 24 clusters were identified (Figure 5.28a), again very closely related with only clear regions of very low PD-L1 expression clearly separating neighbouring clusters (Figure 5.28b). Regions of higher CD11b expression appeared to overlay with higher CD10 expression, and additionally with CD11c expression and to a lesser extent CD66b expression (Figure 5.25b), supporting a correlation between these markers on individual cells.

Quantification of the percentage of neutrophils within each cluster revealed broadly similar numbers between participant groups (Figure 5.29a and b). Three clusters from panel 1 (Figure

5.29a) and two from panel 2 (Figure 5.29b) showed statistically significant differences between groups and are highlighted in Figure 5.29c and d respectively. These differences, however, represent very small changes and are likely due to the lack of spread within the HE participant group. Both cluster 12 from panel 1 (Figure 5.29c) and cluster 4 from panel 2 (Figure 5.29d) show that the most multimorbid patients have higher proportions of neutrophils in these clusters. Comparing the expression profiles of these clusters to the other clusters within the panel, cluster 12 (panel 1; Figure 5.30a) showed slightly lower expression of CD10 and cluster 4 (panel 2; Figure 5.30b) was the higher end for CD11b and CD66b expression. Together, these may suggest more immature or activated neutrophils in the circulation of patients with COPD, CVD and T2D.

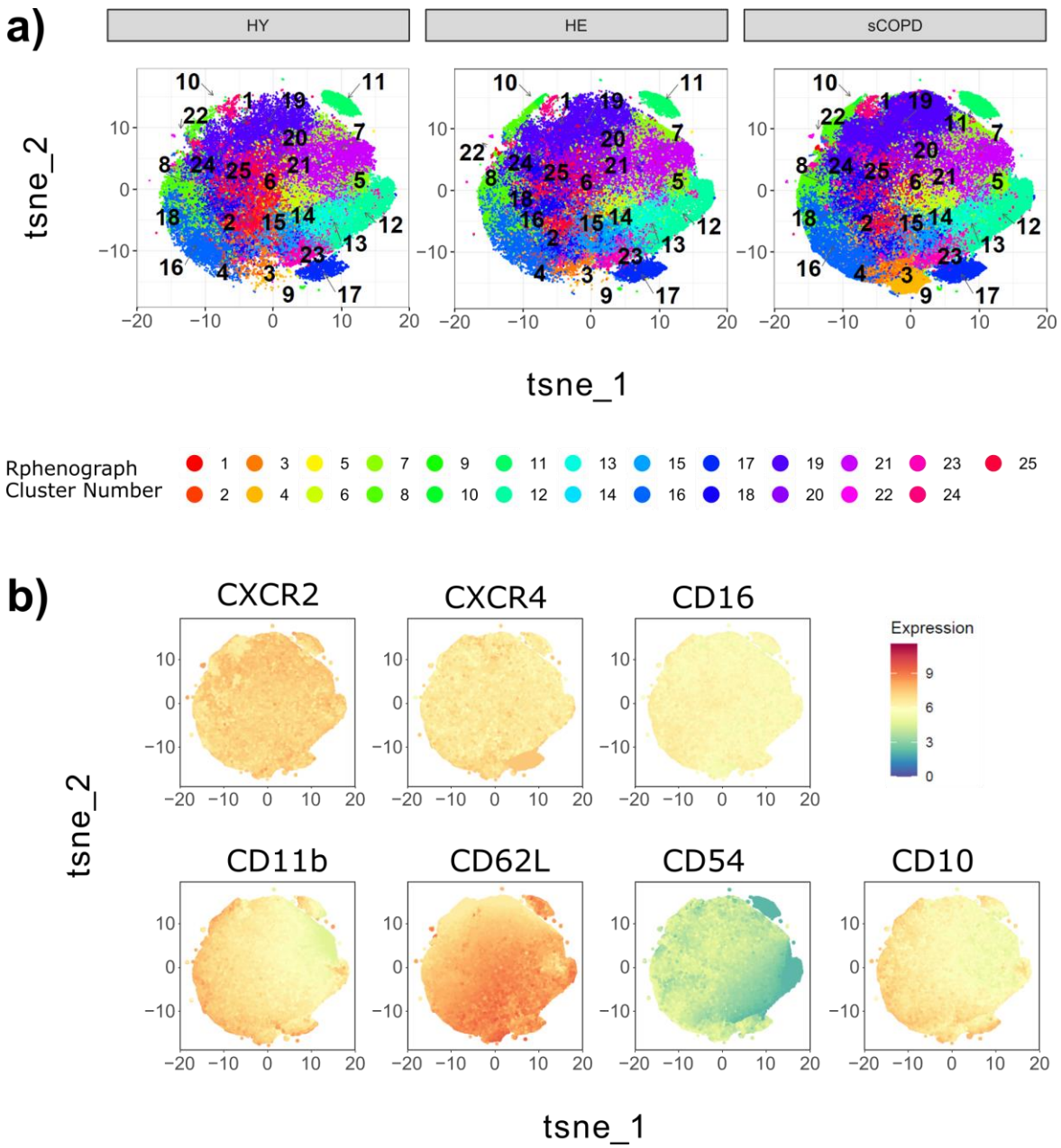

Figure 5.24: tSNE analysis of surface expression of markers from antibody panel 1 for healthy young, elderly and COPD participants clustered using Rphenograph

Neutrophils from whole blood were isolated from healthy young (HY; n=15), healthy elderly (HE; n=15) or patients with stable COPD (sCOPD; n=41), stained with panel 1 antibodies and gated for live cells. Live neutrophils from each sample were then analysed using tSNE and Rphenograph clustering based on surface marker expression. Clusters are presented for **a)** each participant group coloured by cluster number or **b)** combined relative surface expression, where red indicates high expression.

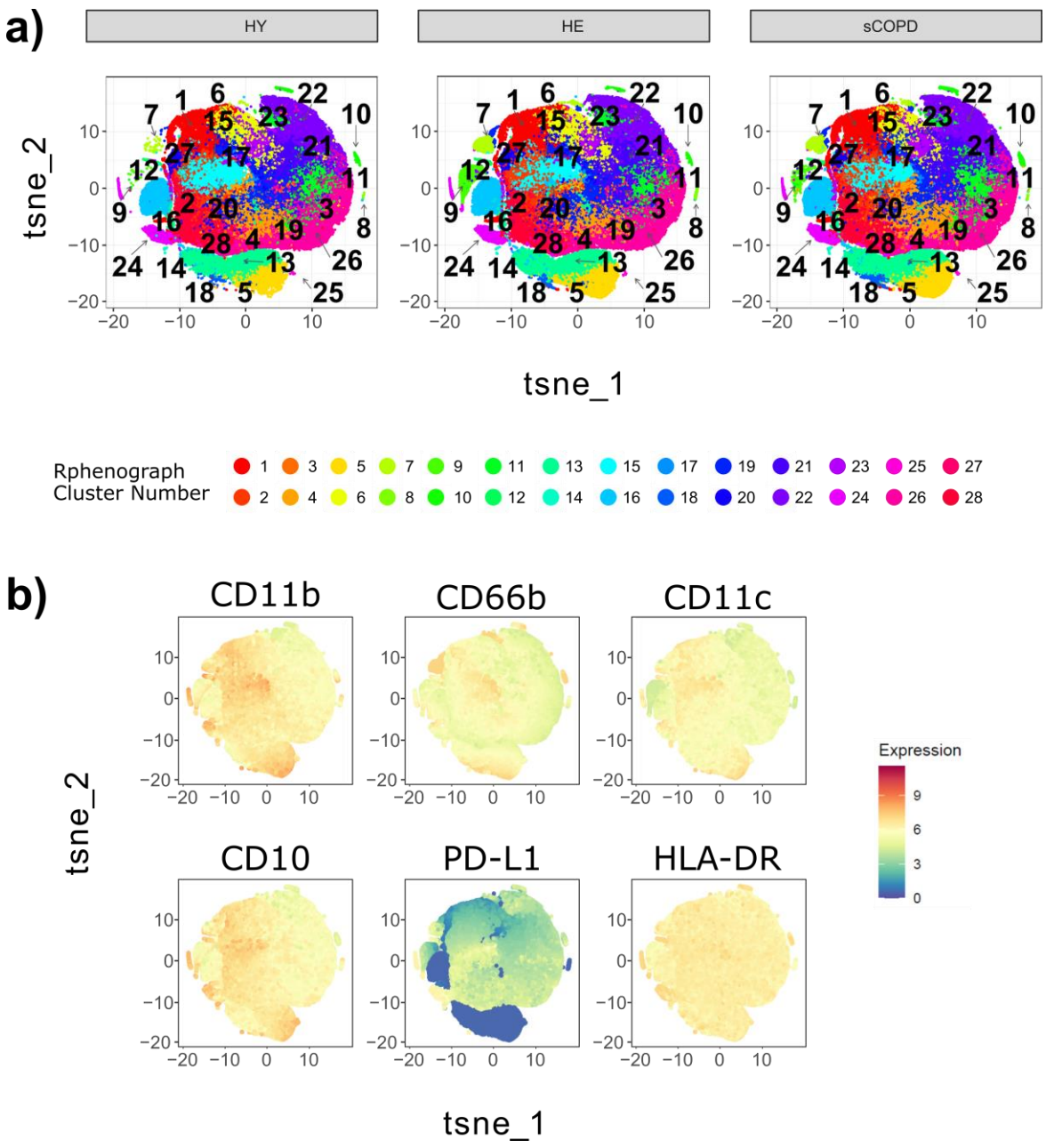

Figure 5.25: tSNE analysis of surface expression of markers from antibody panel 2 for healthy young, elderly and COPD participants clustered using Rphenograph

Neutrophils from whole blood were isolated from healthy young (n=15), healthy elderly (n=15) or patients with stable COPD (n=41), stained with panel 2 antibodies and gated for live cells. Live neutrophils from each sample were then analysed using tSNE and Rphenograph clustering based on surface marker expression. Clusters are presented for **a)** each participant group coloured by cluster number or **b)** combined relative surface expression, where red indicates high expression.

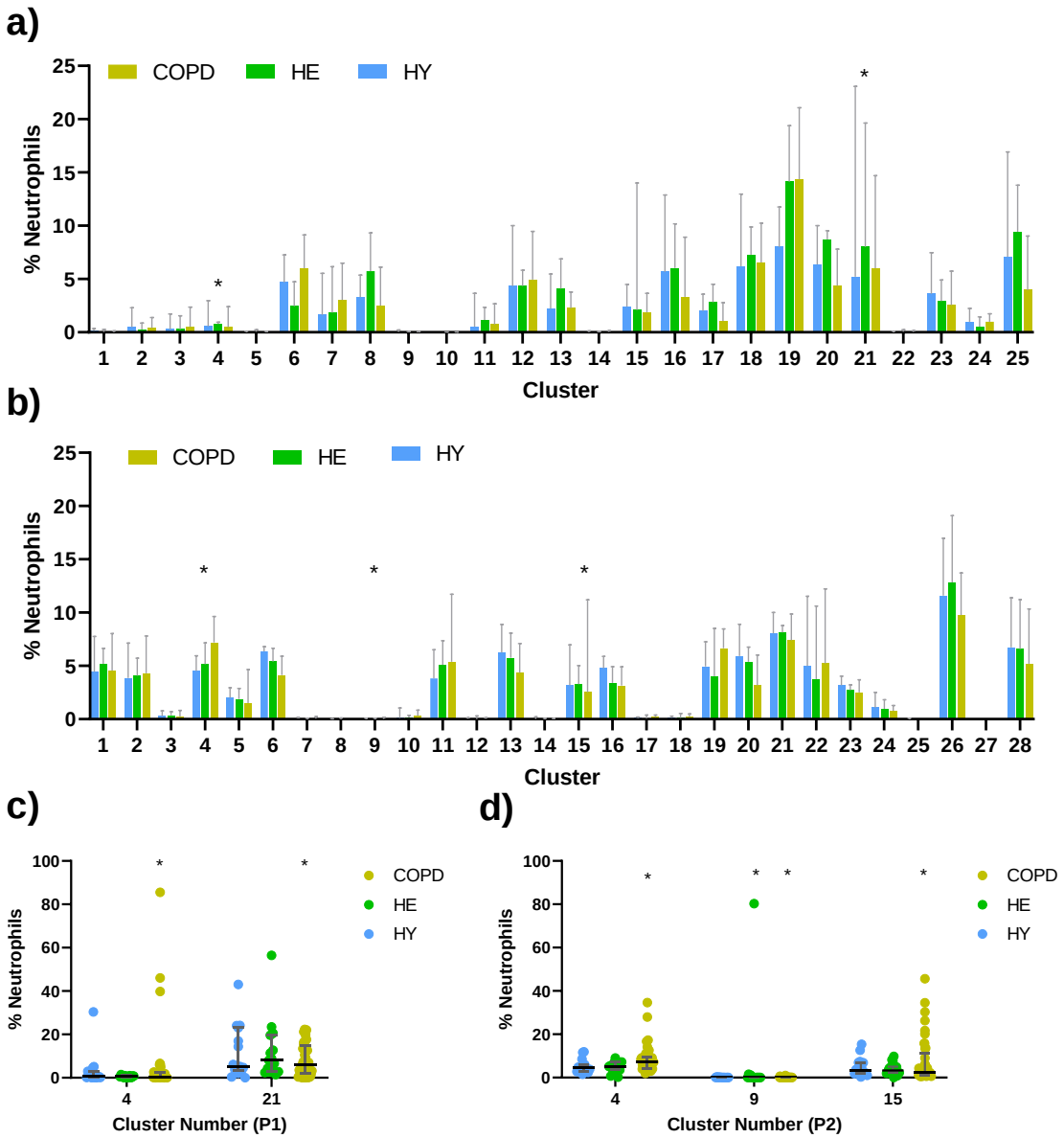

Figure 5.26: Cluster abundance of neutrophils from healthy young, elderly and patients with COPD following Rphenograph cluster analysis

Neutrophils from whole blood were isolated from healthy young (HY; n=15), healthy elderly (HE; n=15) or patients with stable COPD (n=41), stained with panel 2 antibodies and gated for live cells. The percentage of live neutrophils identified within each cluster (Figure 5.24 and Figure 5.25) are shown for **a)** panel 1 and **b)** panel 2. Statistically significant changes in cluster percentages between groups are shown in more details for **c)** cluster 4 and 21 from panel 1 and **d)** cluster 4, 9, and 15 from panel 2. In each case, horizontal lines indicate the median with the interquartile range. Statistical analysis on cluster percentages were performed using two-way ANOVA with Dunnett's multiple comparisons compared to the HE group.

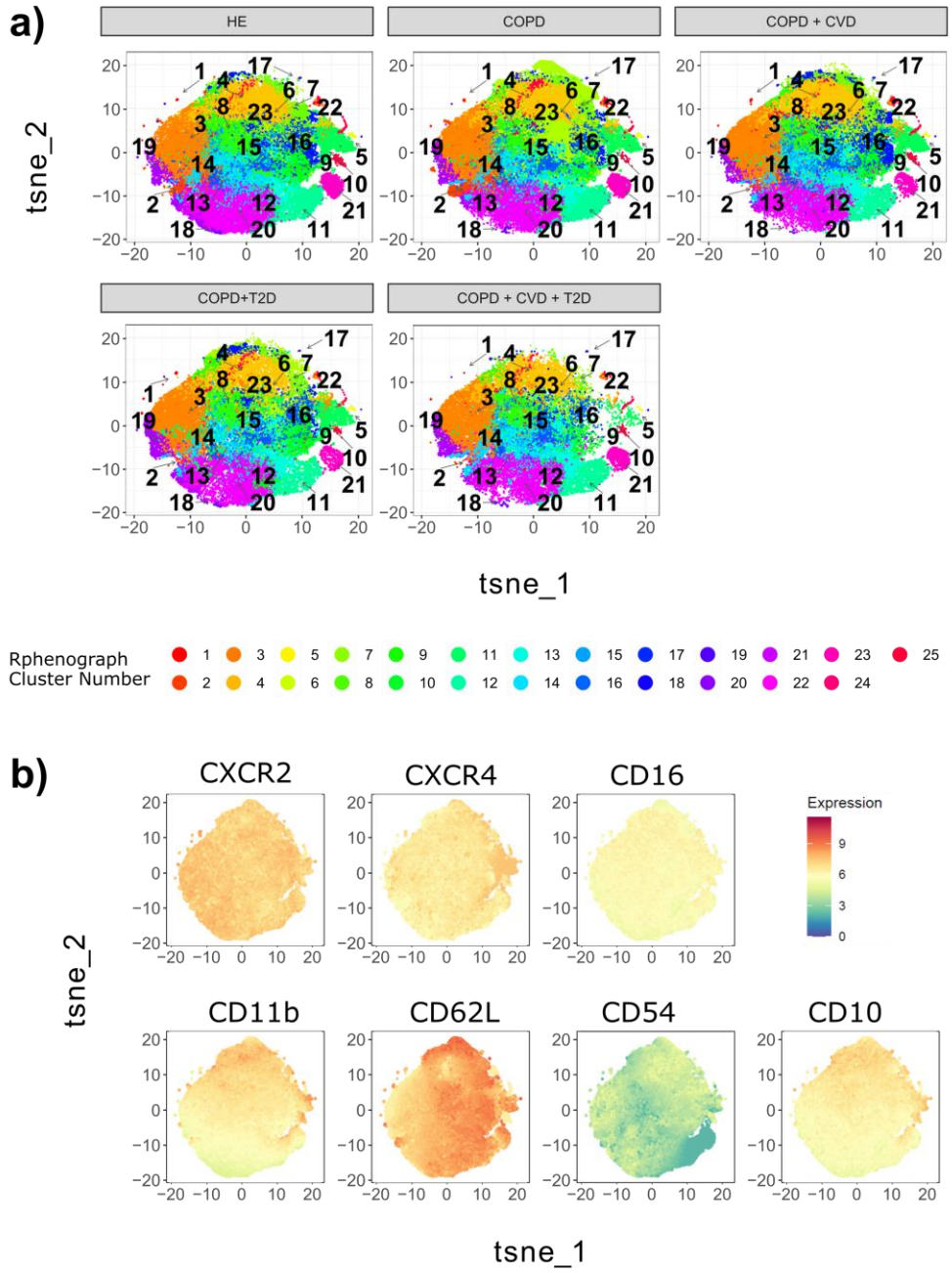

Figure 5.27: tSNE analysis of surface expression of markers from antibody panel 1 for healthy elderly and COPD participants, stratified by multimorbidity, clustered using Rphenograph

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) or patients with COPD and either no co-morbidities (n=15); with CVD (n=12); T2D (n=9) or CVD and T2D (n=6). Neutrophils were stained with panel 1 antibodies and gated for live cells. Live neutrophils from each sample were then analysed using tSNE and Rphenograph clustering based on surface marker expression. Clusters are presented for **a)** each participant group coloured by cluster number or **b)** combined relative surface expression, where red indicates high expression.

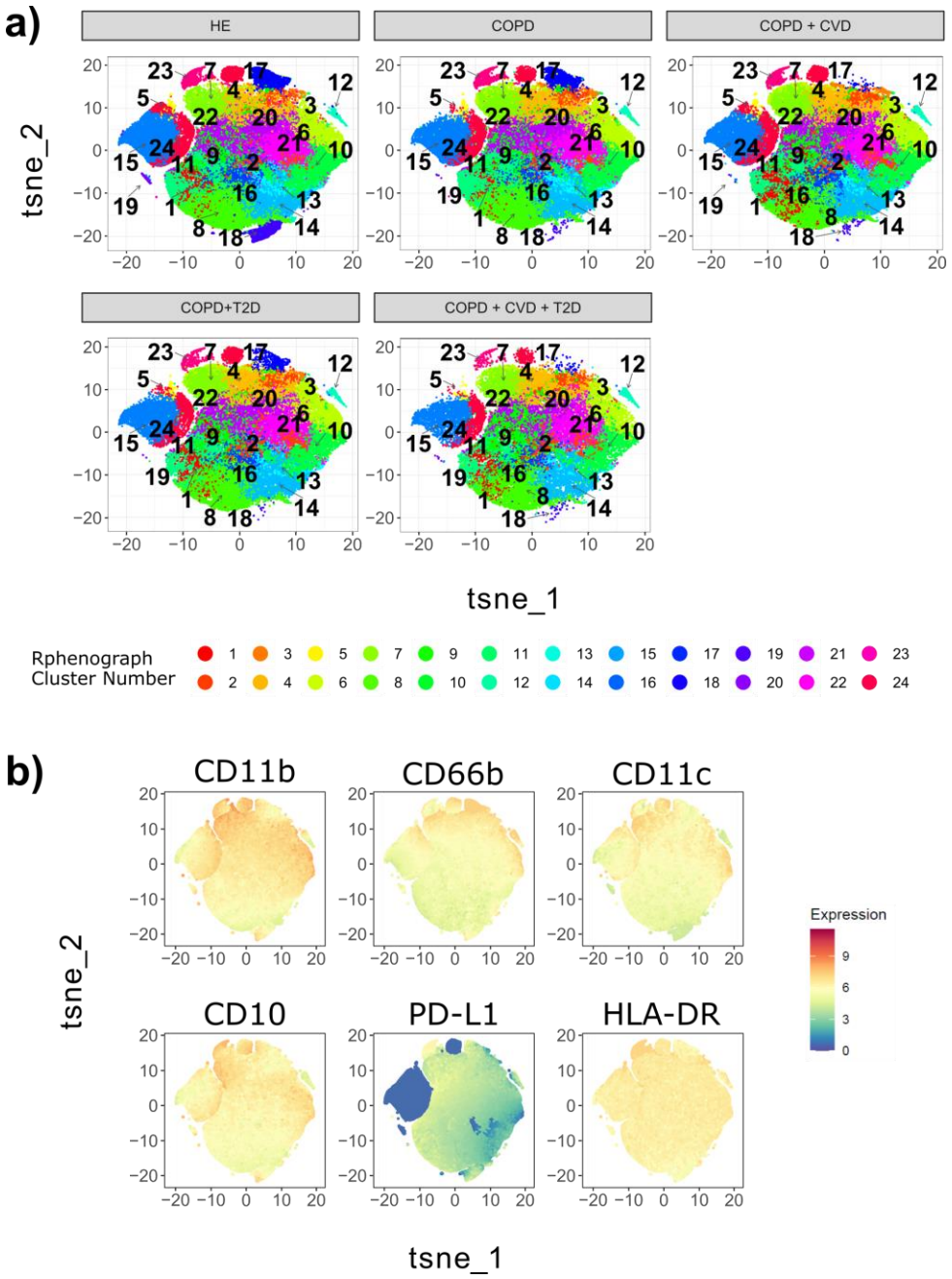

Figure 5.28: tSNE analysis of surface expression of markers from antibody panel 2 for healthy elderly and COPD participants, stratified by multimorbidity, clustered using Rphenograph

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) or patients with COPD and either no co-morbidities (n=15); with CVD (n=12); T2D (n=9) or CVD and T2D (n=6). Neutrophils were stained with panel 2 antibodies and gated for live cells. Live neutrophils from each sample were then analysed using tSNE and Rphenograph clustering based on surface marker expression. Clusters are presented for **a)** each participant group coloured by cluster number or **b)** combined relative surface expression, where red indicates high expression.

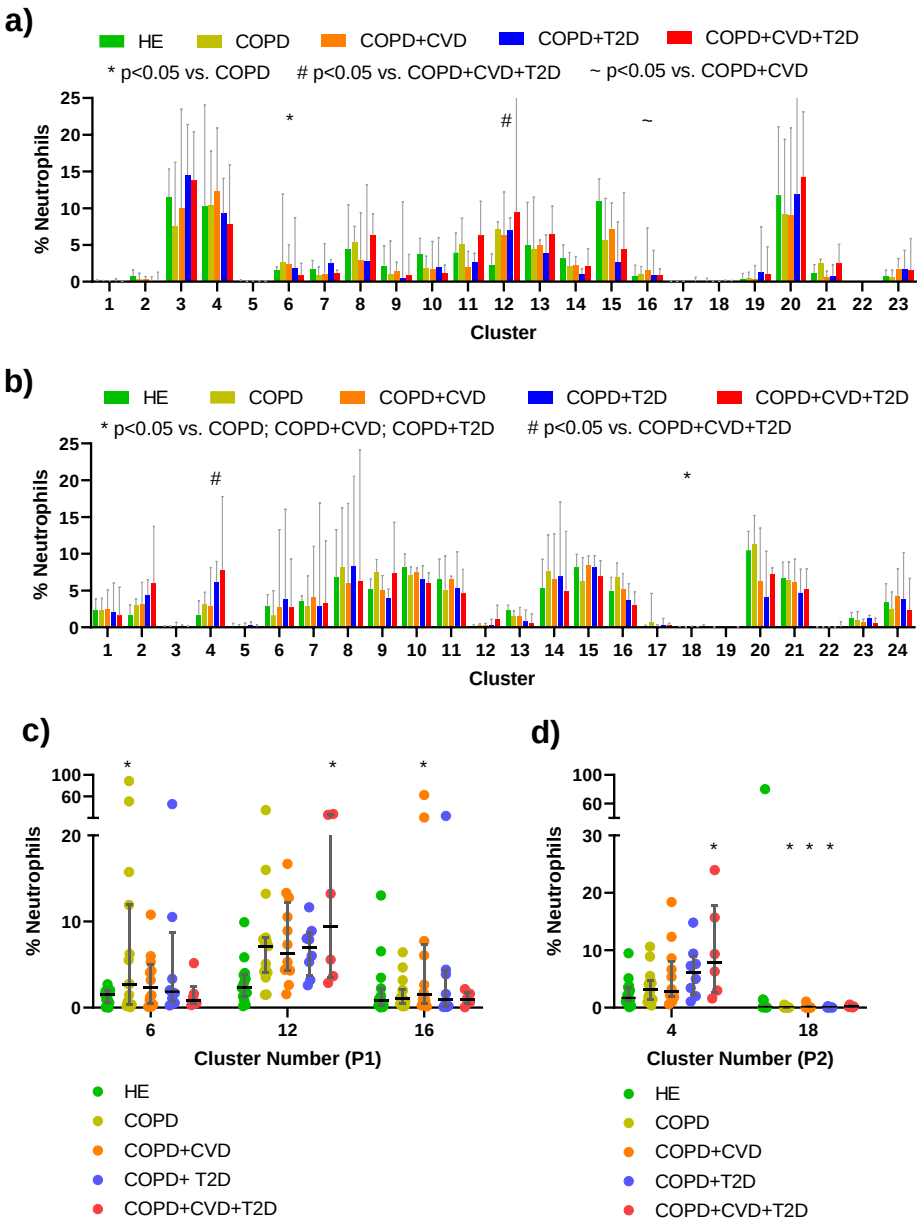

Figure 5.29: Cluster abundance of neutrophils from healthy elderly and patients with COPD, stratified based on multimorbidity, following Rphenograph cluster analysis

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) or patients with COPD with either no co-morbidities (n=15); with CVD (n=12); T2D (n=9) or CVD and T2D (n=6). The percentage of live neutrophils identified within each cluster (Figure 5.27 and Figure 5.28) are shown for **a)** panel 1 and **b)** panel 2. Statistically significant changes in cluster percentages between groups are shown in more details for **c)** cluster 6, 12 and 16 from panel 1 and **d)** cluster 4, and 18 from panel 2. In each case, horizontal lines indicate the median with the interquartile range. Statistical analysis on cluster percentages were performed using two-way ANOVA with Dunnett's multiple comparisons compared to the HE group.

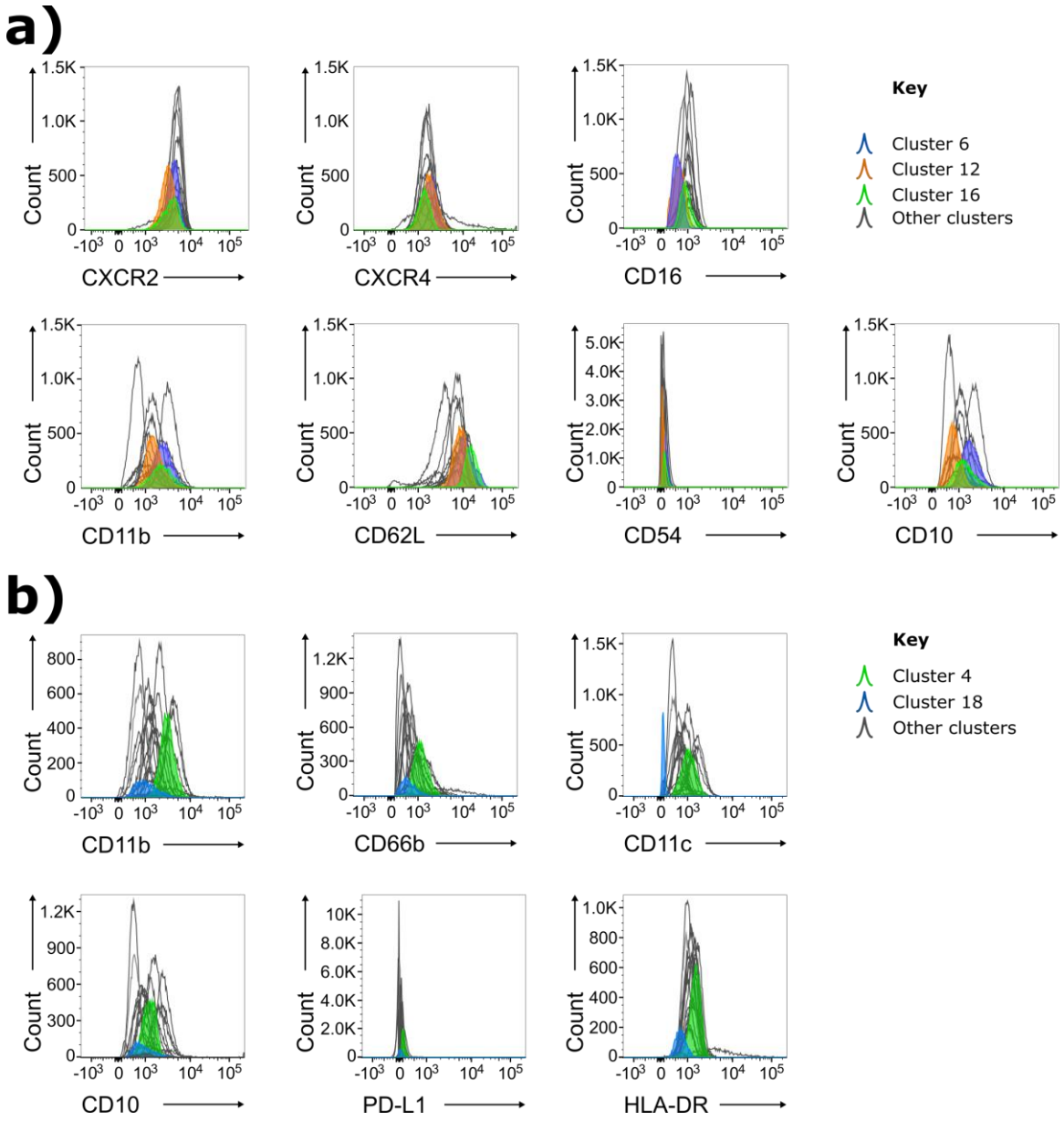

Figure 5.30: Surface expression for neutrophils within each cluster identified using Rphenograph

Neutrophils from whole blood were isolated from healthy elderly (HE; n=15) or patients with COPD with either no co-morbidities (n=15); with CVD (n=12); T2D (n=9) or CVD and T2D (n=6), stained with antibodies and the fluorescence intensity (MFI) measured for each marker (see Table 1). Key clusters highlighted in Figure 5.29 for **a)** panel 1 and **b)** panel 2 are identified to show the expression for each marker compared to the other clusters (grey traces).

5.2.6 Differential gene expression in neutrophils in patients with COPD

As our understanding of neutrophil biology has evolved, it has become clearer that neutrophils are capable of changing their gene expression profile, especially in response to pathogens (Terkawi, Takano and Kato, 2018). RNA was extracted from neutrophils isolated from each participant group, HY, HE, COPD (without stratification for multimorbidity) and prepared for RNA sequencing (RNASeq).

5.2.6.1 Quality control and sample selection

QC steps were carried out prior to shipping of isolated RNA samples. Immediately after extraction, absorbance readings using a nanodrop were performed. Ratios (260nm to 280nm and 260nm to 230nm) above 2.0 indicated good sample purity, between 2.0 and 1.8 indicated adequate purity and below 1.8 indicated poor purity. Using the 260/280 ratio to distinguish RNA from DNA, 23 of 30 samples achieved good purity and 5 adequate purity, with 2 samples failing to contain any RNA (Table 5.3). Using the 260/230 ratio to identify potential contaminants from the isolation buffers, none of the samples achieved good purity and only 6 adequate purity (Table 5.3). These data indicate that whilst pure RNA was extracted with little DNA contamination, potential contaminants that absorb at 230nm were present.

Further QC was carried out on all successful RNA: an RNA integrity score (RIN) was measured using a Tapestation and accurate RNA quantification performed using a Qubit fluorescence reader. For RNA sequencing, a RIN score of 8 or higher is considered good, between 7 and 8 adequate and below 7 as poor. Using these criteria, 5 samples were of good integrity, 10 of adequate integrity and 13 poor integrity (Table 5.3). Across these three quality control measures, 5 samples in the HY, HE groups and 10 samples from the COPD group (not stratified

based on multimorbidity due to sample number) with an adequate quantity of RNA, with the highest purity and integrity scores were chosen for downstream RNASeq.

5.2.6.1 Differentially expressed genes between participant groups

Differential gene expression analysis was performed (see Section 2.10.1 for method) and revealed that there were no significant differences in gene expression between HY and HE. One gene, ARHGAP24, was differentially expressed between HE and stable COPD. Due to only one gene being identified, further analysis was not performed.

5.2.6.2 Does gene expression match surface protein expression?

The genes corresponding to the surface markers investigated in Sections 5.2.3-5.2.4 were identified and plotted on a heatmap for each participant group. Whilst there was sample to sample variation and no statistically significant differences (Figure 5.31a), patterns were observed at a group level (Figure 5.31b). Notably, gene expression of CEACAM8 (CD66b) and ITGAM (CD11b) was relatively higher in stable COPD compared with both healthy groups (Figure 5.31b).

Neutrophils from participants where both surface expression (as shown in Section 5.2.3-5.2.4) and gene expression data were available were used to directly compare these data. The small sample number with both data sets limits the comparison of surface and gene expression due to lack of power, and no statistically significant correlations were observed in all investigated parameters (Figure 5.32 and Figure 5.33). These data may however highlight the differences between gene expression and protein levels on the surface of neutrophils.

Table 5.3: RNA extraction nanodrop quality control results

Sample number	Participant group	260/280	260/230	RIN Score	Concentration (ng/µL)	RNASeq ID
1	COPD	2.08	1.94	6.5	53	RNAC01
2	COPD	2.07	1.98	6.2	66.8	RNAC06
3	COPD	2.05	1.58	7.9	60.6	RNAC03
4	COPD	2.05	1.64	8.7	71	RNAC04
5	COPD	2.06	1.85	8.9	79	RNAC05
8	COPD	1.93	-0.53	6.5	20.2	
9	HY	2.08	1.19	7.7	33.8	RNAY01
10	HY	2.08	1.75	6.4	52.6	RNAY04
11	HE	FAIL	FAIL	N/A	N/A	
12	HE	FAIL	FAIL	N/A	N/A	
13	HE	1.99	1.53	5.7	63.2	
14	HY	2.03	0.96	7	29.8	RNAY02
15	COPD	2.1	1.56	6	69.8	
16	COPD	1.86	1.05	4.9	18.5	
17	HY	2.07	0.18	7	23.8	RNAY05
18	COPD	2.14	0.26	6.6	19.8	RNAC08
19	HY	2.14	0.27	6.3	21.6	RNAY03
20	HY	2.08	0.43	6	37.4	
21	COPD	2.08	0.53	7.7	102	RNAC09
22	COPD	2.08	1.83	7.9	39.6	RNAC07
23	COPD	2.34	1.64	6.2	17.1	
24	COPD	2.07	1.69	7.4	75	RNAC10
25	HE	1.95	1.17	5.9	53.6	
26	HE	2.03	1.76	7.3	41.2	RNAE02
27	HE	2.06	1.82	8	61.8	RNAE03
28	HE	2.02	1.44	7.1	30.2	RNAE04
29	COPD	2.09	1.72	8	42	RNAC02
30	COPD	1.97	1.12	4.3	11.6	
31	HE	2.07	1.99	8.1	126	RNAE05
32	HE	2.06	1.77	7	65.8	RNAE01

Legend: Participant group indicates neutrophil donor: healthy young (HY); healthy elderly (HE); stable COPD (COPD). Ratios based on absorbance readings at 280nm, 260nm and 230nm. RNA integrity score (RIN) measured using a Tapestation and RNA concentration using fluorescence quantification where N/A indicates sample not assessed.

