Examining neutrophil function in acute and chronic inflammation to identify novel therapeutic strategies to treat disease

By:

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A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

The University of Sheffield

Faculty of Medicine, Dentistry and Health.

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Date 6th February 2022

Abstract

Introduction: Neutrophils are professional phagocytes of the host immune system that are important in the clearance of microbial pathogens. To kill pathogens, neutrophils possess a range of antimicrobial effector mechanisms including degranulation of cytotoxic proteins, release of reactive oxygen species and generation of neutrophil extracellular traps (NETs). However, neutrophil activation, and the process NETosis, are implicated in the exacerbation of inflammation and pathology of several diseases including diabetes, chronic obstructive pulmonary disease and COVID-19. The overarching aim of this PhD project was to better understand neutrophil function in disease, focusing on diabetes. People with diabetes suffer severe and chronic infections and the initial aim of the project was investigate neutrophil function in people with diabetic foot disease (DFD). The hypothesis of this work was that neutrophils from people with DFD would have multiple aberrant effector mechanisms, that could present novel therapeutic targets to improve infection resolution. However, because of the Coronavirus pandemic, which began in March 2020, research involving outpatients was suspended. I therefore adapted my PhD project to investigate NETosis in hospitalised patients with COVID-19. Evidence emerged that NETosis played a role in the development of acute respiratory distress syndrome (ARDS), which is a severe complication of COVID-19. The hypothesis therefore was that NETosis would be increased in neutrophils isolated from COVID-19 patients and targeting this pathway could be a potential therapeutic strategy to modify hyper-inflammation in COVID-19. Assays of neutrophil function for the STOP-COVID clinical trial, which investigated the neutrophil serine protease inhibitor brensocatib in hospitalised COVID-19 patients, were also completed.

Methods: The aim of the study of patients with DFD was to phenotype the neutrophil and neutrophil phagocytosis, ROS production, NETosis and apoptosis was investigated. The COVID-19 study focused particularly on NETosis and this was quantified using the cell impermeable DNA dye SYTOX™ Green, in response to NET inducers PMA and LPS. Experimental inhibitors of NETosis were tested, including dexamethasone (10 µM), ruboxistaurin (200 nM) and cl-amidine (200 µM) *ex vivo*. NETosis quantification was supported by immunocytochemistry staining for NETs, using the DNA stain DAPI and a myeloperoxidase antibody. A sub-set of COVID-19 patient samples were analysed at the acute stage of infection and then at follow up time point 3-4 months later. Neutrophil cell surface marker expression, phagocytosis and NETosis was investigated by flow cytometry in neutrophils isolated from patients in the STOP-COVID clinical trial.

Results: Analysing neutrophil function using cells isolated from two patients with DFD was completed prior to the pandemic, therefore these results could not be meaningfully interpretated. NETosis was significantly increased in COVID-19 patients in response to LPS (p=-0.025) but not PMA when compared to healthy controls (n=39). Elevated LPS-induced NETosis was inhibited by ruboxistaurin (p=0.0008) but not dexamethasone or cl-amidine. There was significantly more NETosis at the acute stage of infection, compared to at follow up (n=7) (p=0.0256). Brensocatib was without effect on neutrophil function *ex vivo*.

Conclusions: The study of NETosis in COVID-19 provides support for the role of NETs in the pathology of this disease. This work demonstrated for the first time that ruboxistaurin is a potential therapeutic to reduce aberrant NETosis in COVID-19 and potentially other diseases.

Acknowledgements

First and foremost, I would like to thank my supervisors Dr Lynne Prince and Professor Ian Sabroe, for providing me with the opportunity to complete a PhD and for sharing their expansive scientific wisdom. Navigating a PhD in the throes of a global pandemic has been the most challenging experience of my life and I sincerely thank them both for the kindness and encouragement that I have received. I'm extremely grateful for their unwavering support and solid guidance, but most of all for believing in me, when I did not. Thank you.

Next, I would like to acknowledge Dr Ahmed Iqbal who was crucial to the set up and conducting of the patient study of people with diabetic foot disease. The clinical insight I gained through working with Ahmed and talking directly with patients has been invaluable for my career development and a huge thank you to Ahmed for his support in the publication of our literature review. Also, thank you to Professor Steve Renshaw for his support and guidance on the transcriptomics project.

My PhD has been a hugely collaborative undertaking and I sincerely thank the UK-CIC research group at the University of Sheffield for enabling and supporting my research. I would particularly like to thank Professor Endre Kiss-Toth and Professor Claire Lewis, who led the UK-CIC team in Sheffield. Also, Dr Joby Cole, who recruited the patients and the clinical research facility staff at the RHH who collected the patient samples. This work spanned two national coronavirus lockdowns and in times of so much uncertainty and pressure on the NHS, I am extremely grateful for the access to patient samples.

I would also like to thank the team at University of Dundee, particularly Professor James Chalmers, Dr Holly Keir and Dr Merete Long for enabling and supporting the setup of the assays of neutrophil function for the Sheffield arm of the STOP-COVID clinical trial. I sincerely thank them for allowing me to include the neutrophil function data generated for the whole trial in this thesis. I would like to thank Professor Alison Condliffe, the PI for trial in Sheffield, for continued support in delivering the study data and Dr Roger Thompson for recruiting the participants.

I would like to dedicate this thesis to my mum and dad. The joy I feel in making you proud has inspired me to succeed. I was the first one in our family to move away to university and to pursue a PhD and with your constant love and support I have achieved things and overcome obstacles that I never thought possible. Thank you for always being there for me, for always bringing me chocolate when I was revising and for reminding me to take a break when I was overdoing it. Finally, I would like to acknowledge and thank my partner Matthew, who has been by my side throughout this journey. You have made me laugh in difficult moments and injected fun into every day and I will always be thankful for your amazing cooking. Thank you for your all your support and understanding throughout.

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Abbreviations

ACE2	Angiotensin-converting enzyme 2
AGE	Advanced glycation end-product
ANOVA	Analysis of variance
ANXA1	Annexin A1
AP-1	Activator protein-1
BALF	Bronchoalveolar lavage fluid
BHI	Brain heart infusion
BSA	Bovine serum albumin
cDNA	Complementary DNA
C/EBPα	CCAAT-enhancer binding protein α
CGD	Chronic granulomatous disease
Cmax	Maximum serum concentration
CFU	Colony forming unit
COPD	Chronic obstructive pulmonary disorder
COVID-19	Coronavirus disease 2019
CRF	Case report form
CRP	C-reactive protein
$Cytb_{558}$	Flavocytochrome b558
DAPI	4’,6-diamidino-2-phenylindole
DAG	Diacylglycerol
DAMPs	Damage associated molecular patterns
DCF	2’,7’dichlorodihydrofluorescein
DFD	Diabetic foot disease
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
D.p.f	Days post fertilisation
DPI	Diphenyleneiodonium chloride
DPP-1	Dipeptidyl peptidase 1
DPX	Dibutylphthalate Polystyrene Xylene
EGR-1	Early growth response protein 1
EGR-3	Early growth response protein -3
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-reduced kinases
Ery	Erythromycin
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FSC	Forward scatter
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
GPCRs	G protein coupled receptors

GSE	Gene expression omnibus series
HbA1c	Glycated haemoglobin
HBEC3-KT	Human bronchial epithelial cells
HBSS	Hanks balanced salt solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HLA	Human Leukocyte antigen
HSPCs	Haematopoietic stem and progenitor cells
IC_{50}	Half maximal inhibitory concentration 50
ICAM1	Intercellular adhesion molecule 1
ICAM2	Intercellular adhesion molecule 2
IL-6	Interleukin-6
IRAK-4	Interleukin-1 receptor-associated kinase-4
ITU	Intensive care unit
JNK	c-Jun N-terminal kinase
LDNs	Low density neutrophils
Lin	Lincomycin
LogFC	Log fold-change
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MERS-CoV	Middle East respiratory syndrome coronavirus
mRNA	Messenger ribonucleic acid
MRSA	Methicillin resistant *S. aureus*
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary-response protein
N6/8-AHA	PKA agonist pair- 8-AHA-cAMP and N6-MB-cAMP
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NE	Neutrophil elastase
NHS	Normal human serum
NET	Neutrophil extracellular trap
NLR	Neutrophil lymphocyte ratio
NOX	NADPH oxidase complex
NR4A	Nuclear receptor 4A
NSP	Neutrophil serine protease
OD	Optical density
PAD4	Peptidylarginine deiminase inhibitor 4
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood monocytes
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI	Principle Investigator
PI3K	Phosphatidylinositol 3-kinase
PIS	Patient information sheet
PKA	Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetateate
PPP	Platelet poor plasma
PVL	Panton-Valentine Leucocidin

RBCs	Red blood cells
RHH	Royal Hallamshire Hospital
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SaeS	Sensor histidine kinase
SARS-COV-2	Severe acute respiratory distress syndrome 2
SD/Stdev	Standard deviation
SOD	Superoxide dismutase
SPN	Supernatant
SSC	Side scatter
SSIs	Surgical site infections
SSTIs	Skin and soft tissue infections
STH-Obs	Sheffield Teaching Hospitals Observational Study of Patients with Pulmonary Hypertension, Cardiovascular and other Respiratory Diseases
STOP-COVID	Superiority trial of protease inhibition in COVID-19
STZ	streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TMPRSS2	TM protease serine 2
TNFα	Tumour necrosis factor alpha
TRIF	TIR domain-contain adaptor protein inducing interferon-ß
UK-CIC	United Kingdom COVID immune consortium
UoD	University of Dundee
UoS	University of Sheffield
$_{v}$RNA	Volume of RNA
WHO	World health organisation

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1 Introduction

1.1 Neutrophils

1.1.1 The diverse role of neutrophils

Neutrophils are professional phagocytes of the innate immune system. They are the most abundant leukocyte in the human body, consisting of 60% of the total body leukocytes, and approximately 10^{11} are produced by the bone marrow each day (Dancey et al., 1976). Neutrophils are relatively short-lived cells and have a half-life in the circulation of approximately 6-12 hours (Ackermann et al., 2021; Tak et al., 2013). Neutrophils were classically thought to be the 'infantry' of the innate immune response, having a crucial, yet limited role, constrained to the clearance of microbial pathogens. However, the understanding of neutrophil heterogeneity and plasticity has developed over time (Ng et al. 2019). Quiescent neutrophils are not a homogenous population of cells and aged neutrophils display increased expression of integrin CD11b and CXCR4, with the latter tracking the unstimulated neutrophil back to the bone marrow for removal (Grieshaber-Bouyer and Nigrovic et al., 2019). In response to inflammation activation of circulating neutrophils occurs and low density neutrophils (LDNs), which are neutrophils with an immature nuclear morphology, are released from the bone marrow (Ning et al., 2022). LDNs are pro-inflammatory, displaying elevated cytokine release and neutrophil extracellular trap (NET) formation and are associated with diseases including psoriasis and systemic lupus erythematous (Ning et al., 2022). Furthermore, neutrophil function is not confined to bacterial clearance. Neutrophils are involved in wound healing, tissue repair, communication with the adaptive immune system and have both anti-tumorigenic and pro-tumorigenic roles (Jaillon et al., 2020; Perobelli et al., 2017; Wang, 2018).

1.1.2 Neutrophil maturation

Neutrophils develop from multipotent haematopoietic stem and progenitor cells (HSPCs) in the bone marrow, which is a well characterised process named granulopoiesis (Lawrence et al., 2018). Granulopoiesis is controlled by the cytokine, granulocyte colony stimulating factor (G-CSF), and the transcription factors CCAAT-enhancer binding protein α (C/EBPα) and PU.1 (Mehta and Corey, 2021). For the generation of terminally differentiated neutrophils, HSPCs first differentiate into multi-potent myeloid progenitor cells, then further develop into granulocyte-macrophage progenitors (Akashi et al., 2000). Subsequent neutrophil maturation follows a sequence of developmental stages including myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil, segmented neutrophil and finally, a mature neutrophil (Ackerman, 1964; Fiedler, 2012). Neutrophil granule contents containing an arsenal of potent antimicrobial effector proteins also develop through these stages (Fiedler, 2012). Firstly, the primary (azurophil) granules develop in the promyelocyte stage, including myeloperoxidase (MPO) and neutrophil elastase (NE) (Faurschou and Borregaard, 2003). Next, secondary (specific)

granules form in myelocytes, which contain potent antimicrobial proteins such as lactoferrin, which is followed by tertiary (gelatinase) granules in metamyelocytes (Faurschou and Borregaard, 2003). Tertiary granules are secreted when the neutrophil contacts the endothelium and contain matrix degrading enzymes such as gelatinase (Faurschou and Borregaard, 2003). Finally secretory vesicles form in mature neutrophils, which contain actin binding proteins and membrane associated receptors (Faurschou and Borregaard, 2003). In health, mature neutrophils are released from the bone marrow at a steady, continuous rate and in response to systemic infection emergency granulopoiesis occurs, generating additional neutrophils (Manz and Boettcher, 2014).

1.1.3 Neutrophil recruitment cascade

The neutrophil recruitment cascade defines the mechanism by which circulating neutrophils respond to inflammatory signals and move from the vasculature into the surrounding tissues. Neutrophil recruitment is initiated by the activated endothelium, which presents the adhesion molecules P, E, and L selectin on the endothelial cell surface (Kolaczkowska and Kubes, 2013). Neutrophil interaction with these molecules tethers the cell to the endothelium (Ramachandran et al., 2004). Neutrophils roll along the endothelial cell surface down a chemoattractant gradient, which is facilitated by the rapid breaking and forming of bonds with the selectin molecules (Ramachandran et al., 2004). Neutrophils become primed or partially activated when they interact with pro-inflammatory mediators such as tumour necrosis factor alpha (TNFα) and damage-associated molecular patterns (DAMPs) on the endothelial cell surface (Kolaczkowska and Kubes, 2013). Neutrophil priming occurs when the cell does not produce an effector response upon initial stimulation but generates a much greater response upon subsequent activation (Condliffe et al., 1998). Intracellular adhesion molecules 1 and 2 (ICAM1, ICAM2) and integrins slow the speed of rolling, arresting the neutrophil on the inflamed endothelial cell surface (Kolaczkowska and Kubes, 2013). Neutrophils crawl along the endothelial surface before transmigrating, predominantly between endothelial cell-cell junctions, in a process called diapedesis (Filippi, 2016). Neutrophils migrate via chemotaxis to the site of infection/inflammation, where they become fully activated and undergo their key antimicrobial effector mechanisms (Kitayama et al., 1997). Neutrophils can also reverse migrate back into the vasculature (Ji and Fan, 2021). The mechanisms of reverse migration are still being fully defined but include the downregulation of chemotaxis receptor CXCR1 and upregulation of CXCR4, which traffics the neutrophils back into the circulation (Buckley et al., 2006; Jing et al., 2017). Furthermore, damage to the endothelium, causes leakage of chemoattractant into the bloodstream, altering chemotactic gradients (Owen-Woods et al., 2020). This may promote inflammation resolution or serve to disseminate the inflammatory response throughout the body (Ji and Fan, 2021).

1.1.4 Phagocytosis and phagosomal maturation

Neutrophils internalise particles in a process termed phagocytosis. Phagocytosis is initiated by the detection of foreign particles via non-opsonic (e.g. C-type lectins) and opsonic receptors (Fc receptors and complement receptors) (Uribe-Querol and Rosales, 2020). Opsonization is the decoration of particles in complement proteins and antibodies, which label them for phagocytosis (Uribe-Querol and Rosales, 2020). Neutrophil actin cytoskeleton rearrangement forms protrusions in the cell membrane (pseudopodia), which extend outwards and surrounds the particle to be ingested, forming an intracellular phagosome (Botelho and Grinstein, 2011). The phagosome matures to kill internalised pathogens. The phagosome fuses with endosomes containing cytotoxic granule proteins, and lysosomes containing acidic hydrolases, which forms a phagolysosome (Nordenfelt and Tapper, 2011). The phagolysosome is acidic which aids in pathogen killing. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex 2 (NOX) forms on the phagolysosome membrane, producing reactive oxygen species (ROS), to further kill internalised pathogens (Nordenfelt and Tapper, 2011).

1.1.5 Reactive oxygen species production

Neutrophil ROS production is a key pathogen killing mechanism and is induced in response to a range of pro-inflammatory stimuli such as cytokines and chemotactic factors. The importance of this pathway is exemplified by the development of chronic granulomatous disease (CGD) in people with genetic mutations in NOX (Roos, 2016). Those with CGD suffer from frequent and severe bacterial infections (Roos, 2016). ROS production is also important in neutrophil cell signalling, augmenting neutrophil degranulation, stimulating pro-inflammatory cytokine production, and triggering neutrophil cell death (Fialkow et al., 2007; Pérez-Figueroa et al., 2021). The NOX is a multi-protein complex made up of 6 sub-units: $gp91^{phox}$, $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ and the member of the Rho family of small GTPases- Rac2 (El-Benna et al., 2016; Nguyen et al., 2017; Winterbourn et al., 2016). The mechanism of ROS production is described in Figure 1. $gp91^{phox}$ and $p22^{phox}$ form the catalytic sub-unit, flavocytochrome b558 ($cytb_{558}$) (Nguyen et al., 2017; Winterbourn et al., 2016). $cytb_{558}$ is located in specific granules and secretory vesicles and associates with the cell membrane and phagosomal membrane upon neutrophil activation, for extracellular and intracellular ROS generation (Nguyen et al., 2017; Nordenfelt and Tapper, 2011). The regulatory sub-units $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ form a trimer and are present in the cytosol with Rac2 (Massenet et al., 2005). When the neutrophil is activated, phosphorylation of these sub-units occurs, initiating the association of the cytosol components with $cytb_{558}$, forming the intact NADPH oxidase complex (Massenet et al., 2005). Rac2 binds directly to $p67^{phox}$ causing a conformational change that is required for binding to $gp91^{phox}$ (Dang et al., 2001). The phosphorylation of $P47^{phox}$ is critical for ROS production and is mediated by different kinases including Protein kinase B (PKB), Protein kinase C (PKC), Extracellular signal-reduced kinases (ERK) and p38 Mitogen-activated protein kinase (MAPK), which is dependent

on the cell surface receptor that is activated (El-Benna et al., 2009; Nguyen et al., 2017). Signalling via integrin receptors, Fc receptors and neutrophil stimulation with potent PKC activator Phorbol 12-myristate 13-acetate (PMA) directly activates ROS production and signalling via cytokine receptors or Toll-like receptors (TLRs) can prime the neutrophil to generate ROS (El-Benna et al., 2016; Nguyen et al., 2017). The NOX functions by transferring electrons from NADPH to molecular oxygen, generating superoxide anions ($O2^{-}$) (Winterbourn et al., 2016). This process is called the neutrophil respiratory/ oxidative burst. Superoxide dismutase (SOD) catalyses the formation of hydrogen peroxide (H_2O_2), which is converted to hypochlorous acid (HOCL) by MPO, to kill invading pathogens (Aratani, 2018; Buettner, 2011). ROS generation is a tightly controlled process as excessive production of superoxide anions can lead to oxidative stress, which is an accumulation of ROS that exacerbates inflammation and causes host tissue damage. Neutrophils contain antioxidants such as catalase to maintain the redox balance (Roos et al., 1980). Neutrophils also generate nitric oxide using nitrox oxide synthase, which combines with superoxide anions forming reactive nitrogen species (Mittal et al., 2014).