Table 5.4: Corresponding genes for the surface proteins investigated via flow cytometry

Protein name	Gene ID
PD-L1	CD274
CD66b	CEACAM8
CXCR2	CXCR2
CXCR4	CXCR4
CD16	FCGR3B
HLA-DR	HLA-DRA
	HLA-DRB1
CD54	ICAM1
CD11b	ITGAM
CD11c	ITGAX
CD10	MME
CD62L	SELL

Legend: The associated gene for each surface protein investigated in Section 5.2.3-6.2.2.

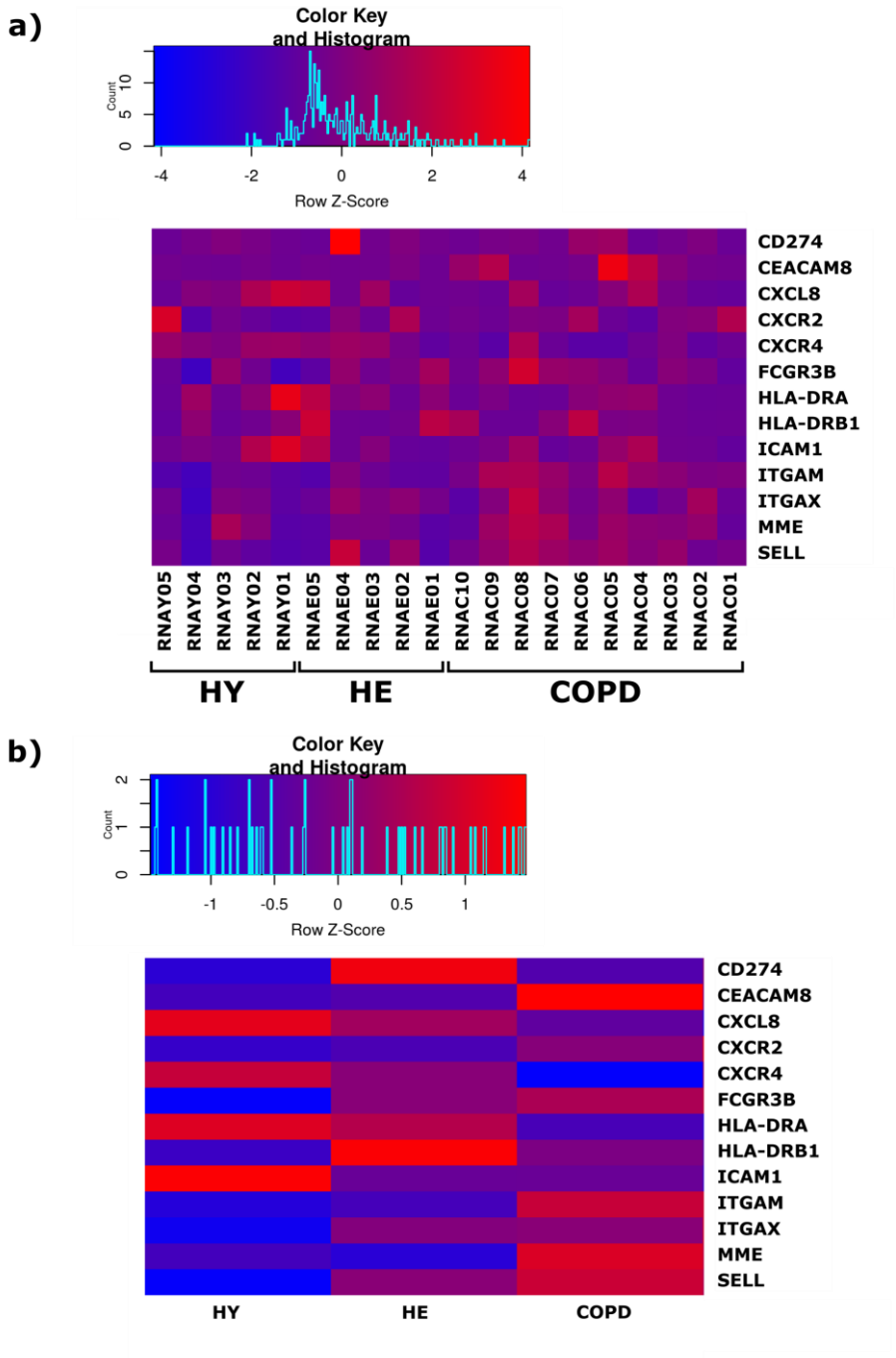

Figure 5.31: Normalised expression of genes corresponding to selected surface markers

Gene counts for mRNA extracted from neutrophils was normalised for gene length and total read number. Gene expression shown for selected markers (**Table** 5.4) for either **a)** each individual participant or **b)** mean expression for each participant group. Genes were clustered using Euclidean spacing and coloured based on the z-score generated from per-gene normalisation.

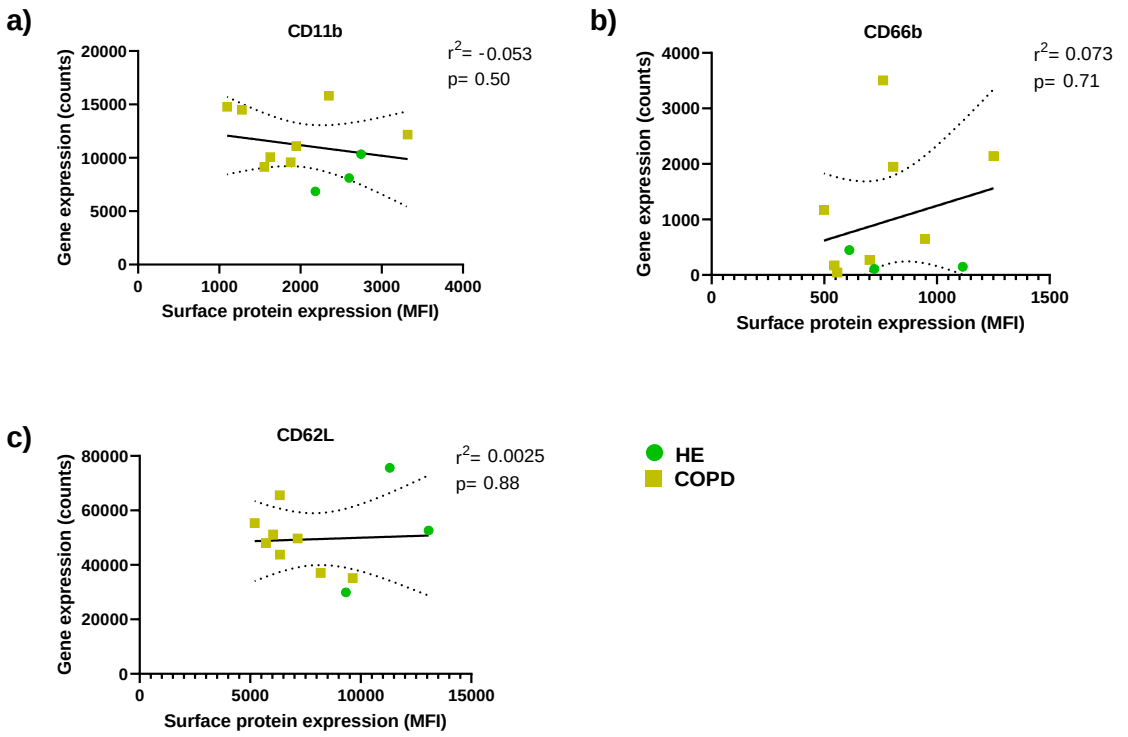

Figure 5.32: Correlation of neutrophil activation markers from surface protein expression and gene expression

Neutrophils from healthy elderly participants (HE, n=3; green circles) and patients with stable (COPD, n=8; yellow squares) were isolated, stained with antibodies and the median fluorescence intensity (MFI) of measured. RNA was also extracted from these samples, processed for RNASeq and gene expression. The correlation between surface and gene expression of **a)** CD11b **b)** CD66b and **c)** CD62L are shown. In each case, linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for each linear regression.

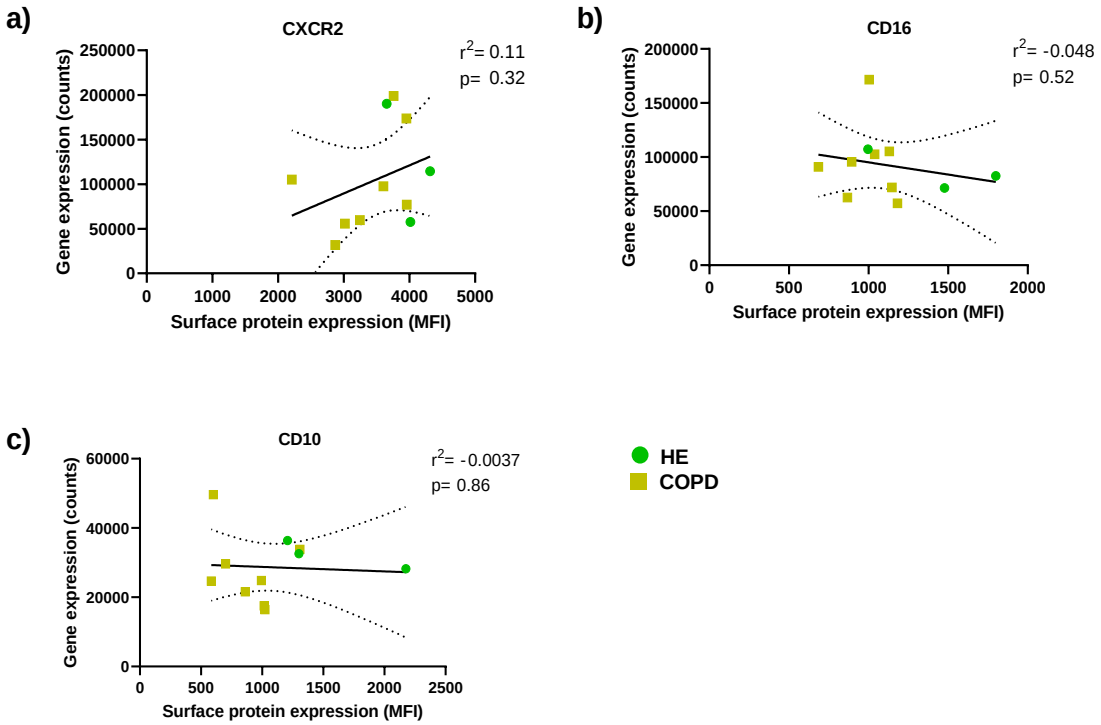

Figure 5.33: Correlation of neutrophil CXCR2, CD10 and CD16 from surface protein expression and gene expression

Neutrophils from healthy elderly participants (HE, n=3; green circles) and patients with stable (COPD, n=8; yellow squares) were isolated, stained with antibodies and the median fluorescence intensity (MFI) of measured. RNA was also extracted from these samples, processed for RNASeq and gene expression. The correlation between surface and gene expression of **a)** CXCR2 **b)** CD16 and **c)** CD10 are shown. In each case, linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for each linear regression.

5.3 Discussion

Many studies have begun to investigate neutrophil phenotypes and how these may be linked to a variety of diseases, however, not much is known about some of these neutrophil phenotypes in COPD. Here, a novel integrated panel was used to assess a variety of neutrophil phenotypes both in healthy ageing, stable COPD and associated co-morbidities.

Broadly speaking, neutrophil phenotypes were similar in both healthy donors and patients with stable COPD regardless of multimorbidity. Notably, a reduction in CXCR2 expression was observed in stable COPD, specifically in patients with COPD and CVD that may suggest a reduced ability of neutrophils to migrate accurately and a differential impact of CVD and T2D in COPD.

5.3.1 The activation status of neutrophils

Neutrophil activation is required for effective neutrophil function, however, chronic activation and systemic activation results in excessive damage without the resolution of inflammation. Whilst many changes occur with neutrophil activation, the expression of CD11b and CD66b have shown to increase and CD62L decrease (Rosales, 2018) when neutrophils are exposed to inflammatory stimuli such as surgery (Orr *et al.*, 2007), exposure to inflammatory cytokines or other activated immune cells (Costantini *et al.*, 2010), or direct receptor engagement (Skubitz, Campbell and Skubitz, 1996). Patients with COPD are also commonly diagnosed with other chronic inflammatory conditions, and given the association of CVD and T2D with systemic inflammation (Hughes, McGettrick and Sapey, 2020b), stratification of patients may help elucidate differences due to multimorbidity.

Based on CD11b, CD66b and CD62L expression, there was no change in neutrophil activation status between either healthy groups, or between HE and COPD patients – regardless of multimorbidity – suggesting there was not systemic activation of neutrophils either with increasing age or presence of stable COPD. In addition, none of these proteins were significantly altered at the gene expression level on the subset of samples tested. Other studies have also suggested that peripheral CD11b expression was not altered in COPD (J. Stockley, 2015; Lokwani, Wark, Baines, Fricker, *et al.*, 2019), yet CD62L expression was shown to be reduced in one of these studies (Lokwani, Wark, Baines, Fricker, *et al.*, 2019). A potential reason for the latter observation was that antibody staining was performed in whole blood in the presence of other cell types that may alter priming signals received by these neutrophils. In addition, studies in patients with coronary artery disease (Särndahl *et al.*, 2007) and patients with T2D (Sampson *et al.*, 2002) detected similar levels of CD11b on neutrophils when compared to healthy controls. A couple of studies have suggested increases in CD11b expression on circulating neutrophils from patients with COPD (Noguera *et al.*, 1998; Yamagata *et al.*, 2007), however, both studies showed large variation between patients, or substantially lower lung function (Noguera *et al.*, 1998) than patients included in this thesis.

Of note, the only statistically significant correlation from the data in this thesis was demonstrated with CD11b and pack year history in the most multimorbid patients (COPD with CVD and T2D), with a lower smoking history indicative of increased activation – a finding that may be due to the low sample number as this was not replicated in any other group. However, the data presented in this thesis and by Lokwani *et al.,* used healthy controls that did not have a comparable smoking history to patients with COPD, nor were controls with a smoking history excluded. This may reduce the ability to detect changes that are caused by smoking, as

another small study of 5 patients with COPD and 9 without, both long-term smokers, demonstrated that CD11b expression was comparable between these two groups, but higher than 8 healthy non-smokers (Stockfelt *et al.*, 2020). One confounder of this study was the isolation of white blood cells using Ficoll gradient separation followed by red blood cell lysis that differs from the methodology described by Lokwani *et al.* and in this thesis. Another study also suggested small increases in surface CD11b expression were observed between healthy non-smokers (n=22) and patients with COPD (n=18) when staining was performed in whole blood (Blidberg *et al.*, 2013).

Differences in the detection of activated neutrophils between studies may, therefore, be explained by differences in blood collection, staining, isolation methods and the patient cohort used. The use of a dextran sedimentation step, used in this thesis, has been suggested to promote neutrophil activation and increase CD11b expression and reduce CD62L expression (Quach and Ferrante, 2017) that may impact on the activation phenotype observed in this study. However, *in vitro* stimulation with a physiological stimulant, fMLP (shown in Section 3.2.7), resulted in increases in surface activation markers, suggesting a lack of activation signal was not due to these cells already being activated. Avoiding cell isolation altogether and using whole blood flow cytometry also has pitfalls as the red blood cell lysis step can also alter neutrophil behaviour and ability to be activated, or fixes the cells preventing any functional analysis (Vuorte, Jansson and Repo, 2001). Given the diversity of neutrophil isolation procedures and the impact these can have on cellular activation, this remains a big problem in comparing the outcomes of studies.

These data suggest peripheral blood neutrophil activation remains unaltered in stable COPD with or without co-morbidities. They also highlight, in combination with previous studies, that experimental procedures and matching of patients with correct controls are confounders in these data and are important considerations when comparing studies.

5.3.2 Accelerated ageing, senescence and changes in chemokine sensing

The concept of accelerated ageing in COPD comes from parallels between 'healthy' ageing and COPD, where processes such as a decline in lung function, cellular senescence and increased oxidative stress are all exaggerated in COPD (Mercado, Ito and Barnes, 2015). Little attention, however, has been given specifically to the neutrophil and the nuanced senescence that neutrophils undergo. As a terminally differentiated cell, neutrophils are already in cell cycle arrest - a feature of cellular senescence (van Deursen, 2014). For neutrophils, experiments in mice describe the neutrophil senescence phenotype as a rise in CXCR4 expression and a decline in CXCR2 expression (Martin *et al.*, 2003).

The low level of CXCR4 expression detected on neutrophils from all participant groups suggested that peripheral neutrophil senescence did not occur with healthy ageing or in COPD. These results are supported by previous studies in healthy individuals (Rankin, 2010), but investigations in either COPD, CVD and T2D are distinctly lacking. In murine studies, it is possible that any $CXCR4^{bright}$ expressing neutrophils are promptly sequestered from the circulation in the bone marrow (Martin *et al.*, 2003), potentially suggesting that $CXCR4^{bright}$ neutrophils may not be detected in the circulation. Indeed, detection of CXCR4 expressing neutrophils has only been robustly detected during *in vitro* ageing of human neutrophils (Yildirim *et al.*, 2005) or at sites of inflammation in humans (Rudd *et al.*, 2019) and zebrafish

(Isles *et al.*, 2019). Combined with the lack of circulating CD54 expressing neutrophils, it seems unlikely that COPD is linked with an increased burden of circulating senescent cells, including the reverse transmigration of any senescent cells from the lungs.

Linked with neutrophil senescence based on CD62L and CXCR4 expression, a previous study described an 'overactive senescence' neutrophil phenotype in patients with ischemic stroke (Weisenburger-Lile *et al.*, 2019), defined as being CXCR4+CD62L^{dim}. Indeed, a small trend (statistically significant at the group level) of an increasing proportion of these neutrophils was seen between HY, HE and patients with COPD - potentially suggesting an age and disease associated increase. Of note, patients with COPD and CVD were not significantly different from other stable COPD patients.

Whilst ageing also had no impact on CXCR2 levels, COPD led to a small but significant decline in surface CXCR2 expression, most marked in patients with both COPD and either CVD alone or with T2D, suggesting chemokine sensing may be altered in these cells. Expression of CXCR2 also negatively correlated with CD11b expression, suggesting whilst activation was not significantly altered between groups, lower CXCR2 levels is associated with activation. These data raise two key points: the lack of change between HY and HE suggest these changes in CXCR2 expression do not reflect accelerated ageing; and CXCR2 expression is not reduced in all patients with COPD, showing heterogeneity in patients with COPD based on the presence of CVD. It cannot be overlooked, however, that these changes represent an overall 12% reduction in CXCR2 expression that is unlikely to completely alter downstream CXCR2 signalling. Whilst further investigation would be required to assess the biological impact of this reduction, existing data on changes in neutrophil responses to CXCL8 (a major ligand of

the CXCR2 receptor) from patients with COPD (Yoshikawa *et al.*, 2007) and blocking of CXCR2 inhibits chemotaxis to CXCL8 (White *et al.*, 1998) may indicate this reduction is able to modulate neutrophil responses.

Several previous studies have investigated CXCR2 expression on circulating neutrophils. One study also showed reduced CXCR2 expression in stable COPD (Pignatti *et al.*, 2005) compared to healthy controls. Other studies have reported lower CXCR2 levels (Traves *et al.*, 2004; Sapey *et al.*, 2011), but without statistical significance – potentially due to sample number, variation within control groups or, as highlighted by this thesis, the heterogeneity in the COPD patient population. Interestingly, CXCR2 expression on neutrophils has not been directly investigated in CVD, atherosclerosis or T2D, despite clinical trials investigating CXCR2 inhibition in CVD (Joseph *et al.*, 2017), linking CXCR2 as an important factor in neutrophil-mediated damage in diabetes (Collier *et al.*, 2017) and linking macrophage CXCR2 with atherosclerosis (Boisvert, Curtiss and Terkeltaub, 2000). However, the suggestion that CXCL2 is raised in the circulation of patients with CVD (Guo *et al.*, 2020) may pose a potential mechanism to reduce neutrophil CXCR2 expression due to receptor internalisation and may also link with the activation markers on these cells.

Collectively, alterations in CXCR2 may suggest a potential mechanism for the reduced migratory accuracy of neutrophils from patients with COPD – a defect that has been previously described (Sapey *et al.*, 2011). Indeed, reduced CXCR2 expression on neutrophils from patients with ANCA-associated vasculitides also reported reduced migration of neutrophils through endothelium *in vitro* (Hu *et al.*, 2011). The inability to accurately sense key chemokines such as CXCL2 and CXCL8 may increase the collateral damage caused by neutrophils that fail to

migrate accurately to inflammatory sites – although the lack of correlation (albeit underpowered) between CXCR2 expression and lung function in patients with COPD weakens the link between this phenotype and lung damage. Further mechanistic studies would be required to determine if this is because of persistent receptor signalling resulting in internalisation, or indeed a different phenotype pre-disposing to these diseases and the clinical impact this may have.

5.3.3 Neutrophil maturity

Immature neutrophils are rarely seen in the circulation of healthy individuals as maturation occurs within the bone marrow, only entering the circulation as CD10-expressing mature neutrophils (Hughes, Sapey and Stockley, 2019). Previous studies have revealed that inflammation, specifically acute inflammation, can lead to premature release of neutrophils into the circulation (referred to as emergency granulopoiesis) and has been seen during sepsis (Taneja *et al.*, 2008), after invasive cardiac surgery (Orr *et al.*, 2005) and in severe COVID-19 (Carissimo *et al.*, 2020).

The data presented here showed that in healthy ageing and the presence of COPD regardless of multimorbidity, there was no change in the maturity of circulating neutrophils, indicating correct regulation of neutrophil maturation. However, neutrophils from multimorbid patients with COPD, and T2D (with or without CVD) potentially showed lower CD10 expression, supported by Rphenograph analysis – but more participants are required to draw this conclusion. There currently is a gap in the current research investigating neutrophil maturity in stable COPD, CVD and T2D, however, the data presented here suggest it may not play a role in these disease areas.

Whilst CD16 expression is commonly used as a marker to identify neutrophils, it has also been linked with changes in neutrophil function and neutrophil maturity (Elghetany *et al.*, 2004; Donnelly and Barnes, 2012). Here, CD16 expression was reduced on neutrophils from patients with COPD compared with HE and to a greater extent in patients with COPD alone or with T2D – suggesting an influence of both multimorbidity and chronic disease. Combined with CD10 expression, there may be evidence of neutrophil immaturity in patients with both COPD and T2D. Studies specifically looking at CD16 expression in COPD, T2D and CVD are, again, lacking, but reduced neutrophil CD16 expression has been reported in several other circumstances: as a response to heat-shock prior to apoptosis (Guzik *et al.*, 2011) and in systemic inflammatory response syndrome as a marker of immature 'banded' neutrophils (Drifte *et al.*, 2013), also described as $CD16^{dim}CD62L^{bright}$ cells (Pillay *et al.*, 2012). Previous studies have offered conflicting reports of the role CD16 may play in neutrophil function: enzymatic removal of CD16 did not appear to impair neutrophil phagocytosis or bacterial killing (Fossati *et al.*, 2002b), but CD16 expression on a cohort of HE volunteers negatively correlated with a reduced phagocytic index (Butcher *et al.*, 2001) – although these studies used different bacteria and opsonisation conditions. Our data may suggest a potential mechanism for reduced phagocytosis observed in patients with COPD (Donnelly and Barnes, 2012), although neutrophils are not the only phagocytes found in the lung.

In summary, these data suggest that immature neutrophils may be present in patients with both COPD and T2D without CVD based on both CD10 and CD16 expression, but these may represent slightly different maturity phenotypes. Reduced CD16 expression might be linked with chronic inflammation seen in COPD that may indicate why patients with COPD have a reduced ability to clear bacteria.

5.3.4 Inflammatory status and reverse transmigration

Neutrophils have also been reported to display pro-inflammatory or anti-inflammatory properties through either the expression of an antigen presentation molecule, HLA-DR (Vono *et al.*, 2017) or inhibiting T cell responses via PD-L1 (Keir *et al.*, 2008; Wei *et al.*, 2013). CD11c has also been linked with a predictive marker in SIRS (Lewis *et al.*, 2015) and CD54 with neutrophil reverse transmigration (Buckley *et al.*, 2006).

In this study, little or no HLA-DR expression was detected on neutrophils from any participant group, with no changes in PD-L1, CD11c expression or CD54 expression in health or stable COPD – suggesting neutrophils do not display pro-inflammatory, anti-inflammatory or reverse transmigration properties in these patients. However, one study showed an increase in HLA-DR-expressing neutrophils in current smokers with COPD compared to both former smokers with COPD and smokers with normal lung function (Scrimini *et al.*, 2013). Differences in the populations used compared to this thesis may, therefore, explain this discrepancy. All these phenotypes have not been described yet in any detail in COPD, T2D or CVD, and the data here suggest that they do not exist in the circulation of patients with COPD regardless of multimorbidity. The lack of CD54 expression also suggests that it is unlikely that pro-inflammatory conditions in the lung could lead to systemic effects by altering the neutrophil phenotype before they return to the circulation – a hypothesis that could link COPD and related co-morbidities.

Another study identified a different potentially immunosuppressive neutrophil phenotype, CD16+CD62L^{dim}, identified to be increased in response to systemic LPS in healthy human volunteers (Pillay *et al.*, 2012). Recently, CD16+CD62L^{dim} neutrophils have also been linked as

a prognostic marker for the development of pulmonary embolisms in COVID-19 patients (Spijkerman *et al.*, 2021). There was a slight suggestion that the most multimorbid patients (COPD and CVD and T2D) may have an increased proportion of these cells, but these findings were not statistically significant, potentially suggesting little involvement of this particular immunosuppressive phenotype in COPD.

5.3.5 Neutrophil heterogeneity

The data discussed so far has focussed on average differences between population groups, but does not fully address the heterogeneity of the neutrophil population; a concept that is being increasingly appreciated. The role different neutrophil populations play in causing disease is largely unknown as it is challenging to determine if changes occur in the neutrophil before disease pathogenesis, or as a result of the disease (Rosales, 2018).

The use of dimension-reduction algorithms, such as t-SNE as utilised in this chapter, aid identification of complex surface expression changes, but highlight that neutrophils are broadly similar within a given population. Even between healthy individuals and those with COPD, broadly similar phenotypes are observed, further illustrating that there is a finer, subtle shift in surface expression rather than dramatic distinctions between these populations. The ability of these tools to dissect complex and non-discrete datasets has been previously investigated, showing that clear separation is not generally seen when PBMCs are investigated in healthy individuals (Toghi Eshghi *et al.*, 2019). Indeed, there are very few neutrophil proteins that have been shown to have bi-modal distributions. CD177, with as yet undetermined functional relevance does show a distinct bi-modal distribution in healthy people and appears to co-localise with bi-modal expression of PR3 on the neutrophil cell

membrane (Bauer *et al.*, 2007). The lack of distinct bi-modal expression in the markers utilised in these panels within this thesis may confound the ability of this technique to identify completely separate neutrophil populations. The usefulness in multi-dimension analysis in neutrophils, therefore, is not in identifying discrete subsets, but suggesting where differences may exist between neutrophil populations in a more subtle manner.

From these data, overlap of neutrophils expressing higher levels of CD11b, CD66b and CD11c builds on the single-marker analysis and suggests a role of CD11c in the activated neutrophil phenotype, despite none of the markers being significantly different when investigated alone. These analyses highlight that neutrophil phenotypes may be more complex than binary positive/negative populations or those identified by expression of a single marker. These data also support that expression of surface molecules, in general, are a gradient with no clear 'subsets' emerging, indicating that therapeutics will need to shift, rather than eliminate, certain neutrophil phenotypes.

5.3.6 The impact of gene expression on neutrophil phenotype

RNASeq analysis of mRNA isolated from neutrophils provides data on the gene expression profile of these cells. Previous work has identified multiple different areas where neutrophil gene expression was altered, including after *in vitro* stimulation of healthy neutrophils with TNF-α leading to increased expression of IL-1β, CXCL8 and CD54, where increased IL-1β expression was also identified in patients with COPD (Oudijk *et al.*, 2005). In the present study, no *ex vivo* stimulation was performed and, therefore, all changes identified were altered by *in vivo* processes. No genes were identified as differentially expressed between HY or HE participant groups, indicating that the gene expression profile of neutrophils does not

significantly change with age. Several other studies have found variable numbers of differentially expressed genes in peripheral blood with age, but none specifically investigate neutrophils in isolation and focused either on whole blood (Nakamura *et al.*, 2012) or PBMCs (Joehanes *et al.*, 2012). It is possible, with the caveat of only 5 samples in each group, that neutrophils in the circulation do not show age-related changes in either gene expression or surface expression at baseline. These analyses were performed using stringent cut-off criteria for p-value adjustment and this may be another possible reason for the failure to detect differentially expressed genes.

Only one gene showed significantly higher expression in neutrophils from patients with COPD compared with HE: ARHGAP24; encoding for filamin A-binding RhoGTPase-activating protein (FilGAP) (Ohta, Hartwig and Stossel, 2006). FilGAP has been shown to control actin remodelling processes (Ohta, Hartwig and Stossel, 2006) vital for cell polarisation and was shown to negatively regulate chemotaxis in lymphocytes by inhibiting lamellae extension (Iida *et al.*, 2016). Therefore, constitutively higher expression of ARHGAP24 may prevent efficient chemotaxis of neutrophils, a feature that has previously been shown in patients with COPD (Sapey *et al.*, 2011), demonstrating a potential mechanism. However, it cannot be overlooked that a complex process such as chemotaxis is not controlled by a single gene and, therefore, further investigation would be necessary to confirm the role of ARHGAP24 in COPD.

The expression of genes linked with anti-ageing processes such as sirtuins - protein deacetylases that are linked with maintaining genomic stability (Bosch-Presegué and Vaquero, 2014) - was also of interest. SIRT1 has previously been shown to be reduced in PBMCs (Nakamaru *et al.*, 2009) and lung tissue (Rajendrasozhan *et al.*, 2008) from patients with COPD.

The data in the present study suggest that sirtuin expression was not altered in peripheral neutrophils in patients with stable COPD, suggesting a similar mechanism of accelerated ageing was not found in neutrophils and may instead be limited to regulation of maturation (X. Chen *et al.*, 2015).

5.3.7 Summary

Previous work has implicated multiple neutrophil phenotypes with COPD. Here, evidence from a novel antibody panel suggests that neutrophils from patients with COPD, on average, do not have higher surface expression of activation markers regardless of multimorbidity, suggesting that neutrophils are not systemically activated, and increased activation does not link the pathologies of COPD, CVD and T2D.

Circulating neutrophils do, however, show lower CXCR2 expression that is enhanced in patients with CVD (compared to healthy controls), suggesting an inflammatory-mediated regulation of CXCR2 expression. In addition, lower CD16 expression was detected on neutrophils from patients with COPD, specifically in patients with COPD alone or with T2D, potentially suggesting a mechanism of reduced bacterial clearance. The number of patients included in these studies means it was underpowered to draw sound comparisons between the differences in neutrophil phenotype and clinical parameters, or between surface and gene expression. However, these data reveal differences in patients based on multimorbidity, further highlighting how not all patients with COPD are the same and provides a basis for consideration in future studies.

Here, for the first time, comprehensive neutrophil phenotyping reveals subtle neutrophil phenotype differences in patient subsets based on multimorbidity in COPD. These changes may provide mechanisms of neutrophil dysfunction and avenues for therapeutics, but further study is required to elucidate the functional and clinical impact of these phenotypes.

CHAPTER 6:

PHENOTYPING OF PERIPHERAL BLOOD NEUTROPHILS IN AECOPD

6.1 Brief introduction

Clinically, an exacerbation of COPD can be defined as "an acute worsening of respiratory symptoms that results in additional therapy" (GOLD, 2021) – a general definition that has not changed for some time. Exacerbations are associated with long term health impacts (Celli and Wedzicha, 2019) and patients who exacerbate frequently are more likely to experience adverse cardiovascular events (Donaldson *et al.*, 2010). In addition, exacerbations are linked with changes in cellular function, such as impaired alveolar macrophage function (Berenson *et al.*, 2014) and reduced phagocytosis by neutrophils (Lavinskiene *et al.*, 2011). Previous data from Professor Sapey's group has also shown that the speed and accuracy of neutrophil chemotaxis is also significantly reduced in AECOPD (awaiting publication; Figure 6.1) These patients represent a distinct population where acute changes occur in a chronic disease and the investigation of neutrophils here allows insight to changes that are because of acute inflammation. The change in phenotype in the systemic neutrophil population may provide evidence that link changes in respiratory symptoms to altered neutrophils in the circulation that could impact on their function at distant sites.

It is well established that neutrophils play a role in COPD (Stockley, 2002), but less is known about neutrophil function during AECOPD. This study aimed to assess multiple neutrophil phenotypes using a combination of markers linked to neutrophil function that may be altered COPD. This was achieved using two well-validated flow cytometry panels as previously described using neutrophils obtained from a cohort of well-characterised patients with either stable COPD or patients currently experiencing an acute exacerbation of COPD (recruited <24 hours after their hospital admission). It was hypothesised that fundamental differences in

neutrophil phenotypes exist between the stable and unstable state, especially regarding activation status and these neutrophils would show systemic activation in patients with AECOPD.

6.2 Results

For clarity, the results in this chapter follow the same format as those in Chapter 5: investigating neutrophil phenotypes based on surface expression following traditional gating; using multi-dimension clustering analysis; and investigation of gene expression. Sample quality control and gating strategies are the same as shown in Chapter 5 (Section 5.2.1).

6.2.1 Sample quality control

SpheroBeads were used to ensure consistent fluorescence detection from the flow cytometer for each sample, based on previously published EuroFlow guidelines (Kalina *et al.*, 2012). Every SpheroBead preparation collected prior to the neutrophil sample was within the 10% tolerance for all channels (Figure 6.2a).

Analysis was performed only on live cells and all samples from patients with AECOPD or the matched stable COPD were included in phenotype analysis as neutrophil viability was greater than 25% in all but 1 sample (3%; Figure 6.2b).

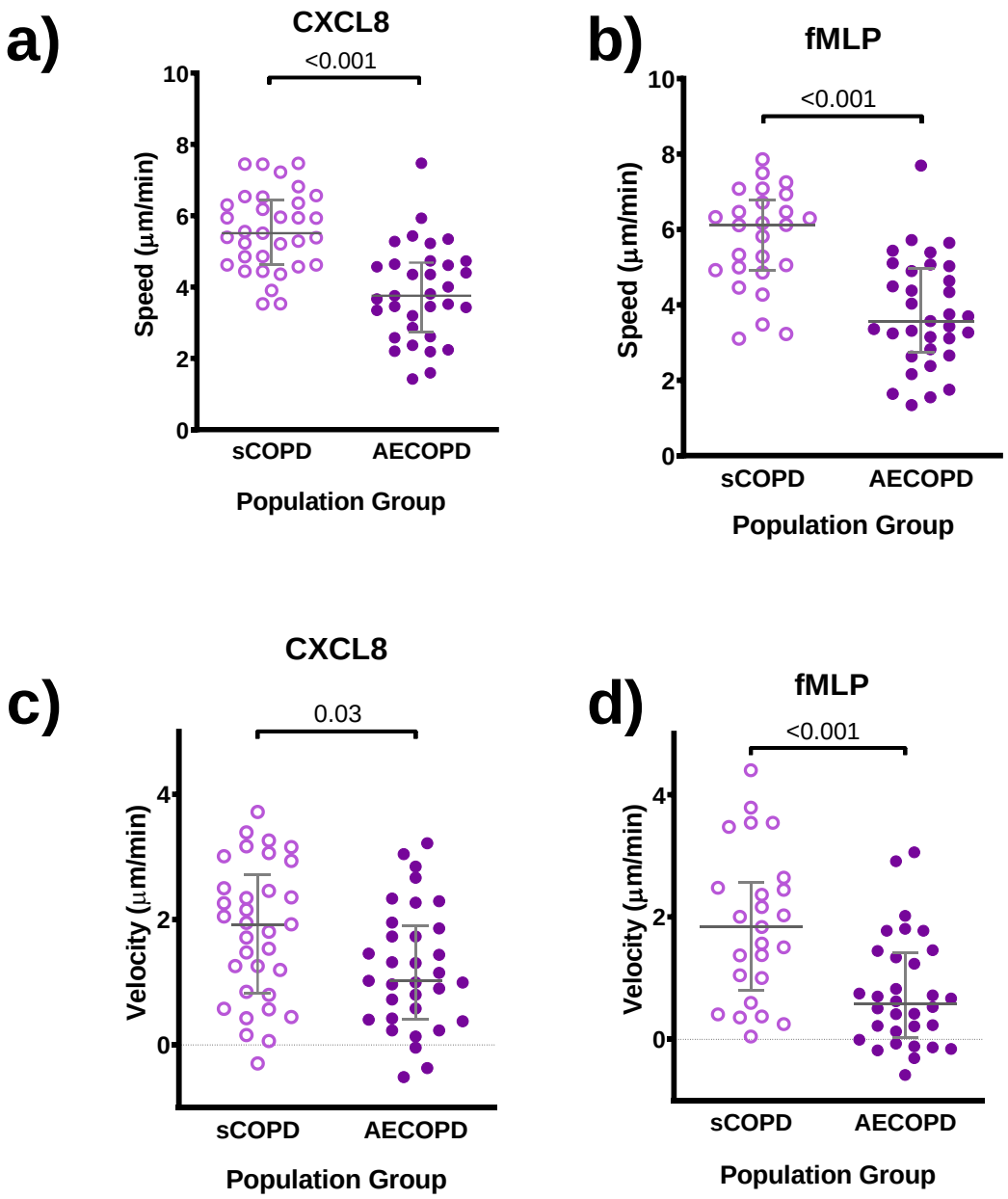

Figure 6.1: The effect of an acute exacerbation of COPD on neutrophil chemotaxis.

Neutrophils from whole blood were isolated, placed in an Insall chamber and chemotaxis towards either **a,c)** CXCL8 or **b,d)** fMLP recorded using time-lapse imaging for 12 minutes (n=33 each). Two parameters are shown: **a,b)** speed and **c,d)** velocity. In each case, the horizontal line indicates the median value with the interquartile range. Statistical analysis performed using a Mann-Whitney test.

These data were collected by other researchers and not the author of this thesis.

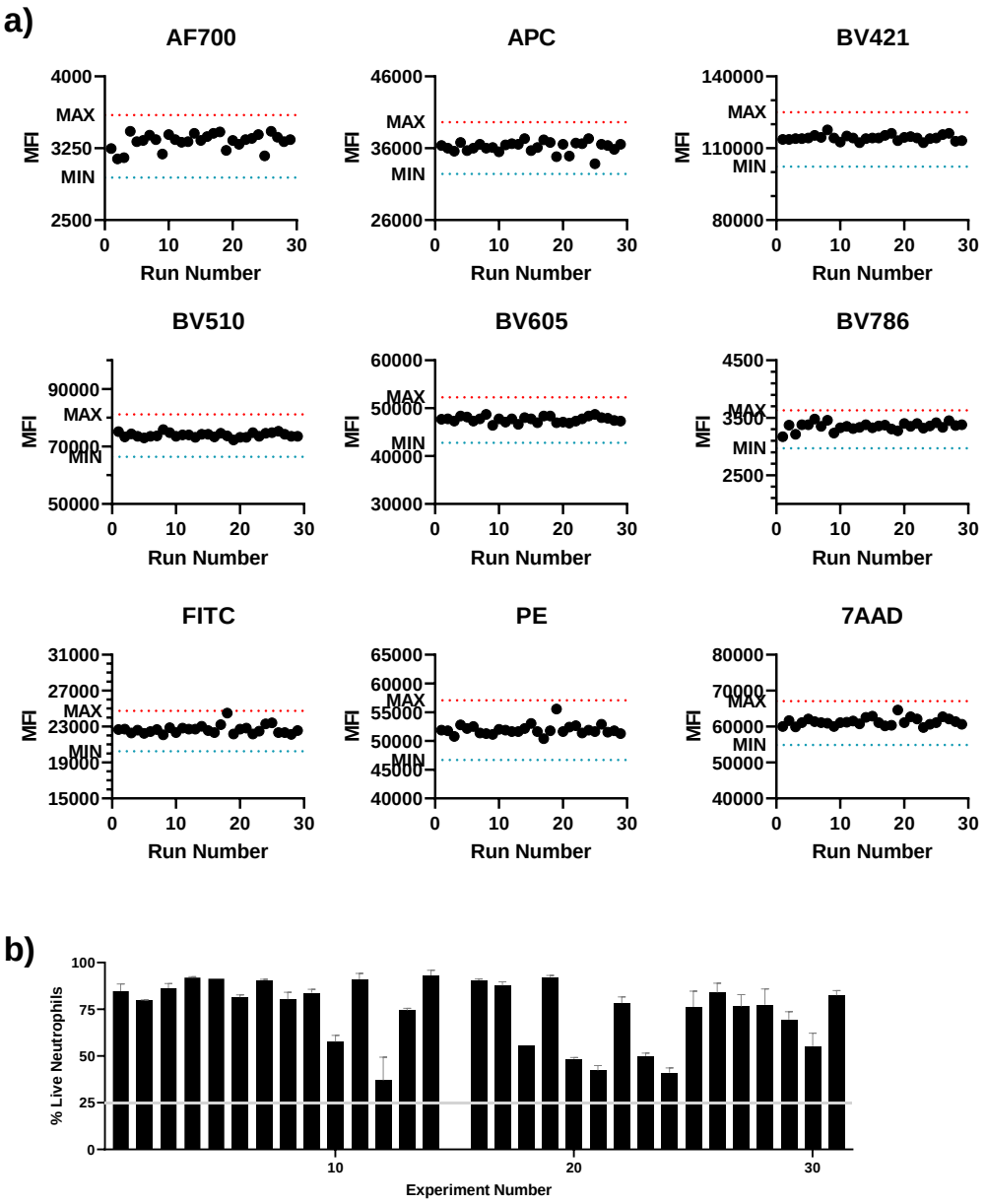

Figure 6.2: Quality control using SpheroBeads and apoptotic exclusion

a) Median Fluorescent Intensity (MFI) was recorded for 4000 events in each fluorescent channel. Each point represents a separate run (n=31). A range of ±10% from the target value (shown in Table 3.7) is shown by the red (+10%) and blue (-10%) dotted lines. **b)** Neutrophils from study participants (n=31) were isolated and viability measured using annexin V and 7ADD staining. Cells negative for both markers were considered live. Bars show mean percentage of live cells for n=1 participant, averaged from samples in panel 1 and panel 2, with standard deviation. Horizontal line indicates the 25% cut-off value for exclusion.

6.2.2 Changes in neutrophil phenotype in patients with an acute exacerbation of COPD

Acute worsening of symptoms in COPD is known to increase the likelihood of hospitalisation and death (Kong and Wilkinson, 2020). Patients that were hospitalised with AECOPD were recruited within 24 hours of admission and their neutrophils isolated. These were matched by age and FEV_1 to 15 patients with stable COPD (from the cohort shown in Section 5.2.3).

6.2.2.1 Patient demographics

There were no significant differences between age, sex, lung function or smoking history between patients with stable or acute exacerbations of COPD (Table 6.1). A greater proportion of patients with an acute exacerbation of COPD were in GOLD Stage 3 than patients with stable COPD, but this was not statistically significant (Table 6.1).

6.2.2.2 Neutrophil activation in AECOPD

In order to assess activation status, the expression of CD11b, CD66b and CD62L were measured. On average, expression of CD11b increased two-fold between patients with AECOPD and stable COPD (Figure 6.3a). However, 4 patients with AECOPD had comparable levels of CD11b to patients with stable COPD. A similar trend was observed with CD66b, however, this increase was not statistically significant (Figure 6.3b). Expression of CD62L was comparable between stable and exacerbation groups (Figure 6.3c). Of note, a higher proportion of apoptotic neutrophils was identified in samples from patients with AECOPD compared with stable COPD. There was also a statistically significant increase in the percentage of $CD11b^{bright}CD66b^{bright}$ neutrophils from patients with AECOPD compared with stable COPD (Figure 6.4). Together these data suggest that neutrophils in patients with AECOPD are systemically activated and may show enhanced levels of apoptosis.

Table 6.1: Basic demographics for study participants during an acute exacerbation of COPD and respective matched stable COPD patients.

	Stable COPD	**Acute exacerbation of COPD (AECOPD)**	**p-value**
Number	15	15	
Age, median (IQR)	72 (71-76)	73 (68-76)	0.86[k]
Sex, M:F	10:5	8:7	0.71[f]
Lung function			
FEV_1 % predicted, median (IQR)	52 (40.0-67.0)	45 (35.8-61.8)[1]	0.43[k]
FVC % predicted, median (IQR)	95.0 (69.0-106)	92 (67.8-100)[1]	0.51[k]
GOLD Stage (1:2:3:4)	2:7:3:3	1:5:7:1[1]	0.40[f]
Smoking			
Smoking status (NS:Ex:C)	0:13:2	0:12:2[1]	1.00[f]
Pack year history, median (IQR)	48.8 (23.0-70.5)	47.5 (34.3-63.8)[1]	0.95[k]
Years ex-smoker, median (IQR)	8 (5.75-17.3)	10 (4-17)[1]	0.89[k]

Patient demographics. Study participants split into those with stable COPD (sCOPD) or with an acute exacerbation of COPD (AECOPD). Median with interquartile range (IQR) to one decimal place are shown where appropriate. **Sex** shown as a ratio of male (M) to female (F). **GOLD Stage** displayed as a ratio of stage 1:2:3:4. **Smoking status** shown as ratio of never(NS):ex(Ex):current(C). Statistical analysis carried out using Kruskal-Wallis test (k) for continuous data and Fishers Exact test (f) for categorical data. FEV_1, forced expiratory volume in one second; FVC, forced vital capacity. [1] Information from one AECOPD patient was unavailable.

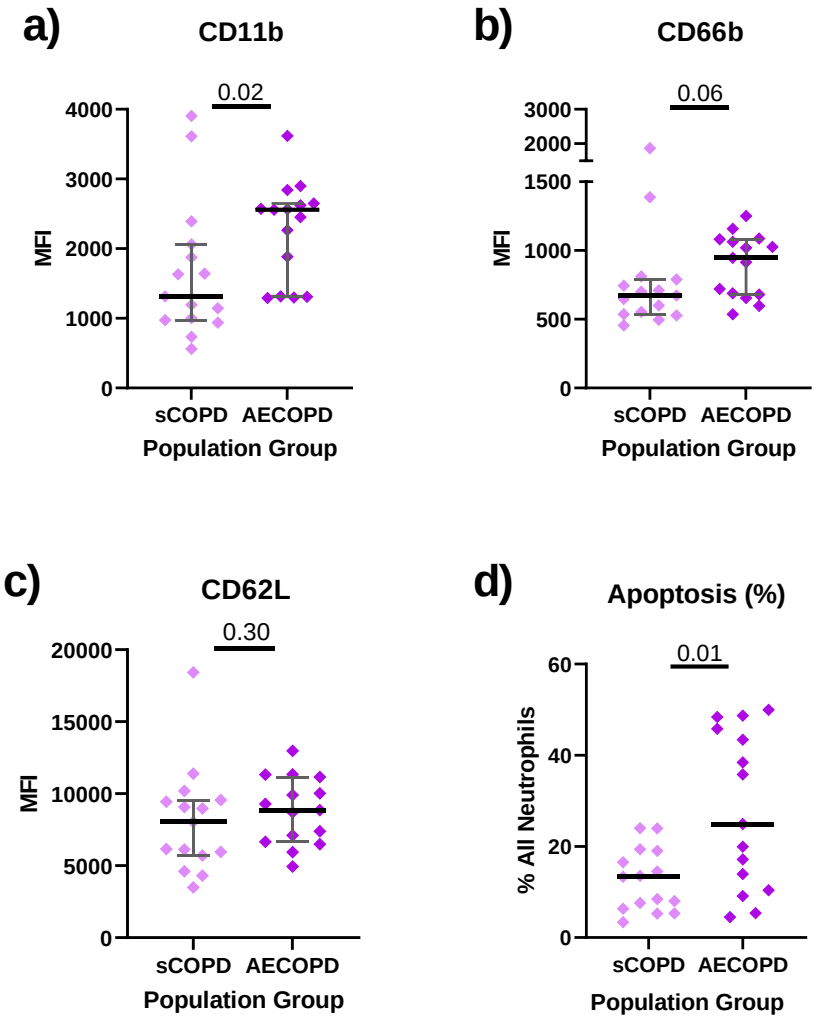

Figure 6.3: Median fluorescence intensity of activation markers of isolated neutrophils from patients with stable COPD or AECOPD

Neutrophils from whole blood were isolated from patients with stable COPD (sCOPD; n=15) or exacerbations of COPD (AECOPD, n=15), stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** CD11b, **b)** CD66b or **c)** CD62L or **d)** the percentage of apoptotic cells. In each case, the horizontal line indicates the median value. Statistical analysis performed using a Mann-Whitney test.

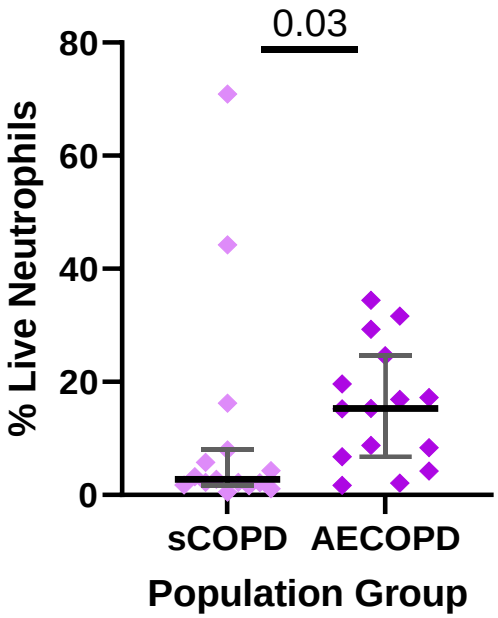

Figure 6.4: The percentage of CD11b^{bright}CD66b^{bright} live neutrophils from patients with stable COPD or AECOPD

Neutrophils from whole blood were isolated from patients with stable COPD (sCOPD; n=15) or exacerbations of COPD (AECOPD, n=15), stained with antibodies and the percentage of CD11b^{bright}CD66b^{bright} live neutrophils recorded. In each case, the horizontal line indicates the median value. Statistical analysis performed using a Mann-Whitney test.

6.2.2.3 Neutrophil senescence in AECOPD

In order to assess neutrophil senescence, the expression of CXCR2 and CXCR4 were measured. There was a significant reduction of CXCR2 expression (on average by 30%) in patients with AECOPD when compared to patients with stable COPD (Figure 6.5a). In contrast, CXCR4 expression was comparable between these two groups (Figure 6.5b). These data suggest an altered neutrophil phenotype between AECOPD and stable COPD – but not one consistent with previously reported neutrophil senescence.

An increase in the proportion of CXCR4+CD62L^{dim} 'overactive senescent' neutrophils was found in patients with AECOPD compared to COPD (Figure 6.5c), suggesting that whilst traditional senescence is not observed in patients with AECOPD, an alternative senescence phenotype may be present.

6.2.2.4 Neutrophil inflammatory status in AECOPD

In order to assess neutrophil anti-inflammatory status, the expression of PD-L1, HLA-DR and CD11c was measured. Whilst CD11c expression was not altered (Figure 6.6c), a significant increase, on average of 85%, in PD-L1 expression was observed in neutrophils isolated from patients with AECOPD compared to stable COPD (Figure 6.6a). The absolute increase, however, was relatively small and may indicate only a small shift towards an anti-inflammatory phenotype and may not be clinically or biologically relevant. No difference was found in the expression of HLA-DR or between AECOPD and stable COPD, suggesting neutrophils do not show evidence of a pro-inflammatory phenotype during AECOPD (Figure 6.6c).

6.2.2.5 Neutrophil reverse transmigration and maturity

Similar levels of CD54 (Figure 6.7), CD10 (Figure 6.8a) and CD16 (Figure 6.8b) expression were observed between neutrophils isolated from patients with AECOPD and stable COPD, suggesting no alteration in neutrophil reverse transmigration of maturity occurred during exacerbation.

6.2.2.6 Hypersegmentation

Neutrophil hypersegmentation based on the percentage of CD16+CD62L^{dim} neutrophils was not altered in AECOPD compared with stable COPD (Figure 6.9). However, substantial patient-to-patient variation was seen in both groups (Figure 6.9). These data suggest the proportion of hypersegmented neutrophils shows considerable variation in patients with COPD regardless of exacerbation status.

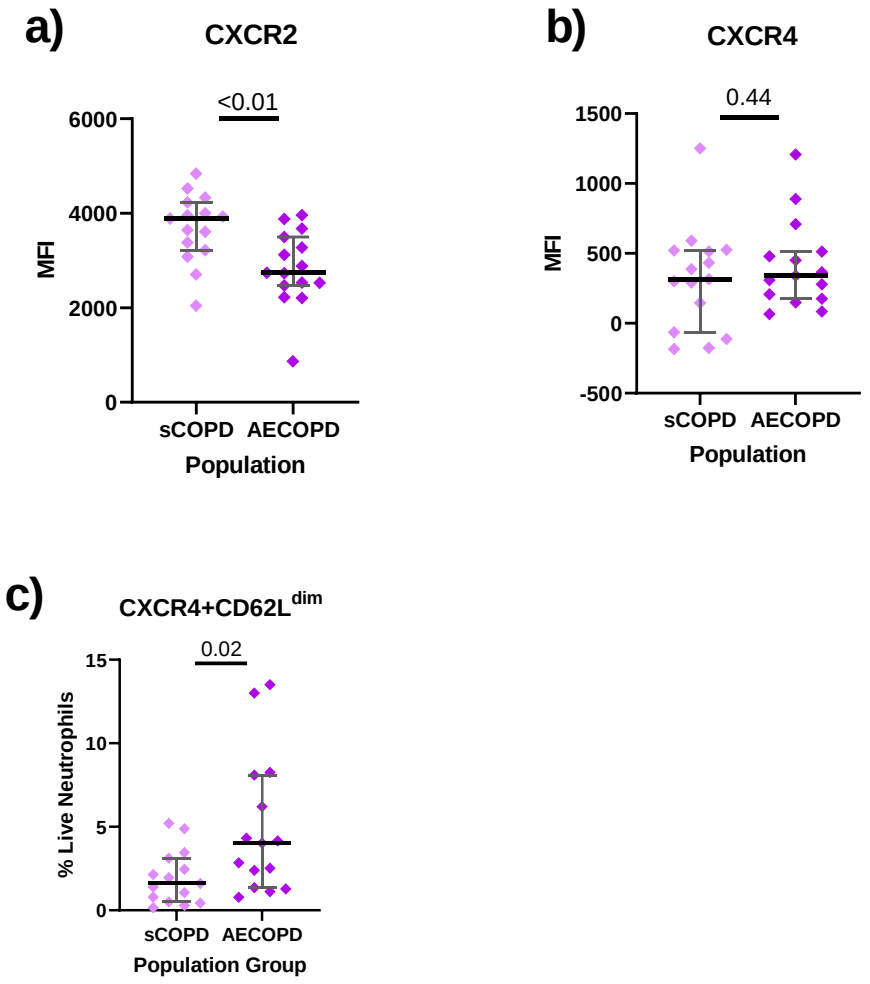

Figure 6.5: Median fluorescence intensity of senescence markers on isolated neutrophils from patients with stable COPD or AECOPD

Neutrophils from whole blood were isolated from patients with stable COPD (sCOPD; n=15) or exacerbations of COPD (AECOPD, n=15), stained with antibodies and the median fluorescence intensity measured for **a)** CXCR2, **b)** CXCR4 or **c)** the percentage of CXCR4+CD62L^{dim} neutrophils. In each case, the horizontal line indicates the median value. Statistical analysis performed using an unpaired t-test.

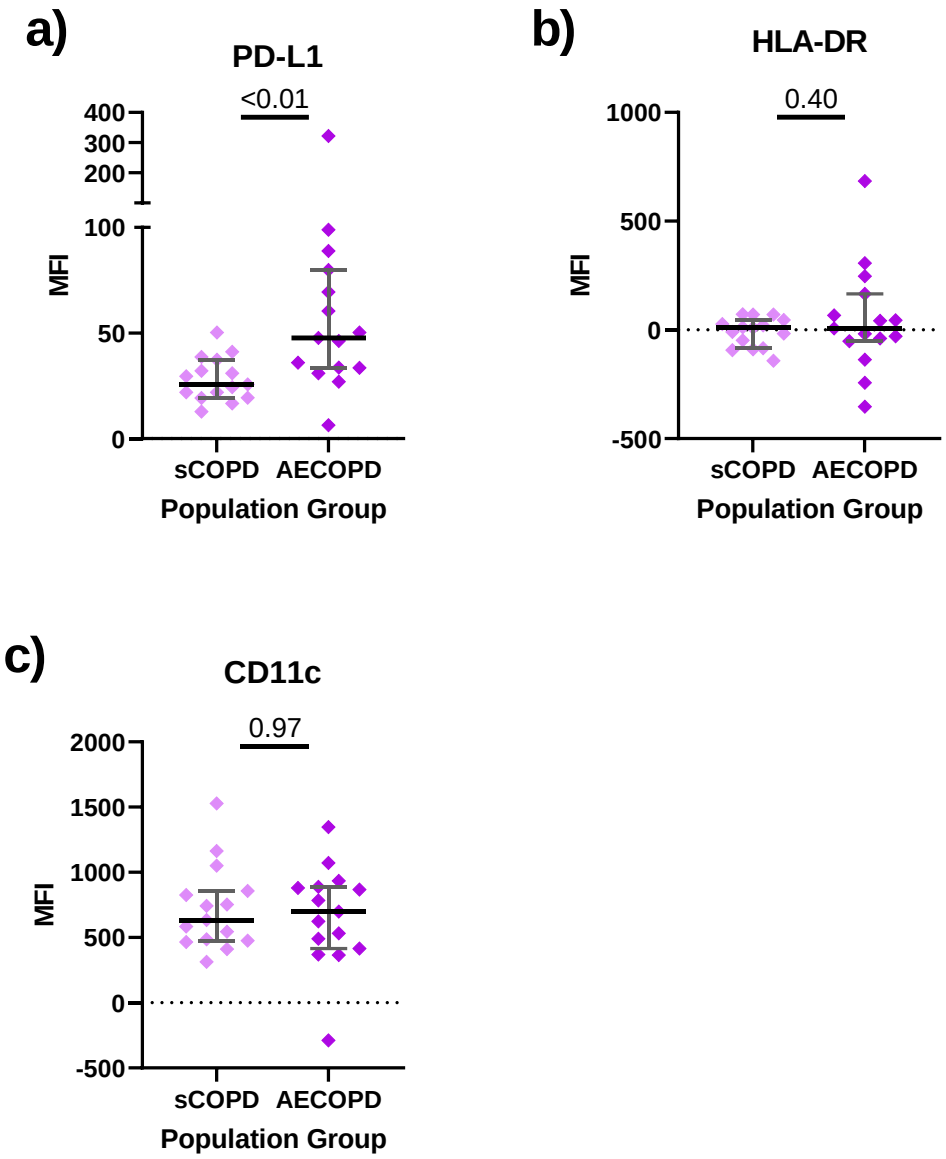

Figure 6.6: Median fluorescence intensity of PD-L1, HLA-DR and CD11c on isolated neutrophils from patients with stable COPD or AECOPD

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) or exacerbations of COPD (AECOPD, n=15), stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** PD-L1 **b)** HLA-DR or **c)** CD11c. In each case, the horizontal line indicates the median value. Statistical analysis performed using a Mann-Whitney test.

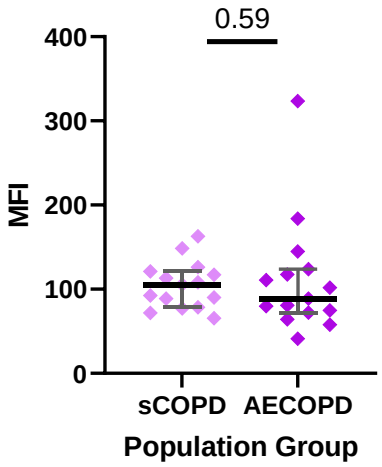

Figure 6.7: Median fluorescence intensity of CD54 on isolated neutrophils from patients with stable COPD or AECOPD

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) or exacerbations of COPD (AECOPD, n=15), stained with antibodies and the median fluorescence intensity (MFI) measured for CD54. The horizontal line indicates the median value with interquartile range. Statistical analysis performed using a Mann-Whitney test.

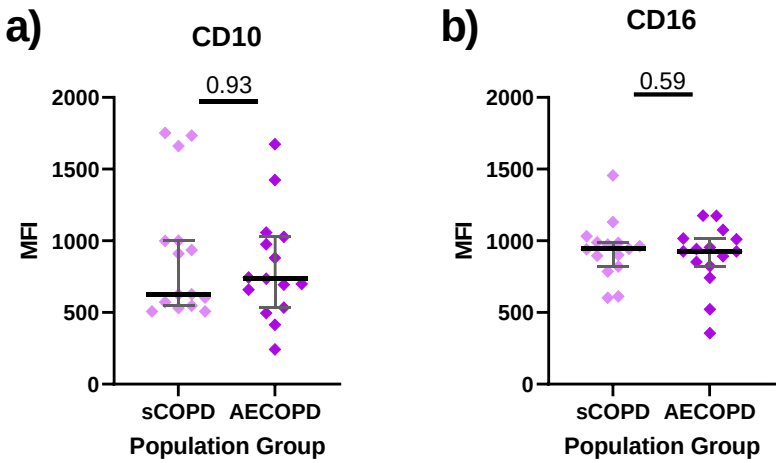

Figure 6.8: Median fluorescence intensity of CD10 and CD16 on isolated neutrophils from patients with stable COPD or AECOPD

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) or exacerbations of COPD (AECOPD, n=15), stained with antibodies and the median fluorescence intensity (MFI) measured for **a)** CD10 or **b)** CD16. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using a Mann-Whitney test.

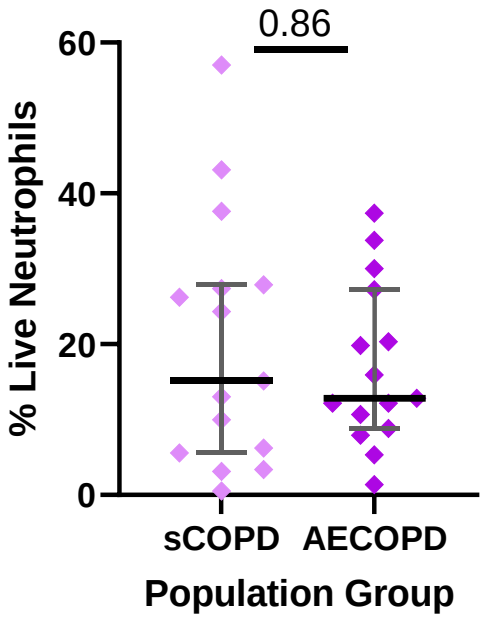

Figure 6.9: Percentage of live CD16^{+}CD62L^{dim} neutrophils isolated from patients with stable COPD or AECOPD

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) or exacerbations of COPD (AECOPD, n=15), stained with antibodies and the percentage of CD16^{+}CD62L^{dim} live neutrophils measured. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using a Mann-Whitney test.

6.2.3 Unassisted analysis using t-Distributed Stochastic Neighbor Embedding (t-SNE) and Rphenograph clustering

As shown in Chapter 5, some of the limitations of conventional gating strategies can be overcome by the use of multi-dimensional clustering and dimension-reduction algorithms – allowing the analysis of all parameters simultaneously. Here, t-SNE visualisation with a clustering algorithm called Rphenograph was utilised to separate live neutrophils (gating strategy shown in Figure 2.3) based on surface expression, where the closer together each cluster, the more similar they are. The percentage of neutrophils in each cluster can then be quantified and the expression of each marker for each cluster can be compared.

6.2.3.1 Rphenograph analysis of patients with stable COPD or AECOPD

Rphenograph analysis identified a total of 22 clusters for panel 1 with no clear separation between clusters (Figure 6.10a). Regions of higher CD11b expression overlayed with higher CD10 expression (Figure 6.10b), with some regions showing greater CXCR2, CD62L and CD54 expression, with homogenous CXCR4 and CD16 expression. Of the 20 clusters identified for panel 2 (Figure 6.11a), there appeared to be some separation in the clusters with one region of lower PD-L1 expression (Figure 6.11b). Regions of higher CD11b, CD66b, CD11c and CD10 appeared to overlay. These data suggest similar CXCR4 and CD16 expression across all the samples, with a link between CD11b, CD66b, CD11c and CD10 expression.

Overall, there were no statistically significant differences between the percentage of neutrophils within each cluster between patients with AECOPD and stable COPD (Figure 6.12a and b). However, there were changes in the median values between groups that, whilst not

statistically significant, approached significance and may still provide biological insight and warrant further investigation.

Neutrophils in cluster 10 and 18 from panel 1 were less abundant in patients with AECOPD compared to stable COPD, contrasting with cluster 21 and 22 that were more abundant in patients with AECOPD compared to stable COPD (Figure 6.12c). From panel 2, the percentage of neutrophils in cluster 11 and 16 was reduced and cluster 4 and 20 increased in patients with AECOPD compared to stable COPD (Figure 6.12d). These changes suggest subtle differences in the neutrophil phenotype between AECOPD and stable COPD.

The expression levels within these clusters were then identified to link them to potential biological differences. For panel 1, the highlighted clusters (10,18,21 and 22) show broadly similar expression profiles for CXCR4, CD16, CD54 (Figure 6.13a), supporting the individual marker analysis that senescence, maturity and reverse transmigration are not altered. Cluster 10 and 18, both reduced in AECOPD, showed lower CD11b and CD10 expression, but similar CD62L expression to the other highlighted clusters (Figure 6.13a). In contrast, cluster 21, increased in patients with AECOPD, had higher CD11b and lower CD62L expression than the other peaks (Figure 6.13a). Cluster 22, again increased in AECOPD, showed lower CXCR2 expression than all other clusters (Figure 6.13a). Together, these support an increase in activated neutrophils in AECOPD, but also highlight how CD10 expression may also play a role in this phenotype. They also suggest that neutrophils with lower CXCR2 may be exaggerated in a subset of activated cells, as cluster 22 showed the lowest CXCR2 expression with intermediate CD11b expression (Figure 6.13a).

Within panel 2, all clusters had similar expression of PD-L1 and HLA-DR (Figure 6.13b). Cluster 11 and 16, reduced in patients with AECOPD compared to stable COPD, showed very similar expression profiles with lower CD11b, CD66b, CD11c and CD10 expression whilst the opposite was true for cluster 4 and 20 (increased in patients with AECOPD) with higher expression of these markers (Figure 6.13b). These data further support that neutrophils with an activated phenotype are present in the circulation of patients with AECOPD in greater frequency than in stable COPD. They also highlight that these cells may also express higher levels of CD11c and CD10 and, therefore, do not represent an immature neutrophil population.

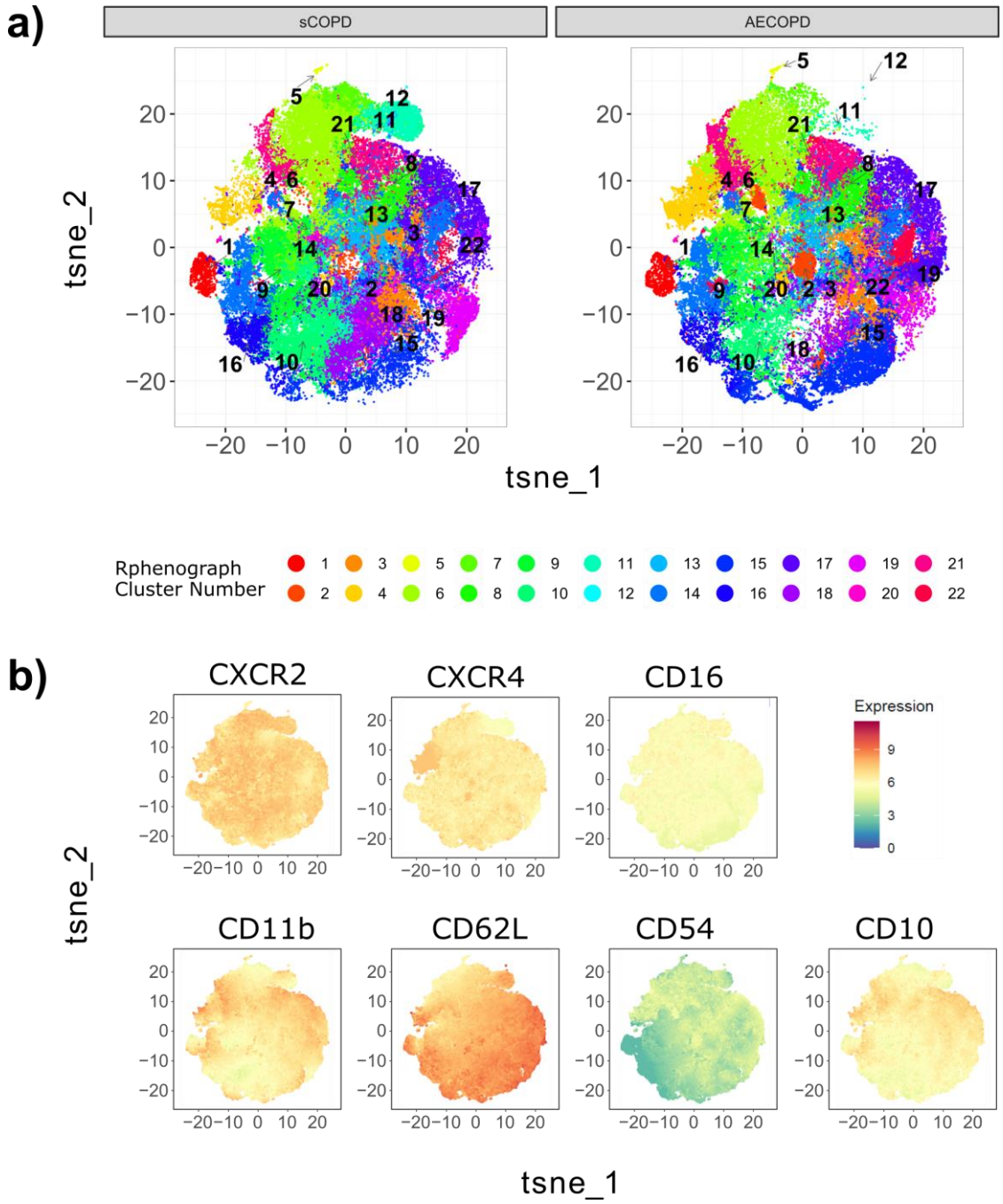

Figure 6.10: tSNE plots of surface expression of markers from antibody panel 1 for patients with stable COPD or AECOPD clustered using Rphenograph

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) or acute exacerbation of COPD (AECOPD; n=15). Neutrophils were stained with panel 2 antibodies and gated for live cells. Live neutrophils from each sample were then analysed using tSNE and Rphenograph clustering based on surface marker expression. Clusters are presented for **a)** each participant group coloured by cluster number or **b)** combined relative surface expression, where red indicates high expression.