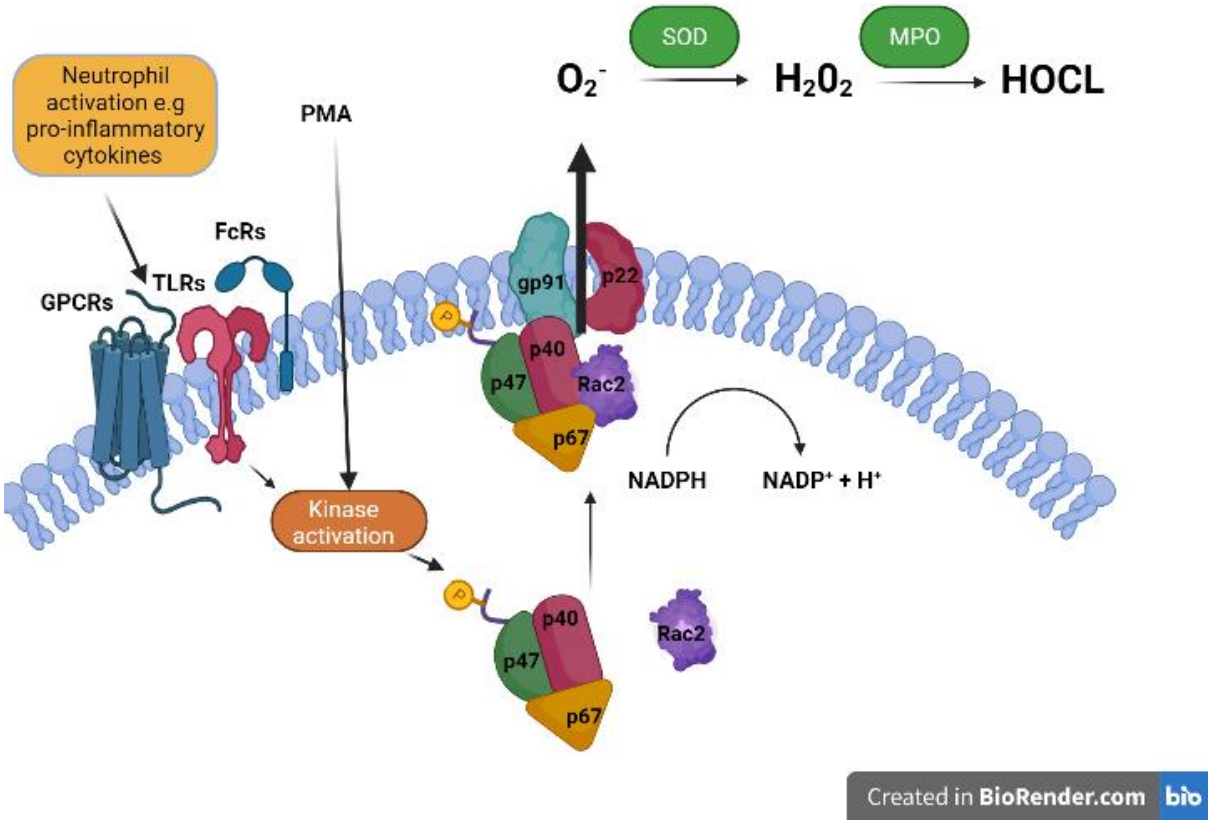


Figure 1-Neutrophil ROS production

The NADPH oxidase complex (NOX) is made up of 6 sub-units: gp91^{phox}, p22 ^{phox}, p40 ^{phox}, p47 ^{phox}, p67 ^{phox} and Rac2. Upon neutrophil activation the NOX forms at the cell membrane and the phagosomal membrane. Superoxide (O_2^-) is produced which is converted to hydrogen peroxide (H_2O_2), by superoxide dismutase (SOD) and then to hypochlorous acid (HOCL) by myeloperoxidase (MPO). Figure created with BioRender.com

1.1.6 NETosis

Neutrophils release NETs via a process named NETosis, which was first discovered in 2004 (Brinkmann et al., 2004). NETs are extracellular web-like structures of DNA, formed from decondensed chromatin, coated in antimicrobial proteins and histones that are released from activated neutrophils (Brinkmann et al., 2004). When NETs were first discovered, they were considered a novel pathogen killing mechanism (Brinkmann et al., 2004). NETs have microbicidal capacity and entrap pathogens for subsequent clearance (Riyapa et al., 2012; Young et al., 2011). However, much of the research in the field over the last decade has demonstrated that NETosis is also pathological, as NETs damage host tissues and exacerbate inflammation and can occur in sterile inflammation (Castanheira and Kubes, 2019; Kaplan and Radic, 2012). Increased NETosis is implicated in the immunopathology of several diseases including COPD, rheumatoid arthritis, diabetes and more recently COVID-19 (Chowdhury et al., 2014; Pedersen et al., 2015; Wong et al., 2015; Veras et al., 2020). The mechanisms of NETosis are not fully elucidated and understanding these pathways is complicated by the high number of stimuli found to induce NETs, meaning variations in the mechanism are found. NETosis is initiated in response to engagement with a range of cell surface receptors, which detect pathogen-associated-molecular patterns (PAMPs) and damage-associated-molecular patterns (DAMPs) (Chen et al., 2021). NETosis results from activation of TLRs, NOD-like receptors, C-type lectin receptors, complement receptors and Fc receptors (Chen et al., 2021). The exact stimulus committing the neutrophil to NETosis is unknown but is thought to be associated with spikes in the concentration of intracellular calcium, which rise in activated neutrophils (de Bont et al., 2018). In this thesis I will describe the widely accepted mechanisms of NETosis.

There are two pathways of NETosis; NOX-dependent and NOX-independent, which both result in chromatin decondensation and DNA release (Kenny et al., 2017; Tatsiy and McDonald, 2018; Thiam et al., 2020). The mechanisms of NETosis are described in Figure 2. The classical pathway of NET formation is a NOX-dependent pathway and is commonly studied *in vitro* using the potent pharmacological PKC activator, PMA (Parker et al., 2012; Hoppenbrouwers et al., 2017). NOX-dependent NETosis is also induced by physiological stimuli such as lipopolysaccharide (LPS), which is a component of the outer membrane of Gram negative bacteria, and by respiratory syncytial virus (Khan et al., 2017; Muraro et al., 2018). ROS production is stimulated via PKC and the Raf-MEK-ERK signalling cascade (Gray et al., 2013; Hakkim et al., 2011). ROS stimulates the release of NE and MPO from the azurophilic granules (Papayannopoulos et al., 2010). These enzymes translocate to the nucleus where they cleave histones to facilitate DNA unfolding (Papayannopoulos et al., 2010). MPO does not to directly degrade histones but instead enhances the activity of NE (Metzler et al., 2014). An influx of calcium ions into the cell is suggested to activate the family of peptidyl arginine deiminase

(PAD) enzymes (Arita et al., 2004; Thiam et al., 2020). PAD-4 is the specific enzyme important in NETosis, which post-translationally modifies histones, causing chromatin decondensation (Leshner et al., 2012; Wang et al., 2009). PAD-4 citrullinates histones, causing the protein to lose its positive charge and association with negatively charged DNA (Wang et al., 2009). Nuclear decondensation in NETosis is a well-defined process and is a key feature of NETosis in comparison to other cell death pathways. The nucleus begins to lose its characteristic multi-lobed structure and nuclear membrane integrity (Neubert et al., 2018). The decondensed chromatin expands into the cytosol, filling the entire cell, until the cell membrane ruptures, and the NETs are released (Neubert et al., 2018). Nuclear and cell membrane rupture is mediated by gasdermin d, which is a pore forming protein activated by NE in PMA-induced NETosis and caspase 11 in LPS-induced NETosis (Chen et al., 2018; Sollberger et al., 2018). Actin and microtubule filaments also disassemble in NETosis, which is thought to aid passage of the NETs out of the cell (Neubert et al., 2018; Thiam et al., 2020). DNA release has also shown to occur via membrane bound vesicles, without cell lysis, allowing the cell to remain functionally active for a short amount of time post exocytosis (vital NETosis) (Fuchs et al., 2007; Pilsczek et al., 2010; Yipp et al., 2012). NETosis can also be NOX-independent and is commonly induced *in vitro* using the calcium ionophore, ionomycin (Parker et al., 2012; Tatsiy and McDonald, 2018). NOX-independent NETosis was shown to occur much quicker than NOX-dependent NETosis and is driven by mitochondrial ROS generation (Douda et al., 2015). Initiation of NOX-independent NETosis is mediated by the potassium ion channel SK3 (Douda et al., 2015). NOX-independent NETosis is also induced by physiological stimuli including *Staphylococcus aureus* (Pilsczek et al., 2010). The DNA in extruded NETs is decorated with a range of histones and proteins, the nature of which is dependent on the stimulant used (Chapman et al., 2019). Abundant NET associated proteins include NE, MPO and citrullinated histones, which are predominantly used to visualise NETosis biochemically (Brinkmann et al., 2016). Other NET associated proteins include annexin, matrix metalloproteinase-8, lipocalin and alarmin S100A8 (Chapman et al., 2019; Urban et al., 2009).

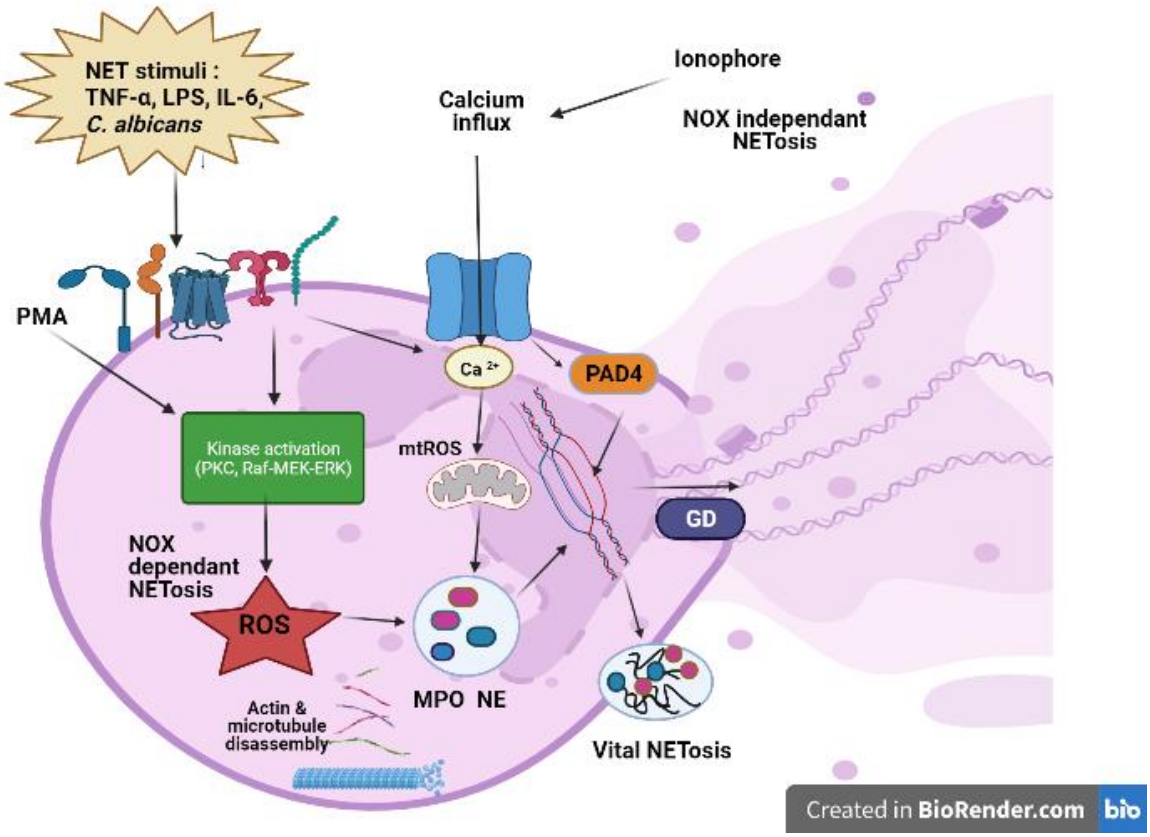


Figure 2-Mechanism of NETosis

NETosis occurs via a NADPH oxidase complex (NOX) dependent or NOX-independent process. NOX-independent NETosis is mediated by mitochondrial ROS (mtROS). DNA is decondensed by myeloperoxidase (MPO), neutrophil elastase (NE) and peptidyl arginine deiminase -4 (PAD4). Gasdermin d (GD) permeabilises the cell membrane for NET release. NETs can also be released in vesicles (Vital NETosis). Figure created in BioRender.com.

1.1.7 Other neutrophil cell death pathways

1.1.7.1 *Apoptosis*

Neutrophil apoptosis is a form of non-inflammatory programmed cell death (Kerr et al., 1972). Apoptosis is mediated by a family of proteases named caspases and is controlled by two key signalling pathways (Fadeel et al., 1998; Geering and Simon, 2011). In brief, the intrinsic pathway is initiated by intracellular factors such as hormones and growth factors, which cause permeabilisation of the mitochondrial membrane and the release of cytochrome c, which activates caspase 9 (Pérez-Figueroa et al., 2021). This is a tightly controlled process regulated by members of the Bcl-2 protein family (Moulding et al., 2001). The extrinsic pathway is initiated by the binding of ligands with neutrophil cell death receptors FAS, TRAIL 1 or TNF receptor 1, which activate caspase 8 (Pérez-Figueroa et al., 2021). Both pathways result in the activation of the effector caspase- caspase 3. Caspase 3 mediates the characteristic nuclear condensation, DNA fragmentation and cell rounding, which is unique to apoptosis (Kerr et al., 1972). Apoptotic neutrophils are unresponsive to subsequent pro-inflammatory stimuli and cannot execute any further antimicrobial effector mechanisms (Ayub and Hallett, 2004;

Whyte et al., 1993). Apoptosis can also be initiated by phagocytosis (Zhang et al., 2003). Apoptotic neutrophils present phosphatidylserine on the cell membrane, signalling to macrophages to engulf the cell for clearance, in a process named efferocytosis (Kourtzelis et al., 2020). Efferocytosis is important for resolving inflammation (Kourtzelis et al., 2020). Apoptosis is modified in activated neutrophils, with pro-inflammatory stimulants such as LPS extending the lifespan of neutrophils (Dick et al., 2009; Sabroe et al., 2003). Delayed apoptosis is associated with chronic inflammation in several diseases including COPD and diabetes (Manosudprasit et al., 2017; Zhang et al., 2012).

1.1.7.2 *Necrosis and Necroptosis*

Unlike apoptosis, necrosis is an unprogrammed and pro-inflammatory form of neutrophil cell death (Iba et al., 2013). Necrosis is induced by noxious stimuli such as cytolytic bacterial toxins (Zysk et al., 2000; Yang et al., 2019). Necrosis results in swelling of the cell and leakage of the intracellular contents into the tissues (Iba et al., 2013). Necrosis release DAMPs, such has heat shock proteins, which exacerbate inflammation (Murao et al., 2021). When apoptotic neutrophils are not rapidly efferocytosed they can undergo secondary necrosis (Rydell-Törmänen et al., 2006). A programmed formed of necrosis, named necroptosis was subsequently discovered (Pasparakis and Vandenabeele, 2015). Necroptosis is primarily initiated by death receptor- TNF1 and is mediated by receptor interacting protein kinases (RIPK) and mixed lineage kinase domain-like protein (MLKL) (Pasparakis and Vandenabeele, 2015).

1.1.8 Neutrophil immunosenescence and inflammageing

Ageing is associated with increased susceptibility to infections (Hazeldine and Lord, 2015). Neutrophil function alters with advancing age, which is a process known as neutrophil immunosenescence (Wessels et al., 2010). Previous literature demonstrates that key antimicrobial effector mechanisms, such as chemotaxis, phagocytosis, ROS production and NET formation, predominantly decline with age (Butcher et al., 2001; Hazeldine et al., 2014; Wenisch et al., 2000). However, neutrophil production is not thought to be reduced in the elderly population, indicating there is a change in neutrophil biology (Born et al., 1995). Changes in neutrophil signal transduction, reduced calcium mobilisation and lipid membrane composition contribute to alterations in neutrophil function (Shaw et al., 2010). Constitutive activation of phosphatidylinositol 3-kinase (PI3K) was found to deregulate chemotactic accuracy in healthy participants aged >65 years, compared to participants <35 years (Sapey et al., 2014; Wilson et al., 2020). Furthermore, reduced expression of the cell surface FC receptor CD16, caused decreased phagocytosis of *E. coli* in participants >65 years (Butcher et al., 2001). However, there is not only a decline in neutrophil function with advanced age, ageing is also associated with an increase in systemic low grade inflammation, termed inflammageing (Ferrucci and Fabbri, 2018). Pro-inflammatory cytokines such as IL-6 , IL-1β, TNFα and inflammatory biomarker C-reactive protein (CRP)

are increased with ageing and are hypothesised to impact neutrophil function (Gonçalves et al., 2016). For example, increased reverse neutrophil migration from inflamed cremaster muscles to the lungs was demonstrated in aged mice, which resulted in increased lung damage (Barkaway et al., 2021). Several host factors associated with increased cytokine generation contribute to inflammageing, including genetic susceptibility resulting from single nucleotide polymorphisms in *IL1RN* and *IL-6* gene promoters and increased cytokine release from adipocytes in obesity (Ferrucci and Fabbri, 2018; Ellulu et al., 2015). Also, reduced efficiency in cellular repair mechanisms increases levels of DAMPs in ageing, further driving inflammation (Ferricci and Fabbri, 2018).

1.2 Neutrophil function in diabetes

The information provided in section 1.2.2, 1.2.4 & 1.2.5 is taken directly from my published, primary author literature review 'A Bittersweet Response to Infection in Diabetes; Targeting Neutrophils to Modify Inflammation and Improve Host Immunity' (Dowey et al., 2021). This article was published in 'Frontiers in Immunology'- doi: 10.3389/fimmu.2021.678771. Extracts of this article are being used in this thesis under the Creative Commons Attribution License (CC BY).

1.2.1 Statement of author contributions

I performed extensive literature searches, created tables and figures and wrote the first draft of the review. Dr Ahmed Iqbal, Professor Simon Heller, Professor Ian Sabroe and Dr Lynne Prince provided intellectual insight into the generation of this manuscript and reviewed and edited subsequent drafts. Dr Ahmed Iqbal particularly supported the writing of the background to diabetes.

1.2.2 Diabetes

The number of people with diabetes globally will exceed 500 million by 2035. Type 1 diabetes (T1D) is an autoimmune condition characterised by T-cell mediated pancreatic β cell destruction and absolute insulin deficiency (Atkinson et al., 2011). T1D represents up to 10% of all diabetes worldwide and a small percentage (<10% type 1B) of affected individuals have no evidence of autoimmunity with the pathogenesis being idiopathic (Epstein et al., 1994; Paschou et al., 2014). A complex interplay of genetic, epigenetic, environmental, and immunologic factors is thought to contribute to the pathogenesis of T1D. Genome-wide association studies have identified more than 50 genetic risk loci to date but the main genes predisposing to T1D are located within the human leukocyte antigen (HLA) on chromosome 6 (Paschou et al., 2018; Størling and Pociot, 2017). Alleles at the HLA locus account for up to 50% of cases with familial clustering (Nerup et al., 1974; Ounissi-Benkalha and Polychronakos, 2008; Polonsky, 2012). Epidemiological studies have implicated a number of environmental factors in the pathogenesis of T1D, including viruses and nutrients such as cow's milk protein (Paschou et al., 2018; Størling and Pociot, 2017). These factors are thought to trigger an autoimmune response consequent upon molecular mimicry in that pancreatic autoantigens that resemble viral or dietary

epitopes undergo cellular destruction (Karlsson and Ludvigsson, 2000; Szopa et al., 1993). Pancreatic β cell destruction involves both cellular and humoral immunity. Autoreactive T-cells are thought to induce apoptosis in a pancreatic islet milieu rich in pro-inflammatory cytokines including IL-1, TNF-α, and IFN-γ (Eizirik et al., 2009). The presence of circulating autoantibodies against proinsulin and other autoantigens in β cells highlights the role of humoral immunity in disease pathogenesis. Indeed, circulating autoantibodies in T1D can occur before the biochemical and clinical manifestations and the presence of two or more autoantibodies in first-degree relatives strongly predicts clinical progression to T1D (Verge et al., 1996).

In type 2 diabetes (T2D), which accounts for 90-95% of all diabetes, a combined resistance to insulin both in skeletal muscle and the liver, in addition to defective insulin production by pancreatic β cells, is present (DeFronzo, 2004). In contrast to T1D, no predominant genetic locus has been found to increase susceptibility to T2D. Genomic studies reveal over 40 genetic variants that increase the risk of T2D, however, overall these genes account for 10% heritability (Ahlqvist et al., 2011; Stolerman and Florez, 2009). A positive family history is important nonetheless with a 38% life-time risk of T2D in individuals who have one parent with T2D with this risk increasing to 60% if both parents have T2D (Pierce et al., 1995; Tattersall and Fajans, 1975). In addition to multiple genes, environmental factors play a critical role in the pathogenesis of T2D. A sedentary lifestyle in addition to consumption of high-fat, high-calorie diets means the majority of individuals with T2D are overweight (Polonsky, 2012). Obesity related insulin resistance together with hypertension, dyslipidaemia, glucose intolerance, and eventually frank hyperglycaemia defines the metabolic syndrome and this clinical phenotype is commonly encountered in many people with T2D (Reaven, 2005). Several mechanisms have been proposed to explain both insulin resistance in T2D which occurs early in the disease and pancreatic β cell dysfunction which is typically a late phenomenon. Increased levels of non-esterified fatty acids, pro-inflammatory cytokines, adipokines, and mitochondrial dysfunction are thought to drive insulin resistance (Stumvoll et al., 2005). Progressive β cell failure is thought to occur due to glucotoxicity, lipotoxicity and direct cytotoxic effects from deposition of islet amyloid polypeptide (Stumvoll et al., 2005). There is accumulating evidence that many of these mechanisms work in concert and are underpinned by low-grade activation of the innate immune system (Pickup, 2004). This not only plays a part in the pathogenesis of T2D but is also causally linked to associated complications including dyslipidaemia and atherosclerosis (Pickup, 2004). Elevated levels of pro-inflammatory cytokines including IL-6 and TNF-α and acute phase markers such as C-reactive protein are thought to disrupt insulin signalling although effects on glucose metabolism remain less clear (Steensberg et al., 2003). Humoral immunity may also play a part in the pathogenesis of T2D. Elevated serum gamma globulin levels, a nonspecific marker of humoral immune activation, have been associated with an increased

risk of T2D in certain populations although the wider significance remains to be elucidated (Lindsay et al., 2001). Despite differences in pathophysiology, chronic hyperglycaemia is a fundamental biochemical abnormality present in both T1D and T2D, which is a key driver of aberrant neutrophil function (Atkinson et al., 2011 & DeFronzo et al., 2004).

1.2.3 Diabetic foot disease

People with diabetes are at an increased risk of infection at various sites including the skin and soft tissue, urinary tract and the respiratory system (Carey et al., 2018). Diabetic foot disease (DFD) is a leading cause of hospitalisation and morbidity (Davis et al., 2018). The pathophysiology of DFD is promoted by poor circulation, neuropathy, which causes both foot deformities and loss of sensitivity, and immune dysfunction (Mishra et al., 2017). DFD can progress from a relatively benign injury, for example from the wearing of ill-fitting shoes to deep-seated bone infection (osteomyelitis) and further to gangrene and sepsis (Davis et al., 2018). Poor wound healing is also a predominant reason for the persistence of DFD (Davis et al., 2018; Nube et al., 2016). DFD is the leading cause of non-traumatic limb amputation in the UK and has a 5-year post diagnosis mortality of 25-48% (Morbach et al., 2012; Young et al., 2008). *S. aureus* is a common pathogen isolated from infected ulcers, as well as *P. aeruginosa* and *E. coli* (Dowd et al., 2008; Neves et al., 2019). Infections are often polymicrobial and form biofilms (Dowd et al., 2008; Pouget et al., 2020). Treatments include antibiotic therapy, wound debridement and off-loading of pressure (NICE, 2019). Reliance on antibiotic therapy means people with diabetes receive increased prescriptions of antibiotics (Barwell et al., 2017; Venmans et al., 2009). Antibiotic resistance is a global health concern and methicillin resistant *S. aureus* (MRSA) was isolated in 15-30% of cases DFD, highlighting the importance of conservative antibiotic usage in this cohort and a need for new therapeutic strategies (Reveles et al., 2016; Stacey et al., 2019; Trivedi et al., 2014). DFD is also a burden on the health service and is estimated to cost approximately £8000 per year per patient (Guest et al., 2018).

1.2.4 The neutrophil phenotype in diabetes

Previous research investigating neutrophil function in T1D and T2D covers an expansive body of literature spanning 60 years, with every function of the neutrophil shown to be dysregulated in T1D or T2D. Neutrophil function in diabetes is described in detail in the published literature review (section 1.2). To summarise, early research in the field focused on neutrophil chemotaxis and phagocytosis, with the weight of evidence demonstrating a reduction of these functions in those with diabetes (Delamaire et al., 1997; Drachman et al., 1966; Gustke et al., 1998; Mowat and Baum, 1971). There were some conflicting findings between early studies, perhaps caused by variations in participant selection, rodent models used and experimental designs (Donovan et al., 1987; Plotkin et al., 1996). More recent research has predominantly focused on neutrophil ROS generation, pro-inflammatory

cytokine production and aberrant neutrophil cell death mechanisms, which are proving to be critical mediators in the weakened response to infection in diabetes (Gyurko et al., 2006; Hand et al., 2007; Manosudprasit et al., 2017; Wong et al., 2015). Extracellular ROS production, pro-inflammatory cytokine release and NET formation are increased in diabetes, whereas neutrophil migration, apoptosis and intracellular ROS production are reduced, which ultimately impairs bacterial killing and inflammation (Hanses et al., 2011; Javid et al., 2016; Karima et al., 2005; Repine et al., 1980; Wong et al., 2015). Phenotypic variations in neutrophil function are supported by transcriptomic data, showcasing a fundamentally altered profile in key pro-inflammatory genes in neutrophils in people with diabetes (Fang et al., 2018; Vecchio et al., 2018). Research aiming to therapeutically modify neutrophil function in response to infection in T1D or T2D lags compared to the volume of studies reporting observational differences between those with and without diabetes. However, research aiming to restore aberrant neutrophil function in diabetes is gaining momentum in the field, with a focus on modifying neutrophil ROS production and NETosis to improve infection outcomes (Cohen et al., 2019; Das et al., 2018; Frydrych et al., 2018; Wang et al., 2019).

1.2.5 Mediators of neutrophil function in the diabetes microenvironment

Hyperglycaemia is a key mediator of neutrophil dysfunction in T1D and T2D. Elevated blood glucose concentrations resulting from insulin insufficiency and tolerance is a core pathology of the disease. The impacts of hyperglycaemia on neutrophils are multi-factorial and present a complex interplay of dysregulated cellular mechanisms. Neutrophil metabolism is altered in response to excess glucose, to ensure intracellular glucose levels do not become toxic (Giacco and Brownlee, 2010). Molecular shunting of glucose from glycolysis into the polyol and hexosamine pathway occurs (Berrone et al., 2006; Giacco and Brownlee, 2010; Kashiwagi et al., 1994). Metabolism via these pathways decreases levels of the intracellular ROS scavenger, glutathione, resulting in increased ROS which activates transcription factors including NF-κB and results in pro-inflammatory cytokine production (Giacco and Brownlee, 2010; James et al., 2002; Kashiwagi et al., 1994). Enhanced generation of cytokines further activates subsequent neutrophils, causing a feed forward loop of excessive inflammation in diabetes (Guest et al., 2008). Furthermore, hyperglycaemia causes de novo synthesis of the PKC activator, diacylglycerol (DAG), upregulating the formation of NADPH oxidase complex at the plasma membrane (Kashiwagi et al., 1994; Xia et al., 1994) and leading to oxidative stress and NET formation (Menegazzo et al., 2015). Hyperglycaemia alters the osmolarity of the body fluids and hyperosmotic stress causes cell shrinkage and calcium influx into neutrophils, leading to derangements in phagocytosis and upregulation of pro-inflammatory cytokines (Demerdash et al., 1996; Otto et al., 2008). High intracellular calcium concentrations deplete available ATP, impacting key energy dependant functions such as phagocytosis (Demerdash et al., 1996; Guerrero-Hernandez and Verkhratsky, 2014). High

glucose also impacts maturing neutrophils in the bone marrow. Hyperglycaemia induced myelopoiesis and leucocytosis in streptozotocin (STZ) and Akita mice (murine models of T1D) is mediated by the production of neutrophil alarmins s100 calcium proteins 8 and 9 (S1008/9) (Nagareddy et al., 2013).

Hyperglycaemia upregulates the receptor for advanced glycation end products (RAGE) on the neutrophil cell surface (Lee and Bergmeier, 2017). Advanced glycation end products (AGE) are formed from the non-enzymatic glycation of proteins (Shi et al., 2003). The pro-inflammatory impacts of AGE, which are extensively reviewed elsewhere, are of particular importance in mediating cardiovascular sequalae in diabetes (Hudson and Lippman, 2018; Nowotny et al., 2015; Yan Shi et al., 2003). In brief, AGEs induce oxidative stress and pro-inflammatory gene expression (NF-κB) in multiple cell types, including neutrophils (Collison et al., 2002; Giacco and Brownlee, 2010; Wong et al., 2003). AGE signals via the RAGE receptor on the neutrophil cell surface, which importantly is a multi-ligand receptor also for the alarmins S1008/9 and high-mobility group box 1 (HMGB1), further perpetuating inflammation (Egaña-Gorroño et al., 2020; Ramasamy et al., 2011). Epigenetic modifications, which are the enzymatic alterations of chromatin to manipulate gene expression, were found in healthy murine macrophages co-incubated with AGE (Ahmed et al., 2017; Jin et al., 2015). Increased methylation of NF-κB and enhanced cytokine transcription was subsequently found (Jin et al., 2015). Neutrophils display 'metabolic memory' in people with diabetes, whereby modified cell phenotypes are maintained after the restoration of normoglycaemia, further prolonging deleterious effects (Carestia et al., 2016; Ceriello et al., 2009; Corgnali et al., 2008). Investigation of epigenetic alterations of neutrophils in T1D and T2D is warranted to provide additional mechanistic understanding of the persisting neutrophil phenotype. Furthermore, whether hypoglycaemia or the oscillations between high and low blood glucose concentrations promotes neutrophil dysfunction is not yet known.

Glucose is not the only pro-inflammatory mediator increased in T1D and T2D. Lipid metabolism is altered in response to insulin deficiency and resistance, which increases lipogenesis and adipose tissue metabolism (Rodrigues et al., 2016; Eid et al., 2019). Circulating levels of free fatty acids and lipoproteins are increased in T1D and T2D, which can be further exacerbated by obesity and poor diet (Radzikowska et al., 2019). The pro-inflammatory impacts of lipids and neutrophils are reviewed elsewhere and have been shown to upregulate key pro-inflammatory neutrophil functions including cytokine generation and ROS production (Jüttner et al., 2008; Palvinskaya et al., 2013;Rodrigues et al., 2016; Eid et al., 2019). Key mediators of neutrophil function in diabetes are summarised in Figure 3.

A gap in the literature remains regarding the impacts, if any, of hypoglycaemia on the neutrophil response to infection. Iatrogenic hypoglycaemia remains one of the major challenges in the treatment of T1D and T2D (Chaudhury et al., 2017). Data from self-reporting studies, which are likely to be

underestimates, suggest people with T1D have approximately two hypoglycaemic episodes per week, with an annual incidence of severe hypoglycaemia, where third party assistance is needed, being 1.15 events per person per year in T1D versus 0.35 events per person per year in T2D (Cho et al., 2012; Goto et al., 2016). Mechanistic studies employing the hyperinsulinaemic-hypoglycaemia clamps in both healthy individuals and those with T1D and T2D, demonstrate that acute moderate hypoglycaemia initiates a pro-longed pro-inflammatory state with upregulation of C-reactive-protein (CRP), increased platelet reactivity and mobilisation of pro-inflammatory leukocyte subsets (Chow et al., 2018; Iqbal et al., 2019; Kahal et al., 2020; Ratter et al., 2017). Additionally, in response to low endotoxin challenge in healthy volunteers, neutrophil counts were significantly increased in those allocated to experimental hypoglycaemia 48 hours earlier when compared to euglycaemic controls (Iqbal et al., 2019). However, whether neutrophils released into the circulation in response to hypoglycaemia have an altered function has not been widely investigated. A small-scale study compared the neutrophil oxidative burst in response to *S. aureus* in people with T1D versus healthy controls, after an insulin induced hypoglycaemic episode (Thomson et al., 1997). A greater reduction in oxidative burst was shown in the healthy control group compared to those with T1D (Thomson et al., 1997). Here marks the end of the work published in the literature review.

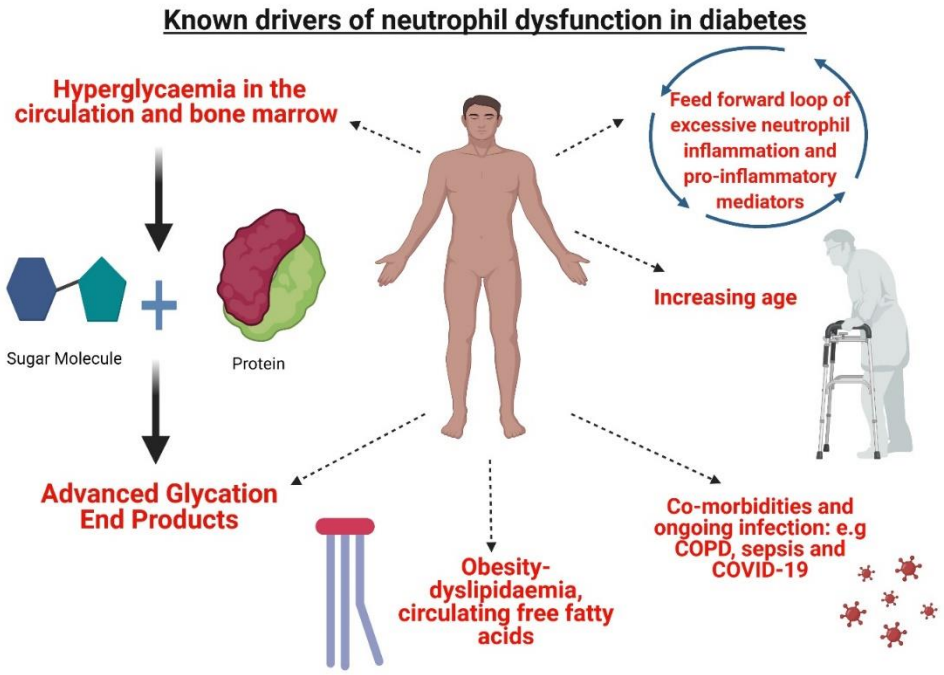


Figure 3-Mediators of neutrophil dysfunction in T1D and T2D.
The microenvironment of T1D and T2D presents a complex interplay of mediators of neutrophil dysfunction. Hyperglycaemia and the formation of advanced glycation end products in the circulation and the bone marrow modify circulating neutrophils and myeloid precursors. Metabolic perturbations in lipid metabolism and increased synthesis of circulating free fatty acids further contribute to aberrant dysfunction. Resulting activated neutrophils produce pro-inflammatory mediators adding to a cycle of inflammation. Increased age further impacts neutrophil function, in addition to co-morbidities and infection, where altered neutrophil functions are previously shown e.g., chronic obstructive pulmonary disease (COPD) sepsis and COVID-19. Figure created with BioRender.com**.** This figure has been re-used in this thesis under the Creative Commons Attribution License (CC BY).

1.2.6 Neutrophil function in the context of DFD and wound healing

Neutrophils are important in the pathology of DFD, with increased neutrophil lymphocyte ratio (NLR) shown to be a predictor of poor wound healing in DFD (Demirdal and Sen, 2018; Vatankhah et al., 2017). There has been significantly less previous research studying neutrophil function in people with DFD, compared to diabetes in general. The discussions informing the decision to focus specifically on people with DFD will be detailed in results chapter 1 (section 3.1.1.1). Early work in the field focused on neutrophil ROS production, with intracellular ROS generation shown to be reduced in cells isolated from patients (Tebbs et al., 1992). Increasing ROS production therapeutically with aldose reductase inhibitors and granulocyte-macrophage colony-stimulating factor (GM-CSF) showed efficacy in people with DFD, but a Cochrane review concluded that GM-CSF was not to be recommended as an adjuvant for DFD treatment and increasing neutrophil ROS production was not widely explored further (Cruciani et al., 2013; Gough et al., 1997; Peck et al., 2001; Tebbs et al., 1992; Yonem et al., 2001).

Subsequent research in neutrophil function in DFD has predominantly focused on the negative impacts of elevated NET formation on wound healing. Wong et al. (2015) were the first to show that NETosis was increased in people with diabetes (without infection) in response to calcium ionophore-ionomycin (Wong et al., 2015). Reduced NET formation in a *Padi4*$^{-/-}$ knockout mouse model resulted in improved wound healing of aseptic wounds (Wong et al., 2015). The importance of NETosis in aberrant wound healing in DFD was supported by a subsequent study using patients with diabetic foot ulcers. NET components, including NE, MPO and histones, were enhanced in wound tissue lysates from non-healing compared to rapid healing ulcers and circulating NET components were higher in people with DFD compared to healthy controls (Fadini et al., 2016). NET biomarker, citrullinated histone 3, was found to be a significant risk factor for impaired healing and major amputation by 12 months in study of 198 patients with DFD (Yang et al., 2020). Inhibiting PAD4 with cl-amidine reduced NETosis and improved wound healing in streptozotocin (STZ) treated diabetic mice (Fadini et al., 2016; Wong et al., 2015). Reducing NETosis to improve wound healing was demonstrated to be efficacious in later studies, utilising rodent models of diabetes and non-infected wounds. Inhibiting NOX-dependent NETosis using the PKCβ-2 inhibitor, ruboxistaurin accelerated wound closure and promoted angiogenesis in STZ treated mice, with similar findings demonstrated when using hydrogen sulfide in a leptin deficient (Lepr$^{db/db}$) mouse model of T2D (Das et al., 2018; Yang et al., 2019). *S. aureus* is a NET inducer and inhibiting *S. aureus* alpha toxin with monoclonal antibody Medi4893, reduced infection burden, increased re-epithelisation and decreased NET formation in a *S. aureus* hind paw infection in a rodent T2D model (TallyHo/JngJ mice) (Ortines et al., 2018). Interestingly, when investigating NETosis *in vitro,* increased spontaneous NETosis but reduced inducible NETosis was demonstrated in peripheral blood neutrophils in response to PMA and calcium ionophore a23187 in

those with DFD compared to healthy controls (Fadini et al., 2016). Furthermore, incubating neutrophils from healthy donors with plasma from diabetic ulcer affected arteries, induced NETs, however plasma from peripheral arteries away from the site of infection did not induce NETs in control neutrophils (Yang et al., 2020).

There are many studies demonstrating the connection between elevated NETosis and poor wound healing in DFD, however limited research has explored pathogen handling by neutrophils. Neutrophil phagocytosis and intracellular killing of *C. albicans* decreased over time in a 4-week longitudinal study of those with non-healing DFD compared to healing DFD (Oncul et al., 2007). However, a previous study investigating the phagocytosis of *S. aureus* found no difference between those with diabetes and active foot infection compared with healthy controls (Peck et al., 2001). Subsequently, the neutrophil phenotype of cells isolated from the peripheral blood and bone marrow were explored in a model of *S. aureus* hind paw infection in $Lepr^{db/db}$ mice (Park et al., 2009). Differences in neutrophil function were only found when using peripheral blood cells, with *S. aureus* killing and ROS production being reduced but there was no difference in phagocytosis of *S. aureus* compared to control mice. Despite a robust neutrophil infiltration to the site of infection in diabetic mice, bacterial burdens were much higher, and infections were prolonged compared to control mice. There has not been a specific neutrophil phenotyping study, exploring multiple neutrophil effector functions, conducted in people with DFD previously. Neutrophil apoptosis is yet to be explored in people with DFD and the assessment of multiple neutrophil functions, would be a novel addition to the field.

1.3 The emerging role of neutrophils in the immunopathology of coronavirus disease 2019

This PhD project was adapted to study NETosis in hospitalised patients with COVID-19. A full explanation of the rationale of these decisions are explained in the relevant results chapters.