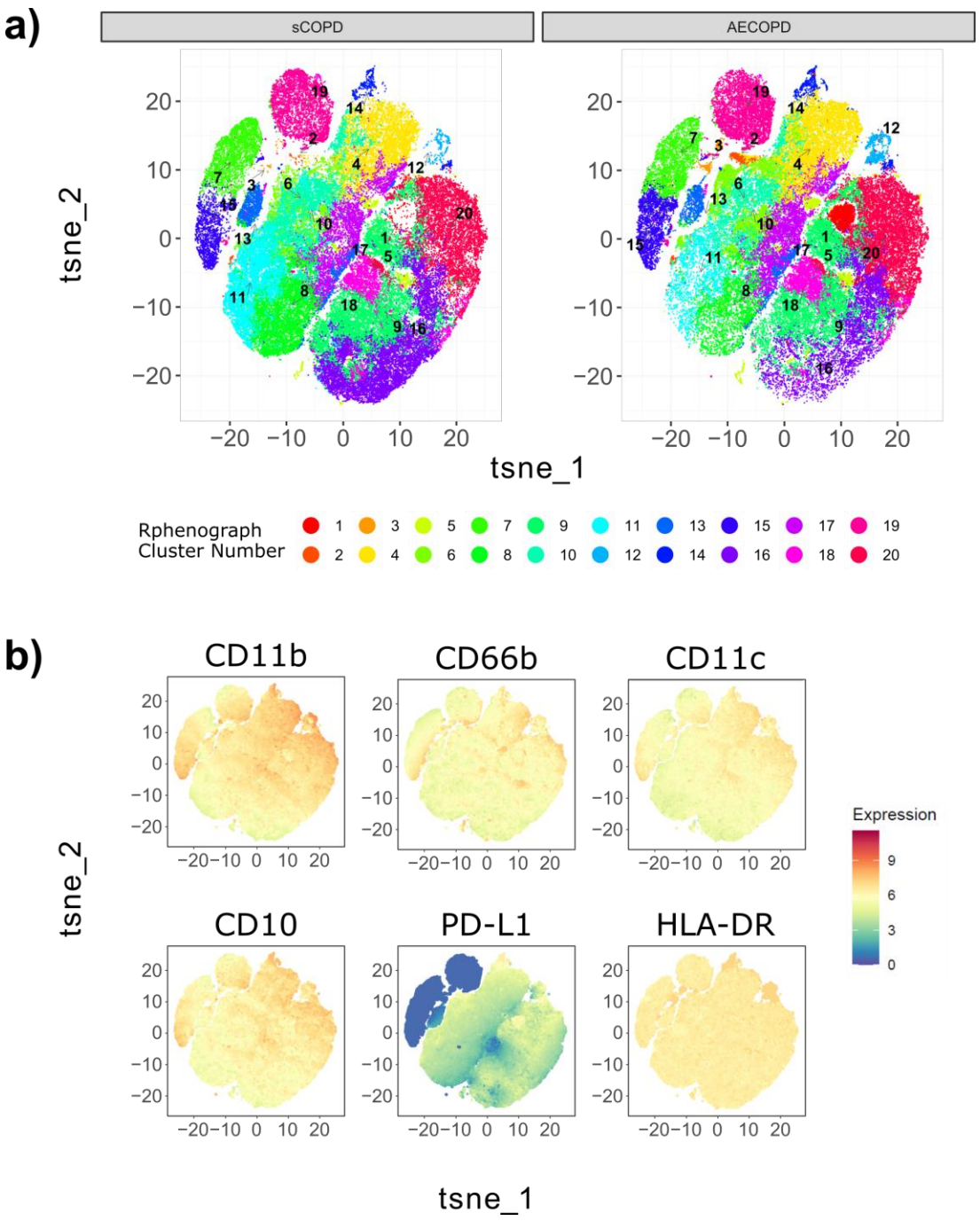

Figure 6.11: tSNE plots of surface expression of markers from antibody panel 2 for patients with stable COPD or AECOPD clustered using Rphenograph

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) or acute exacerbation of COPD (AECOPD; n=15). Neutrophils were stained with panel 2 antibodies and gated for live cells. Live neutrophils from each sample were then analysed using tSNE and Rphenograph clustering based on surface marker expression. Clusters are presented for **a)** each participant group coloured by cluster number or **b)** combined relative surface expression, where red indicates high expression.

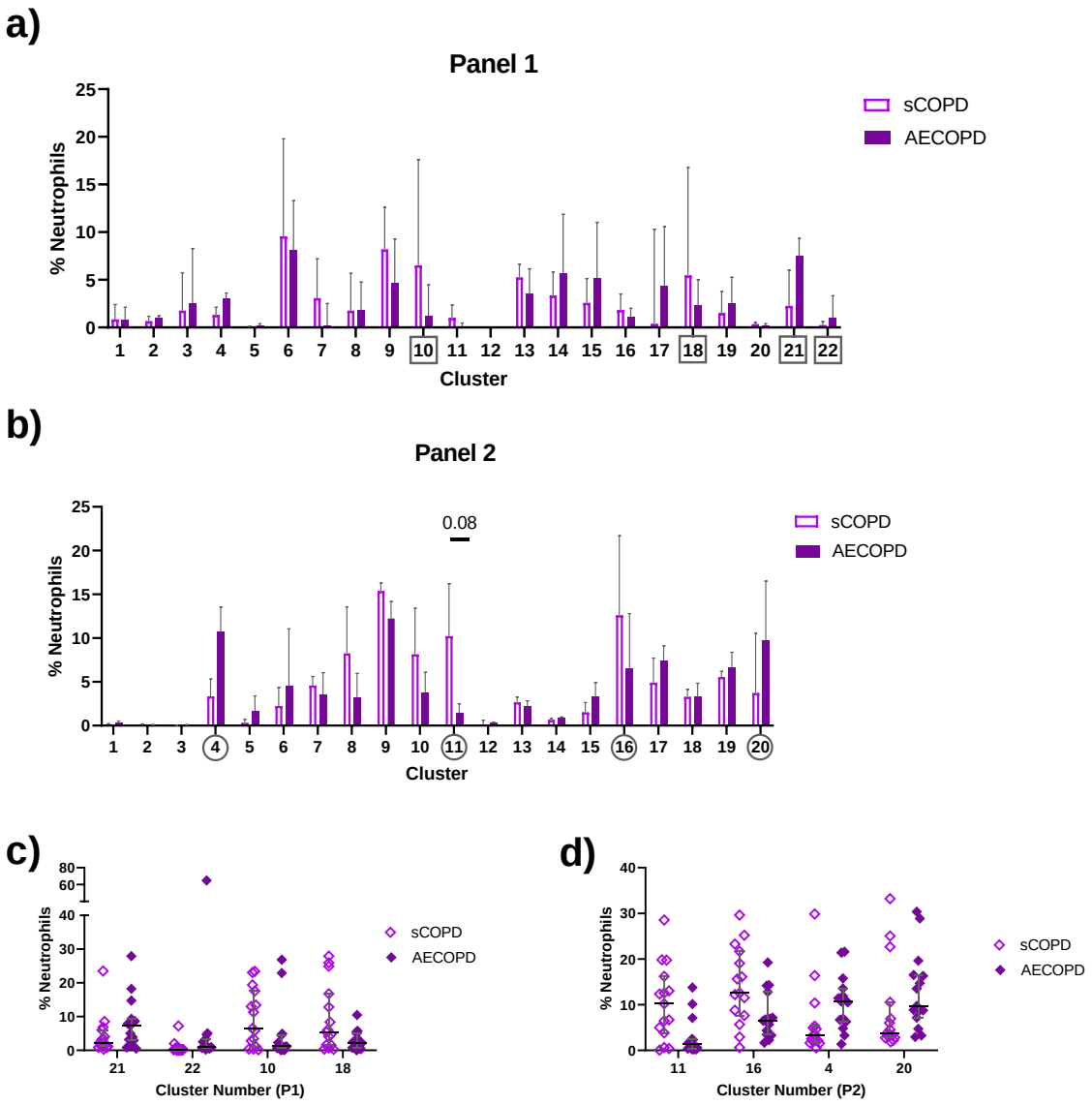

Figure 6.12: Percentage of neutrophils within each cluster following Rphenograph cluster analysis for each antibody panel

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) and AECOPD (n=15), stained with antibodies and the median fluorescence intensity (MFI) measured for each marker. The percentage of live neutrophils identified within each cluster (Figure 6.10 and Figure 6.11) are shown for **a)** panel 1 and **b)** panel 2. **c)** Cluster 10, 18, 21 and 22 from panel 1 and **d)** cluster 4, 11, 16 and 20 from panel 2 are shown in further detail (highlighted in grey squares and circles respectively). In each case, horizontal lines indicate the median with the interquartile range. Statistical analysis on cluster percentages were performed using multiple t-tests using the Holm-Sidak method for multiple analysis.

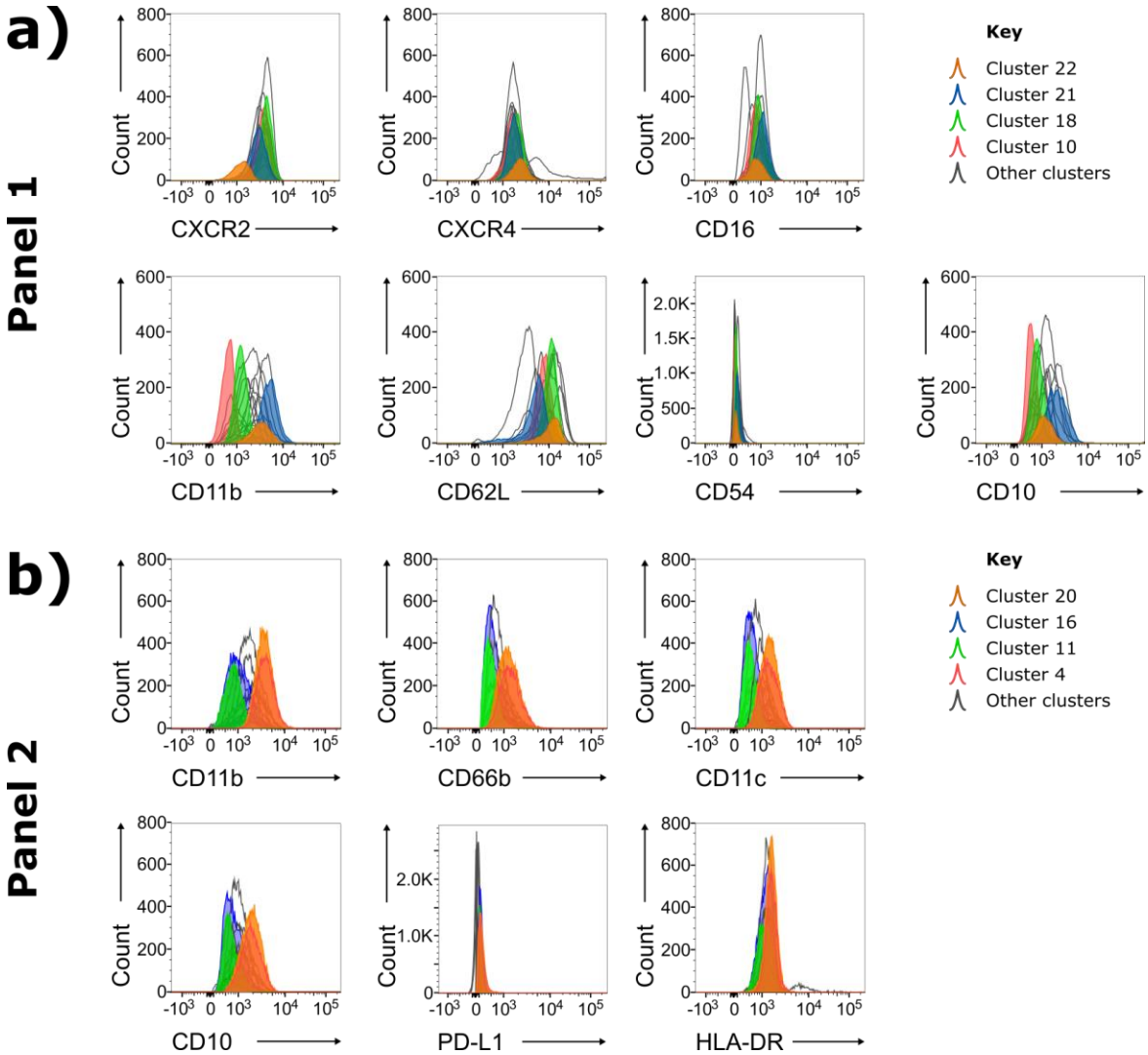

Figure 6.13: Surface expression for neutrophils within each cluster identified using Rphenograph

Neutrophils from whole blood were isolated from patients with stable COPD (n=15) and AECOPD (n=15), stained with antibodies and the fluorescence intensity measured for each marker (see Table 1). Key clusters highlighted in Figure 6.12 for **a)** panel 1 and **b)** panel 2 are identified to show the expression for each marker compared to the other clusters (grey traces).

6.2.4 Differential gene expression in neutrophils in patients with COPD

As our understanding of neutrophil biology has evolved, it has become clearer that neutrophils are capable of changing their gene expression profile, especially in response to pathogens (Terkawi, Takano and Kato, 2018). RNA was extracted from neutrophils isolated from patients with both stable COPD and AECOPD and prepared for RNA sequencing (RNASeq).

6.2.4.1 Quality control and sample selection

QC steps were carried out ensure RNA purity, based on a ratio of absorbance at 260nm and 280nm to determine DNA contamination and 260nm to 230nm to determine salt or organic compound contamination. Ratios above 2.0 indicated good sample purity, between 2.0 and 1.8 indicated adequate purity and below 1.8 indicated poor purity. For 260/280 ratios, all samples achieved good or adequate purity (Table 6.2). For 260/230 ratios, none of the samples achieved good purity and 4 with adequate purity (Table 6.2). These data indicate that whilst pure RNA was extracted with little DNA contamination, potential contaminants that absorb at 230nm were present – but these should not impact on sequencing.

RNA integrity and quantity were assessed using an RNA integrity score (RIN), measured using a Tapestation, and accurate RNA quantification performed using a Qubit fluorescence reader. For RNA sequencing, a RIN score of 8 or higher is considered good, between 7 and 8 adequate and below 7 as poor. Using these criteria, 5 samples were of good integrity, 8 of adequate integrity and 8 poor (Table 6.2). Across these three quality control measures, 5 samples from patients with AECOPD and 10 samples from the COPD group (not stratified based on multimorbidity due to sample number) with an adequate quantity of RNA, with the highest

purity and integrity scores were chosen for downstream RNASeq (shown by the assignment of an RNASeq ID).

6.2.4.2 Differentially expressed genes between participant groups

Differential gene expression analysis was performed (see Section 2.10.1 for method) and revealed 102 genes were differentially expressed between neutrophils from patients with stable COPD and AECOPD (Table 6.3).

Pathway analysis of all 102 differentially expressed genes between neutrophils from patients with stable or acute exacerbations of COPD revealed 7 main processes that were altered: neuroinflammatory response; platelet function; heterotypic cell-cell adhesion; homotypic cell-cell adhesion; regulation of bone development; carbohydrate transmembrane transporter activity and cell-cell adhesion mediator activity (Figure 6.14). The reactome and Kyoto Encyclopedia of Genes and Genomes (KEGG) were also used to incorporate potential disease pathways and molecular interactions (Kanehisa *et al.*, 2017; Jassal *et al.*, 2020).

Platelet function, heterotypic and homotypic cell-cell adhesion grouped together and encompassed the greatest number of processes. Integrin genes (ITG) ITGA7, ITGB3 and ITGA2B featured many links within this group (Figure 6.14). These analyses also identified three cardiac conditions (hypertrophic, dilated and arrhythmogenic right ventricular cardiomyopathy) due to their association with integrins ITGA7, ITGB3, ITGA2B and the gene for Calcium Voltage-Gated Channel Auxiliary Subunit Gamma 6 (CACNG6). These genes were all upregulated in AECOPD (Table 6.3), suggesting a link between neutrophils and the increased risk of these diseases in this patient group. In addition to integrins, CACNG6, SPARC

and SYTL4 were involved in platelet function (Figure 6.14), all of which had higher expression in neutrophils from patients with AECOPD compared with stable COPD (Table 6.3).

Closely linked to cell adhesion was cell-cell adhesion mediator activity. Both PDLIM1 and ESAM were increased in neutrophils from patients with AECOPD compared with stable COPD, but JUP expression (coding for the cell junction protein plakoglobin) was reduced (Table 6.3 and Figure 6.14).

Several genes were also identified that linked to neuroinflammation (Figure 6.14): however, BACE2 expression was lower and three other genes higher in AECOPD compared with stable COPD (Table 6.3 and Figure 6.14), suggesting some evidence of the role of neutrophils in neuroinflammation, but one that requires further investigation.

There were also two other processes identified: regulation of bone development and carbohydrate transmembrane transporter activity (Figure 6.14). Two of the three genes associated with bone development had reduced expression in neutrophils from AECOPD compared with stable COPD (FBN1 and RFLNB, Table 6.3), whereas all genes associated with transporter activity were increased (Table 6.3 and Figure 6.14). Together, these may indicate neutrophils in AECOPD have altered involvement in bone development regulation and changes in carbohydrate transport.

More detailed analysis was performed on the top 50 differentially expressed genes, clustering based on normalised gene expression (Figure 6.15). Four main clusters were identified: two where genes were upregulated (cluster 1 and 3) and two where genes were downregulated (cluster 2 and 4) in neutrophils from patients with AECOPD compared with stable COPD (Figure 6.15).

Gene ontology (GO) analysis of both clusters of genes upregulated in neutrophils from AECOPD patients revealed links with 41 GO biological processes (Table 6.4), including “platelet activation”, “coagulation”, “cell-cell adhesion” and “positive regulation of cell motility”. These genes were also associated with two GO molecular functions (Table 6.4): “fibrinogen binding” and “extracellular matrix binding”, suggesting activation and adhesion processes are increased during an acute exacerbation. Those genes downregulated in neutrophils from patients with AECOPD were associated with the GO biological process “cellular responses to drugs” and GO molecular functions including transmembrane signalling activity and lipase activity (Table 6.4), suggesting neutrophils from patients with AECOPD may have a reduced ability to sense their external environment compared with stable COPD.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) resource can also be used to identify high-level functions of cells related to gene expression. Genes upregulated in patients with AECOPD were associated with several pathways, including PI3K-signalling due to the increased expression of integrin molecules that signal via PI3K (Table 6.4). There were no statistically significant related KEGG-identified functions in genes downregulated in neutrophils from patients with AECOPD.

Together, these data point to several hypotheses: that neutrophils from patients with AECOPD are pro-inflammatory with higher expression of adhesion molecules; they have reduced expression of extracellular sensing receptors; and have increased activity of pathways controlling cell migration.

Table 6.2: RNA extraction nanodrop quality control results

Sample number	Participant group	260/280	260/230	RIN Score	Concentration (ng/µL)	RNASeq ID
1	COPD	2.08	1.94	6.5	53	RNAC01
2	COPD	2.07	1.98	6.2	66.8	RNAC06
3	COPD	2.05	1.58	7.9	60.6	RNAC03
4	COPD	2.05	1.64	8.7	71	RNAC04
5	COPD	2.06	1.85	8.9	79	RNAC05
6	AECOPD	2.05	1.77	8.4	42.6	RNAA01
7	AECOPD	2.05	0.71	7.8	50	RNAA02
8	COPD	1.93	-0.53	6.5	20.2	
15	COPD	2.1	1.56	6	69.8	
16	COPD	1.86	1.05	4.9	18.5	
18	COPD	2.14	0.26	6.6	19.8	RNAC08
21	COPD	2.08	0.53	7.7	102	RNAC09
22	COPD	2.08	1.83	7.9	39.6	RNAC07
23	COPD	2.34	1.64	6.2	17.1	
24	COPD	2.07	1.69	7.4	75	RNAC10
29	COPD	2.09	1.72	8	42	RNAC02
30	COPD	1.97	1.12	4.3	11.6	
33	AECOPD	1.87	0.99	8	60	
34	AECOPD	2	1.75	7.3	45.2	RNAA03
35	AECOPD	1.96	1.39	7.5	35.2	RNAA04
36	AECOPD	1.89	0.98	7.5	20	RNAA05

Legend: Participant group indicates neutrophil donor: healthy young (HY); healthy elderly (HE); stable COPD (COPD) and acute exacerbation of COPD (AECOPD). Ratios based on absorbance readings at 280nm, 260nm and 230nm. RNA integrity score (RIN) measured using a Tapestation and RNA concentration using fluorescence quantification where N/A indicates sample not assessed. Patients with COPD included in this table are the same as shown in Chapter 5 Table 5.3.

Table 6.3: Differentially expressed genes between neutrophils from patients with stable and exacerbations of COPD

Gene ID	Adj. p-value	Gene ID	Adj. p-value	Gene ID	Adj. p-value	Gene ID	Adj. p-value
NAIPP1	<0.001	BACE2	0.006	PTGS1	0.021	GALNT14	0.036
SH3BGRL2	<0.001	OLAH	0.008	LINC01271	0.021	LINC02751	0.036
SPNS3	<0.001	JUP	0.009	LGALS2	0.021	LINC01232	0.037
CYSLTR2	<0.001	CLVS1	0.011	NME8	0.023	PCSK6	0.037
SIGLEC8	<0.001	IL5RA	0.012	PINX1	0.023	LRRC6	0.038
ASPH	<0.001	H2BC11	0.012	CCDC86	0.023	TMEM119	0.038
SYTL4	<0.001	ITPKC	0.012	ADM2	0.023	ZDHHC19	0.040
AC007877.1	<0.001	MYL9	0.013	AP3B2	0.025	SYN2	0.044
ITGB3	<0.001	MIR5690	0.014	PEAR1	0.026	BCAT1	0.044
GUCY1B1	0.001	CLC	0.014	ESAM	0.026	AL049836.1	0.044
TMEM40	0.001	TUBB1	0.015	C2orf88	0.027	RFLNB	0.044
SLC29A1	0.001	CCR3	0.015	FBN1	0.029	CST7	0.046
AL157823.2	0.001	CALHM6	0.016	RPL22P2	0.030	ABLIM3	0.047
LINC01270	0.001	SMPD3	0.018	ZDHHC1	0.030	CROCCP2	0.047
TREML1	0.001	NUDT17	0.018	COX6B2	0.030	MYO10	0.047
TPPP3	0.002	AC020636.1	0.018	SAP30	0.030	SLC2A1	0.047
ITGA2B	0.002	AC093849.4	0.018	AC021594.2	0.030	FLNB	0.047
ADGRE4P	0.002	DTX4	0.018	AL021978.1	0.032	GNG11	0.047
HPGD	0.002	OR7E140P	0.018	KEL	0.032	AC022762.2	0.047
SPARC	0.003	AC127521.1	0.018	AL049651.1	0.034	NDUFB4P11	0.047
CACNG6	0.003	PLD4	0.019	AQP10	0.034	ABCC11	0.047
LYSMD4	0.003	BTBD11	0.020	CAPN13	0.034	SAMSN1	0.047
ITGA7	0.003	PDLIM1	0.021	C5orf67	0.034	TEF	0.047
P2RY2	0.004	SRPX2	0.021	LINC02193	0.034	PLAAT5	0.049
Z85996.2	0.006	PPBP	0.021	EFNA1	0.035		
HRH4	0.006	PRL	0.021	CLU	0.036		

Legend: Normalised gene counts from neutrophils with patients with stable or acute exacerbations of COPD were compared. Each gene with a p-value <0.05 is shown. Genes are highlighted based on upregulation (orange) or downregulation (blue) in neutrophils from patients with AECOPD compared with stable COPD.

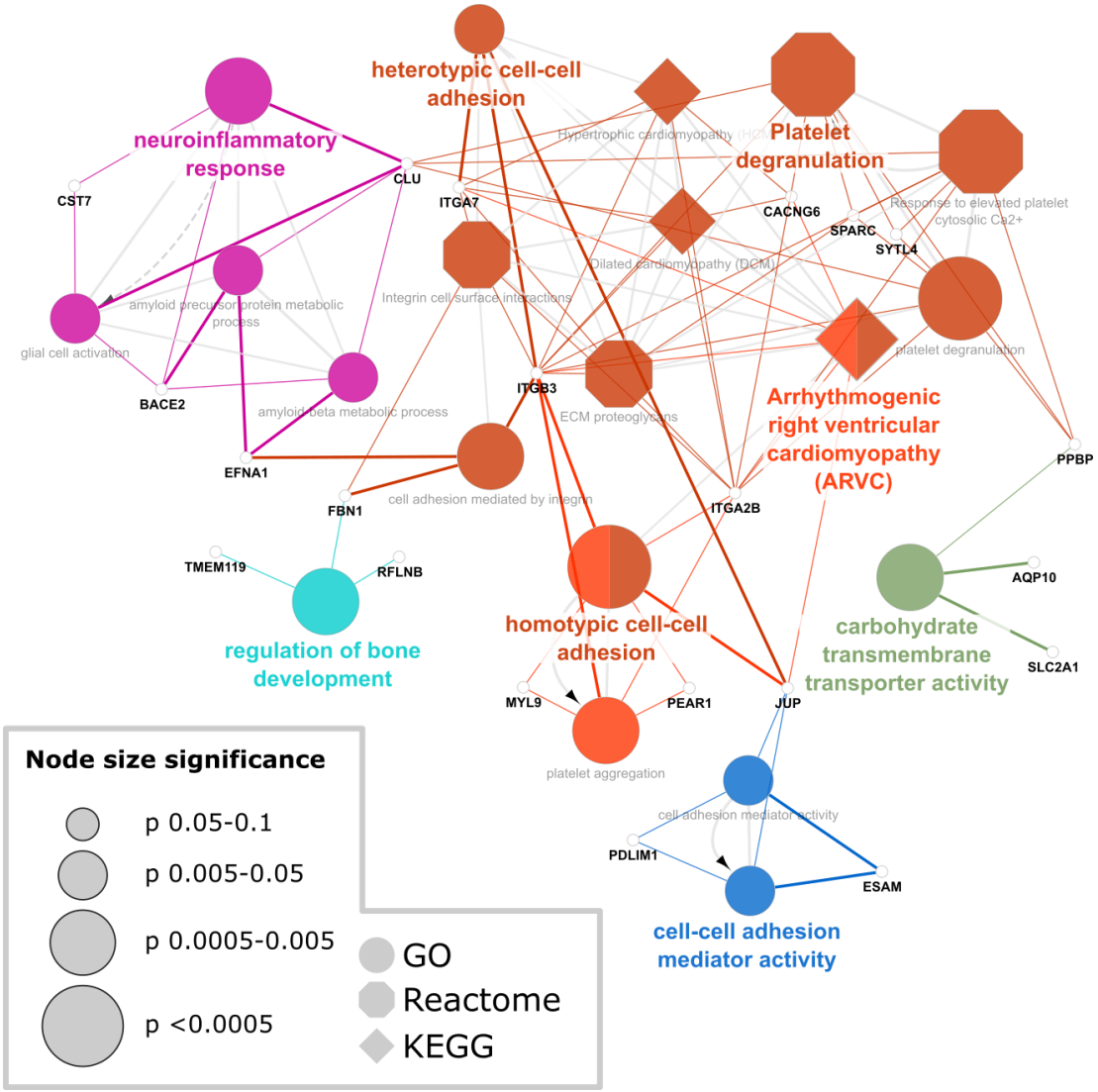

Figure 6.14: ClueGO visualization including gene labels and links of differentially expressed genes between stable COPD and those with an acute exacerbation of COPD

All differentially expressed genes identified between patients with stable and an acute exacerbation of COPD were selected as the input to ClueGO. Gene ontology (GO, circle), Reactome (octagon) and Kyoto Encyclopedia of Genes and Genomes (KEGG, diamond) databases were selected to match genes to biological processes. Shape size corresponds to the significance of association between the gene list and the process. Links between processes are indicated by the grey lines and links between each gene and process are indicated by coloured lines.

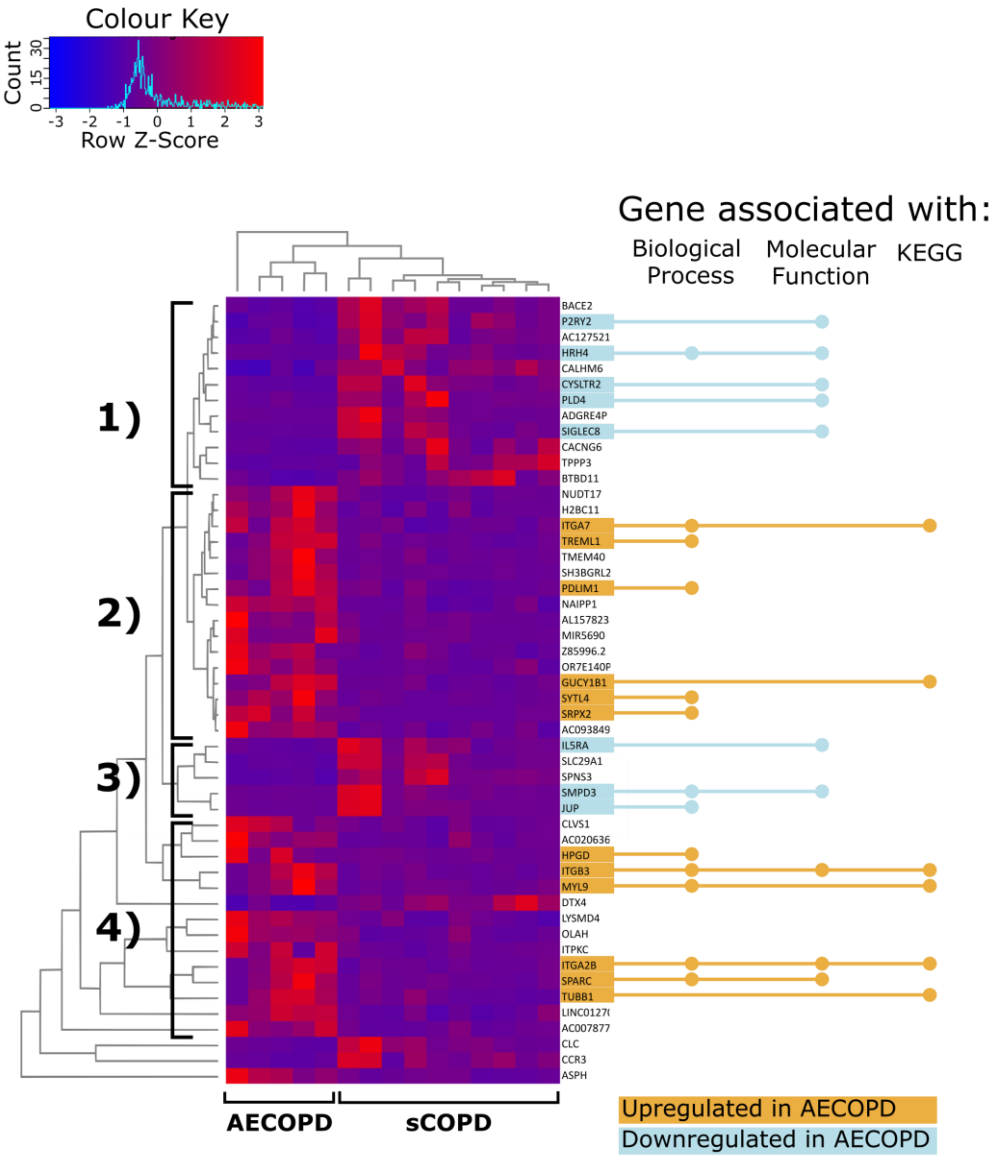

Figure 6.15: Differential gene expression between neutrophils from patients with stable and exacerbations of COPD

Gene counts for mRNA extracted from neutrophils was normalized for gene length and total read number. Differential gene expression analysis performed using DESeq2. The top 50 differentially expressed genes are shown, scaled by gene counts per gene and coloured according to the resulting z-score; clustered using Euclidean spacing. Differentially expressed genes were annotated using ShinyGo to identify potentially altered biological processes. Gene ontology biological processes; molecular functions and Kyoto Encyclopedia of Genes and Genomes databases were selected to match genes to biological processes, where genes significantly associated with these categories are shown by a dot in the corresponding column.

Table 6.4: Gene ontology analysis of differentially expressed genes between neutrophils from patients with stable and AECOPD

Outcome	GO analysis	p-value	GO term	Associated genes	# of associated genes
Upregulated	Biological	Related to chemotaxis			
		0.005	Positive regulation of endothelial cell migration	SPARC SRPX2 ITGB3	3
		0.010	Positive regulation of epithelial cell migration	SPARC SRPX2 ITGB3	3
		0.011	Regulation of endothelial cell migration	SPARC SRPX2 ITGB3	3
		0.012	Extracellular matrix organization	ITGA2B SPARC ITGA7 ITGB3	4
		0.019	Endothelial cell migration	SPARC SRPX2 ITGB3	3
		0.019	Extracellular structure organization	ITGA2B SPARC ITGA7 ITGB3	4
		0.022	Regulation of epithelial cell migration	SPARC SRPX2 ITGB3	3
		0.025	Cell-matrix adhesion	ITGB3 ITGA2B ITGA7	3
		0.026	Positive regulation of cell migration	SPARC ITGA2B SRPX2 ITGB3	4
		0.027	Positive regulation of cell motility	SPARC ITGA2B SRPX2 ITGB3	4
		0.028	Epithelium migration	SPARC SRPX2 ITGB3	3
		0.028	Epithelial cell migration	SPARC SRPX2 ITGB3	3
		0.028	Positive regulation of locomotion	SPARC ITGA2B SRPX2 ITGB3	4
		0.029	Tissue migration	SPARC SRPX2 ITGB3	3
		Related to maturity			
		0.028	Regulation of megakaryocyte differentiation	ITGA2B MYL9	2
		Related to activation			
		0.003	Platelet activation	MYL9 ITGA2B TREML1 ITGB3	4
		0.003	Platelet aggregation	MYL9 ITGA2B ITGB3	3
		0.004	Homotypic cell-cell adhesion	MYL9 ITGA2B ITGB3	3
		0.007	Integrin-mediated signalling pathway	ITGA2B ITGB3 ITGA7	3
		0.010	Cell-cell adhesion	MYL9 SRPX2 ITGA2B PDLIM1 ITGB3 ITGA7	6
		0.011	Blood coagulation	MYL9 ITGA2B TREML1 ITGB3	4
		0.028	Heterotypic cell-cell adhesion	ITGA7 ITGB3	2

Table 6.4: Gene ontology analysis of differentially expressed genes between neutrophils from patients with stable and AECOPD

Outcome	GO analysis	p-value	GO term	Associated genes	# of associated genes
		Other			
		0.003	Wound healing	MYL9 ITGB3 SYTL4 ITGA2B SPARC TREML1	6
		0.003	Platelet degranulation	ITGA2B SYTL4 SPARC ITGB3	4
		0.004	Response to wounding	MYL9 ITGB3 SYTL4 ITGA2B SPARC TREML1	6
		0.011	Haemostasis	MYL9 ITGA2B TREML1 ITGB3	4
		0.011	Coagulation	MYL9 ITGA2B TREML1 ITGB3	4
		0.027	Regulation of body fluid levels	MYL9 ITGA2B TREML1 ITGB3	4
		0.028	Positive regulation of cellular component movement	SPARC ITGA2B SRPX2 ITGB3	4
		0.029	Blood vessel morphogenesis	SPARC SRPX2 HPGD ITGB3	4
	Molecular	Other			
		0.001	Fibrinogen binding	ITGB3 ITGA2B	2
		0.001	Extracellular matrix binding	SPARC ITGB3 ITGA2B	3
	KEGG	Related to chemotaxis			
		<0.001	Focal adhesion	MYL9 ITGA2B ITGA7 ITGB3	4
		<0.001	ECM-receptor interaction	ITGA2B ITGA7 ITGB3	3
		<0.001	Regulation of actin cytoskeleton	MYL9 ITGA2B ITGA7 ITGB3	4
		0.012	PI3K-Akt signalling pathway	ITGA2B ITGA7 ITGB3	3
		Related to phagocytosis			
		0.016	Phagosome	ITGB3 TUBB1	2
		Related to activation			
		0.001	Platelet activation	GUCY1B1 ITGA2B ITGB3	3
		Other			
		<0.001	Hypertrophic cardiomyopathy (HCM)	ITGA2B ITGA7 ITGB3	3
		<0.001	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	ITGA2B ITGA7 ITGB3	3
		<0.001	Dilated cardiomyopathy (DCM)	ITGA2B ITGA7 ITGB3	3

Table 6.4: Gene ontology analysis of differentially expressed genes between neutrophils from patients with stable and AECOPD

Outcome	GO analysis	p-value	GO term	Associated genes	# of associated genes
		0.010	Gap junction	GUCY1B1 TUBB1	2
		0.011	Hematopoietic cell lineage	ITGA2B ITGB3	2
		0.011	Human papillomavirus infection	ITGA2B ITGA7 ITGB3	3
		0.015	Vascular smooth muscle contraction	MYL9 GUCY1B1	2
		0.015	Fluid shear stress and atherosclerosis	ITGA2B ITGB3	2
		0.016	Oxytocin signalling pathway	MYL9 GUCY1B1	2
		0.017	CGMP-PKG signalling pathway	MYL9 GUCY1B1	2
		0.024	Rap1 signalling pathway	ITGA2B ITGB3	2
Downregulated	Biological	Other			
		0.029	Cellular response to drug	HRH4 JUP SMPD3 P2RY2	4
	Molecular	0.016	Phospholipase activity	SMPD3 PLD4	2
		0.016	Transmembrane signalling receptor activity	IL5RA HRH4 CYSLTR2 P2RY2 SIGLEC8	5
		0.016	Phosphoric diester hydrolase activity	SMPD3 PLD4	2
		0.016	Lipase activity	SMPD3 PLD4	2
		0.023	Signalling receptor activity	IL5RA HRH4 CYSLTR2 P2RY2 SIGLEC8	5
		0.023	Molecular transducer activity	IL5RA HRH4 CYSLTR2 P2RY2 SIGLEC8	5

Legend: Complete combined list of differentially expressed genes of neutrophils from patients with stable COPD compared with AECOPD associated with biological processes, molecular functions or (KEGG) pathways using ShinyGO gene ontology (GO) analysis. Terms are grouped based on upregulated (orange) or downregulated (blue) in AECOPD compared to stable COPD and if terms are related to chemotaxis, phagocytosis, maturity or activation.