1.3.1 Severe acute respiratory syndrome coronavirus 2

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel coronavirus first detected in Wuhan in 2019 (Wu et al., 2020; Zhou et al., 2020). SARS-CoV-2 is an enveloped, positive sense single stranded RNA coronavirus. Coronaviruses are a diverse family of viruses able to infect humans and mammals (V'kovski et al., 2021). Most human coronaviruses cause mild respiratory disease and are associated with the 'common cold' (V'kovski et al., 2021). However, some coronaviruses are highly pathogenic and previous outbreaks were caused by SARS-CoV (2002-2004) and Middle East respiratory syndrome coronavirus (MERS-CoV) (2012) (Al-Omari et al., 2019; Cherry and Krogstad, 2004). Previous outbreaks were much smaller than for SARS-CoV-2, which has spread throughout the world and was declared a global pandemic by the World Health Organisation (WHO) on 11th March 2020. SARS-COV-2 has a transmembrane spike glycoprotein

on its cell surface, which binds to the angiotensin-converting enzyme (ACE2), to infect human cells (Renhong et al., 2020). The spike protein is made of two sub-units (S1 & S2) (Jan et al., 2003). S1 is responsible for binding to the ACE2 receptor and entry into the cell is mediated by the cellular serine protease TM protease serine 2 (TMPRSS2) (Hoffmann et al., 2020). TMPRSS2 cleaves the spike protein, allowing the S2 sub-unit to mediate fusion of the viral and cellular membranes (Hoffmann et al., 2020). The primary role of ACE2 is in the maturation of the hormone angiotensin, which controls vasoconstriction and blood pressure (Donoghue et al., 2000). ACE2 is expressed in many cell types throughout the body including the lung, nasal & oral mucosa, kidney, heart, stomach and intestines (Salamanna et al., 2020). Limited research demonstrates that there was no expression of ACE2 on granulocytes or monocytes in peripheral cells isolated from healthy donors (Song et al., 2020). However, there was high expression of ACE2 on LPS-treated type 1 macrophages and tissues macrophages from healthy donors (Song et al., 2020). An opposing study detected ACE2 expression by PCR and Western blot in neutrophils isolated from healthy donors (Veras et al., 2020). Whether SARS-CoV-2 interacts directly with immune cells via the ACE2 receptor is unknown. Prior to vaccination the evolution of SARS-CoV-2 was relatively stable (Harvey et al., 2021). However genetic mutations in the SARS-CoV-2 spike protein have occurred, which yield viral variants capable of higher transmissibility (Harvey et al., 2021). Recently the omicron variant identified in November 2021, has demonstrated escape from neutralising antibodies produced in response to vaccination, and is spreading throughout the globe (Zhang et al., 2021).

1.3.1.1 *Clinical presentation and treatments*

COVID-19 presents a wide spectrum of disease ranging from asymptomatic infection to respiratory failure and death. Asymptomatic infections are thought to be responsible for the high rates of transmission of the virus, and studies show that 25% of cases have no symptoms (Alene et al., 2021). Most cases with symptoms (80%) present as mild upper respiratory tract illness, with the characteristic symptoms being dry cough, loss of taste or smell and a fever (Parasher, 2021). In approximately 20% of cases infection progresses to the lower respiratory tract and patients also present with breathlessness and chest pain (ISARIC Clinical Characterisation Group, 2021; Parasher, 2021). In the UK, which currently has a double vaccination rate of 83% (12th January 2022), 6.4 out of 100,000 people are hospitalised with COVID-19, although this is constantly changing as the pandemic develops (UK government, 2021). There are specific risk factors which pre-dispose people to hospitalisation including advanced age, obesity, cardiovascular disease, diabetes, cancer and pre-existing lung disease such asthma and COPD (CDC, 2021). Those with critical illness can have respiratory failure, multi-organ dysfunction and septic shock (Zaim et al., 2020). In hospital, treatment relies on the potent anti-inflammatory dexamethasone and oxygen therapy (NICE, 2021). Oxygen therapy ranges from

supportive treatment to mechanical ventilation in an intensive care unit (ITU) (NICE, 2021). For severe disease there are multiple anti-inflammatories, monoclonal antibody therapies and antiviral therapies approved for use in the UK (NICE, 2021). New treatments for COVID-19, such as dexamethasone and the IL-6 receptor inhibitor- Tocilizumab, have been successfully trialled via the international clinical trial network, 'Randomised Evaluation of COVID-19 Therapy' (RECOVERY) (The RECOVERY Collaborative, 2021, 2020). Vaccination and subsequent booster vaccines have been successful in reducing hospitalisations and death in the UK.

1.3.2 Pathophysiology of severe COVID-19

1.3.2.1 *Overview of immune cell dysregulation in COVID-19*

In a healthy immune response, the body successfully clears the SARS-CoV-2 infection, by recruiting viral specific T-cells ($CD8^+$) to clear infected cells (Tay et al., 2020). Also, neutralising antibodies are produced by B cells, which prevent viral entry (Tay et al., 2020). Neutralised virus and apoptotic cells are cleared by alveolar macrophages (Tay et al., 2020). However, a dysregulated and uncontrolled immune response is a hallmark of severe COVID-19. The immune landscape in severe COVID-19 is characterised by an impaired protective and adaptive immune response but an over-activated pro-inflammatory innate immune response (Yang et al., 2021). Lymphopenia is found in COVID-19 patients and is associated with disease severity (Ghizlane et al., 2021). There is also dysregulation of monocytes and macrophages with an increase in pro-inflammatory classical $CD14^+$ monocytes and there is a reduction of the antigen presentation molecule, human leukocyte antigen-DR, on monocytes in severe COVID-19 (Schulte-Schrepping et al., 2020). Neutrophilia and aberrant neutrophil function is another defining characteristic of COVID-19 immunopathology (Hazeldine and Lord, 2021). Neutrophils infiltrate the lung in response to SARS-CoV-2, which is driven by chemokine CXCL8 (Park and Lee, 2020). Blood neutrophil counts in hospitalised patients are high and NLR is a marker of disease severity and mortality (Liu et al., 2020). Peripheral blood neutrophils isolated from COVID-19 patients demonstrated a pro-inflammatory gene signature with upregulation in genes encoding NE, MPO, matrix metalloproteinases, and alarmins S100A8/A9 (Aschenbrenner et al., 2021). Increased numbers of neutrophils were demonstrated in both lung autopsy samples and bronchoalveolar lavage fluid (BALF) from patients with COVID-19 (Dentone et al., 2021; Rendeiro et al., 2021). Also, there is a higher proportion of low-density neutrophils (LDNs) in severe COVID-19 patients (Cabrera et al., 2021; Morrissey et al., 2021; Schulte-Schrepping et al., 2020). LDNs are immature neutrophils, which are released during emergency myelopoiesis (Malengier-Devlies et al., 2021). LDNs isolated from COVID-19 patients expressed the ligand for programmed cell death protein 1 receptor (PD-L1), which is found on pro-tumour neutrophils (Schulte-Schrepping et al., 2020; Yajuk et al., 2021). This subset of neutrophils are immunosuppressive to T lymphocytes, which could contribute to lymphopenia in

COVID-19 (Cabrera et al., 2021; Schulte-Schrepping et al., 2020; Yajuk et al., 2021). The role of NETosis in COVID-19 immunopathology is described in section 1.3.2.3.

1.3.2.2 *Acute respiratory distress syndrome and the cytokine storm*

Acute respiratory distress syndrome (ARDS) is a severe complication of COVID-19 and is characterised by hypoxemia and pulmonary oedema (Meyer et al. 2021). Inflammation and fluid in the lungs is accompanied by diffuse alveolar damage, resulting in respiratory failure (Meyer et al., 2021). ARDS is not unique to COVID-19 pathogenesis and can occur in sepsis and severe trauma (Meyer et al., 2021). Mortality in mechanically ventilated patients with COVID-19 associated ARDS is approximately 65-94% (Gibson et al., 2020). In lower respiratory tract infection, SARS-CoV-2 enters alveolar type 2 epithelial cells, resulting in viral replication and cell apoptosis, releasing virus particles (Bridges et al., 2021). Infection of the alveolar epithelium initiates a robust innate immune response and stimulates the formation of the NLRP3 inflammasome (Pan et al., 2021). The NLRP3 inflammasome is a multi-protein complex which catalyses the formation of the mature form of the pro-inflammatory cytokine, IL-1β (Pan et al., 2021). IL-1β activates the NF-κB transcription factor, causing gene transcription of pro-inflammatory cytokines, including IL-6, CXCL8, TNFα and chemokines (Pan et al., 2021). Cytokine and chemokine production stimulates the recruitment of macrophages, neutrophils, and lymphocytes to the alveolus (Yang et al., 2021). Activated immune cells further release cytokines, forming a positive feedback loop of immune activation, inflammation and tissue damage (Yang et al., 2021). This is termed a cytokine storm, which is a characteristic feature of severe COVID-19 (Hojyo et al., 2020; Yang et al., 2021). Anaphylatoxin C5a, which is part of the complement cascade, is increased in patients with COVID-19 and is associated with disease severity (Carvelli et al., 2020). C5a stimulates immune cell recruitment to the lungs further driving the pathology ARDS (Carvelli et al., 2020). Interferon cytokines, which are key anti-viral cytokines, are down-regulated in SARS-CoV-2 infection (Kim and Shin, 2021). Excessive pro-inflammatory cytokine release damages the endothelium and activates the coagulation cascade and platelets, causing disseminated intravascular coagulation, which contributes to multi-organ failure (Bonaventura et al., 2021).

1.3.2.3 *The role of NETosis in COVID-19 associated ARDS*

NETosis has emerged as an important mechanism in the development of COVID-19 associated ARDS. At the start of the pandemic, in the absence of supporting evidence, NETosis was suggested to be a possible contributor to the pathology of COVID-19, due its known role in inflammation and tissue damage in other diseases (Mozzini and Girelli, 2020). Subsequently it was found that NETosis is increased in COVID-19. Serum biomarkers of NETs (cell-free DNA, citrullinated histones & DNA-MPO complexes) and immunohistochemical staining of lung autopsy samples, demonstrated increased NET formation in COVID-19 patients (Radermecker et al., 2020; Zuo et al., 2020). Furthermore, blood

plasma from patients induced NETosis in peripheral blood neutrophils from healthy donors and neutrophils from COVID-19 patients undergo increased NETosis in comparison to controls *ex vivo* (Masso-Silva et al., 2021; Middleton et al., 2020). The importance of NETosis is further supported by the finding that NET biomarkers are an indicator of COVID-19 disease severity (Ng et al., 2021). There are several possible reasons why NETosis is increased in COVID-19. Firstly, SARS-CoV-2 directly induces NET formation *ex vivo,* which was demonstrated to be reliant on active viral replication (Arcanjo et al., 2020a; Veras et al., 2020). NETosis is also hypothesised to be driven by the cytokine storm, as cytokines prevalent in COVID-19 (e.g. IL-6 & CXCL8) induce NETosis *ex vivo* (Borges et al., 2020; Gillot et al., 2021; Gupta et al., 2014; Joshi et al., 2016). ROS production is higher in neutrophils from patients with COVID-19, which could further exacerbate NETosis (Masso-Silva et al., 2021). Also, LDNs, which are increased in severe COVID-19 patients, undergo increased NETosis (Cabrera et al., 2021; Carissimo et al., 2020; Morrissey et al., 2021; Schulte-Schrepping et al., 2020). NETs contribute the development of lung injury and ARDS (Figure 4) by directly causing endothelial and airway epithelial cell damage and death (Narasaraju et al., 2011; Saffarzadeh et al., 2012; Veras et al., 2020). NE, which is abundant on NETs, cleaves the intercellular junction proteins (E-cad) between lung epithelial cells, further contributing to tissue destruction (Boxio et al., 2016). NET bound proteases also degrade the glycosaminoglycan heparan sulfate, which is a structural component of the alveoli and the lung parenchyma (Klebanoff et al., 1993). A key role of NETs in the pathogenesis of COVID-19 associated ARDS is the contribution to immunothrombosis. NETs activate and aggregate with platelets, initiating clot formation and NETs also activate the coagulation cascade (de Bont et al., 2019). NETs are localised to microthrombi in the lungs of deceased COVID-19 patients (Leppkes et al., 2020; Middleton et al., 2020). Microthrombi causes vascular occlusion, further exacerbating lung damage (Leppkes et al., 2020). Children predominantly suffer asymptomatic or mild COVID-19 disease (Seery et al., 2021). Interestingly children infected with SARS-CoV-2 demonstrated a comparable amount of NETs to healthy controls, further demonstrating the importance of NETosis in severe disease (Seery et al., 2021).

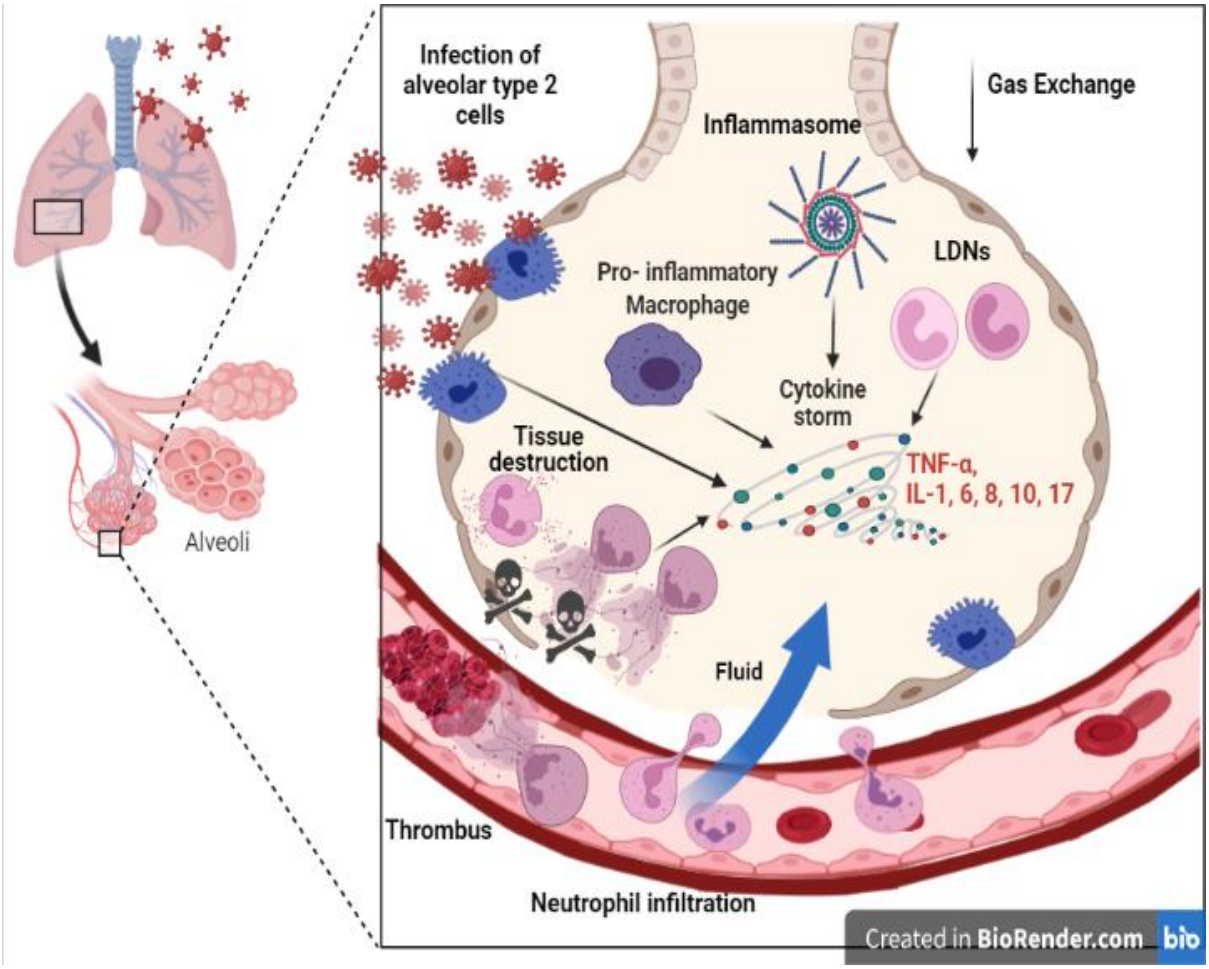


Figure 4-The role of neutrophils and NETosis in the immunopathology of acute respiratory distress syndrome.

Acute respiratory distress syndrome (ARDS) is a severe complication of COVID-19. SARS-CoV-2 infects alveolar type 2 cells. Mature neutrophils are recruited to the alveolus, where they undergo NETosis contributing to tissue destruction and thrombus formation. Low density neutrophils (LDNs) are increased in COVID-19 and contribute to immunopathology. Fluid enters the alveolar space and reduces the capacity for gas exchange. The cytokine storm results from inflammasome activation and immune cell activation, perpetuating inflammation. Figure created with BioRender.com

1.3.2.4 *COVID-19 clinical trials targeting NETosis*

Targeting the host immune response to infection, to improve disease outcomes, is very important in COVID-19, which is exemplified by the effectiveness of dexamethasone and tocilizumab. Due to the importance of NETosis in COVID-19 there are several therapies being explored in human clinical trials to inhibit this pathway. Therapeutic strategies directly target the NETosis pathway but also the pro-inflammatory cytokine signalling pathways associated with the cytokine storm and enhanced neutrophil activation in COVID-19. Unless stated, results from ongoing clinical trials of the drugs described below are not yet publicly available. Dornase alfa (Pulmomzyme, Genentech), is a recombinant DNase enzyme, used to treat cystic fibrosis (Yang et al., 2016). Dornase alfa is a mucolytic, which degrades extracellular DNA from extruded NETs and it is being explored in multiple

clinical trials in hospitalised patients with COVID-19 (e.g. NCT04359654, NCT04432987 & NCT04355364) (Okur et al., 2020). Pilot study data demonstrates that dornase alfa improved oxygenation and decreased NETs in the BALF from COVID-19 patients with ARDS (Holliday et al., 2021). Another therapy being investigated is disulfiram, a gasdermin D inhibitor (Fillmore et al., 2021). Disulfiram is licensed for the treatment of alcohol dependence and there are two clinical trials exploring the impacts of disulfiram in COVID-19 (NCT04594343 & NCT04485130) (Skinner et al., 2014). An observational study demonstrated that disulfiram lowered the risk and severity of COVID-19 (Fillmore et al., 2021). Disulfiram abrogated NET formation and improved survival in a mouse model of sepsis (Silva et al., 2021). Also, disulfiram may be antiviral, as it inhibited SARS & MERS papain-like proteases, which are important proteases in viral replication (Lin et al., 2018). The anti-viral and anti-inflammatory effects of disulfiram in SARS-CoV-2 infection are being explored in NCT04485130. Anakinra is under investigation in multiple clinical trials using COVID-19 patients (e.g. NCT04603742, NCT04443881 NCT04680949). Anakinra is a IL-1 receptor antagonist which inhibited NET formation in neutrophils from patients with pyogenic arthritis, pyoderma gangrenosum and acne syndrome (Mistry et al., 2018). However, a randomised control trial of anakinra in mild-moderate patients with COVID-19 pneumonia demonstrated there were no improvement in outcomes or mortality in the treatment group compared to placebo (NCT04341584) (Tharaux et al., 2021). Targeting oxidative stress in COVID-19 is being investigated using the antioxidant N-acetylcysteine (NCT04792021 & NCT04419025). N-acetylcysteine inhibited neutrophil ROS production and reduced NETosis in response to PMA *ex vivo*, using neutrophils isolated from healthy donors (Kirchner et al., 2013). However, N-acetylcysteine treatment did not improve outcomes (need for mechanical ventilation, mortality, ICU admission) in a placebo-controlled trial in patients with severe COVID-19 (Brazilian Registry of Clinical Trials: U1111-1250-356) (de Alencar et al., 2021). NE is abundantly found on NETs and contributes to the tissue damage found in ARDS. Sivelestat is the most well-known NE inhibitor, and it is used for the treatment of ARDS in Asia (Maki et al., 2020). Sivelestat is undergoing investigation in the context of COVID-19 in a range of trials (NCT05020210, NCT04973670 & NCT04909697). The STOP-COVID clinical trial, which targeted NE using brensocatib (NCT04343898), will be explained in results chapter 2.

1.3.2.5 *Secondary bacterial infections in COVID-19*

Secondary bacterial infections, identified in the blood or sputum occur in approximately 7-12% of hospitalised patients with COVID-19 and they are a significant indicator of morbidity and mortality (Langford et al., 2020; Shafran et al., 2021). Susceptibility to infection is increased due to tissue damage and dysregulation of the immune response. The causative pathogens include *E. coli, S. aureus, H. influenzae* and *P. aeruginosa* (Langford et al., 2020; Shafran et al., 2021). Although not a dominant feature of COVID-19, increased understanding of the immune response to bacterial pathogens would

be of value. There is limited evidence investigating the neutrophil phenotype and neutrophil pathogen handling in COVID-19, aside from the research regarding NETosis. Belchamber et al. (2021) (pre-print), demonstrated that neutrophil phagocytosis of *S. pneumoniae* and ROS generation were reduced in peripheral blood neutrophils from hospitalised non-ITU COVID-19 patients in comparison to aged matched healthy controls (Belchamber et al., 2021). Furthermore, neutrophil migration to CXCL8 was increased, but the expression of cell surface marker, CD11b was reduced in neutrophils from COVID-19 patients.

1.3.2.6 *Long COVID*

The clinical definition of long COVID is the persistence of symptoms for at least 3 months after SARS-CoV-2 infection, which cannot be explained by an alternative diagnosis and which impact daily functioning (Ward et al., 2021). Examples of long COVID symptoms include breathlessness, severe fatigue, cognitive defects and tachycardia (Ward et al., 2021). Causes of long COVID are suggested to include lung fibrosis and damage and prolonged dysregulation of the immune system (Giacomelli et al., 2021; Peluso et al., 2021). The immune landscape in long COVID is only beginning to be understood. Recent research demonstrates there were decreased numbers of natural killer T cells, increased proportion of LDNs and elevated levels of cytokines (e.g. vascular endothelial growth factor) in convalescent patients 39 days after acute infection (Lim et al., 2021). Immunotherapies are likely to be of use in treating long COVID as well as acute infection.

1.4 Hypothesis and aims

The overarching aim of this project is to better understand aberrant neutrophil function in disease, with the goal to seek to therapeutically modify dysfunctional pathways *in vitro*. Neutrophilic inflammation is a central immunopathology in a range of diseases and targeting the host immune response to infection could provide novel therapeutic strategies to treat infections and chronic illnesses. This research was funded by the national SHIELD consortium which aims to tackle the global antibiotic resistance problem by discovering new drugs to improve the host immune response to infection. Previous research demonstrates that neutrophil function is fundamentally altered in diabetes, with all known neutrophil functions shown to be dysregulated, which is covered in a large body of literature spanning 60 years. Despite this, there are no licenced treatments directly targeting neutrophilic inflammation in diabetes. People with DFD suffer severe and chronic infections and previous research shows that NETosis is elevated in DFD, contributing to poor wound healing. However, the neutrophil phenotype in people with DFD is yet to be fully defined. This is an important gap in the literature, as it could provide novel therapeutic targets to control neutrophilic inflammation in DFD. The hypothesis for this work was that people with DFD would have multiple dysfunctional

neutrophil effector functions, which would be amenable to therapeutic modulation with pre-existing therapeutics *in vitro*. To address this hypothesis the following aims were set:

1) Optimise a collection of core assays of neutrophil function to be used in a phenotyping study of neutrophils isolated from people with DFD.
2) Design and set up a pilot study investigating neutrophil function in people with DFD using the assays optimised in aim 1.
3) Explore therapeutic modulation of aberrant neutrophil functions in patients with DFD that were identified in aim 2 *ex vivo*.

The patient study stated in aims 1 & 2 began in January 2020. Unfortunately, the significant danger posed by the COVID-19 pandemic, stopped the study in March 2020, and it was cancelled indefinitely to protect participant safety. Aims 2 and 3 could not be completed. I therefore adapted my PhD project 18 months after starting my PhD. The core aim of my PhD project remained the same, which was to study neutrophilic dysregulation and inflammation in disease and to identify pathways amenable to therapeutic modulation *in vitro.* This required me to adapt the context of the disease that I was investigating. Through the knowledge and skills I obtained from the diabetes project, I understood the importance of NETosis in disease pathology and NETosis was emerging as a key pathological feature of COVID-19 associated ARDS. I therefore decided to study NETosis in neutrophils isolated from hospitalised patients with COVID-19 and explore how this pathway could be modulated *in vitro*. This research was conducted and supported by the UK COVID Immune Consortium (UK-CIC), which is a national consortium set up in response to the COVID-19 pandemic, which aims to understand the immunology of COVID-19 and improve outcomes for patients. In parallel with this, I was integral to the set up and completion of assays of neutrophil function in Sheffield for the national 'Superiority Trial of Protease Inhibition in COVID-19' (STOP-COVID) clinical trial, which aimed to target NE mediated inflammation in COVID-19. I hypothesised that NETosis would be increased in hospitalised patients with COVID-19 and that this would be an important pathway to seek to modulate therapeutically. To meet the aims of the project the following objectives were set:

1) Investigate NETosis in neutrophils isolated from hospitalised patients with COVID-19 as part of the UK-CIC.
2) Explore ways to modify NETosis using patient neutrophils *in vitro*.
3) Set up and conduct assays of neutrophil function, including NETosis, for the Sheffield arm of the national STOP-COVID clinical trial.

2 Materials and Methods

This chapter will detail the materials and methods used for 3 separate patient studies, which investigated neutrophil function in patients with either DFD, COVID-19 or COVID-19 patients enrolled onto the STOP-COVID clinical trial. The patient study design, including participant selection, recruitment protocols and inclusion/exclusion criteria will be explained. Experiments involving human neutrophils, and/or bacteria were conducted inside a category 2 safety cabinet. Handling of samples from patients with acute SARS-CoV-2 infection were handled in a Category 3 containment facility following all health and safety policies. Methodology for the transcriptomics investigation conducted in the first COVID-19 lockdown is incorporated into the relevant results chapter (section 4.2).

2.1 Set up and design of a study investigating neutrophil function in people with DFD

2.1.1 Research ethics

Blood was taken from healthy volunteers according to the protocol approved by the National Research Ethics Committee- Yorkshire & The Humber - Sheffield (05/Q2305/4). Written informed consent was obtained. For volunteers with diabetes ethical approval was granted for the study 'The Control of Innate Immunity, Host-Pathogen Interactions and Leukocyte Function in Disease' by the Health Research Authority. The sponsor was Sheffield Teaching Hospitals NHS Foundation Trust. IRAS project ID 254367 and REC reference 18/EE/0369. Patient information sheets (PIS) (Appendix 1) and participant consent forms were submitted by Professor Ian Sabroe as part of the ethical approval process for the study. Donor numbers were assigned to anonymise volunteers and maintain confidentiality. Professor Ian Sabroe was the principal investigator (PI) for this study.

2.1.2 Research Passport

I was granted my NHS research passport and letter of access to conduct research within the NHS on the 7th of February 2019 (Appendix 2). DBS and occupational health checks were conducted. I completed good clinical practice online training prior to commencing the study.

2.1.3 Recruitment, screening, consent taking and venesection of patients with DFD

In line with the approved study protocol, recruitment was conducted by Dr Ahmed Iqbal (Dept. Infection Immunity & Cardiovascular Disease, University of Sheffield), a diabetes specialist and member of the participant's clinical care team. Dr Iqbal gave the PIS to individuals in his care at the DFD clinic at the Royal Hallamshire Hospital (RHH), Sheffield, who were interested in taking part in the study. PIS sheets were given to individuals who had active diabetic foot infection, were aged between 50-75 years of age, did not have a BMI of greater than 35, were not a smoker (or an ex-smoker with a pack year history of greater than 5 and who had not smoked in the last 5 years) and did not have a contraindication to venesection. The patient's choice to enrol in the study and emphasis that this would have no impact on clinical care was explicitly communicated to the patient, in addition to the

right to withdraw from the study at any time. Dr Iqbal contacted the participant via telephone at least 48 hours after receiving the PIS to answer any questions about the study and confirm that the participant was happy to answer further screening questions. The screening questions addressed the inclusion and exclusion criteria outlined in section 3.1.1.3. Prior to the patient attending the clinical research facility to donate blood, Dr Iqbal confirmed that the patient still met the criteria of having an active diabetic foot infection, as patients were undergoing standard treatment for DFD. Informed consent was obtained by Dr Iqbal prior to venesection. The participant kept a signed copy of the consent form for their records. The consent forms were stored in the site file.

2.1.3.1 *Venesection*

Dr Iqbal conducted the phlebotomy in the clinical research facility. A total of 40 ml of blood was taken from each participant. A phlebotomy record sheet was completed to ensure that the patient had had no change in physical health since study enrolment, and a donation tracking log was kept. The donation tracking log ensured that a patient, if required to donate blood on multiple visits, did not exceed the total blood volume approved in the study protocol. Phlebotomy limits were a maximum donation of 500 ml over 12 months, divided into a maximum of 4 donations, with no individual donation exceeding 150 ml in volume. Patients were reimbursed for travel expenses.

2.1.3.2 *Collection of patient clinical information*

In discussion with the research team, I designed the case report form (CRF) to capture all the relevant information for each participant (Appendix 3). Separate forms were designed for healthy controls as clinical data was not collected (Appendix 4). After informed consent was obtained, Dr Iqbal accessed the relevant patient clinical information outlined in the CRF. The following information was collected to inform data interpretation downstream: type and duration of diabetes, last recorded glycated haemoglobin (HbA1c) to measure diabetes control, comorbidities, names and doses of diabetes medication, antibiotics and any other medications, if not currently on antibiotics- antimicrobial history for the last 3 months, most recent microbiology culture result, antibiotic sensitivity of *S. aureus* isolates (if any) and duration of diabetic foot ulceration. Clinical data was anonymised. A letter informing the patient's GP of the participation in the study was sent (Appendix 5).

2.2 Optimisation and setup of assays of neutrophil function for use in the DFD patient study

2.2.1 Bacterial Culture

2.2.1.1 *Preparing brain heart infusion broth for S. aureus culture*

Brain heart infusion (BHI) powder (Sigma-Aldrich, Missouri, USA) was mixed with sterile distilled water in a Duran bottle as per the manufacturer's instructions and autoclaved.

2.2.1.2 *Preparing BHI agar for S. aureus growth*

BHI powder (Sigma-Aldrich) was mixed with sterile distilled water as per the manufacturer's instructions. Once autoclaved and cooled to a safe temperature liquid agar was poured into sterile Petri dishes (Sigma-Aldrich). Agar was left to solidify for 30 minutes before being stored for one month (4 °C). To select for the *SaeS* mutant strain of *S. aureus* (3.2.6.1) the antibiotics lincomycin (Lin) [12.5 µg/mL] and erythromycin (Ery) [2.5 µg/mL] were used as resistance markers and added at the relevant concentrations to the BHI broth and agar.

2.2.1.3 *Generation of a Staphylococcus aureus growth curve*

A long-term storage bead from a Microbank™ cryovial (Pro-Lab Diagnostics, Merseyside, UK) containing SH1000 methicillin sensitive laboratory strain of *S. aureus* were removed from -80 °C storage and streaked onto BHI agar and incubated overnight at 37 °C. For clonal expansion, a single colony from each BHI plate was placed in 10 ml of BHI broth in a sterile 50 ml Falcon tube (Sarstedt, Nümbrecht, Germany) and incubated overnight (37 °C, 5% CO_2) on a rotary shaker at 350 revolutions per minute (rpm). The lid was kept loose on the Falcon tube but secured with autoclave tape to allow air exchange. The following day, the optical density (OD_{600}) was measured using a spectrophotometer (6100 Jenway). One millilitre of BHI broth was placed in a sample cuvette and used to blank the spectrophotometer. The absorbance of a 1:10 dilution of the bacterial suspension in BHI broth was then quantified. The OD_{600} was used to calculate the volume of suspension required for sub-culture to give a starting OD of 0.05 in 10 ml of BHI broth, using the formula outlined below.

Volume of bacterial suspension required for an OD of 0.05= (0.05/ (OD reading x 10)) x10

The subculture was placed on a rotary shaker, as before, for 9 hours. Once per hour (0-9 hours) the OD was measured. OD and time were plotted to generate the growth curve, shown in Figure 5. *S. aureus* growth curves were in line with published literature (Guido et al., 2012; Singh et al., 2015). Growth curves were not conducted for the other strains of *S. aureus* as they were used in assays directly after overnight culture.

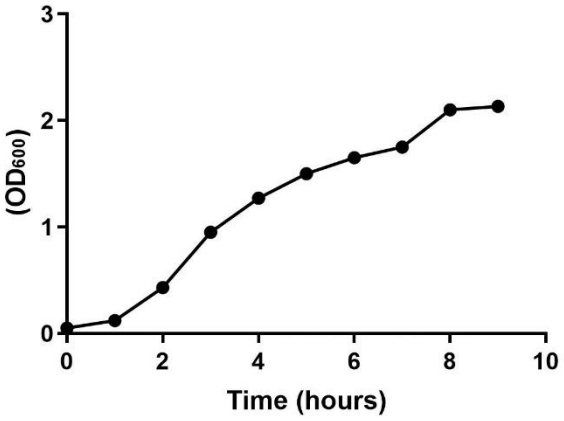


Figure 5-SH1000 strain of *S. aureus* growth curve
S. aureus (SH1000) was grown in BHI broth after an inoculation to an optical density (OD_{600}) of 0.05. The OD_{600} reading was taken once per hour for 9 hours. Data represents n=1.

2.2.1.4 *Growth of S. aureus stocks*

Bacterial stocks were made for the following strains of *S. aureus:* SH1000, SH1000-GFP, JE2 and *SaeS* mutant JE2. Stocks were made using an identical procedure to that used to generate the SH1000 *S. aureus* growth curve (section 2.2.1.3). However, after approximately 2.5-3 hours of sub-culture, when the bacteria were in the mid-exponential phase of growth (OD_{600} 0.7-0.9), 1 ml aliquots of subculture suspension were placed in 1.5 ml Eppendorf tubes (Sarstedt) and stored at -80 °C. Where stationary phase stocks of bacteria were needed for NETosis assays, 1 ml aliquots of *S. aureus* were frozen after overnight culture (section 2.3.2.4).

2.2.1.5 *Calculation of CFU/ml of S. aureus stocks using the Miles and Misra method*

An aliquot of each *S. aureus* strain was removed from -80 °C storage and defrosted at room temperature (RT). Nine hundred microlitres of Gibco™ Dulbecco's phosphate buffered saline (DPBS) (ThermoFisher Scientific) was added to each of 7, 1.5 ml Eppendorf tubes. One hundred microlitres of the bacterial cell suspension was serially diluted across the tubes, vortexing each time. A BHI agar plate was divided into 8 sections, and 10 µl from each Eppendorf was pipetted onto the plate in triplicate. The plate was incubated overnight (37 °C). Growth was counted for dilution ranges containing 20-50 colonies. The average of three replicates was used. The formula below was used to calculate the colony forming unit per ml (CFU/ml).

CFU/ml= (colony count x dilution factor) x 100

2.2.1.6 *Calculation of multiplicity of infection*

A *S. aureus* aliquot was removed from -80 °C storage and defrosted at RT before centrifuging at 6,000 *g* for 7 minutes. Pellets were washed in DPBS, prior to resuspending in 1 ml of the appropriate cell

culture media. The following calculation was used to determine the volume of bacterial suspension to add to neutrophils to achieve the desired MOI for an experiment.

Volume of bacterial suspension = Desired CFU of *S. aureus* per well / CFU/µl of stock

2.2.2 Preparation of cell culture media

2.2.2.1 *Preparing 1 M glucose stock solution*

Five grams of D-(+)-Glucose (Sigma-Aldrich) was mixed with 27.5 ml of pre-warmed sterile distilled water to make a 1 M stock. The solution was filter sterilised using sterile Acrodisc 0.2m Supor Membrane syringe filters (Pall Life Sciences, Portsmouth, U.K) and stored at 4°C for up to 3 months. A 1:100 dilution of the 1 M stock concentration was sent to the Clinical Biochemistry department at the Royal Hallamshire Hospital (RHH), Sheffield for validation of the stock glucose concentration, against the use of a handheld FreeStyle Optium Neo blood glucose meter (Abbott Diabetes Care Inc, Berkshire, UK). Consistency between methods was validated so henceforth the blood glucose meter was used to confirm glucose concentration in media for subsequent preparations.

2.2.2.2 *Preparing cell culture media of different glucose concentrations*

All media, apart from that used in the NETosis experiments, consisted of 10% heat inactivated fetal calf serum (FCS) (ThermoFisher Scientific, Massachusetts, USA). Gibco™ RPMI 1640 containing L-glutamine and phenol red (ThermoFisher Scientific) was used. This media was routinely used in the laboratory for cell culture and in this project is named 'standard' media. Standard media contained 11 mM glucose. Gibco™ RPMI 1640 containing L-glutamine and phenol red without glucose (ThermoFisher Scientific) is referred to as 'low' glucose media. The glucose concentration of low glucose media was 0.3 mM when analysed by the Clinical Biochemistry department at the RHH. This is likely due to the presence of residual glucose in the FCS. To make media containing 20 mM glucose, 1 ml of 1 M glucose solution was added to 50 ml of Gibco™ RPMI 1640 Media without glucose. Twenty millimolar glucose cell culture media is referred to as 'high' glucose media in this thesis. For assays analysing fluorescence output, Gibco™ RPMI 1640 Media, without phenol red was used (ThermoFisher Scientific), as phenol red can increase background fluorescence (Stadtfeld et al., 2005). This formulation did not contain 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) required for pH maintenance, so Gibco™ 1 M HEPES solution was added at a final concentration of 10 mM (ThermoFisher Scientific). Media were stored at 4°C for up to three months.

2.2.3 Neutrophil isolation from whole blood using dextran sedimentation and plasma/Percoll density gradient separation

Forty millilitres of whole blood were added to 5 ml of 3.8% tris-sodium citrate (Ethypharm, Buckinghamshire, UK) in a 50 ml sterile Falcon tube. The tube was inverted slowly to aid mixing of the anticoagulant. The blood was centrifuged at 177 *g* for 20 minutes at 20 °C. The platelet rich plasma

was removed and centrifuged at 493 *g* for 20 minutes at 20 °C to form platelet poor plasma (PPP). To the cell fraction, 6 ml of 6% dextran (Sigma-Aldrich) (3 g dextran 500,000 mw in 50 ml saline) was added and made up to 50 ml with sterile saline (Baxter, Illinois, USA). The tube was gently inverted, air bubbles removed, and the Falcon tube lid left loose. Dextran sedimentation of the red blood cells (RBCs) proceeded for 20 minutes at room temperature until a clear interface was visible. The leukocyte containing upper layer was aspirated and spun at 123 *g* for 6 minutes at 20 °C. The white blood cells were resuspended in 2 ml of PPP and layered onto the plasma/Percoll (GE medical systems Ltd, Buckinghamshire, UK) gradient. The plasma/Percoll gradient consisted of a lower phase made up of 51% Percoll and 49% PPP and the upper phase made up of 42% Percoll and 58% PPP. The upper phase was carefully layered on to the lower phase so as not to mix the layers. The resuspended peripheral blood cells were layered on top of the Percoll gradient. The gradient was spun at 149 *g* for 11 minutes at 20 °C to yield three layers of cells, which were peripheral blood monocytes (PBMCs), granulocytes and RBCs. The granulocytes were located at the interface of the two Percoll densities (Figure 6). A Pastette was used to remove the PBMCs and granulocytes, which were placed in separate 50 ml Falcon tubes, containing 10 ml of PPP. The cell suspension was topped up to 50 ml with Hanks balanced salt solution 1X without magnesium and calcium (HBSS) (ThermoFisher Scientific) for cell counting, using a glass haemocytometer. Ten microliters of cell suspension was added to the haemocytometer and all neutrophils in a 4x4 square were counted using an inverted light microscope and a manual cell counter. The following calculation was used to determine the volume of cell suspension needed for 5 x 10^6 cells/ml, which was the concentration of cells used in subsequent experiments.

Volume of cell suspension required:

5 x 10^6/ Haemocytometer count (cells/ml)

After cell counting, the calculated volume of cell suspension was spun at 277 *g* for 6 minutes at 20 °C and the pellet was gently resuspended in 1 ml of the appropriate cell culture media.

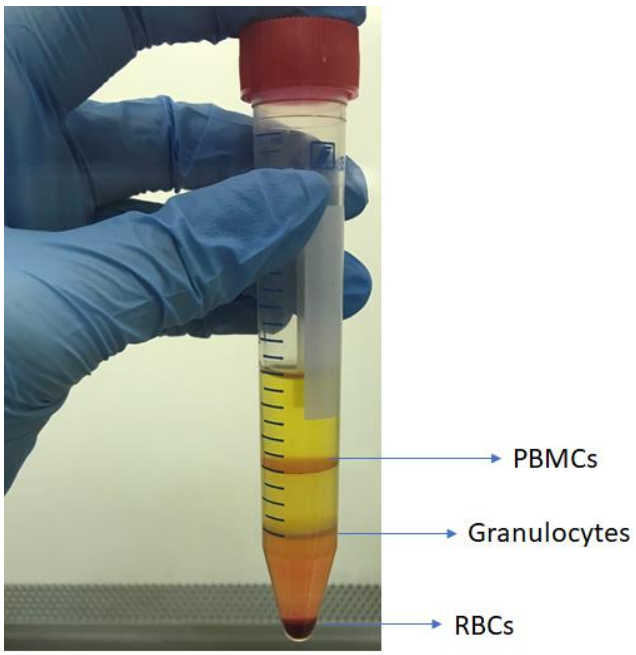


Figure 6-Percoll density gradient separation of cells.
Three bands of cells were visible after separation. The upper phase peripheral blood mononuclear cells (PBMCs), the granulocytes in the middle and red blood cells (RBCs) pelleted at the bottom.