6.2.4.3 Does gene expression match surface protein expression?

The genes corresponding to the surface markers (shown in Chapter 5 Table 5.4) investigated in Section 6.2.2 were identified and plotted on a heatmap for both participant groups (Figure 6.16). There were no statistically significant changes in expression of these genes, although visually it appeared CEACAM8 expression was reduced and CXCR2 expression increased.

Due to the limited sample number, correlations between surface and gene expression were not included.

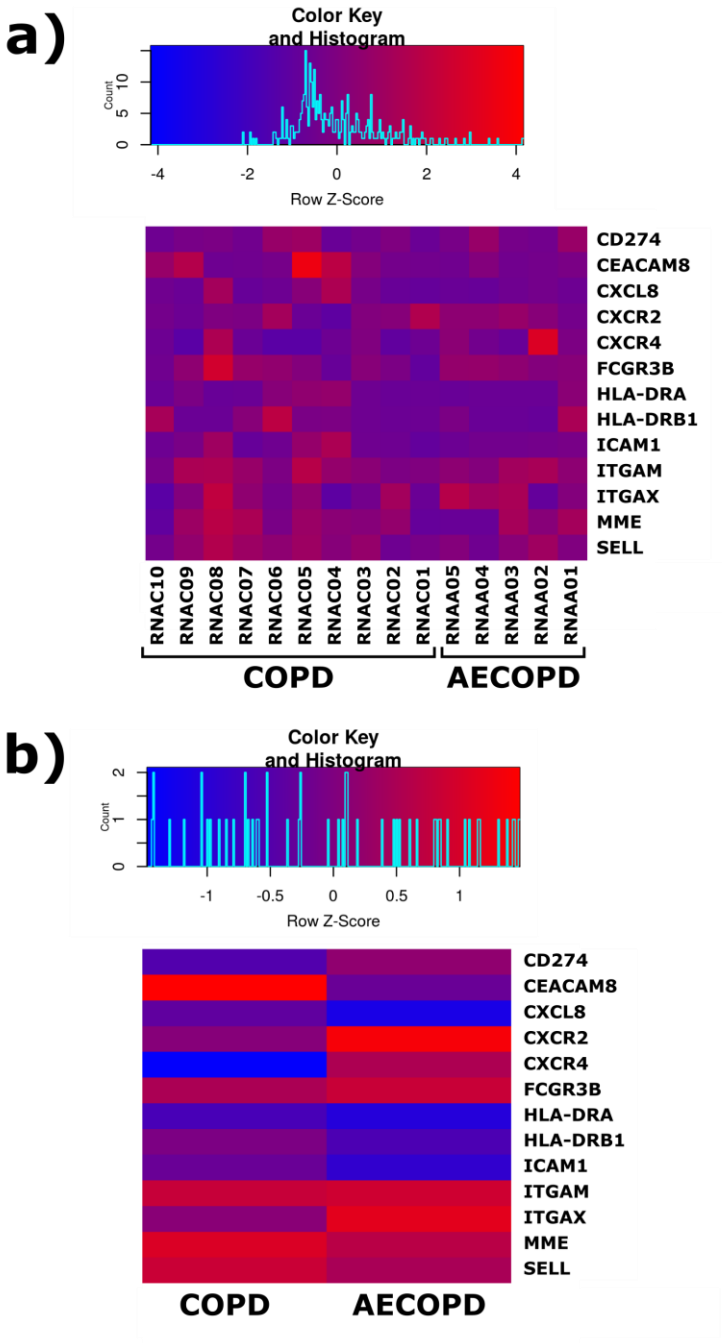

Figure 6.16: Normalised expression of genes corresponding to selected surface markers

Gene counts for mRNA extracted from neutrophils was normalised for gene length and total read number. Gene expression shown for selected markers (Table 5.4) for either **a)** each individual participant or **b)** mean expression for each participant group. Genes were clustered using Euclidean spacing and coloured based on the z-score generated from gene normalisation.

6.3 Discussion

Many studies have begun to investigate neutrophil phenotypes and how these may be linked to a variety of diseases, but less is known about these neutrophil phenotypes change in AECOPD. Here, a novel integrated panel was used to assess a variety of neutrophil phenotypes both in stable COPD and during an exacerbation, based on evidence that neutrophil migration was reduced in AECOPD (McIver *et al.*, 2019).

Neutrophils from patients with AECOPD showed increases in the expression of activation markers, increased apoptosis, a reduction in CXCR2 expression, and increased expression of genes linked to chemotaxis and activation.

6.3.1 The activation status of neutrophils

Based on expression of CD11b, CD66b and CD62L, previously linked with neutrophil activation (Rosales, 2018), neutrophils from patients with AECOPD were systemically activated; showing higher expression of CD11b and CD66b (although CD66b expression did not quite achieve statistical significance). Notably, a reduction in CD62L was not observed. A similar activation pattern has been observed in patients with sepsis (Lewis *et al.*, 2015) and following cardiac surgery (Orr *et al.*, 2007), where CD62L expression was not lower, potentially suggesting that these changes in systemic neutrophil activation are in response to the acute increased inflammatory burden in AECOPD rather than chronic disease. A subset of patients also showed increased levels of apoptosis that may partly link to increased activation as previously observed (Noguera *et al.*, 2004) and could suggest altered mechanisms of neutrophil clearance or survival.

6.3.2 Senescence and changes in chemokine sensing

Previous studies have described neutrophil senescence based on the increase of chemokine receptor CXCR4 (Martin *et al.*, 2003; Weisel *et al.*, 2009). The low level of CXCR4 expression detected on neutrophils patients with COPD and AECOPD in this thesis suggest that increased senescence is not observed in the circulating neutrophil population and These results are supported by previous studies in healthy individuals (Rankin, 2010), but has not previously been reported in AECOPD.

Linked with neutrophil senescence, a previous study described an 'overactive senescence' neutrophil phenotype in patients with ischemic stroke (Weisenburger-Lile *et al.*, 2019), defined as being CXCR4+CD62L^{dim}. Indeed, there was some evidence this may be increased in AECOPD - potentially suggesting this population is enhanced by acute systemic inflammation. The increase during acute inflammation, including from the previous study in ischemic stroke (Weisenburger-Lile *et al.*, 2019), suggests a role of non-traditional senescent cells in acute inflammation and to a lesser extent in chronic inflammation. It cannot be overlooked, however, that only a small absolute percentage of neutrophils display this phenotype, and further investigation would be required to determine if this is of clinical importance.

In contrast, levels of CXCR2 were reduced on neutrophils from patients with AECOPD, suggesting chemokine sensing may be altered in these cells. In addition, a similar decrease in surface CXCR2 on neutrophils has been observed in patients with sepsis (Rios-Santos *et al.*, 2007), suggesting another similarity between systemic inflammation and the impact on neutrophils in both sepsis and AECOPD. Alterations in CXCR2 may suggest a potential mechanism for the reduced migratory accuracy of neutrophils from patients with COPD and

AECOPD – a defect that has been previously described (Sapey *et al.*, 2011). The reduction in CXCR2 expression described here may also explain the lack of clinical efficacy in some studies of CXCR2 inhibitors in COPD (Lazaar *et al.*, 2020), as these may be targeting a mechanism that is already downregulated in patients with AECOPD. Furthermore, this thesis demonstrated that several genes were upregulated in neutrophils from patients with AECOPD that were linked to positive regulation of cell chemotaxis (including SPARC, SRPX2 and ITGB3). Whilst relatively under-investigated in neutrophil chemotaxis, previous studies have suggested SRPX2 increases cell motility in cancer (Tamaka *et al.*, 2009) and SPARC appears to reduce clustering and activation of several integrins in ovarian cancer (Said, Socha and Motamed, 2007). Upregulation of these genes may link to changes to a loss in cell polarity leading to reduced migration accuracy and the potential for increased collateral damage. However, further investigation would be required to identify the direct functional impact of the increased expression of these genes in human neutrophils.

6.3.3 Neutrophil maturity

Acute inflammation has previously been reported to lead to premature neutrophil release into the circulation (referred to as emergency granulopoiesis), seen during sepsis (Taneja *et al.*, 2008), after invasive cardiac surgery (Orr *et al.*, 2005) and in severe COVID-19 (Carissimo *et al.*, 2020).

The data presented here showed that in AECOPD, there was no change in either CD10 or CD16 expression, and therefore maturity of circulating neutrophils, indicating an absence of emergency granulopoiesis. The lack of circulating immature neutrophils in AECOPD is perhaps surprising given the acute inflammatory stimulus, but may suggest chronic inflammation

prevents or significantly diminishes emergency granulopoiesis and shows divergence in AECOPD compared with sepsis.

6.3.4 Inflammatory status and reverse transmigration

Neutrophils have also been reported to acquire a pro-inflammatory phenotype through the expression of an antigen presentation molecule, HLA-DR (Vono *et al.*, 2017) or anti-inflammatory properties by inhibiting T cell responses via PD-L1 (Keir *et al.*, 2008; Wei *et al.*, 2013). CD11c has also been linked with a predictive marker in SIRS (Lewis *et al.*, 2015) and CD54 with neutrophil reverse transmigration (Buckley *et al.*, 2006).

In this study, little or no HLA-DR expression was detected on neutrophils from any participant, with no changes in CD11c or CD54 expression in AECOPD – suggesting neutrophils do not display pro-inflammatory or reverse transmigration properties in these patients. The similar low expression of CD54 expression also suggests that it is unlikely that pro-inflammatory conditions in the lung could lead to systemic effects by altering the neutrophil phenotype before they return to the circulation.

However, a small yet significant change in PD-L1 was observed in patients with AECOPD compared with stable COPD, suggesting an appearance of an anti-inflammatory neutrophil phenotype. A similar phenotype of neutrophils has been reported in patients with SLE, showing increased PD-L1 expression that correlated with disease severity scores (Luo *et al.*, 2016), providing some parallels to the impact of systemic inflammation. However, increasing PD-L1 expression on neutrophils in both AECOPD and SLE does not demonstrate these populations are beneficial. Given the small change in PD-L1 expression in AECOPD, further investigation is required to determine firstly if this has functional relevance, and indeed if this

neutrophil population exhibits immunosuppressive and indeed protective properties. The lack of increase in CD11c expression suggests a divergence from the potential anti-inflammatory phenotype observed in SIRS and sepsis (Lewis *et al.*, 2015).

A further potentially immunosuppressive neutrophil phenotype, defined as CD16+CD62L^{dim}, has been identified in healthy human volunteers injected with systemic LPS (Pillay *et al.*, 2012). There was no increase in this population of neutrophils in AECOPD compared with stable COPD, suggesting this immunosuppressive phenotype does not appear in AECOPD.

6.3.5 Neutrophil heterogeneity

Going beyond the constraints of traditional gating, dimension-reduction clustering and visualisation revealed broadly similar phenotypes are observed in both stable COPD and AECOPD, suggesting whilst differences are observed in individual markers, there is a fine and subtle shift in surface expression across individual cells. The lack of statistical significance between the proportion of neutrophils within each cluster may be a result of the number of clusters and number of neutrophils selected for analysis – however, subtle distinctions could be identified that built upon the individual marker analysis: two clusters, increased in AECOPD, showed higher CD11b and CD66b expression compared to two clusters, reduced in AECOPD, that had lower CD11b and CD66b expression.

Taking both traditional and multi-dimensional analyses together suggest systemic activation of neutrophils does occur in AECOPD, but neutrophils are not activated equally. Furthermore, overlap of neutrophils expressing higher levels of CD11b, CD66b and CD11c builds on differences seen in the single-marker analysis and suggests a small role of CD11c in the activated neutrophil phenotype. The sorting of these cells may reveal true heterogeneity

between neutrophil populations and may provide insight to why neutrophils may respond to systemic signals differently. These analyses highlight not only that neutrophil phenotypes are complex, but further suggest a sepsis-like phenotype in AECOPD, as CD11c expression was increased on neutrophils from patients with sepsis (Lewis *et al.*, 2015).

6.3.6 The impact of gene expression on neutrophil phenotype

A total of 102 differentially expressed genes between stable COPD and AECOPD, providing evidence that neutrophils are transcriptionally active cells. Many genes associated with functional processes were upregulated in neutrophils from patients with AECOPD, showing increased expression of genes related to cell adhesion, a function of activated neutrophils and could potentially explain increased influx of neutrophils into inflamed tissue, such as the lung, in these patients. Another study comparing ICU vs non-ICU patients with COPD suggested that multiple neutrophil proteases were upregulated (Almansa *et al.*, 2012), although the comparison was done on whole blood and not isolated neutrophils, potentially explaining the lack of overlap in differentially expressed genes identified between their study and the data in this thesis.

Whilst genes associated with activation were increased, several surface G-protein coupled receptors (GPCRs) linked with detection of external chemical signals, referred to as drugs in the GO database, were downregulated. Further investigation is necessary to assess the impact of reduced expression of chemokine receptors. For instance, CCR3 was found to be expressed at a lower level in neutrophils AECOPD compared with stable COPD, but the surface expression has been suggested to increase in neutrophils in the lung during influenza pneumonia (Rudd

et al., 2019). Together, these data may point to an inability of neutrophils in patients with AECOPD to mount appropriate responses to infection.

In addition, upregulation of integrins ITGA7, ITGB3, ITGA2B and the gene for a calcium channel CACNG6 (linked with hypertrophic, dilated and arrhythmogenic right ventricular cardiomyopathy) may suggest evidence for the role of neutrophils in right-sided heart failure seen in patients with COPD (Díez, Morgan and García, 2013; Mullerova *et al.*, 2013) and the increased cardiovascular burden during an exacerbation.

Despite the identification of alteration in processes linked with activation and chemical sensing, genes linked with the surface markers investigated within the phenotyping panel (6.2.2) related to these processes were not significantly altered – but are underpowered to draw conclusions and requires further investigation. However, changes in gene expression may present novel avenues for future research, as altered gene expression may suggest an inability of neutrophils to respond effectively when stimulated by inflammatory or pathogenic cues. Indeed, as RNASeq becomes more accessible, the ability to use this technique to provide insight to neutrophil biology at the clinical interface becomes a greater reality – a recent study of whole blood RNASeq in COVID-19 patients demonstrated neutrophil-activation signatures associated with disease severity (Aschenbrenner *et al.*, 2021).

One key caveat may exists with all these gene expression analyses: neutrophil preparations will not have been 100% pure and, therefore, results could be skewed by contaminating cell types with higher levels of RNA than neutrophils. A previous study compared the effect on RNASeq data when neutrophil purity was 95-99% compared with 82-90% and found only 23 genes were reported as differentially expressed between these two methods (Thomas *et al.*,

2015). Three of these genes, SIGLEC8, CLC and SMPD3, were also identified in this thesis as being differentially expressed – however, none of these genes were independently associated with changes in biological or molecular processes. Overall, neutrophil purity is unlikely to have substantially impacted the findings from these analyses.

6.3.7 Summary and limitations

Here, evidence from a novel antibody panel suggests that neutrophils from patients with AECOPD show systemic neutrophil activation and could link to an increased cardiovascular burden in AECOPD. In addition, lower CXCR2 expression was observed, suggesting an inflammatory-mediated regulation of CXCR2 expression that could influence neutrophil migration and increased collateral damage in AECOPD. During an acute exacerbation, a small (but significant) increase in PD-L1 expression on neutrophils from patients with AECOPD provides evidence of an immunosuppressive response in these patients compared with stable COPD. Together, these changes have similarities with neutrophils in patients with sepsis – potentially impacting clinical care of these patients.

The nature of COPD exacerbations, along with the recruitment of patients once admitted to hospital, present several challenges and limitations. All patients recruited were hospitalised and, therefore, likely reflect only the most severe exacerbations - although these events do represent the greatest burden of disease with the greatest impact on patient morbidity and mortality, as well as healthcare costs. Furthermore, many patients who have previously experienced an AECOPD are provided with 'rescue packs' – steroids and antibiotics that can be taken at the onset of an exacerbation to help prevent further symptoms and hospitalisation. It was not recorded which patients had used their rescue packs, or how long

ago, and presents a possible source of variation within these data as glucocorticoids have been suggested to promote neutrophil survival (Saffar, Ashdown and Gounni, 2011).

Here, for the first time, comprehensive neutrophil phenotyping reveals subtle neutrophil phenotype differences in patients with an exacerbation, including changes at the gene expression level, that do not appear to be due to increased immaturity of neutrophils or classical senescence. Instead, changes were consistent with activation and migration. These changes may provide mechanisms of neutrophil dysfunction, such as migration, and potential for therapeutics that could promote anti-inflammatory phenotypes – but further investigation would be necessary to determine if this provided clinical benefit.

CHAPTER 7:

FUNCTIONAL ANALYSIS OF NEUTROPHILS IN COPD

7.1 Brief Introduction

Neutrophils carry out a number of functions that protect from pathogen attack and in response to tissue damage (Hughes, Sapey and Stockley, 2019). In order to perform these roles, they must first successfully navigate to the correct area of the body, following either inflammatory signals of damage or infection – a process known as chemotaxis (Wilkinson, 1985). In the absence of any chemoattracting agent, human neutrophils still move in a process called chemokinesis (Campoccia *et al.*, 1993) - random and non-specific movement. The accuracy of neutrophil chemotaxis has previously been shown to decline with age and is impaired in patients with COPD compared to age-matched healthy individuals (Yoshikawa *et al.*, 2007). It has been previously shown that neutrophils incubated with PI3K inhibitors can rescue the age-related decline in neutrophil chemotaxis (Sapey *et al.*, 2014). Accurate migration is not only essential to neutrophils successfully getting to the site of inflammation, but has been suggested as a potential mechanism for increased lung tissue damage (Naccache and Lefebvre, 2014).

Much is known about neutrophil chemotaxis to common chemical mediators of inflammation such as CXCL8, which activates neutrophils seen both as increases in CD11b and CD66b surface expression (Detmers *et al.*, 1990) and an increase in neutrophil chemotaxis (Moore and Kunkel, 2019). However, far less attention has been given to their response to CXCL12 – the major ligand of CXCR4. As discussed, CXCR4 expression on neutrophils has been linked to senescence (Martin *et al.*, 2003). Previous work has indicated HeLa cells over-expressing CXCR4 are capable of sensing and migrating towards CXCR4 (Dillenburg-Pilla *et al.*, 2015) and that CXCL12 can retain neutrophils at sites of inflammation in zebrafish (Isles *et al.*, 2019) and

in the bone marrow of mice (Martin *et al.*, 2003) and humans (Kawai and Malech, 2009). The CXCR4/CXCL12 axis may, therefore, be a potential mechanism for neutrophil retention in the lungs of patients with COPD.

Specific patterns of protein surface expression by neutrophils, referred to as the neutrophil phenotype, can be altered by the inflammatory environment and subsequently impact on neutrophil chemotaxis; for example, human serum altered neutrophil migration behaviour though agarose gel *in vitro* (Dahl and Lindroos, 1979). The neutrophil phenotype is also influenced by the local environment, with an intense interest in the cause or effect relationship between chronic disease and neutrophil phenotype (Hellebrekers, Vrisekoop and Koenderman, 2018; Rosales, 2018; Hughes, Sapey and Stockley, 2019; Maréchal *et al.*, 2020). Understanding how the inflammatory environment in patients with COPD influences changes in neutrophil function is of paramount importance to assist the development of potential therapeutics.

7.1.1 Aims and hypothesis

To help address some of these questions, healthy volunteers and patients with COPD (but without stratification based on multimorbidity) were recruited and the levels of CXCL12 in peripheral blood samples were measured using an ELISA. Whilst the previous two chapters revealed no differences in CXCR4 expression on neutrophils between healthy volunteers and patients with COPD, it was hypothesised that functional differences may exist in the receptor or downstream signalling, and chemotaxis of isolated neutrophils towards CXCL12 using Insall chambers (Muinonen-Martin *et al.*, 2010) and time-lapse light microscopy was assessed. In addition, isolated neutrophils were pre-incubated with serum and plasma from both healthy

volunteers and patients to determine its impact on both neutrophil phenotype and subsequent chemotaxis.

It was hypothesised that patients with COPD would show increased migration towards CXCL12 compared with healthy controls. Due to potentially higher levels of inflammatory cytokines in peripheral blood of patients with COPD, neutrophils exposed to serum and plasma from these patients would show increased activation and mimic changes in CXCR2 expression observed in the previous chapters.

7.2 Results

7.2.1 CXCL12 and neutrophil chemotaxis

7.2.1.1 Plasma CXCL12 concentration

CXCL12 is the major ligand for CXCR4 – a receptor implicated in neutrophil senescence (Martin *et al.*, 2003). Using an ELISA, plasma CXCL12 concentrations from study participants ranged from 0.685ng/mL to 2.1ng/mL, with a significantly lower CXCL12 concentration in both COPD groups compared to healthy (Figure 7.1). These data indicated CXCL12 levels in these healthy volunteers was consistent with previously published findings and provided evidence that CXCL12 was reduced in patients with both stable and acute exacerbations of COPD, potentially having implications for the control of neutrophil homeostasis in these patients.

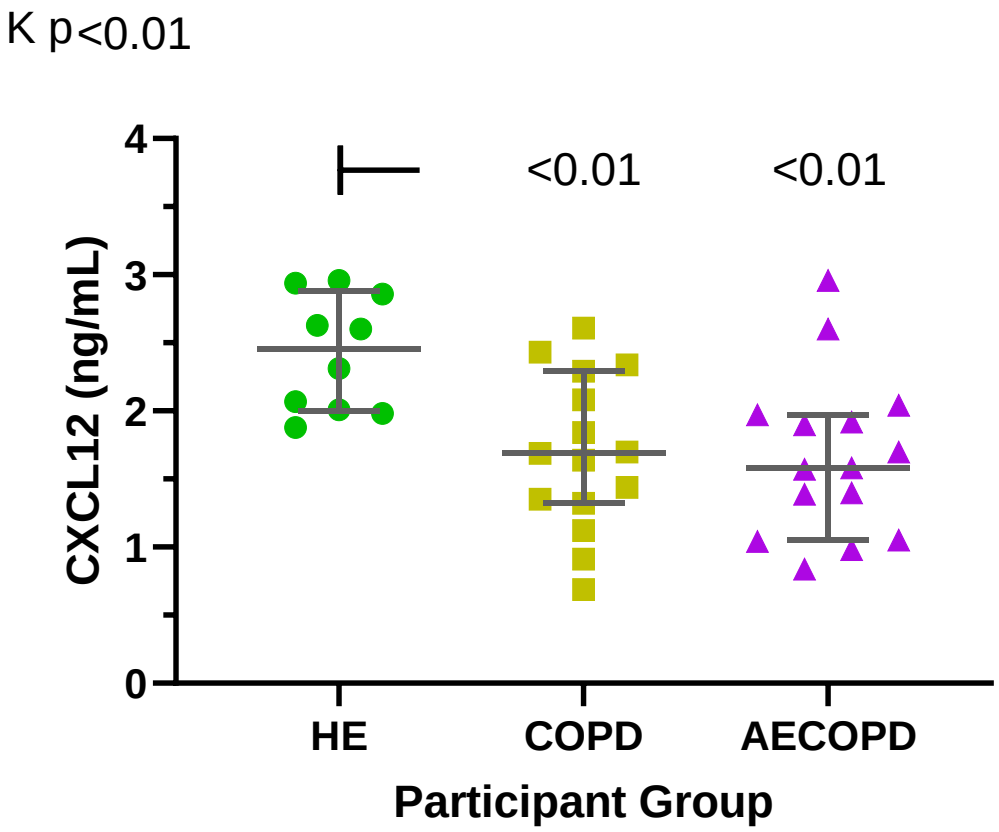

Figure 7.1: Plasma concentration of CXCL12 from healthy individuals and patients with stable and exacerbations of COPD

Plasma was separated from whole blood from healthy elderly participants (HE, n=10), patients with stable COPD (n=15), or patients with an acute exacerbation of COPD (AECOPD, n=15) and frozen prior to testing. Vials were thawed and plasma CXCL12 levels measured using an ELISA. In each case, the horizontal lines indicate the median value with interquartile range. Statistical analysis performed using a Kruskal-Wallis test (K) with Dunn's multiple comparison, compared to the HE group.

7.2.1.2 Healthy young neutrophil chemotaxis towards CXCL12

Using the concentration of CXCL12 from plasma samples in both healthy volunteers and patients with COPD, neutrophil chemotaxis towards three concentrations of CXCL12 was assessed: 1, 10 and 100ng/mL; where RPMI served as the negative control and CXCL8 as the positive control. Increases in speed, velocity and chemotaxis index were observed towards CXCL8 compared with RPMI (Figure 7.2a, b and c), albeit only reaching statistical significance for velocity. There was also an increase in displacement (significant; Figure 7.3a) and distance travelled (not significant; Figure 7.3b) between CXCL8 and RPMI, but no change in persistence (Figure 7.2d) or directness (Figure 7.3c).

Unlike with CXCL8, neutrophils from HY individuals showed no change in speed, velocity, chemotaxis index towards CXCL12 when compared to RPMI (Figure 7.2), although statistical analysis of persistence could not be completed due to inadequate data for one sample (Figure 7.2d). The was also no difference observed in displacement, distance travelled or directness of neutrophils from HY participants towards CXCL12 compared to RPMI (Figure 7.3). Together, these data support that neutrophils from healthy individuals could migrate towards CXCL8, but not CXCL12.

When the same experiment was performed with neutrophils from patients with stable COPD, again, a significant increase in speed (Figure 7.4a), chemotaxis index (Figure 7.4c) displacement (Figure 7.4a), distance travelled (Figure 7.4b) and directness (Figure 7.4c) was observed towards CXCL8 compared to RPMI control. No significant change between CXCL8 and RPMI was observed in velocity (Figure 7.4b) or persistence, although complete statistical analysis could not be performed due to a missing data point (Figure 7.4d).

In contrast, no significant differences in any parameter were observed in neutrophils from patients with COPD towards CXCL12 compared with RPMI (Figure 7.4 and Figure 7.5). There was some evidence of a response at 10ng/mL based on both chemotaxis index (Figure 7.4c) and directness (Figure 7.5c), but did not reach statistical significance.

Overall, these data suggest neutrophils from patients with COPD responded to CXCL8, albeit less robustly than neutrophils from HY participants. There was also a greater variation in the response to CXCL12 of neutrophils from patients with COPD compared with HY, suggesting neutrophils from some patients may show a small response to CXCL12.

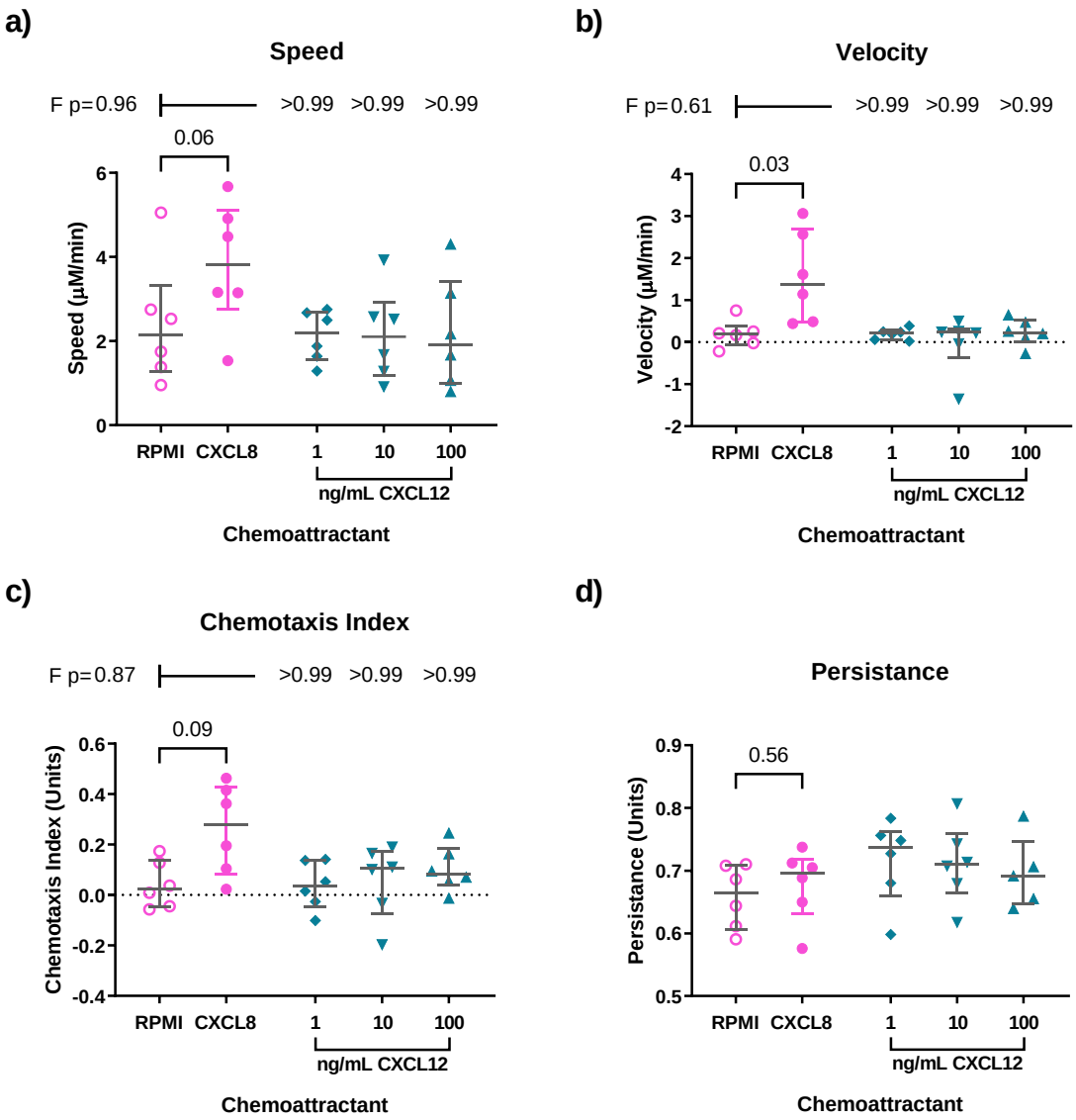

Figure 7.2: Average speed, velocity, chemotaxis index and persistence of neutrophils isolated from healthy individuals towards CXCL12

Neutrophils were isolated from healthy young participants (n=6) and incubated on glass coverslips before inverting onto an Insall chamber with each chemoattractant as indicated. Roswell Park Memorial Institute (RPMI) media was used as a negative control and 100nM CXCL8 as the positive control. Ten neutrophils were selected for analysis from each 12-minute time lapse and results averaged to give **a)** speed, **b)** velocity, **c)** chemotaxis index and, **d)** persistence, per sample. Solid horizontal lines indicate the median value with interquartile range. Statistical analysis performed using a Wilcoxon matched-pairs signed rank test between RPMI and CXCL8 or a Freidman test with Dunn's multiple comparison compared to RPMI and doses of CXCL12. *Indicates statistical analysis could not be performed due to missing values for one sample at 100ng/mL CXCL12.

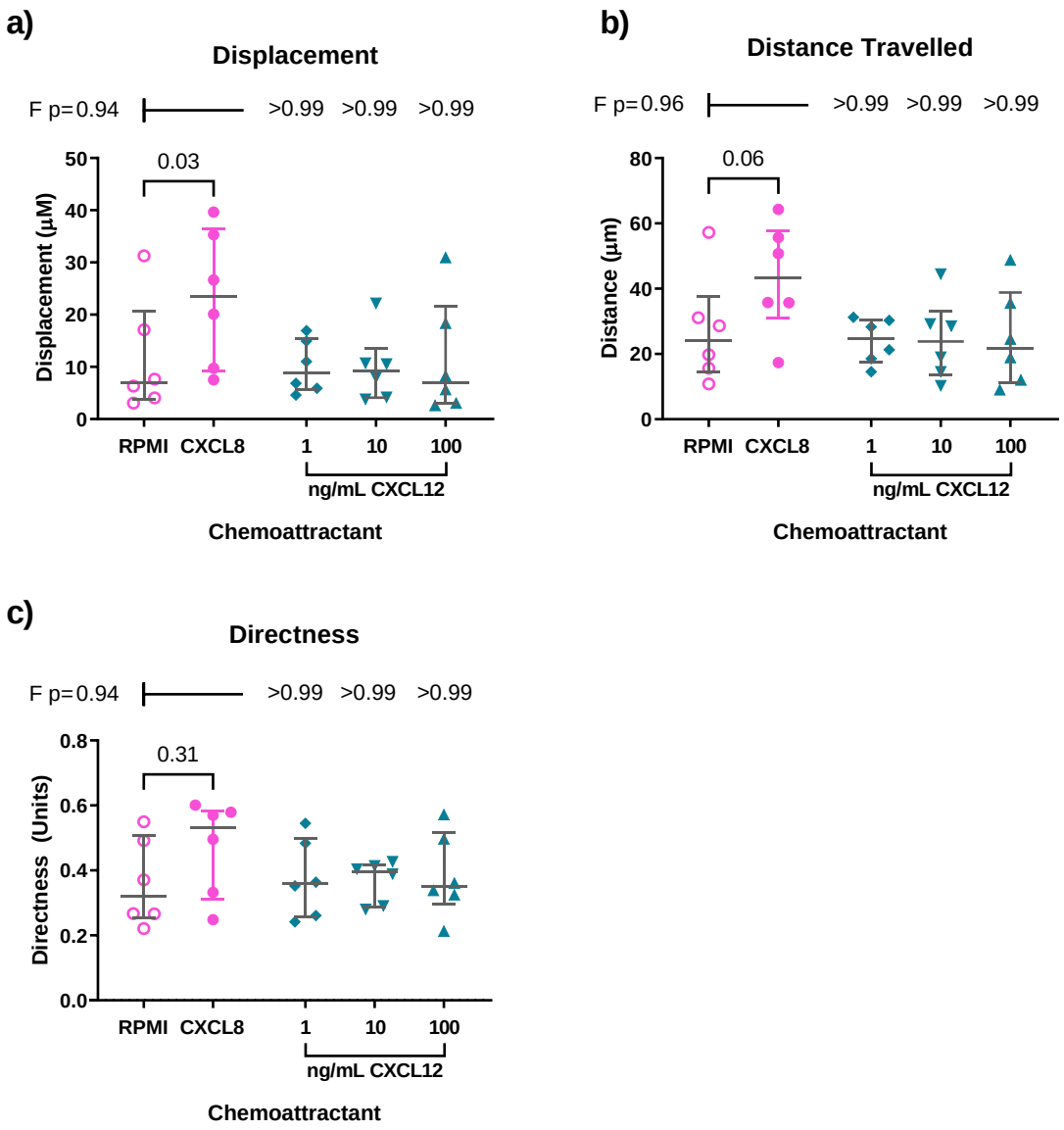

Figure 7.3: Average displacement, distance travelled and directness of neutrophils isolated from healthy individuals towards CXCL12

Neutrophils were isolated from healthy young participants (n=6) and incubated on glass coverslips before inverting onto an Insall chamber with each chemoattractant as indicated. Roswell Park Memorial Institute (RPMI) media was used as a negative control and 100nM CXCL8 as the positive control. Ten neutrophils were selected for analysis from each 12-minute time lapse and results averaged to give **a)** displacement, **b)** total distance travelled and **c)** the directness, per sample. Solid horizontal lines indicate the median value with interquartile range. Statistical analysis performed using a Wilcoxon matched-pairs signed rank test between RPMI and CXCL8 or a Freidman test with Dunn's multiple comparison compared to RPMI and doses of CXCL12.

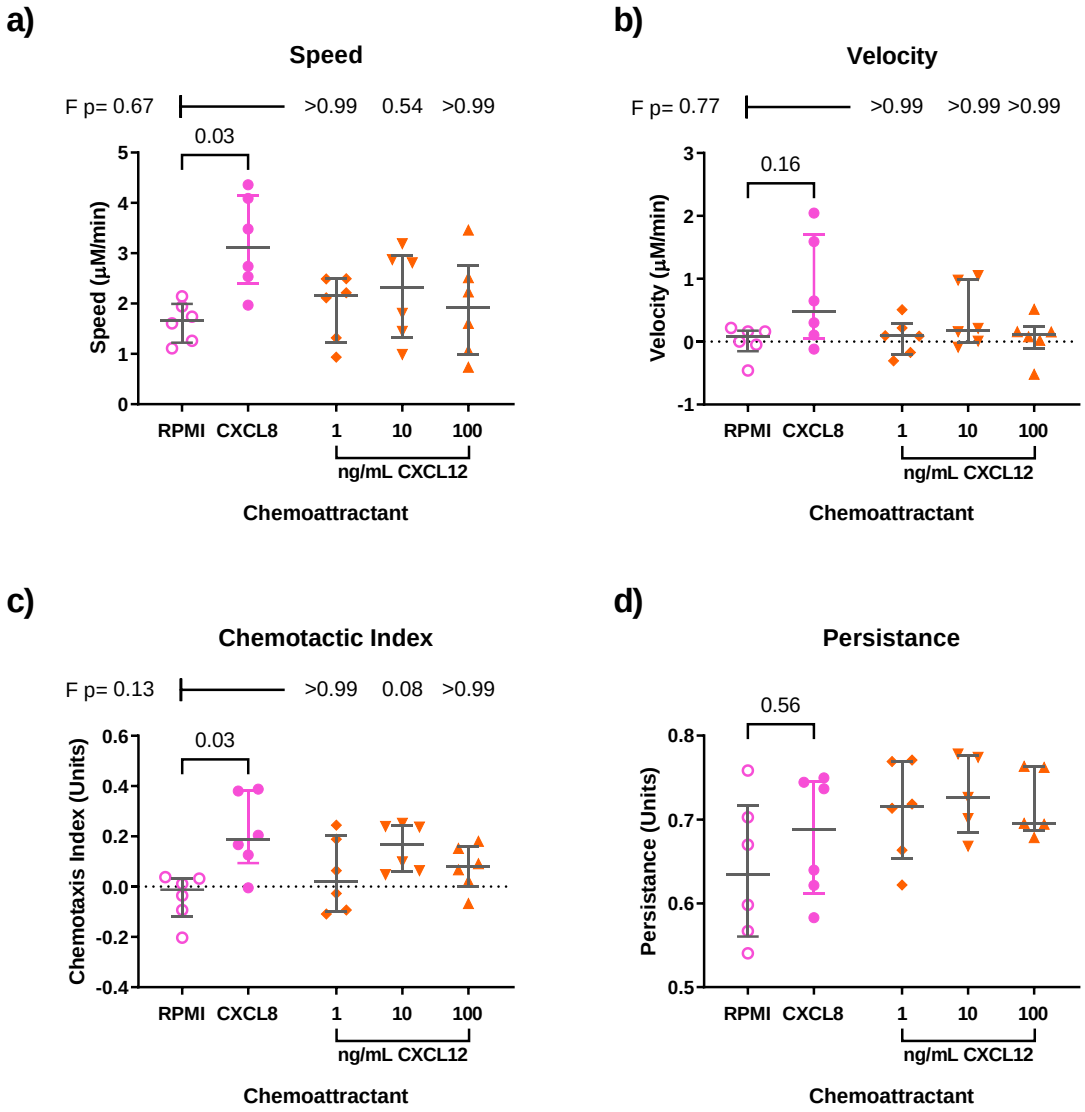

Figure 7.4: Average speed, velocity, chemotaxis index and persistence of neutrophils isolated from patients with COPD towards CXCL12

Neutrophils were isolated from patients with COPD participants (n=6) and incubated on glass coverslips before inverting onto an Insall chamber with each chemoattractant as indicated. Roswell Park Memorial Institute (RPMI) media was used as a negative control and 100nM CXCL8 as the positive control. Ten neutrophils were selected for analysis from each 12-minute time lapse and results averaged to give **a)** speed, **b)** velocity, **c)** chemotaxis index and, **d)** persistence, per sample. Solid horizontal lines indicate the median value with interquartile range. Statistical analysis performed using a Wilcoxon matched-pairs signed rank test between RPMI and CXCL8 or a Freidman test with Dunn's multiple comparison compared to RPMI and doses of CXCL12. *Indicates statistical analysis could not be performed due to missing values for one sample at 100ng/mL and 10ng/mL CXCL12.

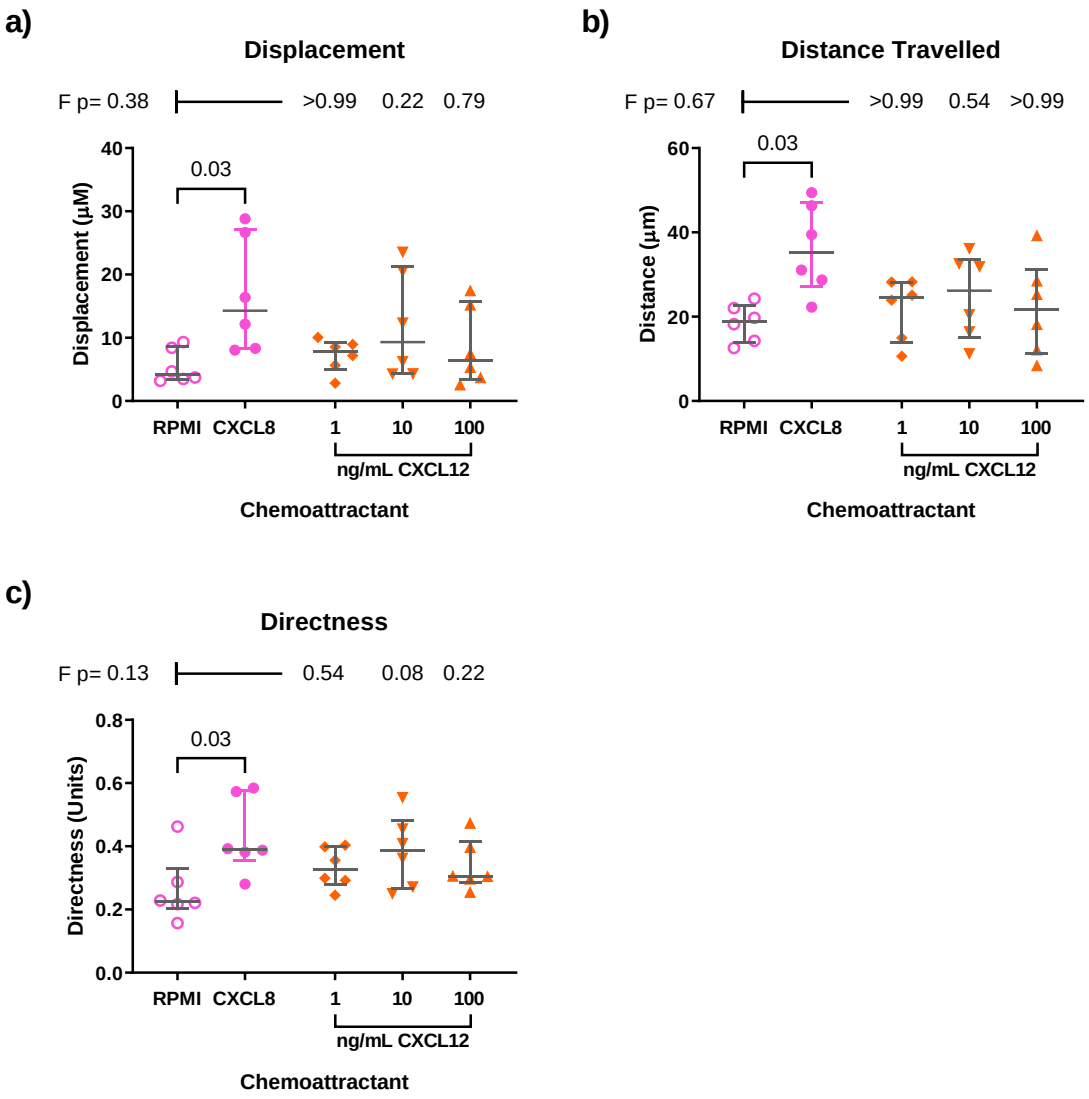

Figure 7.5: Average displacement, distance travelled and directness of neutrophils isolated from patients with COPD towards CXCL12

Neutrophils were isolated from patients with COPD (n=6) and incubated on glass coverslips before inverting onto an Insall chamber with each chemoattractant as indicated. Roswell Park Memorial Institute (RPMI) media was used as a negative control and 100nM CXCL8 as the positive control. Ten neutrophils were selected for analysis from each 12-minute time lapse and results averaged to give **a)** displacement, **b)** total distance travelled and **c)** the directness, per sample. Solid horizontal lines indicate the median value with interquartile range. Statistical analysis performed using a Wilcoxon matched-pairs signed rank test between RPMI and CXCL8 or a Freidman test with Dunn's multiple comparison compared to RPMI and doses of CXCL12.

7.2.2 Neutrophil chemotaxis following phosphoramidon treatment

Initial data from the phenotyping panel (Section 5.2.3.7) suggested there may be altered surface expression of CD10 and this may play a role in controlling chemotaxis towards fMLP. As CD10 can be pharmacologically blocked using phosphoramidon, neutrophil chemotaxis following phosphoramidon incubation was assessed. Previous data have shown that phosphoramidon achieves 50% inhibition (IC_{50}) of CD10 at 0.034 µM (Kukkola *et al.*, 1995), but can also inhibit endothelin-converting enzyme and angiotensin-converting enzyme with an IC_{50} of 3.5 µM and 78 µM respectively (Kukkola *et al.*, 1995).

Chemotaxis of neutrophils following pre-treatment with various concentrations of phosphoramidon was assessed towards fMLP, where RPMI served as a negative control and CXCL8 and fMLP the positive control. Statistically significant increases in speed, velocity, chemotaxis index (Figure 7.6a, b and c), displacement and distance travelled (Figure 7.7a and b) towards fMLP was observed when compared with RPMI. No change was seen in persistence (Figure 7.6d) or directness (Figure 7.7c) between fMLP and RPMI.

A potentially small concentration-dependent decline in overall speed was observed with phosphoramidon treatment compared to RPMI treatment, approaching statistical significance at 50µM (Figure 7.6a), but not with velocity, chemotaxis index or persistence (Figure 7.6b, c and d) or displacement and directness (Figure 7.7a and c) – although sample-to-sample variation may impact this assessment. Again, there was a suggestion that 50µM of phosphoramidon reduced the total distance travelled compared to untreated neutrophils towards fMLP, reaching significance at the group level (Figure 7.7a and b).

Together, these data suggest phosphoramidon may alter overall neutrophil speed and distance at 50µM, however, did not impact accuracy or reach statistical significance at any dose and requires further samples to fully investigate the potential effect on neutrophil migration.

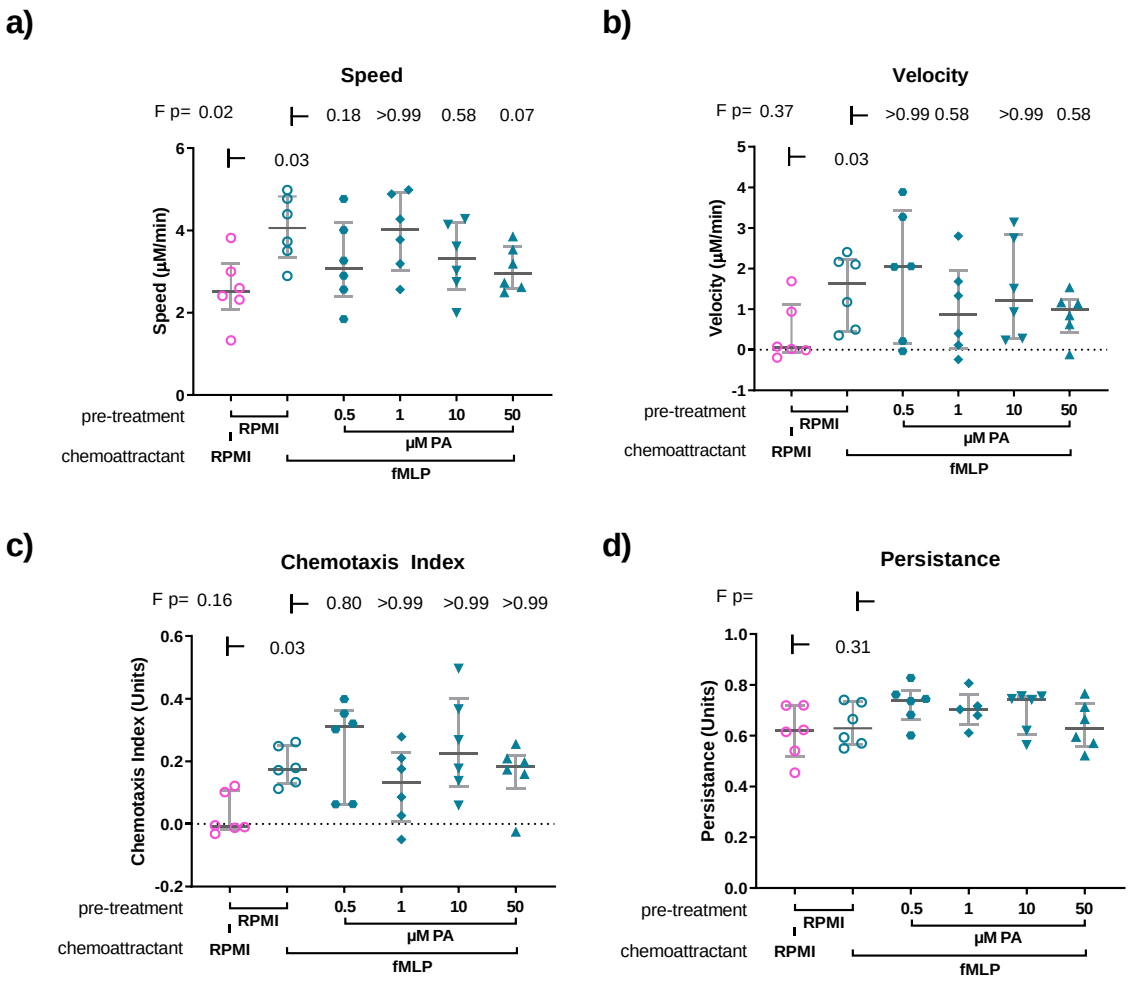

Figure 7.6: Impact of phosphoramidon treatment on the speed, velocity, chemotaxis index and persistence of neutrophils isolated from healthy individuals towards fMLP

Neutrophils were isolated from healthy young participants (n=6) and pre-treated with Roswell Park Memorial Institute (RPMI) media or phosphoramidon (PA) for 30 minutes before incubation on glass coverslips and inverting onto an Insall chamber. Ten neutrophils were selected for analysis from each 12-minute time lapse and results averaged to give **a)** speed, **b)** velocity, **c)** chemotaxis index and, **d)** persistence, per sample. Solid horizontal lines indicate the median value with interquartile range. Statistical analysis performed using a Wilcoxon matched-pairs signed rank test between RPMI and fMLP or a Freidman test with Dunn's multiple comparison compared to fMLP for doses of PA.

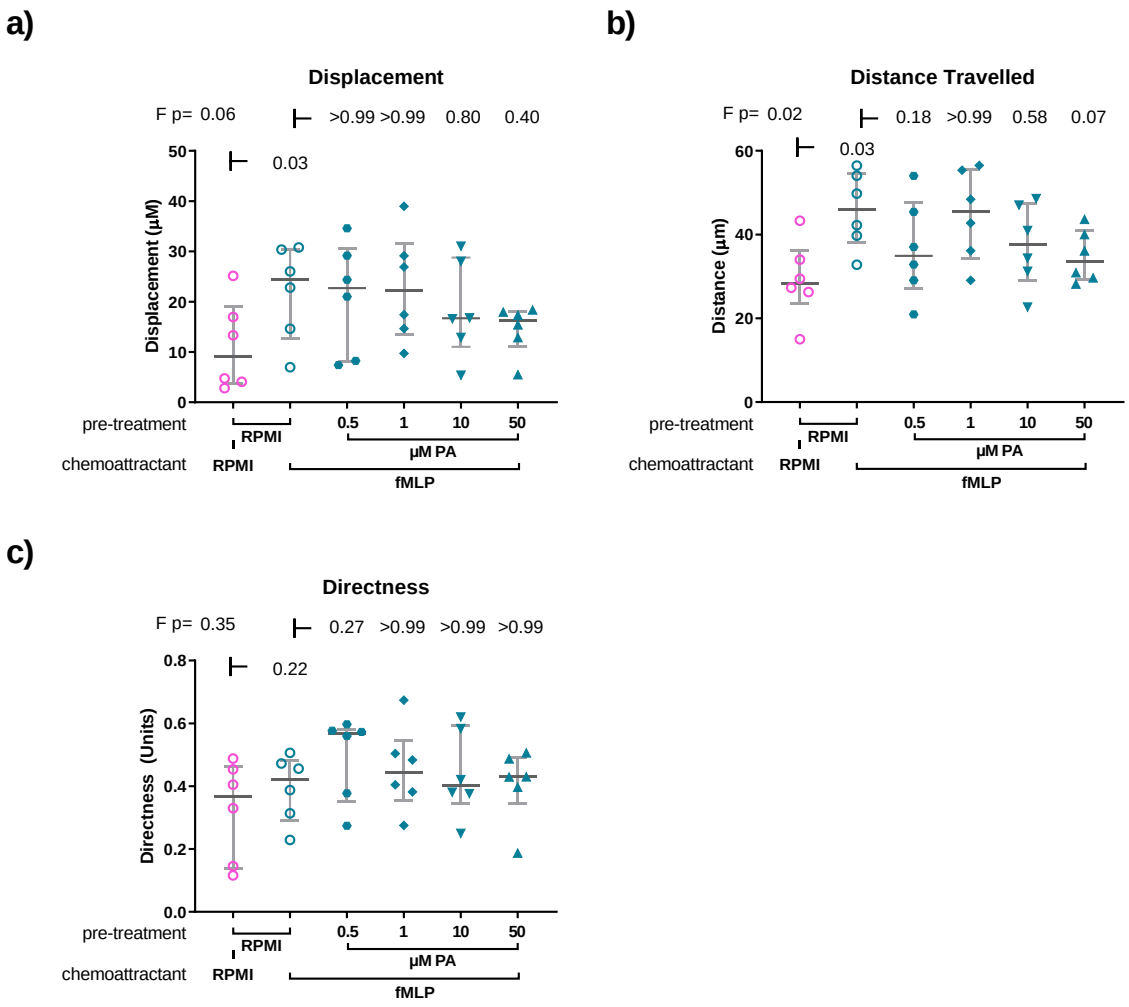

Figure 7.7: Impact of phosphoramidon treatment on the displacement, distance travelled and directness of neutrophils isolated from healthy individuals towards fMLP

Neutrophils were isolated from healthy young participants (n=6) and pre-treated with Roswell Park Memorial Institute (RPMI) media or phosphoramidon (PA) for 30 minutes before incubation on glass coverslips and inverting onto an Insall chamber. Ten neutrophils were selected for analysis from each 12-minute time lapse and results averaged to give **a)** displacement, **b)** total distance travelled and **c)** the directness, per sample. Solid horizontal lines indicate the median value with interquartile range. Statistical analysis performed using a Wilcoxon matched-pairs signed rank test between RPMI and fMLP or a Freidman test with Dunn's multiple comparison compared to fMLP for doses of PA.

7.2.3 Inducible phenotype using pooled plasma

In patients with stable COPD, neutrophils will be exposed to potentially raised levels of cytokines for a prolonged period due to chronic inflammation in the lungs. To test if neutrophils from healthy individuals exposed to potentially inflammatory conditions would display an altered phenotype, plasma from patients with COPD was collected and pooled before incubating with isolated neutrophils from HE participants. Equally, to test if the altered neutrophil phenotype identified in patients with AECOPD could be replicated *ex vivo*, neutrophils from patients with stable COPD were exposure to pooled plasma from patients with AECOPD. These experiments are summarised in Figure 7.8.

7.2.3.1 Validation of serum and plasma exposure

Neutrophils isolated neutrophils from HY participants incubated with 10-50% autologous serum resulted in a significant decline in the percentage of live neutrophils (Figure 7.9). To determine if this effect was replicated when using plasma, neutrophils from HY participants were incubated with autologous plasma (AP) at the same concentrations, which had less of an effect on neutrophil viability (Figure 7.9). Therefore, plasma was used to investigate the influence of chronic inflammation on the phenotype of circulating neutrophils.

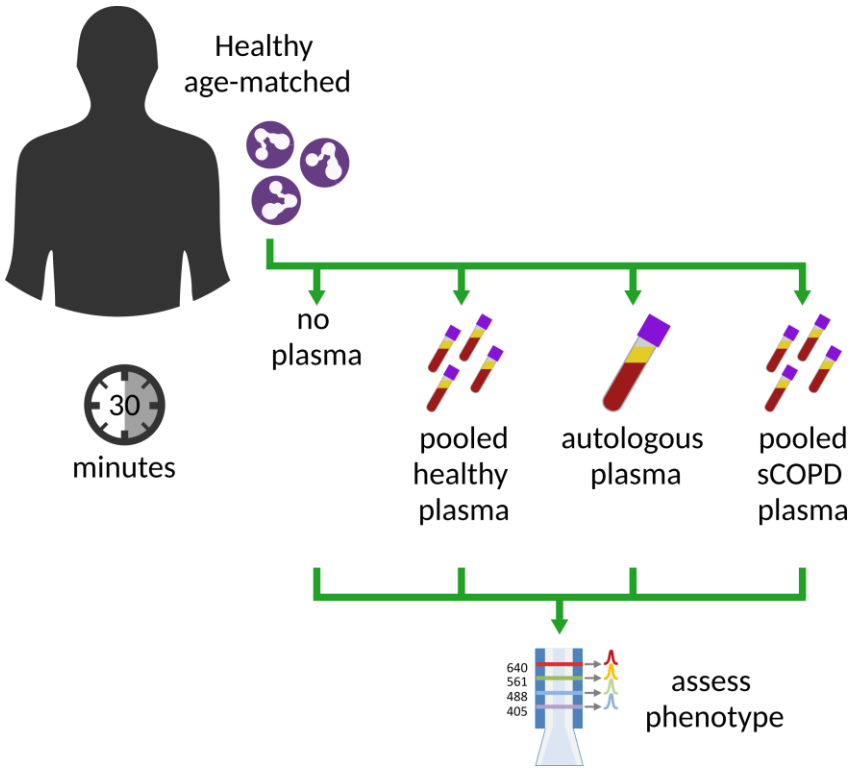

Figure 7.8: Schematic overview of pooled plasma experiments

Neutrophils from healthy elderly volunteers were isolated from whole blood and incubated for 30 minutes with either no plasma, autologous plasma, pooled healthy plasma or pooled plasma from patients with COPD. Neutrophil phenotypes were then assessed using surface expression of key markers as detailed in Section 2.6.

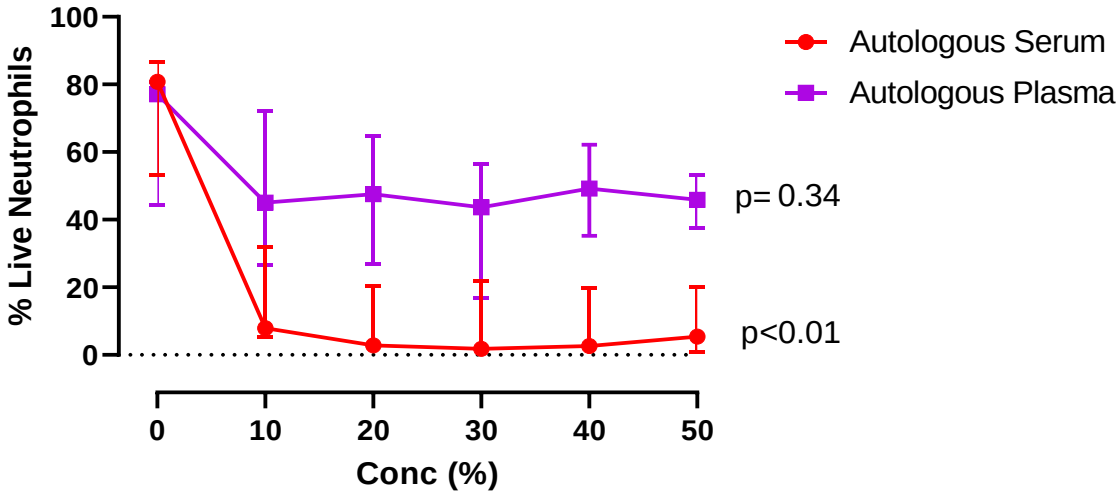

Figure 7.9: Effect of autologous serum and plasma on isolated neutrophils from healthy young participants

Neutrophils from whole blood were isolated and incubated for 30 minutes with either autologous serum (n=6) or autologous plasma (n=3) at various concentrations before staining with viability dyes. The percentage of live neutrophil events were recorded for each participant. For each concentration, the point indicates the median value with interquartile range. Statistical analysis performed using a Freidman test for both conditions.

7.2.3.2 Effect of stable COPD plasma on neutrophils from healthy age-matched participants

Previous investigations revealed that activation markers were not significantly altered in neutrophils from healthy participants and patients with stable COPD, but there was a significant decrease in CXCR2 expression (Section 5.2.3). To investigate if the change in neutrophil phenotype is blunted or otherwise altered due to the chronic exposure to inflammatory stimuli, acute exposure of neutrophils from HE donors to pooled plasma from patients with stable COPD was carried out.

Despite validation experiments revealing limited impact of pooled plasma incubation on neutrophil viability with neutrophils from HY participants, neutrophils from HE participants showed a marked reduction in the percentage of live cells per sample (Figure 7.10), with the majority of non-live neutrophils being apoptotic. It was then assessed if inclusion of apoptotic cells in the calculation of MFI differed from only including live cells to negate samples where very few cells would be included in the live cell gate. A good correlation was observed in all markers across all conditions (Figure 7.11), with outliers occurring when the percentage of live cells was very low. Therefore, only dead or necrotic cells were excluded from analysis allowing a more accurate MFI to be calculated from both live and apoptotic cells.

Incubation of neutrophils from HE participants in pooled plasma from stable COPD patients resulted in a significant increase in CD11b expression (Figure 7.12a) and decline in CD62L expression (Figure 7.12c) compared to AP. With both CD11b and CD62L, no significant change in expression was seen on neutrophils incubated with no plasma or pooled HE plasma compared to AP, indicating the changes observed were specifically caused by plasma from patients with COPD (Figure 7.12a and c). In contrast, a significant change in CD66b expression

was observed between no plasma and AP, but not between AP and pooled plasma from either group (Figure 7.12b), indicating a non-specific change due to incubation of neutrophils with plasma.

No change in CXCR2 expression was observed between AP and pooled COPD plasma treatment, however, significant changes were observed between AP and both no plasma and pooled HE plasma controls (Figure 7.13a). In contrast, CD10 expression was significantly higher in pooled COPD plasma compared to AP (Figure 7.13b), although there did also appear to be a slight, but not statistically significant, increase even with AP treatment. No difference was seen in CD16 expression between AP and pooled COPD plasma treatment, however treatment with pooled HE plasma showed a significant reduction compared with AP treatment – albeit a very small absolute change (Figure 7.13c).

Together, changes in CD11b and CD62L suggest increased activation of healthy neutrophils when incubated in the presence of pooled COPD plasma, with a small increase in CD10 expression. Changes in CD66b and CXCR2 and CD16 appear related to plasma treatment rather than specifically to COPD plasma.

As shown previously, neutrophils from HE participants incubated with AP showed a significant decrease in viability compared to neutrophils incubated with no plasma (Figure 7.10). To investigate if the viability impacted on the surface expression, linear regression analysis was performed of viability against surface expression for all participants and conditions (Figure 7.14). A weak, but statistically significant, correlation was observed with viability and CD66b, CD62L (Figure 7.14b and c), CXCR2 and CD10 (Figure 7.14d and e). There was not a statistically significant correlation between viability and CD11b (Figure 7.14a) or CD16 (Figure 7.14f)

expression. These data suggest that cell viability may be a contributing factor to the change in surface expression, although increased apoptosis was observed in treatment conditions.

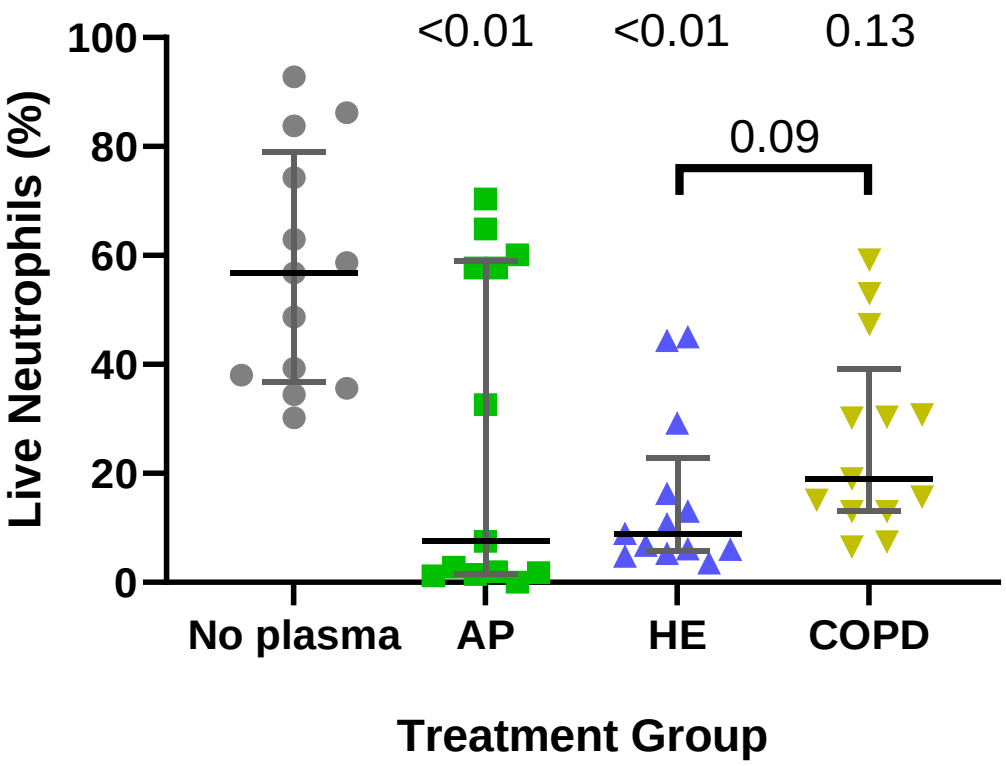

Figure 7.10: The effect of pooled plasma from patients with stable COPD on neutrophil viability from healthy participants

Neutrophils from healthy elderly participants (n=13) were isolated from whole blood and incubated with either autologous plasma (AP), pooled plasma from patients with stable COPD (COPD), no plasma (control) or pooled plasma from healthy elderly participants (HE, control). The percentage of live neutrophil events were recorded for each participant. In each case, the horizontal line indicates the median value with interquartile range. Statistical analysis performed using a Freidman's test (F) with Dunn's multiple comparison, compared to the no plasma group.

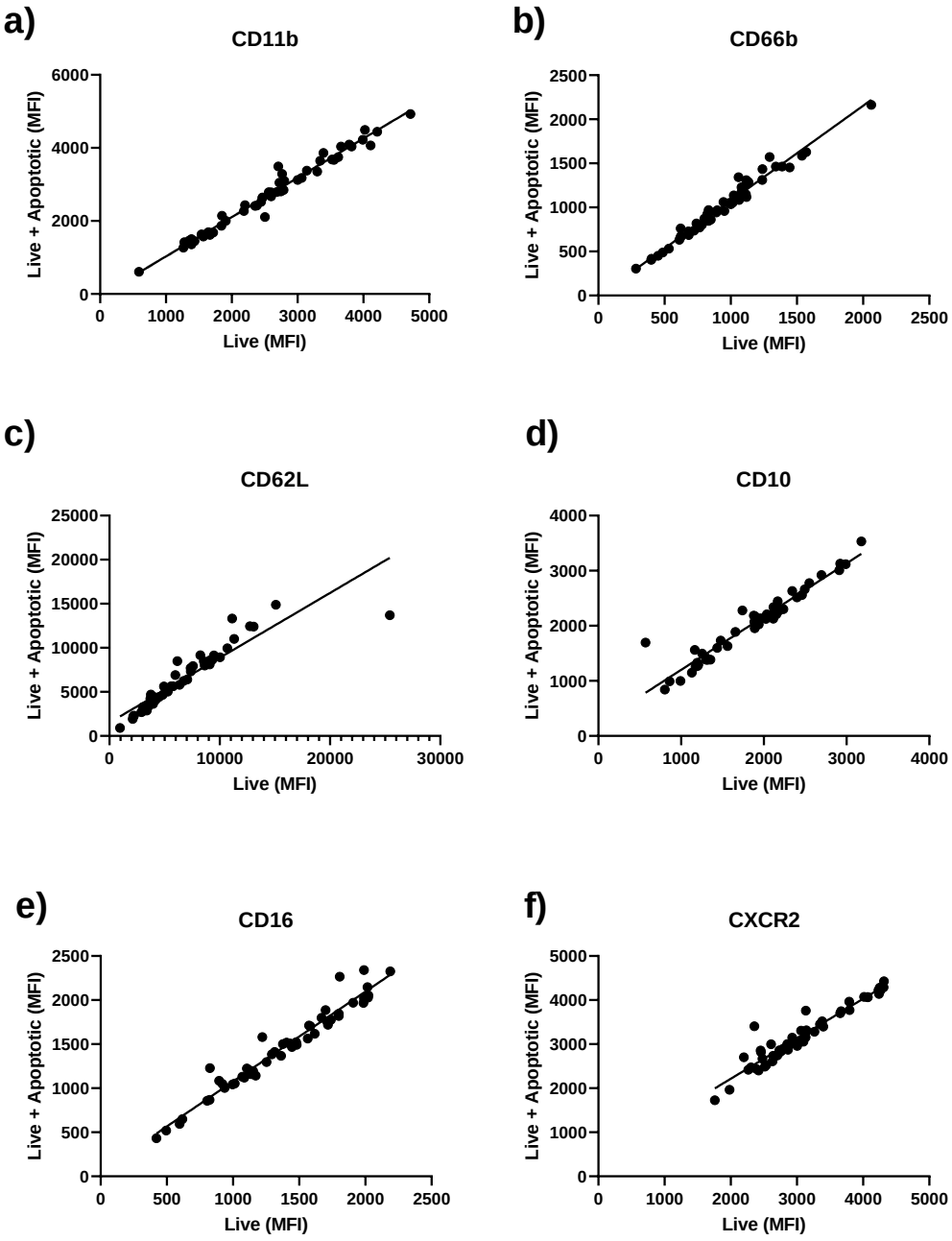

Figure 7.11: Comparison of the median fluorescence intensity of surface markers between only live or live and apoptotic neutrophils from healthy elderly participants

Neutrophils from healthy elderly participants (n=13) were isolated from whole blood and incubated with either autologous plasma, pooled plasma from healthy elderly participants or pooled plasma from patients with stable COPD. The median fluorescence intensity (MFI) for **a)** CD11b, **b)** CD66b, **c)** CD62L, **d)** CD10, **e)** CD16 and **f)** CXCR2 were recorded for either only live cells, or both live and apoptotic cells. The line of best fit using linear regression is shown.