2.2.4 Neutrophil purity- Preparing and analysing cytocentrifuge slides

For each neutrophil isolation, the purity of the cell preparation was determined by making cytocentrifuge slides and analysing using light microscopy. One hundred microlitres of cell suspension was placed inside a cytospin funnel (ThermoFisher Scientific) and centrifuged (Shandon Cytospin 3) at 300 rpm onto a glass microscope slide (Academy Science, Kent, UK). Cells were fixed with methanol and left to air dry before staining with ReaStain Quick-Diff Red (Reagena, Toivala, Finland) for 3 minutes and then Kwik Diff Solution blue (ThermoFisher Scientific) for 3 minutes. Once dry, coverslips were mounted onto the slides using Dibutylphthalate Polystyrene Xylene (DPX) mountant (ThermoFisher Scientific) and left overnight to dry. Microscope slides were analysed using the oil immersion inverted light microscope (Nikon Eclipse TE300,) with the 100x objective lens. To calculate the purity of neutrophils a total of 300 cells were counted on the slide and the relative number of neutrophils, eosinophils and PBMCs was noted. To calculate the neutrophil purity the formula below was used. A purity of >95% was aimed for.

% Purity of neutrophils= (neutrophil count/300) x 100

2.2.5 Measuring neutrophil apoptosis

Fifty microlitres of neutrophils (2.5 x 10^5 cells) were seeded in quadruplicate wells for each media condition (low, standard, or high) in a 96 well flexi plate (Corning, New York, USA). Forty microlitres of the corresponding cell culture media was added. Ten microlitres of lipopolysaccharide (LPS) from *E.*

coli, serotype 515 (Enzo Life Sciences, Exeter, UK) were added to half of the wells at a final concentration of 100 ng/ml. Cells were incubated for 6 and 22 hours (37°C, 5% CO_2). Cytocentrifuge preparations were made and imaged as outlined in section 2.2.4. Apoptotic neutrophils were identified by their typical morphology of a deeply stained, round nucleus. Three hundred neutrophils were counted (both live and dead) and the calculation outlined below was used to quantify % apoptosis in the different media conditions.

% neutrophil apoptosis = apoptotic neutrophils/300 x 100

2.2.6 Flow cytometry analysis of ROS production

2',7'-dichlorodihydrofluorescein (DCF) (Sigma-Aldrich) was used to assess intracellular ROS production in neutrophils. To optimise the MOI of S. *aureus* to use in the assay, 50 µl of neutrophils (2.5 x10^5) were seeded in duplicate wells in standard media in a 96 well flexi plate. Two additional wells were seeded for the bacteria negative control. Neutrophils were incubated for 30 minutes (37 °C, 5% CO2) before 10 µl of DCF, to give a final concentration of 5 µM, was added to all wells. To confirm that ROS production was being measured the NADPH inhibitor, diphenyleneiodonium chloride (DPI) (Cayman Chemical, Michigan, USA) was also added to duplicate wells at a final concentration of 10 µM and the plate was re-incubated for 30 minutes. *S. aureus* (SH1000), diluted in standard media was added to the cells to give either an MOI of 1, 5 or 10 and co-cultures were incubated for 30 minutes. An MOI 5 of *S. aureus* was added to the duplicate wells containing DPI. After incubation the co-cultures were placed in 1.5 ml Eppendorf tubes and centrifuged at 300 *g* for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 500 µl of ice-cold PBS containing gentamicin [20 µg/ml] (ThermoFisher Scientific) and penicillin [40 units/ml] (Sigma-Aldrich) and transferred to a 5 ml round bottom flow cytometry tube (ThermoFisher Scientific). The BD™ LSRII (BD Biosciences, San Jose, California) flow cytometer, and the BD FACSDiva™ software was used to analyse the samples. The 488 nm blue laser and 530 nm filter were employed to detect the fluorescence from DCF inside neutrophils. Neutrophils were gated using forward and side scatter (FSC and SSC) and 10,000 events were analysed per sample. The raw data were interpreted using the FlowJo data analysis program. The geometric mean of the 530 nm peak was compared between media conditions. The assay was repeated using an MOI of 5 of *S. aureus* using neutrophils and bacteria cultured in either low, standard or high glucose media.

2.2.7 Neutrophil phagocytosis of *S. aureus*

Fifty microliters of neutrophils (2.5 x 10^5) in either low, standard, or high media were added to a 96 well flexi plate and pre-incubated for 60 minutes. GFP- labelled *S. aureus* suspended in the appropriate cell culture media was added to the neutrophils to give an MOI of 5 and co-cultures were incubated for 30 minutes (37 °C, 5% CO_2). Co-cultures were placed in 1.5 ml Eppendorf tubes and centrifuged at

300 *g* for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 500 µl of ice-cold PBS containing gentamicin [20 ug/ml] and penicillin [40 units/ml] in 5 ml round bottomed flow cytometry tubes. Prior to analysis extracellular fluorescence was quenched using 0.2 % trypan blue (ThermoFisher Scientific). The BD™ LSRII flow cytometer, and the BD FACSDivaTM software was used to analyse the samples. The 488 nm blue laser and 530nm filter were employed to detect the fluorescence of ingested GFP-labelled *S. aureus* inside neutrophils. Neutrophils were gated using FSC and SSC and 10,000 events were analysed per sample. The raw data were interpreted using the FlowJo data analysis program. The percentage of GFP positive neutrophils, based on absolute number and geometric mean of the 530 nm peak were compared between media conditions.

2.2.8 Neutrophil intracellular killing of *S. aureus* in different glucose containing media

2.2.8.1 *Preparation of pH 11 alkali water for neutrophil lysis*

Alkali water was chosen for lysing neutrophils, which was based on previous optimisation by Natalia Hajdamowicz in the laboratory group and is supported by published findings (Decleva et al., 2006; Mashruwala et al., 2015). Forty-five millilitres of distilled water were placed in a 50 ml Falcon tube. Five microlitres of NaOH were pipetted into the solution and the pH was measured using a pH probe (Mettler Toldeo). A pH of 11 was required (+/- 0.05 error). The alkaline solution was filter sterilised and made fresh before each assay.

2.2.8.2 *Intracellular killing assay*

The aim of the intracellular killing assay was to enumerate the number of *S. aureus* (SH1000) that were internalised and killed by neutrophils and to determine if this was impacted by the concentration of glucose in the cell culture media. Neutrophil cell pellets were resuspended in 1 ml of either standard, low or high glucose media. Neutrophils (2.5×10^5) were seeded in duplicate wells for each media condition in a 96 well flexi plate. For each media condition, three sets of duplicate wells were seeded, which were used to quantify 'internalisation' and killing after 30 or 120 minutes of incubation. Cells were left to equilibrate for 60 minutes in the different media conditions (37 ºC, 5% CO_2), before, *S. aureus* (SH1000) diluted in the corresponding media, were added at an MOI of 5 and co-cultures were incubated for 30 minutes. Next, neutrophils from the 'internalisation wells', were aspirated from the plate and centrifuged at 400 *g* for 3 minutes, before resuspending in 1 ml of alkali water. Neutrophils were lysed for 10 minutes in alkali water, with intermittent vortexing and then placed on ice. This was conducted to quantify the number of bacteria phagocytosed by neutrophils (internalisation). Alkali water does not affect *S. aureus*; therefore, this allows quantification of live bacteria that are released from inside neutrophils. Gentamicin [40 µg/ml] was added to the remaining wells to kill extracellular bacteria and the plate was re-incubated. After 30 minutes (30-minute killing timepoint) the contents of two wells were removed, centrifuged and re-suspended in alkali water, as previously described and

then placed on ice. Gentamicin can permeate neutrophils after prolonged incubation; therefore, the gentamicin was removed from the remaining wells, which was conducted by centrifuging the co-culture at 400 *g* for 3 minutes. The cell pellets were resuspended in the corresponding media to wash the neutrophils and placed back in the 96 well plate. After a further 90-minute incubation (120-minute killing-timepoint), cells were aspirated and resuspended in alkali water as before. The alkali water samples were stored on ice until ready for quantification of the released bacteria at the three timepoints. Viable bacteria were quantified using the Miles & Misra technique (section 2.2.1.5). The CFU/ml at the different timepoints was calculated the following day.

2.2.9 Inducing and imaging neutrophil extracellular trap (NETs)

2.2.9.1 *Sterilising and coating coverslips*

Square 20 mm coverslips (Agar Scientific Ltd, Essex, UK) were UV sterilised for 15 minutes and placed in a 12 well plate (Corning) and 500 µl of Poly-L-lysine solution (0.01%) (Sigma-Aldrich) was added. The coverslips were immersed for 5 minutes before the solution was aspirated and the coverslips were stored for 3 months at 4°C.

2.2.9.2 *Quantifying NETs using DNA area*

One million neutrophils were resuspended in 900 µl of RPMI 1640 cell culture media (standard glucose, without FCS) in an Eppendorf tube. Phorbol 12-myristate 13-acetateate (PMA) (Sigma-Aldrich) was added to give a final concentration of either 10, 25, 50 or 100 nM and the tube volume was made up to 1 ml with cell culture media. An equivalent amount of DMSO (0.001%) (Sigma-Aldrich) was used as the vehicle control. In later experiments, media only was used as the negative control, once it was confirmed that DMSO did not have any impact on the assay. DPI [10 µM] was added to neutrophils stimulated with 25 nM PMA. One hundred microlitres of the stimulated neutrophil suspension (1 x 10^5 cells) were seeded onto poly-l-lysine coated coverslips placed in a 12 well plate and incubated for 3 hours (37 ^{o}C, 5% CO_2). Cells were fixed with 4% paraformaldehyde (PFA) for 15 minutes at RT. PFA was gently aspirated and cells were washed 3 times with PBS for 5 minutes. NETS are fragile; therefore, PBS washes were done by floating the coverslips on drops of PBS placed on parafilm. A drop of ProLong™ Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (ThermoFisher Scientific) was added to a microscope slide and the coverslips were placed cell face down onto the mountant. DAPI was used to stain DNA. Slides were stored overnight at 4°C in the dark before imaging. To investigate the impacts of glucose on NET formation the experiment was conducted as described above using only 25 nM PMA and with neutrophils cultured in either low, standard, or high glucose containing media. Slides were imaged using the Nikon Widefield Fluorescent microscope. The 395-455 nm wavelength filter set was used for the detection of DAPI signal. The 40x oil immersion objective lens was used. Between 3-5 fields of view were imaged at random across

duplicate slides for each condition. Approximately 200 neutrophils were analysed per condition. DNA area was used to quantify NET formation using the FIJI image analysis software. The thresholding function in FIJI was used quantify the area of each image that was positive for DAPI signal above background (Figure 7). The area positive for DAPI was adjusted for the number of cells in the field of view and the average area across multiple images was reported. This was used as a surrogate marker of NET formation. This technique was modified from previously published protocols with support from the Light Microscopy Facility at the UoS (Halverson et al., 2015; Rebernick et al., 2018).

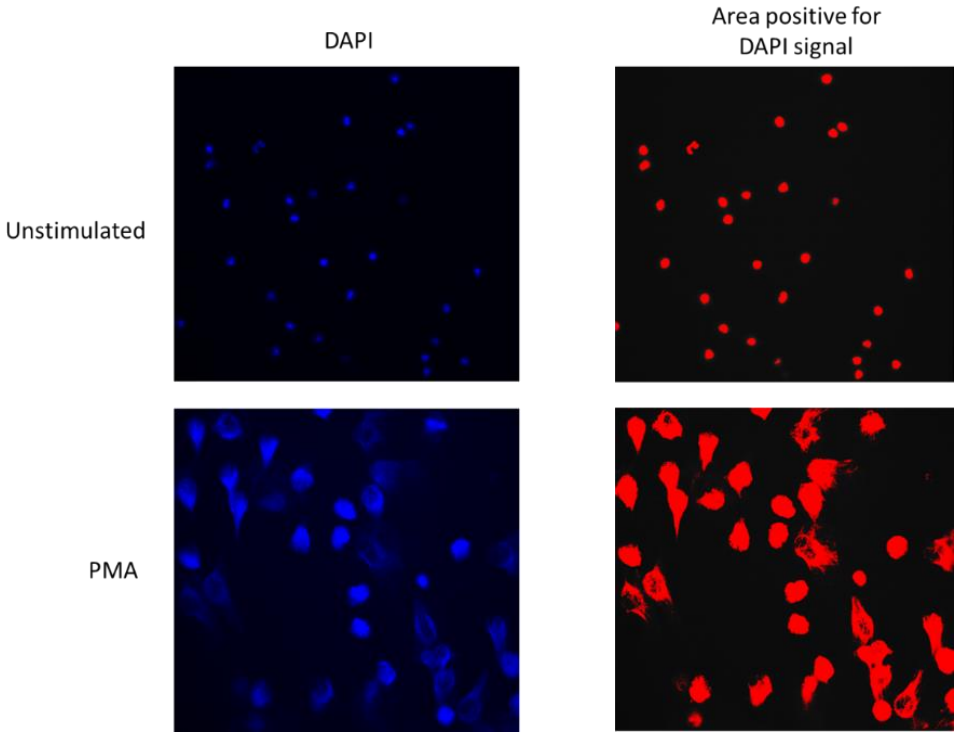


Figure 7-Quantifying DNA area.
Images of neutrophils stimulated with PMA to induce NETs and stained with DAPI (blue) were uploaded into the FIJI image analysis software. The thresholding function (red) was used to identify the area of each image positive for DAPI signal, which was used as a surrogate marker of NET formation. The total area was adjusted for the number of cells per image.

2.2.9.3 *Using S. aureus to induce NETs in primed neutrophils*

One million neutrophils were resuspended in 900 µl of RPMI 1640 cell culture media (without FCS) in an Eppendorf tube. Neutrophils were primed with granulocyte-macrophage colony-stimulating factor (GM-CSF) [20 ng/ml], LPS [100 ng/ml] or Formyl-Methionyl-Leucyl-Phenylalanine (FMLP) [1 µM] and the suspension volume was made up to 1 ml with cell culture media. Unprimed neutrophils were used as the control. Neutrophils were primed for 1 hour (37 ^{o}C, 5% CO_2), before stimulation with *S. aureus* (SH1000) (MOI 10). Co-cultures (1 x 10^5) were seeded onto poly-l-lysine coated glass coverslips. Co-cultures were incubated for a further 3 hours. Cells were fixed and prepared for imaging as described above (section 2.2.9.2).

2.2.9.4 *Immunocytochemistry*

2.2.9.4.1 *Blocking Buffer*

A 5% saponin (Sigma-Aldrich) stock solution was first made by adding 2.5 g of saponin to 50 ml of sterile distilled water. To make a 25 ml stock of blocking buffer a 5% bovine serum albumin (BSA) (Melford, Ipswich, UK) solution was made by adding 1.25 g to 23.25 ml of PBS in a 50 ml Falcon tube. Then, 1.25 ml of normal goat serum (Sigma-Aldrich) (final concentration 5%) and 0.5 ml of saponin (Sigma-Aldrich) (final concentration 0.1%) was added to give a total volume of 25 ml.

2.2.9.4.2 Antibody diluent

To make a 25 ml stock solution of antibody diluent 0.25 g of BSA was dissolved in 24.5 ml of PBS in a 50 ml Falcon tube. Then, 0.5 ml of a 5% saponin solution was added (final concentration 0.1%) to give a total volume of 25 ml.

2.2.9.4.3 Immunocytochemistry procedure

Neutrophils (5 x 10^5) were seeded into IBIDI™ µ-slide 8 well chambers with ibiTreat coated coverslips (Ibidi, Gräfelfing, Germany). NETosis was induced using the relevant stimulus. Media was removed and replaced with 250 µl of 4% PFA and incubated for 15 minutes at RT. Cells were then washed three times with PBS (250 µl) and stored at 4 ^oC until antibody staining. Blocking buffer (200 µl) was added to all wells and incubated on a rotating platform (350 rpm) for 1 hour (37 ^oC). Blocking buffer was removed and primary rabbit anti-human myeloperoxidase (MPO) antibody (A0398) (Agilent, California, USA), was diluted 1:500 in antibody diluent and 100 µl was added to each well. Chamber slides were re-incubated on the rotating platform as before, for 90 minutes. Cells were washed three times for 5 minutes in PBS (250 µl) on the plate shaker. Goat anti-rabbit IgG Heavy & Light chains Alexa Fluor® 594 antibody (ab150088) (Abcam, Cambridge, UK) was diluted 1:1000 in antibody diluent and 100 µl was added to all wells (including IgG control wells) and incubated for 45 minutes. Cells were washed three times for 5 minutes in PBS (250 µl) on the plate shaker. Two drops of ProLong™ Gold Antifade Mountant with DAPI was placed into each well. Rabbit IgG control antibody (I-1000-5) (Vector laboratories, San Francisco, USA) was used in three independent optimisation experiments to validate the anti-MPO staining, using the same method described. Slides were imaged using the Nikon Widefield fluorescent microscope, using the x60 or x40 oil immersion objective lens and the DAPI (excitation/emission 395/455 nm) and Texas red filter sets (excitation/emission 555/605 nm). Image analysis was conducted using the FIJI image analysis software. DAPI (blue) and MPO (red) channels were merged to produce composite images.

2.2.10 Workflow of neutrophil function assays in the study of people with DFD

The apoptosis, phagocytosis, ROS generation and NETosis (PMA-induced) assays were designed to be run simultaneously on the same day for the patient study. This design would enable neutrophil phenotyping in those with DFD, without requiring repeat venesection. Due to the labour-intensive methodology the intracellular killing assay was not included in this workflow. Different protocols were trialled to enable the apoptosis, ROS, phagocytosis and PMA-induced NETosis experiments to be conducted simultaneously without conflicting incubation times and in a timely manner after isolation. The method for conducting the assays is detailed in Figure 8. In brief, after neutrophil isolation the apoptosis plates were set up first due to the long incubation times of 6 and 22 hours. Then, the NETosis assay was initiated and in the 3-hour NETosis incubation the ROS and phagocytosis experiments were completed simultaneously. The completion of the flow cytometry assays was timed to coincide with the end of the NETosis incubation, when slides were then prepared, prior to finishing the apoptosis experiments. Microscopy for of the fixed apoptosis and NETosis slides were analysed the following day. For patient samples, Dr Nick Van Hateren, the laboratory manager at the UoS light microscopy facility, captured the images at random for the NETosis slides, so there was no bias in image selection for analysis.

Figure 8-Workflow for assays of neutrophil function to be completed on the same day.
Phenotyping of neutrophil function from cells isolated from people with diabetic foot disease and age-matched healthy controls was designed to be run simultaneously. Assays of neutrophil function included analysis of apoptosis, reactive oxygen species (ROS), phagocytosis and NETosis.

2.3 Methods used for investigating function of neutrophils from hospitalised COVID-19 patients

Two patient studies using neutrophils from COVID-19 patients were conducted; these were the STOP-COVID clinical trial and the NETosis study which was conducted as part of the UK-CiC. Experimental procedures were adapted to allow safe working with COVID-19 positive blood samples, which included the removal of any glassware from experimental protocols.

2.3.1 Study design and experimental protocols used for the STOP-COVID clinical trial

STOP-COVID was a multi-centre randomised control double blinded clinical trial of the neutrophil elastase inhibitor brensocatib, in hospitalised patients with COVID-19, led by Professor James Chalmers at the University of Dundee (UoD). As a participating centre in the study, COVID-19 patients were recruited and consented at the RHH, Sheffield, and I performed a number of pre-defined neutrophil assays in order to determine the effect of brensocatib on immune cell function. The trial was blinded at the time of experimentation (August 2020- February 2021) and unblinded in August 2021. Fully risk assessed protocols for the STOP-COVID clinical trial were designed and optimised by the team at the UoD and were approved for use in Sheffield. The experimental protocols required for the clinical trial involved a mixture of whole blood sample processing to obtain serum and cells for storage and subsequent transfer to Dundee at the end of the study for further processing (which is not included in this thesis) and also *ex vivo* assays of neutrophil function. These assays included assessment of NETosis (section 2.3.1.4), phagocytosis (section 2.3.1.5) and cell surface marker expression (section 2.3.1.6).

2.3.1.1 *Research ethics*

The study was approved by the South of Scotland Research Ethics Committee, approval number 20/SS/0057; all patients provided written informed consent.

2.3.1.2 *Participant recruitment and inclusion/exclusion criteria*

In Sheffield, participants were recruited to the trial by Dr Roger Thompson and the staff at the clinical research facility at the RHH. The PIS is provided in Appendix 6. Participants were recruited within the first 96 hours following hospital admission. Patients were randomised to receive either brensocatib (25 mg daily) or placebo for 28 days using a web-based randomisation software, which stratified patients on age, with 65 being the cut-off. The inclusion criteria for the trial were patients aged ≥16 years, who had a confirmed or highly suspected clinical case of SARS-CoV-2 infection and who had the capacity to give informed consent. Patients needed at least one risk factor for severe disease such as the requirement of supplemental oxygen. Exclusion criteria included, but was not limited to, history of liver disease, stage 4 severe chronic kidney disease or receiving HIV treatment. Patient blood

samples were taken, and neutrophil assays were conducted on day 1, day 15 if patients were still hospitalised and day 29.

2.3.1.3 *Isolation of neutrophils from COVID-19 patients using negative magnetic selection*

Whole blood was received in 2 x 10 ml EDTA vacutainers (k2) (MedicalWorld, West Bromwich, UK). Neutrophils were isolated from whole blood using EasySep™ Direct Human Neutrophil Isolation Kit (Stemcell™ Technologies, Vancouver, Canada). Whole blood was transferred to a 50 ml blue topped Falcon tube (ThermoFisher Scientific) and 1 ml per 20 ml of whole blood of the EasySep™ Direct Human Neutrophil Isolation Cocktail was added. EasySep™ Direct RapidSpheres™ were vortexed for 30 seconds and an equivalent amount to the neutrophil isolation cocktail was also added to the whole blood. The whole blood was gently mixed by inversion and incubated for 5 minutes. DPBS (without Ca^{+2} or Mg^{+2}) containing 1 mM ultrapure EDTA (ThermoFisher Scientific) was added to the whole blood to make a volume of 50 ml and was gently mixed by inversion again. The tube was placed inside the Easy 50 EasySep™ magnet (Stemcell™ Technologies) for 10 minutes with the Falcon tube lid left loose (Figure 9A). Using a stripette the neutrophil containing layer was removed and placed in a new 50 ml Falcon tube. The addition of vortexed EasySep™ Direct RapidSpheres™ was repeated twice more, with a subsequent 5 minute and then 10-minute incubation in the magnet. After the neutrophil containing layer was removed for the third time, 20 µl of cell suspension was placed inside a plastic haemocytometer (NanoEntek, Seoul, South Korea) and sealed with a gene frame (ThermoFisher Scientific). The neutrophils were centrifuged at 300 *g* for 6 minutes and then washed in 10 ml of DPBS (without Ca^{+2} or Mg^{+2}). The cells were centrifuged again at 300 *g* for 6 minutes and then resuspended in DPBS to give a final concentration of 5 x 10^6 neutrophils/ml. The following equation was used to calculate the volume of DPBS or cell culture media to resuspend the pellet in for use immediately in following experiments.

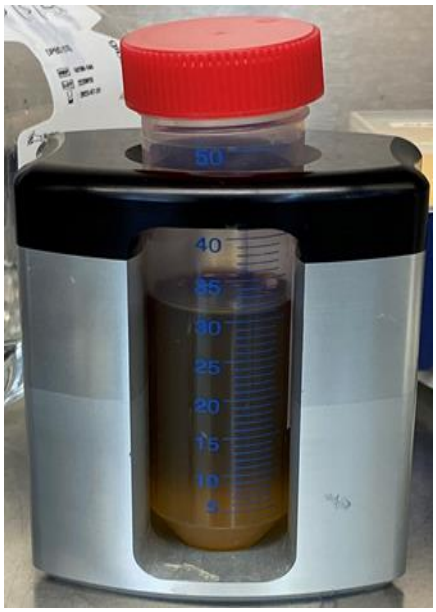
Volume of DPBS required to obtain 5 x 10^6 cell/ml:

A) Haemocytometer count (cells/ml) x volume of original cell suspension = total cell number

B) Total cell number / 5 x 10^6

Assessment of neutrophil purity was not conducted on samples from COVID-19 patients, due to the use of glass microscope slides and the cytocentrifuge being prohibited in the Category 3 laboratory. However, a high neutrophil purity (> 98%) was obtained when using this isolation method on healthy donor samples (Figure 9B).

A)



B)

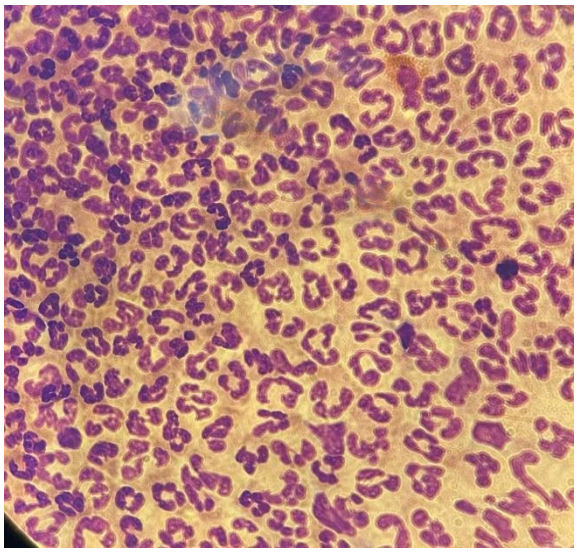


Figure 9-Neutrophil isolation using negative magnetic selection.
Neutrophils were isolated from whole blood using EasySep™ Direct Human Neutrophil Isolation Kit (A). Neutrophil purity was >98% (B).

2.3.1.4 *STOP-COVID SYTOX™ green NETosis assay*

Neutrophils (5 x 10^4), resuspended in RPMI 1640 cell culture media (no phenol red) and 10 mM HEPES, were seeded in quadruplicate into 2 separate Nunc™ MicroWell™ 96 well flat bottom microplates. One row on each plate was for the media only (no cells) control. Neutrophils were stimulated with either 100 nM PMA (plate a) or 5 µg/ml LPS (plate b) (*E. coli* O111:B4) (Sigma-Aldrich) in the appropriate wells and plates were incubated (37 °C, 5% CO_2). After 1 hour, plate b was removed from the incubator and SYTOX™ Green nucleic acid stain (555 nM) (ThermoFisher Scientific), was added to all wells, apart from the cells only control row. SYTOX™ Green is a cell impermeable stain for extracellular DNA and is used a surrogate for NET formation. The plate was sealed using VIEWseal™ transparent plate seals (Greiner Bio-one, Kremsmüster, Austria) and immediately read on the VarioSkan Flash plate reader (version 4.00.53) using an excitation/emission of 490/537 nm. Median fluorescent values were used to generate results. Plate b was re-incubated for a further three hours. SYTOX™ green was added to plate a, and both plates were analysed as before.

2.3.1.5 *Neutrophil Phagocytosis of opsonised heat-killed E. coli*

Vials of fluorescein isothiocyanate (FITC) labelled heat-killed *E. coli* were received from the UoD. First, the bacteria were opsonised using 10 % normal human serum (NHS) (aliquots of NHS were shipped from UoD) for 30 minutes at 37 °C. NHS negative controls were made by using sterile HBSS 1X (without Ca^{+2}, Mg^{+2} or phenol red) (ThermoFisher Scientific). Neutrophils (0.5 x 10^6) were added to labelled 1.5

ml Eppendorf tubes in a volume of 100 µl and opsonised *E. coli* were added at an MOI of 10. The co-cultures were incubated for 30 minutes (37 °C, 5% CO_2) prior to addition of 300 µl of 2% BSA and centrifugation of the samples at 500 *g* for 5 minutes. The cells were washed with 500 µl of DPBS and centrifuged again at 500 *g* for 5 minutes. The cells were resuspended in 250 µl of 4% PFA and incubated at 4 °C for 90 minutes. After fixation, neutrophils were centrifuged and washed again with DPBS. The cells were resuspended in 500 µl of 2% BSA and transferred to a 5 ml round bottomed flow cytometry tube. The BD™ LSRII flow cytometer was used to analyse the samples. The 488 nm argon blue laser and 530 nm filter were employed to detect fluorescenct bacteria inside neutrophils. Neutrophils were gated based FSC and SSC and 10,000 events were analysed per sample. Raw data files were analysed by the UoD.

2.3.1.6 *Cell surface marker expression of neutrophils from patients with COVID-19*

Neutrophils (0.5×10^6) were added to 100 µl of 2% BSA in 1.5 ml Eppendorf tubes. Cell surface marker antibodies CD63, CD66b, CD11b, CD88 and CXCR2 were added at the required volume, as detailed in Table 1. Unlabelled neutrophils were used as the control. Samples were incubated for 30 minutes at 4 °C, then a further 300 µl of 2% BSA solution was added to each Eppendorf tube. Cells were centrifuged at 300 *g* for 5 minutes, before washing in 500 µl of DPBS and being centrifuged again at 300 *g* for 5 minutes. Supernatant was removed and cells were fixed with 250 µl of 4% PFA for 90 minutes at 4 °C. Subsequently, samples were centrifuged at 500 *g* for 5 minutes and washed once with 500 µl DPBS, before resuspension of the pellet in 500 µl of 2% BSA solution and transferred to 5 ml round bottomed flow cytometry tubes. The BD™ LSRII flow cytometer using the 488 nm blue laser**,** and either the 575 nm filter (phycoerythrin- PE) or the 690 nm filter (PerCP-Cy™5.5) were employed to detect cell surface marker expression. Neutrophils were gated using FSC and SSC and 10,000 events were analysed per sample. Raw data files were analysed by the UoD.

Table 1- Antibodies used for cell surface marker expression

Antibody	Supplier	Volume added to cells
BD Pharmingen™ PerCP-Cy™5.5 Mouse Anti-Human CD66b (562254)	BD Biosciences, New Jersey, USA	5 µl /tube
BD Pharmingen™ PE Mouse Anti-Human CD11b (557321)	BD Biosciences	10 µl /tube
BD Pharmingen™ PE Mouse Anti-Human CD182 (555933)	BD Biosciences	2.5 µl /tube
BD Pharmingen™ PE Mouse Anti-Human CD63 (555933)	BD Biosciences	10 µl /tube
PE anti-human CD88 (C5aR) (344303)	BioLegend, California, USA	1.25 µl /tube

2.3.2 Study design and experimental protocols used to investigate NETosis in the UK-CIC study of hospitalised patients with COVID-19

The UK-CIC is a national consortium that aimed to understand the immunology of SARS-CoV-2 and COVID-19 (UK-CIC, 2020). Sheffield was one of 19 centres in this consortium. UK-CIC received 12 months of funding from the UK Research and Innovation (UKRI) to investigate 5 keys areas of immunology in COVID-19 including primary immunity, protective immunity, immunopathology, cross-reactive coronavirus immunity and immune evasion. This project was carried out in collaboration with Professor Endre Kiss-Toth (Dept. Infection, Immunity & Cardiovascular Disease, UoS) and Professor Clare Lewis (Dept. Oncology & Metabolism, UoS) and patient sampling ran between November 2020-June 2021.

2.3.2.1 *Research ethics*

Hospitalised patients with COVID-19 admitted to the RHH, Sheffield, were recruited to the study and provided fully informed consent via The Sheffield Teaching Hospitals Observational Study of Patients with Pulmonary Hypertension, Cardiovascular and other Respiratory Diseases (STH-Obs), REC 18/YH/0441, IRAS 248890, project title: Establishing the magnitude, breadth and durability of SARS-CoV-2 induced activation of innate immune blood cells (COVID-19 INNATE). Ethical approval was given by the Yorkshire & The Humber - Sheffield Research Ethics Committee. Specific project approval was given by the STH-Obs Scientific Advisory Board.

2.3.2.2 *Patient recruitment and inclusion/exclusion criteria*

Hospitalised COVID-19 patients were recruited to the study by Dr Joby Cole on average 3 days (range 1-17 days) after hospital admission. A copy of the PIS is provided in Appendix 7. Blood (5-10 ml) was taken by Dr Joby Cole and the clinical research staff at the RHH. The inclusion criteria for the study were age ≥18 years, with COVID-19 (identified by clinical presentation or positive SARS-CoV-2 PCR test) and have the capacity to give informed consent. All patients were subsequently confirmed to be SARS-CoV-2 positive by PCR test. There were no specific exclusion criteria for the study. Blood samples were collected for seven patients who returned for a follow-up clinic visit 3- 4 months post-acute sampling. Neutrophils were also isolated from 9 healthy controls. We were not able to recruit healthy controls of a similar age range to the COVID-19 patients, or controls with co-morbidities found in the COVID-19 patient population, due to COVID-19 lockdown restrictions and the importance of maintaining participant safety.

2.3.2.3 *Collection and analysis of anonymised clinical data*

Clinical data was collected for all patients by Dr Joby Cole, Chenghao Huang and Jacob Whatmore, who anonymised these data for use. Patient clinical information collected included age, co-morbidities, O_2 requirement, length of hospital admission and medications. These data were used to understand if there were any associations between patient characteristics and markers of disease severity with the results of the study.

2.3.2.4 *SYTOX™ Green NETosis assay and imaging used in the UK-CIC study*

This SYTOX™ green method was adapted from the procedure used in the STOP-COVID clinical trial (section 2.3.1.4). Neutrophils were treated with either ruboxistaurin [20 nM & 200 nM] (Selleckchem, Texas, USA)**,** dexamethasone [1 µM & 10 µM] (Sigma- Aldrich), Cl-amidine [20 µM] (Selleckchem), DPI [10 µM] or DMSO (vehicle control) (Sigma- Aldrich) and incubated for 1 hour (37 ^{o}C, 5% CO_2). Then, neutrophils were stimulated with PMA [100 nM] or LPS (*E. coli* O111:B4) [5 µg/ml] and re-incubated for 3 hours. Quantification of SYTOX™ green fluorescence was conducted, as described in section 2.3.1.4. To obtain images of the wells, cell culture media was removed, and the cells were fixed with 4% PFA for 15 minutes at RT. Plates were then imaged using the Nikon Widefield fluorescent microscope. The x10 objective lens and the FITC filter (excitation/emission 470/525 nm) set was used. Images were processed using the FIJI image analysis software. This method was also used to investigate *S. aureus* induced NETosis in the optimisation of assays for the diabetes study. Neutrophils were infected with *S. aureus* strains SH1000 (MOI 5 or 10), JE2 (MOI 5 or MOI 10) or *SaeS* mutant JE2 (MOI 5) for 2 hours. Overnight cultures of bacteria were used for NETosis assays.

2.3.3 RT-qPCR for IL-6 gene expression

2.3.3.1 *mRNA Extraction and purification*

Neutrophil cell pellets (5 x 10^6) stored in Tri-reagent® at -80 °c were thawed and kept at RT for 5 minutes, before vortexing for 1 minute. Messenger RNA (mRNA) was extracted using the RNeasy UCP Micro kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Firstly, RNA was separated from DNA and protein using phase separation. Chloroform (Sigma) (200 µl) was added to the Tri-reagent® suspension and shaken vigorously for 15 seconds, before centrifugation at 4°C for 15 minutes at 12,000 *g.* The RNA containing aqueous phase was removed and mixed with an equal volume of 70% ethanol and placed into a RNeasy MiniElute spin column. The column was spun for 15 seconds at 8000 *g* and the flow through was discarded. The spin column was washed with RUWT buffer (Qiagen) prior to on-column DNase digestion for 15 minutes at RT. The column was washed again with RUWT and RPE buffer (Qiagen). Ethanol (80 %) was added to the column and centrifuged at 12, 000 *g* for 2 minutes. The flow-through was discarded and the column was spun with the lid open to dry the membrane for 5 minutes at 12, 000 *g*. RNase-free water (14 µl) was used to elute the RNA. The TURBO DNA-*free*™ kit (ThermoFisher Scientific) was used to remove any remaining contaminating genomic DNA, as per the manufacturer's instructions. To the sample, 0.1 volume of 10X TURBO DNase buffer and 1 µl of TURBO DNase were added and the sample was incubated at 37°C for 25 minutes. DNase Inactivation Reagent was then added (2 µl) and incubated at RT for 5 minutes, with intermittent agitation. The sample was then centrifuged at 10,000 *g* for 1.5 minutes to pellet the DNase Inactivation Reagent and the RNA containing supernatant was removed. It was not possible to accurately determine the yield and purity of the RNA using the Geneflow nanophotometer (N60) due to the abundance often reading <10 ng/µl. Instead, a 'no reverse transcriptase control' was used in qPCR to confirm there was no contaminating genomic DNA.

2.3.3.2 *cDNA synthesis*

Complementary DNA (cDNA) was synthesised using the iScript™ cDNA synthesis kit, as per the manufacturer's instructions (BioRad, California, USA). An RNA concentration of 2.5 ng per reaction was used. For cDNA synthesis a total sample volume of 20 µl was required. The following equation was used to calculate the volume of RNA (V_{RNA}) needed.

V_{RNA} (µl) = Total amount of RNA needed per sample (ng)/ RNA concentration (ng/ µl)

Four microliters of x5 IScript Reaction mix and 1 µl iScript Reverse Transcriptase was used per sample. The total volume was made to 20 µl using RNAse free water. To a PCR tube (STARLAB, Hamburg, Germany) water and IScript reaction mix were added first prior to the RNA and iScript Reverse Transcriptase, which were kept on ice until needed. Samples were placed in a thermal cycler (Bioer Technology, Life ECO) and cDNA was synthesised using the protocol outlined in Table 2. cDNA was

used straight away for quantitative reverse transcription polymerase chain reaction (RT-qPCR). A no reverse transcriptase control was used.

Table 2- cDNA synthesis protocol

Stage	Time (minutes)	Temperature (°C)
Priming	5	25
Reverse Transcription (RT)	20	46
RT inactivation	1	95

2.3.3.3 *Primer Design*

Primers were designed using Primer-BLAST (NIH). Primers were designed to span an exon-exon junction and have an amplicon length of less than 200 base pairs. Splice variants were allowed. To check that primers would not self-dimerise the primer sequences were run through online oligonucleotide properties calculator- Oligo Calc (Kibbe, 2007). Primers outlined in Table 3 were used for qPCR (Sigma-Aldrich).

Table 3- Specification of PCR primers

Primer	Target Name	Sequence (5'-3')	Length	Tm °C	GC%
Human *GAPDH* FWD	GAPDH (Housekeeping gene)	ATTGCCCTCAACGACCACTTT	21	66.7	48
Human *GAPDH* RV		CCCTGTTGCTGTAGCCAAATTC	22	66.5	50
Human *IL-6* FWD	IL-6	AGACAGCCACTCACCTCTTCAG	22	64.9	54.5
Human *IL-6* RV		TTCTGCCAGTGCCTCTTTGCTG	22	70.8	54.5

2.3.3.4 *SYBR Green RT- qPCR*

SYBR Green Precision®PLUS master mix (Primer Design, Camberley, UK) was added to the required wells of a 384 well PCR microplate (Greiner), in addition to the appropriate forward and reverse primers (10 µM) for the target gene. The required volume of cDNA to achieve 2.5 ng per reaction was made up to 5 µl with RNase-free water (Qiagen) and added to appropriate wells. Each gene was run in triplicate for individual samples. The plate was sealed with Polyolelin Film (STARLAB, Hamburg, Germany) and spun at 1500 *g* for 3 minutes. The plate was inserted into the qPCR machine (BioRad CFX384 Real Time System) and the following programme was used:

- 95 °C for 2 minutes
- 95 °C for 10 seconds *

- 60 °C for 1 minute *
- Plate read*

* 40 cycles

Ct represents the qPCR cycle number and to analyse these data the ΔCt values were calculated, using the formula below and these were used for statistical analysis.