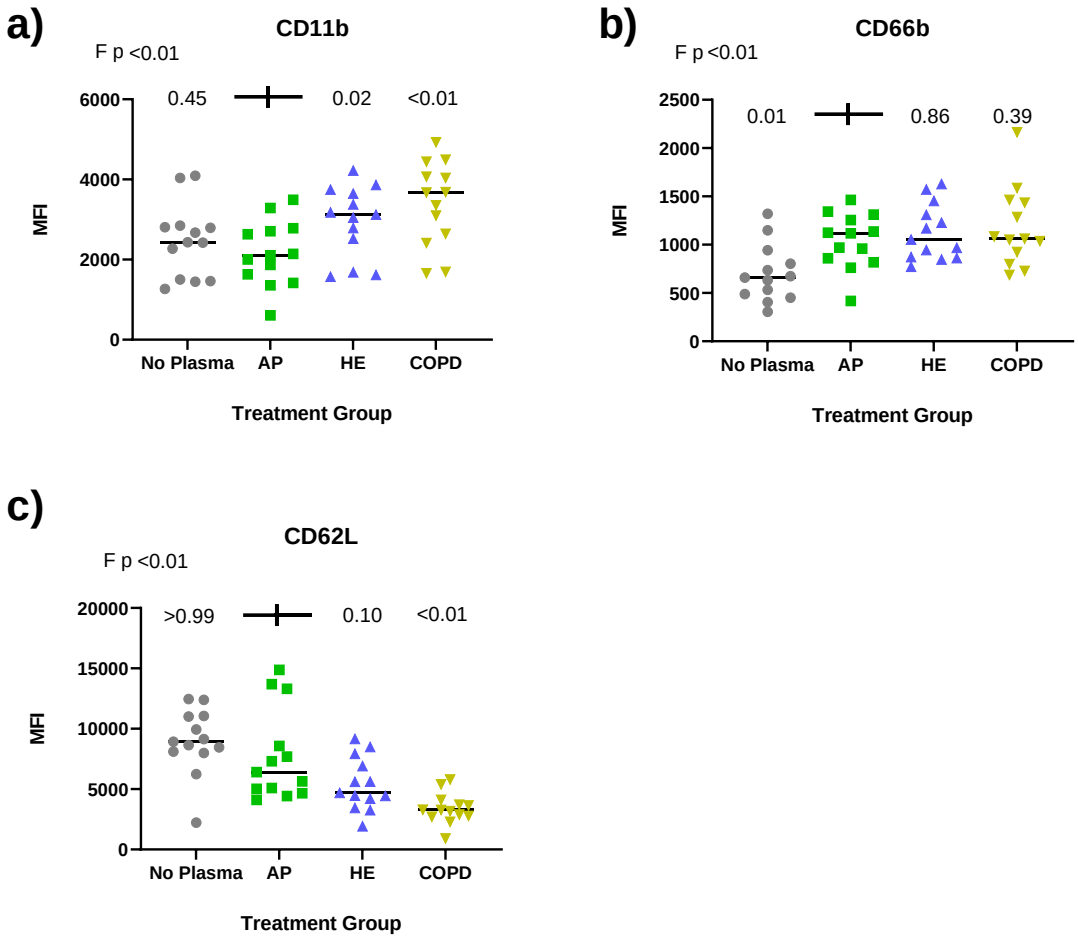

Figure 7.12: The effect of pooled plasma from patients with stable COPD on neutrophil activation markers from healthy participants

Neutrophils from healthy elderly participants (n=13) were isolated from whole blood and incubated with either autologous plasma (AP), pooled plasma from healthy elderly participants (HE) or pooled plasma from patients with stable COPD (COPD). The median fluorescence intensity (MFI) of **a)** CD11b, **b)** CD66b, and **c)** CD62L were measured. In each case, the horizontal line indicates the median value. Statistical analysis performed using a Freidman's test (F) with Dunn's multiple comparison, compared to the AP treatment group.

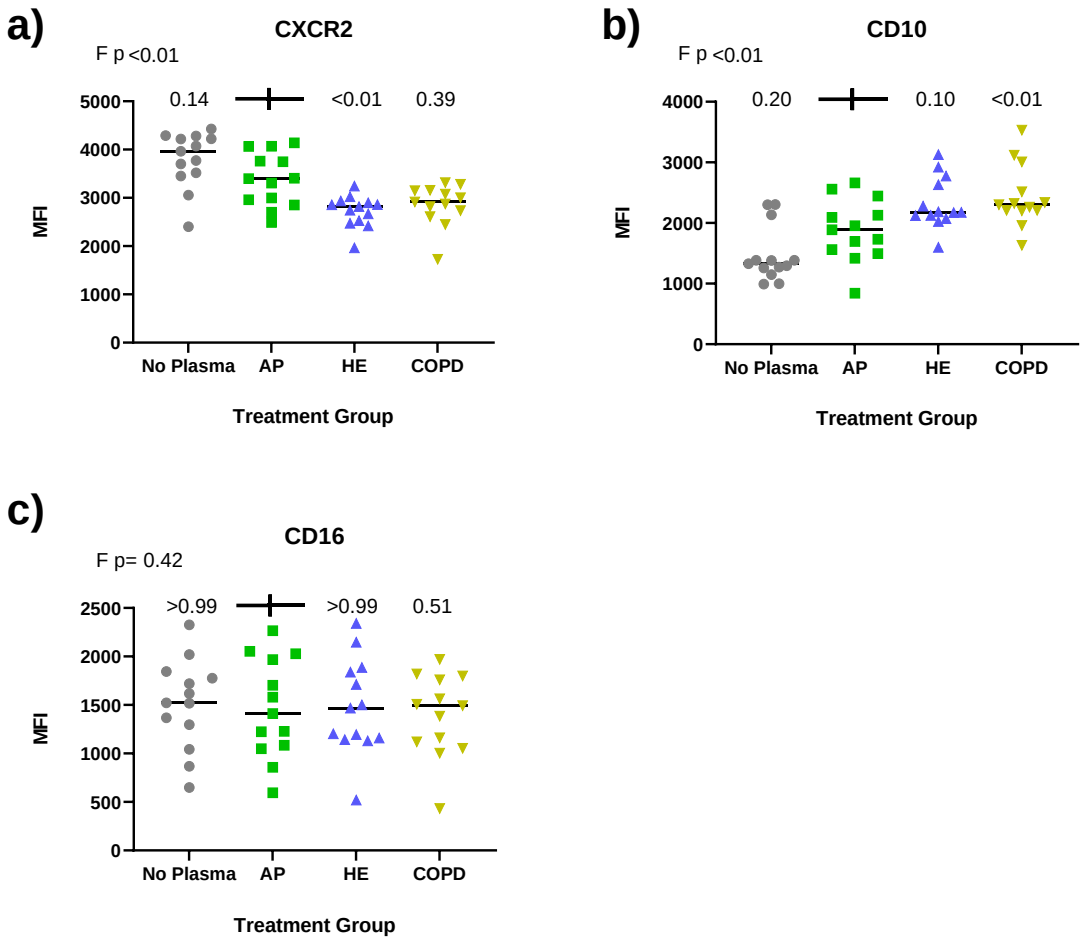

Figure 7.13: The effect of pooled plasma from patients with stable COPD on neutrophil chemokine and maturity markers from healthy participants

Neutrophils from healthy elderly participants (n=13) were isolated from whole blood and incubated with either autologous plasma (AP), pooled plasma from healthy elderly participants (HE) or pooled plasma from patients with stable COPD (COPD). The median fluorescence intensity (MFI) of **a)** CD11b, **b)** CD66b, and **c)** CD62L were measured. In each case, the horizontal line indicates the median value. Statistical analysis performed using a Freidman's test (F) with Dunn's multiple comparison, compared to the AP treatment group.

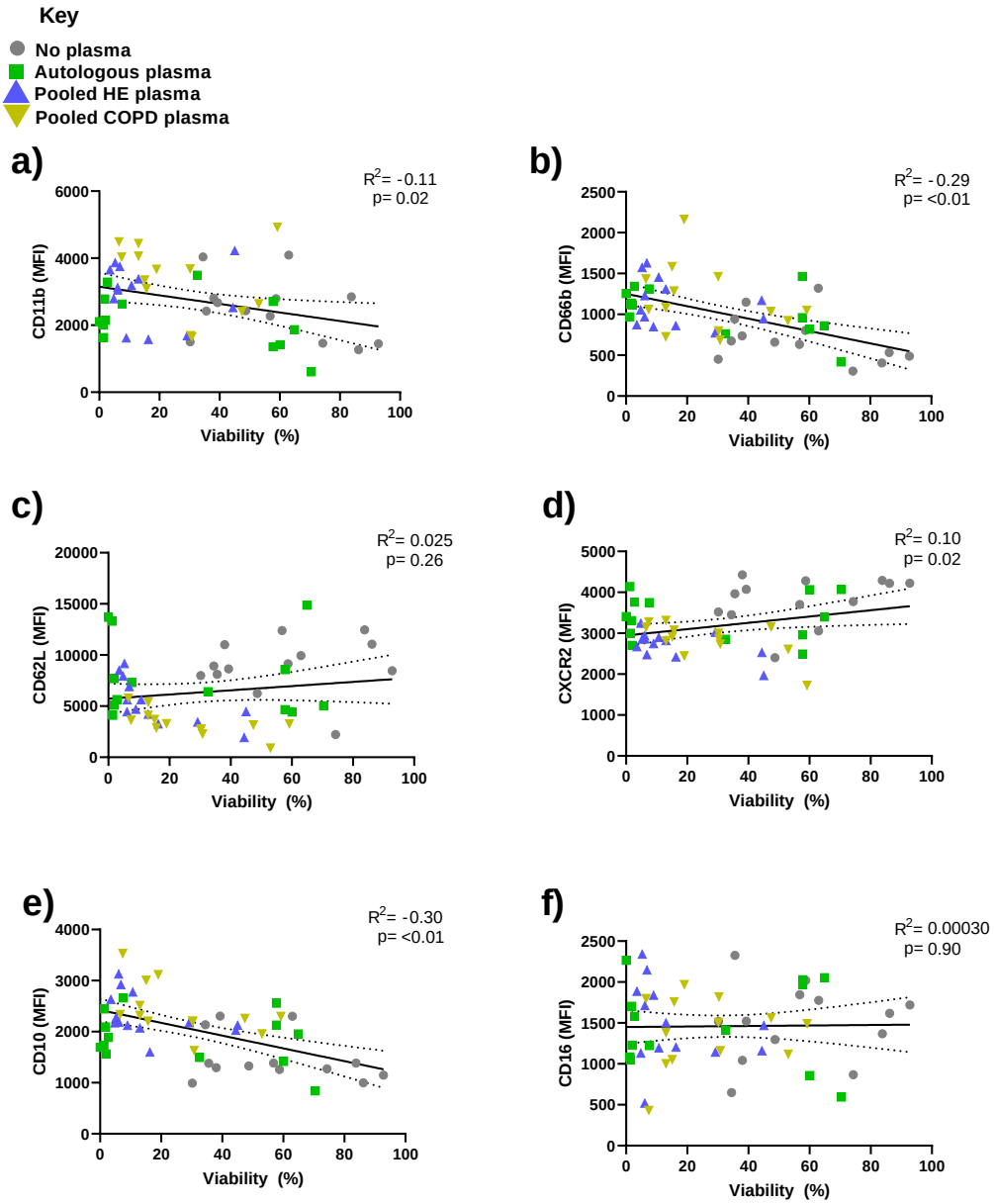

Figure 7.14: Correlation between neutrophil viability and surface expression of activation, chemokine and maturity markers on neutrophils from healthy participants following plasma treatment

Neutrophils from healthy elderly participants (n=13) were isolated from whole blood and incubated with either autologous plasma, pooled plasma from patients with stable COPD, no plasma (control) or pooled plasma from healthy elderly participants (control). The percentage of live neutrophil events was plotted against the median fluorescence intensity (MFI) of **a)** CD11b, **b)** CD66b, **c)** CD62L **d)** CXCR2, **e)** CD10 and **f)** CD16. In each case, linear regression (solid line) is shown with 95% confidence intervals (dotted line). The goodness of fit (r^2) and p-values are indicated for each linear regression.

7.2.3.3 Effect of patient plasma on neutrophil chemotaxis

To further evaluate changes in neutrophils in response to plasma, the chemotaxis of isolated neutrophils towards CXCL8 using Insall chambers was assessed. It was noted that significantly less neutrophils adhered to the coverslips after pre-treatment with either AP or pooled plasma from patients with COPD compared to RPMI treated neutrophils (Figure 7.15). This resulted in less than 10 cells on some coverslips, meaning these conditions could not be analysed. These results suggest plasma pre-treatment altered the ability of neutrophils to adhere to the coverslip.

7.2.3.4 Time-lapse microscopy of neutrophils during plasma incubation

Due to the reduced numbers of neutrophils bound to glass coverslips during plasma incubation, time-lapse imaging was used to visualise the binding dynamics of neutrophils incubated in human plasma. Cells kept in RPMI showed little signs of activation (spreading out of the cell), but adherence after 10 minutes (Figure 7.16). In contrast, cells incubated in pooled plasma show flattening of the cell, indicating activation, but do not appear to properly bind the coverslip (Figure 7.16). After a single wash, as usually performed in these experiments, neutrophils show a less extreme flattening morphology compared to pooled plasma alone, but still do not appear to bind to the coverslip (Figure 7.16). These results suggest incubation with plasma alters the ability of neutrophils to properly adhere to the coverslip.

Due to the lack of adherence and number of cells to track, chemotaxis was not analysed further.

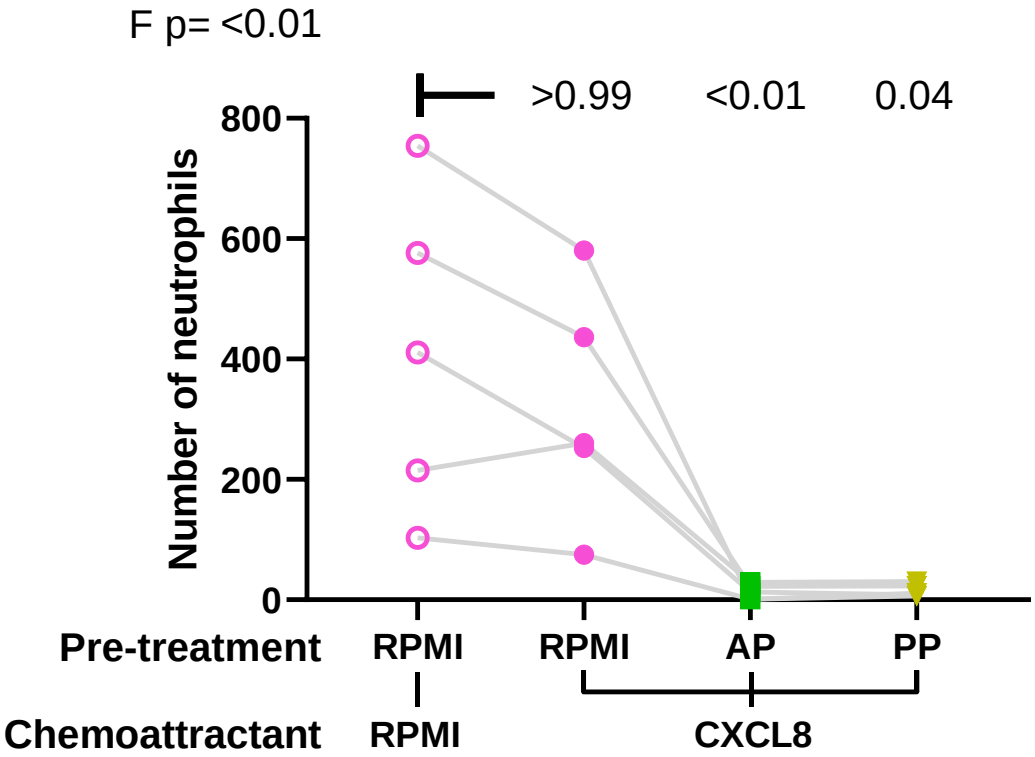

Figure 7.15: Number of neutrophils within the viewing frame of the Insall chamber for each slide imaged

Neutrophils from whole blood from either healthy elderly (HE, n=4) or patients with COPD (n=1) were isolated and incubated with either RPMI, autologous plasma (AP), or pooled plasma (PP) from patients with stable COPD (in the case of HE donors) or an acute exacerbation of COPD (in the case of COPD donors) and allowed to migrate towards either RPMI or CXCL8. The total number of neutrophils within the microscope view were counted for each slide. Each participant is linked by the grey line. Statistical analysis performed using a Freidman's test (F) with Dunn's multiple comparison, compared to the RPMI-RPMI treatment group.

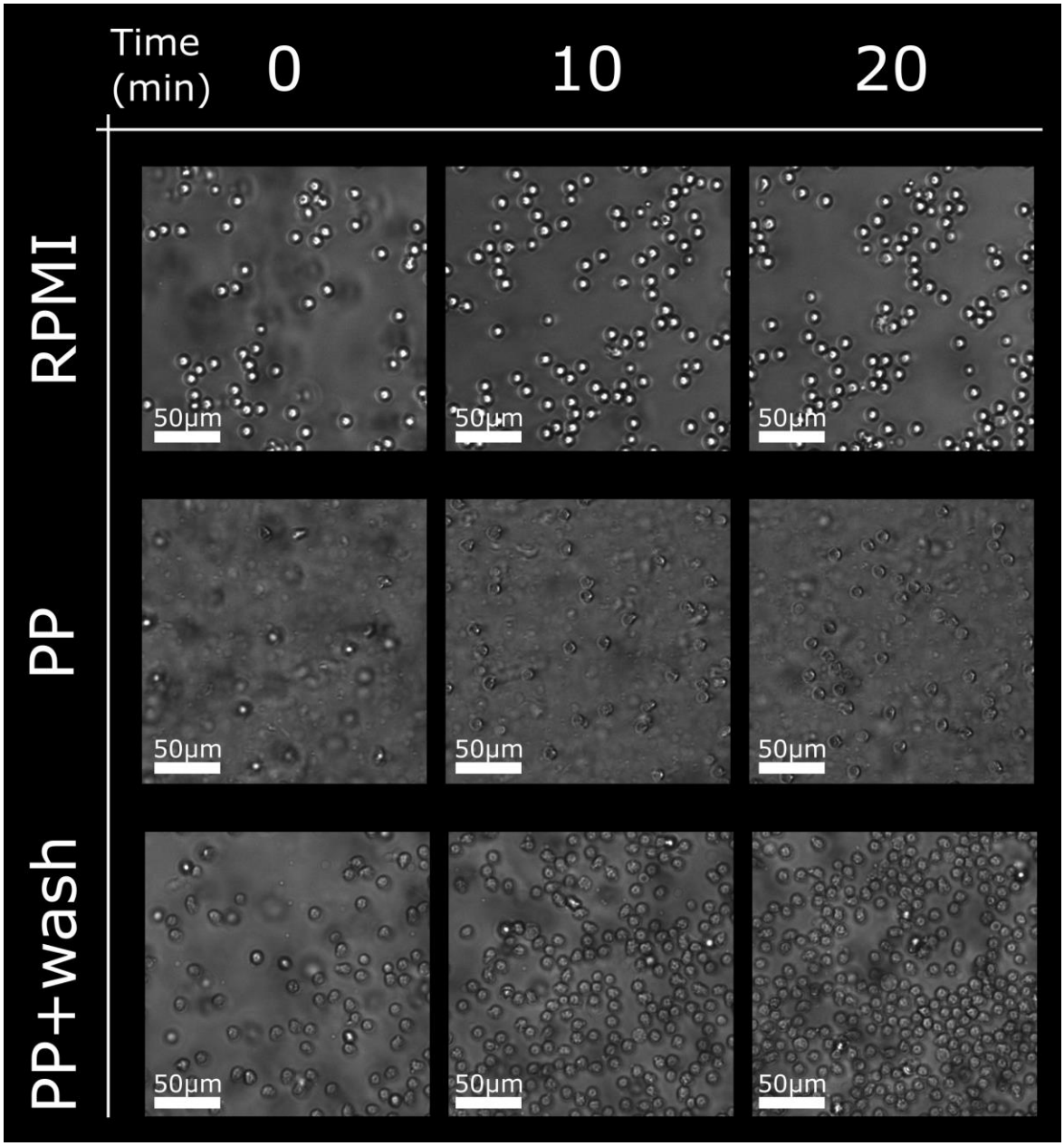

Figure 7.16: Time-lapse microscopy of neutrophils in pooled plasma

Neutrophils from whole blood from healthy young participants (n=2) were isolated and incubated on BSA-coated slides with either RPMI, pooled plasma (PP) from healthy elderly (HE) participants without washing, or after washing once in RPMI. Images were acquired every 20 seconds for 20 minutes during incubation with RPMI or plasma to observe adherence to the slide. Representative images for 0 minutes, 10 minutes and 20 minutes are shown. Scale bar indicates 50µm in length.

7.3 Discussion

Chemotaxis is fundamental process by which neutrophils can migrate towards signals of damage, inflammation and infection, or respond to signals to remain or leave the bone marrow. Here, it was shown that circulating neutrophils from both healthy volunteers and patients with stable COPD are not able to migrate towards CXCL12, the major ligand for CXCR4. As CXCL12 allows retention in the bone marrow and return after senescence, these data suggest that circulating neutrophils in COPD do not show increased senescence. In addition, inhibition of CD10 was not able to significantly alter neutrophil chemotaxis towards fMLP, suggesting a limited role for targeting CD10 to modify neutrophil chemotaxis.

In addition, the increase in activation markers on healthy neutrophils exposed to plasma from patients with stable COPD suggest that systemic inflammatory conditions are present in stable COPD that are capable of activating neutrophils.

7.3.1 Circulating neutrophils show no migratory response to CXCL12

There is interest in the role of different cytokines in patients with COPD as potential therapeutic avenues (Henrot *et al.*, 2019). Neutrophil responsiveness to CXCL12 may alter homing of neutrophils to the bone marrow following senescence and several studies that have reported on neutrophil migration towards CXCL12, with one study suggesting a functional response to synthetic CXCL12 (Ueda *et al.*, 1997), but another study showing no response to natural CXCL12 (Bleul *et al.*, 1996). Here, Insall chambers were used to assess neutrophil chemotaxis.

The Insall chamber provides a unique assessment of neutrophil chemotaxis, allowing cells to be tracked under brightfield microscopy in a stable chemical gradient (Muinonen-Martin *et al.*, 2010). From time-lapse microscopy, multiple parameters can be derived to assess different aspects of cell movement as described in Section 2.7.2. Derivation of these parameters is unique to slide-based migration chambers, as Transwell migration chambers, commonly used in multiple studies (Mócsai *et al.*, 2006; Németh *et al.*, 2010) only allow counting of neutrophils that pass through small membrane pores into a chemoattractant well. In addition, relatively steep chemoattractant gradients are formed with pore-based migration chambers (Wilkinson, 1988; Shutt *et al.*, 1998) compared to shallow gradients formed in the Insall chamber (Muinonen-Martin *et al.*, 2010). Not only are shallow gradients likely more physiologically relevant, but some studies have also suggested that gradient steepness impacts on cell migration and reliance on PI3K signalling. In shallow gradients there was no reliance on PI3K for migratory accuracy (Andrew and Insall, 2007), in contrast to migration in steep gradients (Huang *et al.*, 2003) – where both studies used the model organism *Dictyostelium*. Whilst these may not directly show differences with human neutrophils, the chemoattractant gradient could also influence neutrophil migration. However, Insall chambers do not allow recovery of cells after chemotaxis, whereas migrated cells in Transwell systems can be used for further analysis.

As previously discussed, neutrophil CXCR4 (the receptor for CXCL12) can be increased on the surface of neutrophils when left to 'age' *ex vivo* (Weisel *et al.*, 2009). The data presented in this chapter assesses chemotaxis within a short timeframe (less than 3 hours after blood collection) to assess the functional response with native CXCR4 receptor levels and demonstrated that neutrophils from HY individuals do not show a chemotactic response to

CXCL12. These results are consistent with previous data with freshly isolated human neutrophils from peripheral blood, albeit using a Transwell system (Martin *et al.*, 2003). In addition, data here showed limited evidence that neutrophils from patients with COPD could respond to CXCL12, suggesting unaltered dynamics of neutrophil responses to CXCL12 in COPD compared with health, although further investigation may reveal some response. These analyses are hindered, however, by the reduced signal window as neutrophils from patients with COPD migrate less accurately to fMLP. These data may contrast to neutrophils in the lungs as neutrophils have been reported to have increased CXCR4 expression in both LPS-treated mice (Yamada *et al.*, 2011) and patients with COPD (Hartl *et al.*, 2008). Given the possible differences between circulating and tissue neutrophils, the ability of neutrophils to increase CXCR4 expression in both health and disease may be worth investigating as these may alter dynamics within the tissue.

The concentration of CXCL12 in plasma from healthy and various disease groups has previously been shown to be around 1-2ng/mL (Mehta *et al.*, 2014; Łukaszewicz-Zając *et al.*, 2016). Identification of reduced levels of CXCL12 was perhaps surprising given previous studies suggesting circulating CXCL12 levels were either unaltered in both stable and AECOPD (Dupin *et al.*, 2016) or raised in these patients (Kuźnar-Kamińska *et al.*, 2016). Both of these previous studies utilised different methods for measuring CXCL12 and the impact of using non-platelet poor plasma may have impacted the accuracy of determining CXCL12 levels. Given the low responsiveness to CXCL12, it is unlikely the difference in circulating CXCL12 alters neutrophil dynamics, but may have implications for other cells expressing CXCR4. However, circulating levels does not necessarily relate to bioavailability on endothelial surface and lower circulating levels may, therefore, be due to CXCL12 being bound to endothelial cells.

7.3.2 CD10 inhibition may alter speed, but not accuracy, of neutrophils from healthy donors to fMLP

The pharmacological inhibition of CD10 (e.g. by phosphoramidon) has been shown previously to increase neutrophil transmigration either across cell monolayers (Hofman *et al.*, 1998) or polycarbonate membranes (Shipp *et al.*, 1991) to fMLP. The current study identified that the accuracy of neutrophil chemotaxis was unaffected by CD10 inhibition, but that higher concentrations of phosphoramidon may reduce the overall speed of migration and distance travelled, indicating subtle control of neutrophil migration responses by CD10.

It is hard to directly compare these results to previous studies, as both previous studies mentioned (Shipp *et al.*, 1991; Hofman *et al.*, 1998) measured total neutrophil migration in a binary manner instead of migration characteristics as investigated here. However, it has been shown that the primary fMLP receptor, formyl peptide receptor 1, is rapidly internalised and recycled to the leading edge of neutrophils during chemotaxis (Subramanian, Moissoglu and Parent, 2018). Furthermore, neutrophils showed greater movement even in RPMI than observed during validation work (not shown here), suggesting a reduced signal window to detect changes due to phosphoramidon treatment here. There was also a large variation in responses from individual donors and may indicate large donor variability in this assay.

In theory, blockade of CD10 activity would increase the local concentration of fMLP, as CD10 also functions to hydrolyse fMLP. This may then increase receptor internalisation and slow the response to fMLP without altering directionality. Further studies would be required to identify if indeed inhibition of CD10 significantly altered neutrophil chemotaxis and if it occurred by this mechanism, especially in a population of patients with COPD. Limited use of

phosphoramidon is reported in clinical trials. Two studies have used nebulised phosphoramidon in patients (at a concentration of 1µM) with asthma, showing some increase in bronchodilation, but did not investigate any impact specifically on neutrophils (Crimi *et al.*, 1994; Polosa *et al.*, 1997). The fine-tuning of the neutrophil response to fMLP may provide therapeutic benefits, as multiple attempts have been made to modulate neutrophil migration to reduce the overall neutrophil load in the lungs of patients with COPD (Mårdh *et al.*, 2017), where subtle changes in chemotaxis may be more beneficial than those that directly block chemokine receptors.

7.3.3 Serum and plasma alter neutrophil phenotype

7.3.3.1 Apoptosis

The data presented here show that incubation with autologous serum resulted in a rapid increase in apoptotic neutrophils, identified by annexin V binding, and a subsequent drop in cell viability. Initially, this was thought to be due to serum preparation increasing platelet constituents (Garcia-Aguilar, Lanser and Brown, 1988) or complement proteins (such as C4d and C3a) in serum (Yang *et al.*, 2015). These factors may potentially alter neutrophil behaviour and viability (Nabizadeh *et al.*, 2016), as a similar effect was not replicated with autologous plasma.

However, neutrophils isolated from HE participants and used to assess surface expression in response to plasma demonstrated a similar rapid increase in apoptotic neutrophils and reduction in the percentage of live neutrophils, both with autologous plasma and pooled plasma. These results potentially contrast to other studies where 10% autologous plasma supplementation resulted in prolonged neutrophil survival and resistance to apoptosis

(Alipour *et al.*, 2020). Another study, although not specifically investigating apoptosis, suggested incubation with 50% human serum resulted in reduced neutrophil chemotaxis (Keller, Hess and Cottier, 1974), determined using a Boyden chamber (a pore-based chamber). The reduction in migration of cells across the membrane may in part be due to a reduced ability to bind the membrane (similar to what was demonstrated in Section 7.2.3.4), as neutrophils require a surface to migrate along.

Only a slight decrease in neutrophil viability was observed with HY donors incubated with autologous plasma, two clear populations could be seen when neutrophils from HE donors – 7 donors showing less than 10% live neutrophils and a further 5 showing similar viability to no plasma controls. The differences in response to plasma *ex vivo* may further highlight heterogeneity in the participant population. It is unclear if the increased apoptosis in these neutrophils was in response to initial serum starvation followed by reintroduction to processed serum, as previously reported using a neutrophil cell line with foetal bovine serum (Park *et al.*, 2012). It calls into question whether the observed increase in annexin V binding to the neutrophil surface, in this case, was indeed due to neutrophil apoptosis. Previous work has suggested that neutrophil apoptosis, after incubation at 37^{o}c for 18-24 hours, was also accompanied by a loss in the surface expression of CD16 (Dransfield *et al.*, 1994; Homburg *et al.*, 1995; Noguera *et al.*, 2004). As demonstrated here, similar levels of CD16 expression were observed in both annexin V negative and positive neutrophils. In addition, previous studies investigating neutrophil apoptosis report apoptosis after 4-24 hours (Afford *et al.*, 1992; Coxon *et al.*, 1996; Zhang *et al.*, 2003), suggesting the increase in annexin V binding observed here may represent very early apoptosis prior to the alteration in other surface markers. A study of neutrophils in endometriosis also reported only around 65% apoptotic neutrophils

after incubation of control neutrophils with 10% autologous plasma (Kwak *et al.*, 2002), but this was assessed using neutrophil morphology and not annexin V staining.

Whilst it was not part of the primary investigation, neutrophils from HE participants incubated in pooled plasma from patients with COPD showed consistently higher viability than with pooled plasma from HE participants. These results suggest that, whilst apoptosis was increased compared to no plasma, pooled COPD plasma could partially prevent neutrophil apoptosis – a process that has been described in neutrophils obtained from the sputum in patients with COPD when compared with healthy non-smokers (Brown *et al.*, 2009). Indeed, previous studies have indicated prolonged neutrophil survival after migration into tissue and exposure to cytokines such as IL-1β and G-CSF (Taneja *et al.*, 2004). The identification of apoptotic neutrophils here may represent increased sensitivity of neutrophils to *ex vivo* manipulation but did not prevent identification of the neutrophil surface phenotype.

7.3.3.2 Neutrophil activation

As discussed in Chapter 5, classical neutrophil activation has been previously described by increases in CD11b and CD66b (Costantini *et al.*, 2010), along with shedding of CD62L (Mann and Chung, 2006). Treatment of neutrophils from HE donors with pooled plasma from patients with stable COPD showed significant increases in CD11b expression with a reduction of CD62L expression. This alteration in neutrophil phenotype closely resembles more classical neutrophil activation in response to inflammatory cytokines (Wittmann *et al.*, 2004), suggesting that the systemic conditions in patients with stable COPD are capable of causing neutrophil activation *in vitro.* However, these observations must be held in balance with the significant change in CD11b expression also observed when neutrophils from the same donors

were incubated with pooled plasma from HE volunteers, suggesting partial activation in response to pooled HE plasma. Therefore, isolation of neutrophils may influence their sensitivity to activation.

Regardless of treatment condition, CD11b, CD66b, CXCR2 and CD10 expression showed weak correlations with neutrophil viability in neutrophils from HE volunteers in all treatment conditions – whilst this may suggest a link between these changes and apoptosis, these may be an artefact due to the increase in apoptosis observed in all plasma treatment conditions.

7.3.3.3 Neutrophil chemokine sensing and maturity

Incubation of neutrophils from HE donors with both pooled plasma from other HE donors or patients with stable COPD resulted in a decline in CXCR2 expression – although this did not reach statistical significance in the COPD plasma-treated group. The reduction in CXCR2 expression appears to be associated with neutrophil activation. A previous study also identified that CXCR2 expression was downregulated on neutrophils from patients with ANCA-associated vasculitides that correlated with serum levels of CXCL8 and TNF-α (Hu *et al.*, 2011), suggesting a link with activation status. It is possible, therefore, that the decline in CXCR2 levels observed here is due to receptor internalisation, a process that has been shown to occur rapidly upon neutrophil activation with CXCL8 (Nasser *et al.*, 2007).

Lower CD10 expression is associated with neutrophil immaturity, observed in acute inflammation due to the rapid release of neutrophils that have not yet reached full maturity (Manz and Boettcher, 2014). The data in this thesis suggested that a step-wise increase in CD10 expression was observed in neutrophil CD10 expression following incubation of neutrophils from HE participants in pooled plasma from other HE volunteers or patients with

stable COPD – reaching significance in the latter condition. Clearly, changes *ex vivo* cannot be due to changes in the release of granulocytes and may not necessarily represent previously studied changes in neutrophil maturity. However, these data could suggest that, along with increased neutrophil activation, factors in the circulation of patients with COPD may promote the maturation of neutrophils in the circulation.

7.3.3.4 Changes due to autologous plasma

It was expected that incubation of neutrophils with autologous plasma would not result in significant phenotypic changes in neutrophils. However, neutrophils from HE participants showed significant changes in CD66b and patients with COPD showed significant changes in CXCR2 with autologous plasma incubation. A previous study investigating neutrophils in patients with sepsis and healthy controls identified that the addition of autologous plasma at low concentrations (5%) was able to induce a ROS response in as little as 30 minutes in both cases (Pascual *et al.*, 1998). Another study, incubating neutrophils from healthy individuals in 100% autologous plasma, showed an increase in spontaneous IL-6 production (Oishi and Machida, 1997). These data highlight caution is required when using autologous plasma in neutrophil studies.

In addition, the binding properties of neutrophils to BSA-coated glass slides were altered, demonstrated by a reduction in adherent cells and visualisation of morphological changes in neutrophils whilst incubating on these slides. Therefore, autologous plasma is likely to alter neutrophils even over short periods of time and this may be concentration-dependant. A follow-on study comparing both early cellular apoptosis and surface phenotype over a broader range of plasma concentrations may elucidate neutrophil phenotype changes further. These

results may have broader implications for the study of neutrophils *in vitro*, especially if media is routinely supplemented with autologous plasma.

7.3.4 Summary and limitations

This chapter demonstrates the use of an Insall chamber to assess various aspects of neutrophil chemotaxis towards physiological neutrophil chemoattractants. These data showed that patients with both stable COPD and AECOPD have lower circulating levels of CXCL12 that may have potential implications for neutrophil trafficking, However, it was also demonstrated that neutrophils from healthy volunteers and patients with stable COPD did not migrate towards CXCL12 at any concentration tested.

There was also limited evidence that neutrophil chemotaxis could be altered using a readily available CD10 inhibitor, phosphoramidon, but further investigation is required to determine if subtle changes are present that may be of therapeutic benefit – balancing altered migration without complete inhibition of neutrophil chemotaxis. Future work could address the responses of neutrophils from patients with COPD in response to phosphoramidon inhibition, as well as altered downstream signalling pathways that may be implicated in this response.

These data also highlight caution when using autologous serum or plasma to supplement neutrophil media (Murray *et al.*, 1996; Gray *et al.*, 2018), as this may alter neutrophil viability. The detection of a high percentage of apoptotic neutrophils following serum and plasma incubation potentially confounded the changes in surface expression of other markers, however, the inclusion of apoptotic cells did not appear to alter the detected surface expression. In addition, the lack of binding of these neutrophils to BSA-treated glass slides provided a major limitation to the assessment of chemotaxis after plasma treatment. Further

wash steps, increased incubation time or an alternative format to assess chemotaxis may overcome this limitation.

Key to deciphering if neutrophils in patients with COPD are fundamentally altered or represent a plastic population of cells altered by an inflammatory environment, neutrophils from healthy volunteers show an altered phenotype when incubated in pooled plasma from patients with stable COPD. These changes reflect increased activation, loss of CD62L and CXCR2 and an increase in CD10 expression and represent systemic conditions that may promote neutrophil activation and maturation in patients with COPD. The step-wise increase from incubation with other pooled healthy plasma and plasma from patients with COPD also suggests an element of accelerated ageing based on increasing circulating factors that influence neutrophil phenotypes. Importantly, these results highlight that plasma from patients with COPD altered the phenotype of neutrophils from healthy donors *in vitro* to show increased activation and potentially enhanced maturity.

CHAPTER 8:

GENERAL DISCUSSION

Neutrophils are known to play a role in the pathogenesis of COPD and are the most abundant white blood cells found in lung secretions of patients diagnosed with COPD (Pesci *et al.*, 1998) and both the number and products of neutrophils are linked with multiple aspects of disease in animal and cell models (Shapiro *et al.*, 2003; Singh *et al.*, 2010; J. Liu *et al.*, 2017). There is still a need for disease-modifying therapies and therapeutics targeting neutrophils may allow for fine-tuning of the immune response to reduce collateral damage whilst still maintaining good immune protection from pathogens. The fact many patients with COPD experience persistent and recurrent infection (Patel *et al.*, 2002) despite the presence of neutrophils in the lungs suggests that neutrophil function is altered in COPD, and changes in the circulating neutrophil population may influence the development of other related conditions such as CVD and T2D.

This thesis aimed to investigate the phenotype of neutrophils in healthy ageing, stable COPD and during an exacerbation- the hypothesis that changes in phenotype, specifically increased systemic activation, accelerated ageing and senescence, may enhance our understanding of COPD pathogenesis and progression. Furthermore, stratification of patients based on the presence of co-morbidities may reveal nuanced changes in neutrophil phenotypes that may help guide more tailored therapies and provide a possible explanation to the lack of efficacy of neutrophil-targeted therapeutics in the general COPD population.

The phenotypes investigated here were linked with several previously described changes related to key neutrophil functions, including activation (Särndahl *et al.*, 2007), senescence (Martin *et al.*, 2003), maturity (Marini *et al.*, 2017), pro-inflammatory or anti-inflammatory (McNab *et al.*, 2011; Scrimini *et al.*, 2013) and reverse transmigration (Buckley *et al.*, 2006).

These phenotypes may have functional consequences in disease, including chronic inflammatory conditions, such as COPD and multimorbidity (Hughes, Sapey and Stockley, 2019).

Overall, there was no indication of an accelerated-ageing phenotype in patients with COPD – in part due to the lack of changes during healthy ageing and the absence of a senescence signal and in contrast to the hypothesis. There was also no evidence of increased systemic activation in stable COPD compared with health, however, this signal did exist in patients experiencing an acute exacerbation of COPD. In both cases, there was also no evidence of increased senescence, but a reduction in the expression of CXCR2 that was exaggerated with multimorbidity and during an acute exacerbation, specifically in patients with COPD and CVD with or without T2D, highlighting the differential influence of CVD and T2D on neutrophil phenotype. Indeed, changes in CXCR2 may be influenced by the systemic nature of CVD, highlighting an important mechanism that may increase the disease burden on patients with COPD and CVD. Furthermore, incubation of neutrophils from healthy individuals with pooled plasma from patients with stable COPD resulted in marked increases in activation and maturity markers, suggesting neutrophils from patients with COPD are fundamentally resistant to systemic inflammatory signals. Together, these results reveal subtle changes in the neutrophil phenotype due to both chronic and acute inflammation and the impact of multimorbidity that may direct neutrophil-targeted therapeutics.

8.1 Investigating neutrophils

First and foremost, careful handling of neutrophils *ex vivo* is essential to ensure reliable and accurate conclusions can be made from the data. Here, neutrophils were isolated from

heparinised whole blood by dextran sedimentation of red blood cells followed by discontinuous Percoll gradient isolation, allowing for isolation of live neutrophils and the ability to compare surface phenotype with functional data. Recently published data has shown that Percoll gradient isolation resulted in a higher neutrophil purity compared with similar gradient-based Ficoll isolation following dextran sedimentation (Alipour *et al.*, 2020) and that a priming response can be stimulated *in vitro* with LPS, TNF-α and PAF (Condliffe *et al.*, 1996), suggesting this isolation method does not cause substantial alteration of the neutrophil priming response.

8.2 Neutrophil heterogeneity and phenotypes

Neutrophil heterogeneity has become a topic of debate over several years (Hellebrekers, Vrisekoop and Koenderman, 2018; Rosales, 2018; Silvestre-Roig *et al.*, 2019). Importantly, altered neutrophil phenotypes have been identified independently in the circulation in patients with COPD, CVD and T2D (Reviewed in Hughes, McGettrick and Sapey, 2020). These changes may provide insight into both disease pathogenesis, outcomes, and avenues for therapeutic intervention. Whilst changes in individual markers between HY, HE and patients with stable COPD were limited, multi-dimensional cluster analysis highlighted a gradient of expression of each marker rather than distinct subpopulations. These subtle gradient-like changes suggest neutrophil phenotypes are fluid and less discrete than absolute changes in a single marker. The implications of this are two-fold: it is unlikely a pathogenic subpopulation of neutrophils exist in COPD and therapeutics targeting neutrophils that cause small shifts in neutrophil behaviour may provide the most clinical benefit – allowing neutrophils to perform their protective role and reduce collateral damage.

8.2.1 Systemic activation of neutrophils

Given that many inflammatory cytokines have been shown to be raised in COPD, AS and T2D, including TNF-α (Fahim, Halim and Kamel, 2004; Hatanaka *et al.*, 2006; Singh *et al.*, 2018) and IL-6 (Barnes and Celli, 2009), it would not be surprising if there was increased systemic neutrophil activation in these patients, especially those with multimorbidity. Several studies have suggested systemic activation of neutrophils in COPD based on increased ROS production (Noguera *et al.*, 2001; Vaguliene *et al.*, 2013) and the presence of systemic NE or MPO (Vaguliene *et al.*, 2013), however, these do not provide direct evidence of neutrophil activation.

The data presented in this thesis shows that systemic neutrophil activation, measured using CD11b, CD66b and CD62L, was not significantly altered either with increasing age or the presence of stable COPD – regardless of multimorbidity. These observations are supported by similar data generated from Dr Sapey's group where comparable levels of CD11b and CD62L were observed on neutrophils from HE donors and patients with stable COPD (J. Stockley, 2015). Whilst overall no increase in activation was observed, a subset of patients demonstrated raised levels of CD11b and CD66b – however, these did not correlate with clinical severity of COPD or smoking history, suggesting other factors may play a role in causing increases in systemic neutrophil priming and activation that requires further investigation. Indeed, direct measurement of circulating cytokines in these patients may explain these differences in activation status. All these observations were supported by no overall increase in the percentage of $CD11b^{bright}CD66b^{bright}$ neutrophils between groups. Whilst not statistically significant, it appeared that gene expression of both CD11b (ITGAM) and CD66b (CEACAM8)

were increased in patients with COPD compared to both healthy groups, suggesting a potential to upregulate these surface proteins. There was no correlation between gene expression and surface expression data, albeit only available for a subset of samples, that may suggest increased intracellular stores of these proteins – however further investigation would be necessary to draw this conclusion.

Specifically addressing surface marker expression, a previous study suggested that neutrophil priming occurs in peripheral neutrophils from patients with COPD, identified by a reduction in CD62L expression, but no change in CD11b expression (Lokwani, Wark, Baines, Fricker, *et al.*, 2019). Notably, this study performed antibody staining in whole blood before red blood cell lysis. Another study again indicated similar CD11b expression levels between patients with COPD and healthy controls (both smokers and non-smokers), although data was only shown for apoptotic neutrophils (Noguera *et al.*, 2004). The same group had, however, previously shown an increase in CD11b expression in neutrophils from patients with stable COPD (Noguera *et al.*, 1998) - although these patients had lower lung function and neutrophils were isolated by a different isolation procedure. Collectively, the impact of isolation method and patient group examined may explain subtle differences between both the data in this thesis and other studies, further highlighting the necessity for careful handling of cells *ex vivo* and the need for multiple studies to build a clear picture. As *ex vivo* stimulation of neutrophils from healthy individuals to pooled plasma from patients with COPD demonstrated a loss of CD62L expression – exposure of neutrophils to activation signals during handling may cause the decline in CD62L in some previous studies.

Direct comparison of neutrophils from both healthy donors and patients with COPD suggest that systemic neutrophil activation is not a predominant feature of stable COPD and partial priming or activation of neutrophils may be differentially impacted by the isolation technique employed. However, neutrophils from HE volunteers incubated with pooled plasma from COPD showed changes in the surface expression of CD11b and CD62L that appear consistent with classical neutrophil activation (Wittmann *et al.*, 2004; Vogt *et al.*, 2018). Together these data suggest that factors, such as cytokines and other signalling molecules, are present in the circulation of patients with COPD and are capable of activating healthy neutrophils *ex vivo*. The lack of systemic activation in patients with COPD, therefore, may be due to a reduction in the sensitivity of these neutrophils to pro-inflammatory stimuli. Another possible hypothesis is that activated neutrophils in patients with COPD leave the circulation and enter the lung or other inflammatory compartment, following chemokine gradients – although an increase in total circulating neutrophils in these patients makes this explanation less likely (Günay *et al.*, 2014).

Furthermore, during an acute exacerbation, increased systemic neutrophil activation was identified by increased expression of CD11b and CD66b (including the percentage of $CD11b^{bright}CD66b^{bright}$ neutrophils) compared with stable COPD. The observation of increased neutrophil activation in AECOPD is in line with the systemic inflammation seen in other studies of AECOPD (Aaron *et al.*, 2001; Hurst, Perera, *et al.*, 2006; Sapey and Stockley, 2006). In addition, neutrophils stimulated *in vitro* with GM-CSF and TNF-α (Koenderman *et al.*, 2000) showed a similar phenotype to neutrophils in patients with AECOPD shown in this thesis and a previous study (Koenderman *et al.*, 2000). It should be noted that it has been suggested these changes in CD11b and CD66b expression systemically are more aligned with neutrophil

priming prior to extravasation, as CD54 expression, a marker not altered in these samples, represents the final stages of neutrophil activation after extravasation into the tissue (Pillay *et al.*, 2012).

Of note, CD62L expression remained similar between both stable and AECOPD, suggesting priming or activation similar to that identified *in vitro* with PAF (Condliffe *et al.*, 1996). A lack of CD62L shedding, but an increase in other neutrophil activation markers, has also been observed in patients with sepsis (Lewis *et al.*, 2015), potentially suggesting the increased systemic inflammatory burden in AECOPD contributes to maintenance of CD62L.

Notably, a subset of patients did not show an increase in these activation markers, potentially suggesting not all patients presenting with AECOPD have the same level or type of systemic inflammatory burden. An increase in the inflammatory burden, especially one seen systemically here in neutrophils, may link to the increased mortality due to cardiovascular complications (Austin *et al.*, 2016; El-Shabrawy and Eldamanhory, 2017). In addition, similarities to patients with sepsis may suggest similar mechanisms of peripheral neutrophil activation, allowing aspects of the clinical management of patients with sepsis to be applied to patients with AECOPD.

8.2.2 Changes in other neutrophil markers

Novel phenotypes of neutrophils may provide further insight into the dysfunction of these cells in patients with COPD. The data presented here demonstrated similar expression of HLA-DR, CD11c, CD54 and CD10 on neutrophils from HY, HE, stable COPD and in AECOPD. As well as surface expression, no statistically significant differences in gene expression for these markers was observed.

Both HLA-DR and PD-L1 expression on neutrophils have been linked with inflammatory status, where HLA-DR indicates pro-inflammatory and PD-L1 anti-inflammatory neutrophils. Neither marker has been shown to be expressed by neutrophils in the circulation taken from healthy volunteers (Reinisch *et al.*, 2003; Luo *et al.*, 2016). Expression of HLA-DR by neutrophils has been linked with exposure to GM-CSF both *in vitro* (Gosselin *et al.*, 1993) and *in vivo* (Mudzinski *et al.*, 1995) that can cross-present antigens to T cells – although the *in vivo* study only included two patients. A single study reported increased HLA-DR expression in the circulating neutrophils compared with healthy non and current smokers in a large cohort of patients (n=53), but showed large variation in HLA-DR expression in each group (Scrimini *et al.*, 2013). Nevertheless, the data in this thesis shows almost complete lack of surface expression on neutrophils from any group tested, suggesting neutrophils in COPD do not acquire cross-presentation properties that activate T cells in the circulation.

Similarly, PD-L1 expression was only detected at very low levels on all healthy control and stable COPD samples. Neutrophils from patients with AECOPD showed a small, but statistically significant, increase in PD-L1. PD-L1 expression has previously been shown on neutrophils in mice and patients with sepsis (Wang *et al.*, 2015) and may represent an anti-inflammatory response as PD-L1 supresses T cell responses (Keir *et al.*, 2008). Combined with the changes in the activation of neutrophils also showing a similar phenotype to sepsis, the increase in PD-L1 may, therefore, represent a balancing mechanism in patients with COPD during increased in systemic inflammation, one that could be enhanced to reduce further inflammatory responses in other compartments of the immune system. These data provide an exciting potential avenue for therapy that requires more investigation to identify if indeed such low expression of PD-L1 by neutrophils provides any clinical benefit, or if it could be increased.

An increase in the presence of CD54 expressing neutrophils in the circulation may have indicated neutrophils that had previously been within the inflamed lung re-entering the circulation – providing a link between COPD, CVD and T2D whereby neutrophils could be activated in one compartment and move back into the circulation, causing damage within another organ. Previous work has indeed linked an increase of reverse transmigrated neutrophils with increased lung injury during acute pancreatitis in human (Wu *et al.*, 2016) and with atherosclerosis (Buckley *et al.*, 2006) – although in both cases the percentage of neutrophils with this phenotype was very low. In this thesis, there was no increase in this population in patients with COPD compared to healthy volunteers, suggesting reverse transmigration of neutrophils is not enhanced in patients with COPD.

Equally, the maturity status of circulating neutrophils appeared similar in all population groups measured in contrast to previously published studies where inflammatory signals seen during sepsis (Taneja *et al.*, 2008), invasive cardiac surgery (Orr *et al.*, 2005) or severe COVID-19 (Carissimo *et al.*, 2020) promoted the release of immature neutrophils. There does not appear to be previous studies that have investigated the expression of CD10 on neutrophils in the circulation in COPD, CVD or T2D.

Of note, incubation of neutrophils from HE donors with pooled plasma from patients with COPD appeared to increase CD10 expression. These data may suggest circulating factors that may promote an acceleration in maturation in patients with COPD, allowing for unaltered maturity status of neutrophils in the circulation despite the potential for emergency granulopoiesis.

Together, these suggest that neutrophils in patients with COPD, regardless of exacerbation or the presence of multimorbidity, do not show evidence of increased immaturity, reverse transmigration or a pro-inflammatory phenotype. Here, there is some evidence that an anti-inflammatory phenotype of PD-L1 expressing neutrophils may emerge during AECOPD.

8.3 Neutrophil senescence in COPD

The use of senolytics, drugs that selectively remove senescent cells, have been discussed as potential therapies for patients with COPD as senescent cells show, in general, reduced useful functionality compared to non-senescent cells (Drew, Wilson and Sapey, 2018; Baker, Donnelly and Barnes, 2020). Both CVD and T2D have also been linked with an increased burden of senescent cells with β-cell replication slowing past the age of thirty, limiting repair (Perl *et al.*, 2010) with a reduction in functional β-cells (Butler *et al.*, 2003), and vascular smooth muscle cells and endothelial cells around atherosclerotic plaques becoming more senescent (Vasile *et al.*, 2001; Libby *et al.*, 2002). However, as mature neutrophils do not replicate (Klausen *et al.*, 2004), neutrophil 'senescence' requires a more complex interpretation than replicative senescence as coined by Hayflick and Moorhead (Hayflick and Moorhead, 1961; Kaplon *et al.*, 2013). CXCR2 and CXCR4 are both key chemokine receptors for neutrophils and have repeatedly been shown to control neutrophil retention and subsequent release from the bone marrow into the circulation and then migration to sites of inflammation (Strydom and Rankin, 2013). It is these two receptors that have been implicated in neutrophil senescence, where CXCR4 is increased and CXCR2 reduced, allowing 'homing' back to the bone marrow as a mechanism of neutrophil clearance from circulation (Martin *et al.*, 2003; Eash *et al.*, 2010; Casanova-Acebes *et al.*, 2013).

However, there is very little evidence for neutrophil senescence *in vivo* in human, with previous studies observing this phenotype during *in vitro* ageing (Nagase *et al.*, 2002; Yildirim *et al.*, 2005; Weisel *et al.*, 2009). The findings from murine studies may, therefore, not directly relate to neutrophil behaviour or phenotype in human. Nevertheless, assessment of these two receptors may identify a senescent phenotype and indicate if an increased burden of senescent neutrophils in the circulation may impact on COPD and link with accelerated ageing and multimorbidity.

8.3.1 Chemokine sensing via CXCR2 and CXCR4

As CXCR4 expression remains low and unaltered in all groups tested and neutrophils from both HY and patients with stable COPD show no functional chemotaxis to CXCL12, these data do not support a change in neutrophil senescence in COPD or multimorbidity. Low levels of CXCR4 expression on mature neutrophils has previously been shown to be unresponsive to CXCL12 (Martin *et al.*, 2003), supporting the findings here. Despite some studies suggesting that the CXCL12-CXCR4 axis may play a role in COPD in other cell types (Henrot *et al.*, 2019), it appears CXCR4 expression by circulating neutrophils has not been investigated in COPD, CVD or T2D.

Here, CXCR2 expression was not altered during healthy ageing, but is reduced in stable COPD and further reduced in AECOPD. These changes suggest that neutrophils in these patients have a reduced ability to sense chemokines, impacting on the ability of these neutrophils to appropriately respond to these inflammatory signals. Further investigation revealed that CXCR2 expression was not significantly reduced in patients with COPD alone, but in patients that also had CVD as a co-morbidity, revealing a role of multimorbidity in neutrophil phenotype. Previous observations of increased CXCL2 levels, another cytokine that signals

through CXCR2, in patients with CVD (Guo *et al.*, 2020) may suggest why CXCR2 levels are reduced specifically in patients with COPD and CVD, as CXCL2 binding may increase CXCR2 internalisation. However, the same reduction in CXCR2 was not induced to the same extent in neutrophils from HE volunteers with pooled plasma from patients with COPD. Together, these data are suggestive of altered chemokine sensing, but not of senescence, and that there may be a chronic change in CXCR2 expression by neutrophils from patients with COPD.

Reduced CXCR2 surface expression may pose a possible explanation to reduced migratory accuracy observed previously in patients with COPD (Sapey *et al.*, 2011), and patients with AECOPD are likely, therefore, to have even more collateral damage caused by ineffective sensing of chemokines. RNASeq performed in this thesis highlighted multiple genes linked with chemotaxis were also altered in the neutrophils of patients with AECOPD compared with stable disease, further providing evidence that neutrophil chemotaxis is significantly impacted during exacerbations.

Altered CXCR2 expression may also have implications for therapeutics targeted at this receptor. CXCR2 inhibitors have been repeatedly shown capacity to reduce neutrophil infiltration into the tissue in animal models, first in reduced neutrophil margination in rabbits (White *et al.*, 1998). Several other papers have supported that blockade of CXCR2 reduced neutrophilic inflammation: in CS-exposed rats (Stevenson *et al.*, 2005); in an acute lung injury model in mice (Lomas-Neira *et al.*, 2004); in an LPS airway challenge model in guinea pigs (Gordon, 2005); and recently in murine models of neutrophilic airway inflammation (Mattos *et al.*, 2020). Despite *in vitro* and *in vivo* biological evidence, trials in humans have yielded little success, either due to lack of clinical efficacy (Keir *et al.*, 2020; Lazaar *et al.*, 2020), or safety

concerns linked with neutropenia (Aaron, 2015; Rennard *et al.*, 2015). Targeting a receptor that has reduced expression on neutrophils in COPD compared with health may provide a contraindication to the use of these therapies in COPD, especially those patients with underlying CVD.

The data within this thesis suggests that a decline in CXCR2 receptor expression may impact on both the ability of these drugs to perform well in patients with COPD, but also that blocking CXCR2 may, in part, further reduce the ability of neutrophils to accurately migrate towards the inflammatory site, inadvertently increasing long-term tissue damage. Overall, reduced expression of CXCR2 by neutrophils in patients with COPD, particularly those with CVD or during an exacerbation, could result in inaccurate migration and increased collateral damage, both in the lungs and if recruited to other sites of inflammation.

8.4 Do these changes represent accelerated ageing?

One key aspect to investigate was the concept of accelerated ageing specifically for neutrophils in COPD. Previous work have indeed suggested that COPD is a disease of accelerated ageing, showing many aspects of ageing such as a decline in lung function, increased cellular senescence and loss of anti-ageing processes (Ito and Barnes, 2009; Barnes, 2017). For neutrophils, it was expected that an accelerated ageing phenotype would be shown by either: a change occurring between healthy young and elderly populations that is exaggerated in patients with COPD; or an increase in the proportion of senescent cells in patients with COPD. The data presented in this thesis do not convincingly suggest either. Neutrophils from healthy young and elderly volunteers showed very similar phenotypes in

terms of surface expression, one that is carried through to patients with COPD with only one notable exception – CXCR2 expression.

Previous data has suggested that an age-related decline in neutrophil chemotaxis does exist and is further reduced in COPD – supporting accelerated ageing (Sapey *et al.*, 2011, 2014). In this thesis, reduced CXCR2 expression was observed in patients with COPD, further reducing during an exacerbation, but there was not evidence of this change with 'healthy' ageing. These data suggest changes in CXCR2 expression are, therefore, a contributary factor in reduced migratory accuracy, but not wholly responsible for age-related declines in neutrophil migration accuracy.

It must be stated, however, that these data only highlight changes specifically in neutrophils and do not conflict with evidence of accelerated ageing in other cell types (such as endothelial cells and fibroblasts) or processes that have been previously described (Ito and Barnes, 2009; MacNee, 2009). Indeed, they suggest that neutrophils themselves only have very a limited role in the overall accelerated ageing phenotyping in COPD, further supported by the lack of changes in gene expression with ageing.

8.5 Study limitations

In all the elements of the study shown in this thesis, neutrophils were isolated from peripheral blood. This enables specific investigation of neutrophil function and identification of systemic neutrophil phenotypes that may link multiple diseases. However, it is known neutrophils within the lungs are fundamentally altered compared to the periphery (Fortunati *et al.*, 2009) and are where many pathogenic effects of neutrophils are observed within COPD. Therefore,

the study of neutrophils in the periphery only provides one view on neutrophil function and phenotype.

The process of isolating neutrophils for *in vitro* study also has the possibility to alter neutrophil phenotypes, including the methodology employed in this thesis. The sedimentation of red blood cells with dextran was previously shown to increase neutrophil activation, potentially due to monocyte interactions with neutrophils during this procedure (Quach and Ferrante, 2017) and gradient-based isolation may reduce the ability of neutrophils to respond to subsequent stimulation (Mosca and Forte, 2016). These changes may influence the data in this thesis, although a consistent method should not alter comparisons between groups. Whilst a limitation, common alternatives such as red blood cell lysis (Vuorte, Jansson and Repo, 2001), fixation of samples from whole blood (van Staveren *et al.*, 2018) or antibody-based isolation (Hasenberg *et al.*, 2011) can also alter neutrophil phenotype, inherently precludes functional analysis or have a large cost implication.

In addition, the study of neutrophils in isolation removes the influence of other cell types. This may be of particular consideration in a complex disease such as COPD, as other cell types, especially macrophage, are known to influence the severity and progression of COPD and the interaction of neutrophils with the lung is likely to alter their phenotype.

Whilst every effort was made to appropriately age and sex-match HE participants with the relevant patient populations, it was not possible to provide a good match on smoking history. As smoking is known to have multiple impacts both on neutrophils, other immune cells and the inflammatory environment of the lung, better matching patients and controls based on smoking history would provide a more robust comparison.

In addition, during the stratification based on multimorbidity, it was not possible to match all patient groups based on lung function or GOLD Stage due to the patient pool available and possibly also due to the impact multimorbidity may have on the severity of COPD. Therefore, it is not possible to rule out the effect of reduced lung function as a confounder to the differences observed – however, it may also be a feature of interest that multimorbid patients present with the most severe disease.

It is important to consider the impact neutrophil function and phenotype has on clinical parameters and disease progression. Whilst generally smoking history and lung function data was available for patients with COPD (both stable and exacerbating), other clinically important metrics, such as symptom scores and exacerbation history, were not available for all patients. The collection of these data at appointments may have provided further insight to the impact of the investigated neutrophil phenotypes on disease-relevant parameters. In addition, it was not possible to obtain lung function data on healthy controls due to technical limitations, that may have elucidated some variation observed in the control group.

The inhibition of neutrophil binding during the assessment of neutrophil chemotaxis prevented the analysis of pooled plasma treated neutrophils. Therefore, it was not possible to investigate the link between the neutrophil phenotype and migration in these conditions.

Inevitably, conclusions drawn from some of the data presented were limited due to the number of patients recruited – particularly those with multimorbidity and for samples used to investigate the effect of pooled plasma. Unfortunately, the research study was halted prematurely due to the COVID-19 pandemic. In addition, correlations of surface expression

with clinical metrics are underpowered and would require a greater number of patients to be investigated fully.

8.6 Future work

Multiple aspects of neutrophil phenotypes have been investigated in this thesis, building upon data both from other members of this research group, and the increasing wealth of information regarding neutrophil phenotypes. However, it also raised additional questions that would require further investigation.

1. As neutrophils will primarily cause damage when in the lung of patients with COPD, it is important to understand the phenotype and function of these cells in the lung and collection of both blood and lung secretions would enable more detailed phenotyping in patients with COPD, especially as lung neutrophils would be the primary target for therapeutics being administered by inhalation (Axson *et al.*, 2020). Previous comparisons of neutrophils isolated from sputum and peripheral blood in healthy volunteers identified that neutrophils in the sputum (and therefore had undergone migration into the lung) displayed higher expression of CD11b and HLA-DR, suggestive of an inflammatory phenotype (Alexis *et al.*, 2000).
2. Investigating how migration through the lung tissue alters the neutrophil phenotype may further reveal differences in healthy ageing and in COPD. Studies using a lung slice model would allow this to be investigated and assess the influence of other physiologically relevant cell types on neutrophil phenotype. In addition, an alternative model of neutrophil chemotaxis could be investigated both to alleviate the lack of

neutrophil binding to glass slides after incubation with plasma and provide further confirmation of the findings in this thesis.

3. Investigating if the shift in neutrophil phenotype seen in AECOPD recovers following the exacerbation by following up patients to at least 56 days (generally accepted when a patient is clinically stable) and comparing this to patients with stable COPD.
4. Furthermore, whilst technical challenges exist, it would be beneficial to monitor the neutrophil phenotype in COPD prior to an exacerbation to determine if fundamental differences exist in neutrophils either prior to an exacerbation and may be predictive of frequent exacerbators.
5. Lung function decline in patients with COPD is variable and has a large quality of life impact on patients. Repeat sampling of the same patients over time would allow for longitudinal studies on neutrophil phenotypes to determine if these change over time, and if this correlates, or could be predictive of, lung function decline. Expanding the number of patients recruited would also allow for better matching of patients based on both lung function and disease severity parameters.
6. As CXCR2 was a major receptor altered both in stable COPD and during an exacerbation, further work could build on both this and previous investigations of receptor signalling to uncover potential downstream alterations in neutrophil behaviour due to reduced CXCR2 expression and the impact therapeutic blockade. In addition, expression of receptors for other chemoattractants such as fMLP could be investigated to identify if a similar alteration is observed that may be suggestive of a common mechanism of reduced migrational accuracy.

7. Further targeted gene expression analysis could be conducted following on from the data presented here, using more concise gene expression arrays to allow for increased sample number, especially between HE and stable COPD samples. This could also be expanded to allow for stratification of patients with COPD based on multimorbidity to further investigate the role multimorbidity may play in neutrophil gene expression, revealing potentially discrete neutrophil populations.
8. The identification of ARHGAP24 as a differentially expressed gene in neutrophils between HE participants and patients with COPD warrants further investigation to determine if this has any functional relevance to the role of neutrophils in COPD.
9. Aspects of accelerated ageing were investigated, however, the impact of a loss in anti-ageing processes was not investigated. These analyses, including looking for epigenetic changes, may reveal an aspect of accelerated ageing not yet studied in both neutrophils and multimorbidity within COPD.

8.7 Overall findings and conclusion

There is an abundance of evidence to support that neutrophils play a critical role in COPD. Here, a robust method for examining the surface expression of key functional markers on neutrophils is described using a well-validated flow cytometry panel, providing a platform for future studies.

Broadly, neutrophils from patients with COPD do not show evidence of increased systemic activation, immaturity, pro or anti-inflammatory phenotypes or increased reverse transmigration compared with healthy age-matched controls. These phenotypes were not

altered by the presence of multimorbidity nor do they show elements of accelerated ageing, but heterogeneity within the study population was identified.

Instead, a reduction in CXCR2 expression was observed on neutrophils from patients with COPD with CVD, with or without T2D, suggesting that neutrophil chemokine sensing through CXCR2 may be altered specifically in these multimorbid patients. Furthermore, a reduction in CD16 was observed in patients with COPD with or without T2D and without CVD, suggesting a potential banded neutrophil population that may have functional consequences such as reduced phagocytosis. These data begin to highlight the importance of multimorbidity in differentially altering systemic neutrophil phenotypes that may also have broader implications for future therapeutics targeting neutrophils, allowing more tailored options by understanding the influence inflammatory diseases have on each other.

However, an activated phenotype could be induced in healthy neutrophils *in vitro* by pooled plasma from stable COPD patients – suggestive that a fundamental alteration in circulating neutrophils from patients with COPD may make them less sensitive to low-level inflammatory mediators, or represent a form of exhaustion.

In contrast, multiple changes occur both at the gene level and surface expression level during an acute exacerbation. Increased systemic activation, like that seen in sepsis, reduced CXCR2 expression and a small, but significant increase in PD-L1 expression, indicative of anti-inflammatory activity, are seen in neutrophils from patients with AECOPD. Combined, these results show both plasticity in the circulating neutrophil population, but also insights into altered neutrophil function in patients with AECOPD.

Together, these data suggest that subtle heterogeneity exists in circulating neutrophils, and that different phenotypes of neutrophils are differentially observed in multimorbid patients and further altered in AECOPD compared to stable disease. Accelerated ageing of neutrophils was not observed based on either stepwise changes in phenotype during ageing and the presence of COPD, or by increased senescence. These data highlight the importance of understanding patient heterogeneity in COPD based on multimorbidity and exacerbation status. They also draw attention to alterations in circulating neutrophil phenotypes in COPD that do not necessarily manifest by direct comparisons of health to COPD, but instead by the potential of healthy neutrophils to be altered by exposure to the inflammatory conditions in COPD.

CHAPTER 9:

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CHAPTER 10:

APPENDICES

10.1 Appendix 1: Participant exclusion and inclusion criteria

Participants without COPD

Inclusion for Participants without COPD
1. Provision of informed written consent
2. Age 18 years or over

Exclusion for Participants without COPD
1. Significant respiratory symptoms including breathlessness and cough
2. Diagnosis of COPD, Asthma, Interstitial Lung disease, Bronchiectasis, Lung Cancer, Previous Lung resection
3. Haematological malignancy
4. Immunosuppressive medications
5. Lack of capacity to provide informed consent

Participants with COPD

Inclusion Criteria for stable COPD patients
1. Diagnosis of COPD with post bronchodilator FEV1/ FVC ratio <0.7 according to GOLD guidance
2. At least 10 pack year history of smoking
3. Provision of informed written consent
4. Age 40 years or over
5. For COPD patients with Cardiovascular disease*: a physician confirmed diagnosis of previous myocardial infarction, angina or left ventricular dysfunction thought secondary to atherosclerosis
6. For COPD patients with Diabetes**: a physician confirmed diagnosis of type 2 diabetes.

Exclusion Criteria for stable COPD patients
1. Diagnosis of Asthma, Interstitial Lung disease, Bronchiectasis, (fibrosis related to emphysema or bronchiectasis related to COPD is not an exclusion), Lung Cancer, Previous Lung resection
2. Lack of capacity to provide informed consent
3. Age 39 years or under
4. Participation in CTIMP in past 6 months*
5. Taking oral prednisolone for COPD or immunosuppressive medications
6. Haematological malignancy
7. Exacerbation within preceding 6 weeks

* To assess previous participation in a CTIMP, the potential participant will be asked if this is the case (all participants will have capacity and therefore will be able to recall).

Participants with acute COPD exacerbations:

Inclusion Criteria

1. Suspected or proven clinical diagnosis of COPD
2. At least 10 pack year history of smoking
3. Provision of informed written consent
4. Diagnosis of exacerbation of COPD being primary reason for admission to hospital
5. Consent and first blood samples taken within 48 hours of admission to hospital
6. Age 40 years or over

Exclusion Criteria

1. Diagnosis of Asthma, Interstitial Lung disease, Bronchiectasis, (fibrosis related to emphysema or bronchiectasis related to COPD is not an exclusion), Lung Cancer, Previous Lung resection
2. Lack of capacity to provide informed consent
3. Participation in CTIMP in past 6 months
4. Taking immunosuppressive medications (excluding oral prednisolone for exacerbation which is not an exclusion)
5. Haematological malignancy
6. Age 39 years or under

10.2 Appendix 2: Work during the COVID-19 pandemic

As the laboratory closed and the study was paused, I joined the COVID-19 taskforce producing reports and infographics for frontline healthcare staff and the general public on the latest scientific research on SARS-CoV-2 and COVID-19.

These reports and graphics are available to view at https://rapidcomms.wordpress.com/. Reports were generated as part of a team, with graphical concept and design completed by myself with input from the team.

In 2020, the site was accessed over 2,500 times from 58 different countries, with over 400 downloads of the available infographics. These infographics were also adopted by multiple NHS trusts outside of UHB.

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The following document pertains to the use of original figures by the author of this thesis that have also been published as referenced in the forwards of this thesis.

I acknowledge that Figure 1.1, Figure 1.2, Figure 1.3 and Figure 1.5 are also, in part, published in "Neutrophil Phenotypes of Chronic Lung Disease" (October 2019) by Taylor and Francis, available online http://www.tandfonline.com/doi/abs/10.1080/17476348.2019.1654377.

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