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

2.3.4 Investigating the impacts of ruboxistaurin on neutrophil recruitment in a zebrafish tailfin injury model

Zebrafish larvae (*Danio rerio*) are an established model used to investigate the neutrophil response to injury and inflammation *in vivo* (Isles et al., 2021; Renshaw et al., 2006). Zebrafish husbandry was managed by the aquarium staff at the Bateson Centre, UoS. Zebrafish larvae were used 3 days post fertilisation (d.p.f). At this stage zebrafish larvae are not protected under the Animals (Scientific Procedures) Act 1986, meaning a home office licence and ethical permissions were not required to conduct experiments. A transgenic zebrafish line (TgBAC(mpx:EGFP)i114), which have GFP inserted within the neutrophil specific MPO gene promoter were used (Renshaw et al., 2006). Amy Lewis handled the zebrafish, and I conducted the imaging and cell counts. Zebrafish were first anaesthetised using 4% tricaine in E3 media. Tailfin transection involved removing the tailfin immediately posterior to the circulatory loop using a scalpel. Zebrafish were then incubated in 12 well plates containing 3 ml of E3 media containing DMSO [0.5%] or ruboxistaurin [200 nM & 100 µM], using duplicate wells per condition. Fifteen zebrafish larvae were used per well. Amy Lewis randomised the wells containing ruboxistaurin to blind the cell counting. After a 4-hour incubation (28 °C), zebrafish larvae were anesthetised, as before, and I counted the number of neutrophils that had been recruited to the tailfin injury site. Imaging was conducted the Lecia MDG41 stereo microscope with a Lecia EL6000 fluorescent light source and an 80x objective lens. Plates were re-incubated and after a further 20 hours (24 hours total), imaging was repeated. Total whole body neutrophil counts were conducted on uninjured zebrafish larvae after 4 hours incubation in E3 media containing 100 µM ruboxistaurin.

2.4 Statistical analysis

Statistical analysis was completed by using GraphPad Prism Version 9.2, where data represents n ≥3. A Shapiro-Wilk normality test was conducted on these data, where n= ≥6 to determine the use of parametric and non-parametric analyses. When experiments used neutrophils from the same donor in different treatment groups, repeated measures analyses were used. When comparing between different groups, e.g patients and healthy controls, unmatched analyses were used. When there were

more than two treatment conditions being investigated, such as glucose concentration and LPS stimulation, a two-way ANOVA was employed and when there was only one variable being examined between multiple conditions, a one-way ANOVA was used. Multiple comparison post-tests were employed to analyse statistical differences between groups including a Bonferroni's, Šidák's, Tukey's and Dunnett's post-test. Due to missing values in some data sets a mixed-effect analysis was used instead of an ANOVA. When comparing two groups, a student's t-test (parametric) or Wilcoxon matched-pairs signed rank test (non-parametric) was employed. For analysing correlations, a Spearman's rank correlation coefficient was computed, and a line of best fit was plotted using linear regression. Significance asterisks represent * >0.05, ** >0.01, *** >0.001 and **** >0.0001.

3 Setup and execution of a patient study investigating neutrophil function in people with diabetic foot disease

An understanding of the aberrant neutrophil phenotype in diabetes has developed over the last 60 years and generated a significant body of literature. In previous studies, key neutrophil functions including chemotaxis, phagocytosis, apoptosis and intracellular ROS production were mostly observed to be downregulated in people with diabetes, whereas pro-inflammatory neutrophil pathways such as extracellular ROS production and NETosis were largely upregulated (Dowey et al., 2021; Gustke et al., 1998; Hanses et al., 2011; Ihm et al., 1997; Shah, 1983; Ueta et al., 1993; Wong et al., 2015). Limited previous research has shown that modulating neutrophil effector mechanisms therapeutically can improve infection resolution in diabetes, with modulating ROS production a central pathway frequently explored (Das et al., 2018; Frydrych et al., 2018; Hand et al., 2007; Wang et al., 2019). However, there are no licensed treatments to improve neutrophil function in people with diabetes, with antibiotics and wound management, alongside routine blood glucose lowering medicine used for treatment of infections in this cohort (Schaper et al., 2020). The neutrophil phenotype has not been fully defined in those with diabetic foot disease (DFD), a specific complication of diabetes explicitly associated with chronic infection and poor wound healing. The rationale for choosing to investigate DFD specifically is described in section 3.1.1.1. In this chapter the work leading up to March 2020 is described.

The hypothesis for the work in this chapter was '**Neutrophil function in patients with DFD is dysregulated in comparison to age-mached healthy controls and defining the neutrophil phenotype could identify new therapeutic targets to improve the host immune response to infection'**

To address this hypothesis, the following aims were set:

1. Design and set up a pilot neutrophil phenotyping study of 10 patients with DFD and age-matched controls.
2. Setup and optimise a collection of assays to investigate neutrophil apoptosis, phagocytosis, ROS production, intracellular killing and NETosis, using neutrophils isolated from healthy donors in different concentrations of glucose.
3. Complete the patient study designed in aims 1 and 2.

3.1 Designing a patient study investigating neutrophil function in people with DFD

3.1.1 Participant selection

People with diabetes comprise an heterogenous population. When selecting the participants for the study there were multiple co-morbidities and variables to consider such as the type of diabetes and presence of diabetic complications. In-depth discussions with my supervisors and clinical collaborators

(including Dr Ahmed Iqbal, Department of Infection, Immunity & Cardiovascular Disease, University of Sheffield), to choose the most suitable cohort of patients to investigate, were ongoing in the first 6 months of the PhD project.

3.1.1.1 *Focusing on neutrophil function in people with DFD*

The initial project plan was to investigate neutrophil function in people with diabetes, without focusing on a sub-set of the population. Investigating participants specifically with DFD was a suggestion I presented to my supervisors after completing a thorough literature review, which gained their full support. There were several reasons this would be an appropriate group of patients to study in the project. Previous literature exploring neutrophil function in people with diabetes is substantial. Early work in the field focused on phagocytosis and chemotaxis, and studies frequently reported contradictory results, although differences in participant inclusion criteria and experimental techniques make study comparison challenging (Donovan et al., 1987; Mowat and Baum, 1971; Sabioncello et al., 1981; Wilson and Reeves, 1986). Over time, a more consistent phenotype was observed, with extracellular ROS production and NETosis mostly increased in people with diabetes, apoptosis often decreased, and neutrophils shown to have LPS tolerance (Dowey et al., 2021; Fadini et al., 2016; Karima et al., 2005; Manosudprasit et al., 2017; Ridzuan et al., 2016; Yang et al., 2019). However, these studies did not often investigate neutrophil function in participants with diabetes who had known susceptibility to infection or had ongoing bacterial infections. Investigating neutrophil function in people with diabetes who suffered repeat and chronic bacterial infections was a logical progression from the previous literature. People with diabetes are at increased risk of a range of infections at multiple sites including surgical site infections, UTIs, respiratory infections and a spectrum of skin and soft tissue infections including abscesses, carbuncles and DFD (Abu-Ashour et al., 2017; Lipsky et al., 2010). There were several reasons why investigating people with DFD was chosen. Firstly, previous literature has predominantly focused on the negative impacts of NETosis on poor wound healing in DFD. These studies suggest that neutrophils undergo increased NETosis at these sites, contributing to chronic inflammation (Fadini et al., 2016; Yang et al., 2019, Yang et al., 2020). Also, there are some data to suggest that neutrophil antimicrobial functions may be negatively affected in DFD, with a small number of studies demonstrating aberrant ROS production and phagocytosis (Oncul et al., 2007; Peck et al., 2001). However, this has not been explored in detail before, and phenotyping multiple neutrophil effector functions in people with DFD would be a valuable addition to the field. DFD is also an important clinical manifestation of diabetes to study as it is a chronic bacterial infection that relies heavily on antibiotic therapy and where current treatment strategies can fail, with limb amputation occurring in 11-33% of patients (Rodrigues et al., 2016; Shatnawi et al., 2018). An aim of this project, and of the MRC SHIELD Consortium ('Optimising Innate

Host Defence to Combat Antimicrobial Resistance') who co-funded the research, is to uncover novel therapeutic targets to restore host immunity. Reducing the reliance on antibiotics is important to reduce the emergence of antimicrobial resistant pathogens, with vancomycin resistance found in 21% of *S. aureus* isolates in a study of 120 hospitalised patients with diabetic ulcers in Italy (Caruso et al., 2021). The microbiology of DFD also supports that it is a key infection to study in this project. A predominant pathogen isolated in DFD is *S. aureus,* and neutrophils are a key immune cell in the clearance of this pathogen (Guerra et al., 2017; Jenkins et al., 2014; Lipsky et al., 2010). Finally, patients with DFD were the most amenable to investigate practically, due to the chronicity of infection, giving a wider window for patient recruitment, the high number of patients we would have access to through the diabetes foot clinics and since many patients manage their condition outside of hospital, making the logistics of screening telephone calls and arranging venesection possible.

3.1.1.2 *Recruitment target for the study*

The design of this study was to be a small-scale pilot study of 10 patients with DFD compared to an equal number of age-matched healthy controls. This number fits with a published 'rule of thumb' of around 12 subjects for a pilot study and would allow calculation of statistical power if indicated for a larger study (Julious, 2005). This would be the first phenotyping study of neutrophils from people with DFD, which explored multiple different neutrophil functions from the same patient. Depending on the results from this pilot study, a follow up study on a larger scale was to be planned, which would have a narrower focus and seek to modulate aberrant neutrophil pathways *in vitro*. Furthermore, when designing the study, we discussed whether more groups should be included, such as people with diabetes and no infection and people with foot ulcers and no diabetes. We discussed the relative importance of recruiting individuals in these groups. I concluded that we would not extend the pilot study further than those with DFD and healthy controls, as this was the key comparison I was investigating. However, these additional control groups would be useful in future follow-up studies.

3.1.1.3 *Deciding participant Inclusion and Exclusion Criteria*

Once patients with DFD were chosen as the study group, the inclusion and exclusion criteria for patient recruitment were determined (Table 4). This involved many in-depth discussions due to the heterogeneity of the population with DFD. The inclusion criteria were patients aged 50-75 years old, a diagnosis of diabetes and an active foot ulceration. The age range was included as aging can impact upon neutrophil function, however as the average age of an individual with diabetes is often >50, it was unlikely to be a barrier for an individual to enrol in the study (Alosaimi et al., 2019; Butcher et al., 2000). Participants were excluded for: recent diagnosis of non-DFD sepsis, use of oral steroids and known immunosuppressants or immunomodulators (e.g. macrolide antibiotics), COPD, morbid obesity and smoking, since these treatments or diseases are established modulators of neutrophil

function (Brotfain et al., 2015; Demaret et al., 2015; Hoenderdos and Condliffe, 2013; Ronchetti et al., 2018; Sugihara, 1997; Zhang et al., 2018). A significant contraindication to venesection, such as severe anaemia, would also warrant exclusion from the study. Statin use was considered as an exclusion criteria, as previous research shows it exerts anti-inflammatory effects on neutrophils (Greenwood et al., 2014; Guasti et al., 2008). After discussion it was decided to not exclude statin use as it would be extremely difficult to recruit patients who were not taking statins, since it is a frequent medication in this group of patients (Elnaem et al., 2017). Depending on the result of the pilot study, I anticipated this may need additional controlling in later work. Anti-diabetic therapies such as metformin and insulin can also modulate neutrophil function, but they were not an exclusion point, as DFD is often a late-stage clinical manifestation of diabetes and all patients will be prescribed anti-diabetic medication (Cameron et al., 2016; Yano et al., 2012). The presence of chronic DFD indicates that despite these treatments, susceptibility to infection remains, so a possible immune defect could still be discoverable in the study. We obtained ethical approval and patient informed consent to collect anonymised clinical data such as wound culture results, antibiotics and HbA1c, to inform the analysis of results downstream. Participant selection for this study was in line with previous patient research investigating neutrophil function in patients with DFD (Gough et al., 1997; Peck et al., 2001; Top et al., 2007; Yang et al., 2019; Yonem et al., 2001). These studies did not tightly control for a variety of patient factors such as diabetes type and duration and significant differences were found in neutrophil function, despite the heterogeneity of the study population. The inclusion/exclusion criteria for healthy controls were the same as people with DFD. However, healthy controls were excluded for a diagnosis of T1D, T2D or pre-diabetes.

Table 4- Inclusion and exclusion criteria for participants with DFD

Inclusion Criteria	Exclusion Criteria
Aged 50-75 years inclusive	BMI > 35
A diagnosis of diabetes	Admission to hospital with sepsis or a serious infection from any cause in the last 6 weeks
An active foot infection	Current or recent use (last 3 months) of steroids
	Current or recent use (last 3 months) of known immunosuppressants (e.g. macrolide antibiotics)
	A diagnosis of COPD
	A smoker or if an ex-smoker to have stopped 5 years ago with a maximum 5 pack year history
	Any significant co-morbidities that in the opinion of the investigator would be associated with substantial changes in neutrophil function e.g. malignancies
	Any significant contraindication to venesection (e.g severe anaemia)

Body mass index **(BMI)**, Chronic Obstructive Pulmonary Disorder **(COPD)**

3.2 Setting up assays of neutrophil function for use in the patient study using healthy donor neutrophils

In parallel with the design of the study, assays of neutrophil function were optimised using healthy donor neutrophils, this was important to establish a robust set of experiments that could be conducted simultaneously in the subsequent patient study. Blood glucose concentrations vary in health and vary much more in people with diabetes. I therefore undertook assays in a range of glucose concentrations to understand if transient variation in glucose levels was likely to affect neutrophil behaviour. Glucose is an important energy source for neutrophils and glycolysis is a key metabolic pathway (Kumar and Dikshit, 2019). The importance of glucose in fuelling neutrophil effector mechanisms supports previous work demonstrating that increasing glucose concentrations *in vitro* increased ROS production and NETosis, in cells isolated from healthy donors (Mohanty et al., 2000; Rodríguez-Espinosa et al., 2015; Van Oss, 1971). Most studies investigating neutrophil function in people with diabetes *ex vivo*, conduct assays in standard glucose media, therefore this work would provide further insight into the effects of glucose on neutrophils in diabetes (Delamaire et al., 1997; Hand et al., 2007; Manosudprasit et al., 2017). Furthermore, very few studies have previously investigated how low glucose conditions impact neutrophil function *in vitro* (Thomson et al., 1997). In each assay, media containing either low [0.3 mM], standard [11 mM] or high [20 mM] glucose was employed. The standard [11 mM] glucose media was selected as the control in the experiments, as this was the concentration present in the formulation of the Gibco RPMI 1640 routinely used for cell

culture in the laboratory. A normal blood glucose ranges from 4-5 mmol/L when fasting and up to 7.8 mmol/L 90- minutes after meals (NICE, 2015). The standard glucose condition [11 mM] was higher than a normal blood glucose value, however it was chosen as the control in the experiments as almost all laboratory experiments of neutrophil function use standard media, and thus this would allow some referencing to published studies of normal neutrophil function. For the NETosis assays, there was no FCS present in the media which may contain residual glucose, therefore instead of being classed as 'low' glucose, this is referred to as the 'no glucose' condition.

3.2.1 Neutrophil apoptosis in varying concentrations of glucose

Neutrophil apoptosis has not been investigated in people with DFD previously, therefore, to understand whether aberrant apoptosis is a contributory immune defect in DFD, an apoptosis assay employing multiple different glucose concentrations (low, standard and high), was set up. Apoptosis is important for inflammation resolution and previous research shows that apoptosis was delayed in people with T2D (Manosudprasit et al., 2017). LPS is a well-established pro-survival stimulus of neutrophils and LPS [100 ng/ml] was included in this assay as previous studies demonstrated tolerance to LPS in neutrophils from humans and rodents with diabetes (Kuwabara et al., 2018; Tennenberg et al., 1999). Apoptosis was assessed after 6 and 22 hours and was quantified based on characteristic alterations in cell morphology, which were imaged via light microscopy of prepared slides. Six and 22-hour time points were chosen as neutrophils die by constitutive apoptosis *in vitro* over a period of 24 hours and these timepoints allow us to observe a potential changes in apoptosis (Sabroe et al., 2004). This would also reveal whether a short or prolonged exposure to different glucose concentrations is required to mediate an effect, if any. These data demonstrated there was a significant decrease in neutrophil apoptosis in unstimulated cells cultured in low glucose media compared to standard or high glucose media at 6 hours (Figure 10A), but not at 22 hours (Figure 10B). Also, LPS induced a significant survival effect on neutrophils at 6 hours in standard and high glucose media but not in low glucose media (Figure 10A). A darkly stained, rounded nuclear morphology represents an apoptotic neutrophil (Figure 10C).

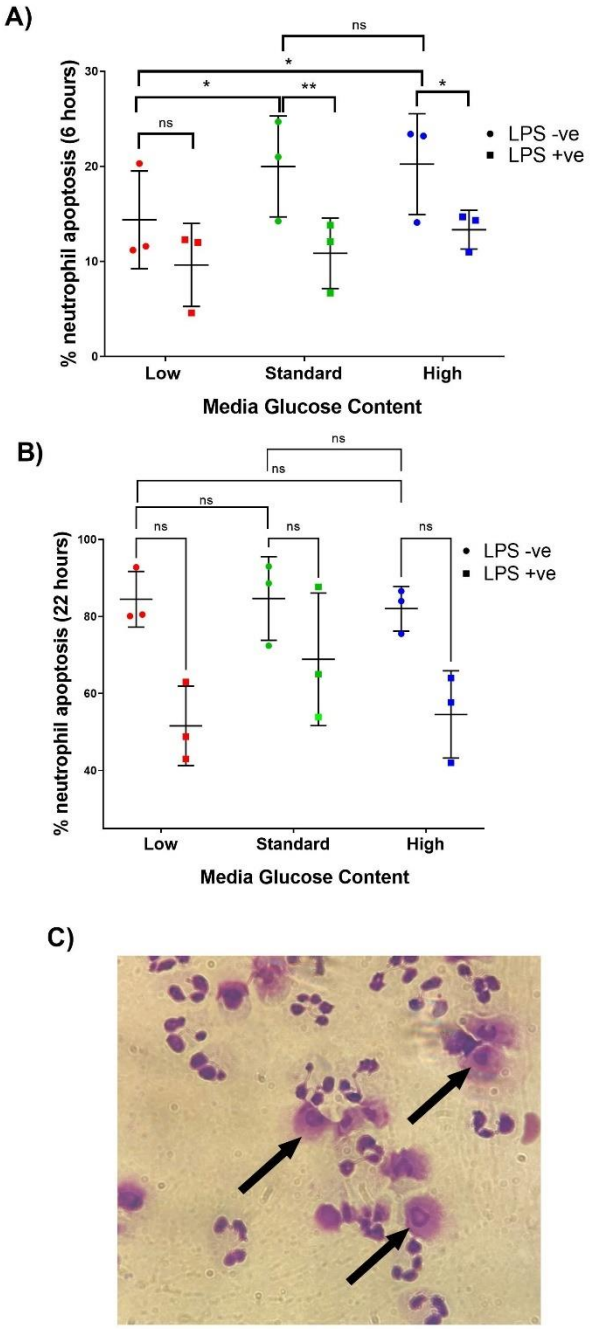


Figure 10-Apoptosis of neutrophils in varying concentrations of glucose.

Neutrophils (2.5 x 10^5 cells/well) from healthy donors were cultured for either 6 (A) or 22 (B) hours in media containing low [0.3 mM] (red), standard [11 mM] (green) or high [20 mM] (blue) concentrations of glucose. The effect of LPS [100 ng/ml] (squares) was compared in each of the media conditions to unstimulated neutrophils (circles). A) There was a significant decrease in neutrophil apoptosis in unstimulated cells cultured in low glucose after 6 hours incubation. Stimulating cells with LPS significantly reduced apoptosis in standard and high glucose media. B) There were no significant differences in neutrophil apoptosis at 22 hours between the different media conditions. LPS did not impact apoptosis at 22 hours. C) Apoptosis was measured based on cell morphology using an oil immersion light microscope. A total of 300 cells were counted in each condition and the % apoptosis calculated. Arrows highlight apoptotic neutrophils. Error bars display SD. The mean values of duplicate samples of an n=3 is shown. Statistical analysis was by repeated measures two-way ANOVA. A Tukey's post-test was used for comparing apoptosis between the three media conditions. A Bonferroni's post-test was used when comparing neutrophils cultured with or without LPS. Significance asterisks represent *p< 0.05 & **p< 0.01 and (ns) denotes not significant.

3.2.2 Flow cytometry analysis of neutrophil intracellular ROS production

Oxidative stress is a central pathway in the pathology of diabetes. Glucose mediates increased ROS production in a variety of cell types, due to mechanisms including the activation of PKC and increased formation of advanced glycation end products (AGEs) (Giacco and Brownlee, 2010). However, neutrophil intracellular ROS production, as opposed to extracellular ROS generation, was significantly reduced in multiple studies of diabetes and DFD (Delamaire et al., 1997; Karima et al., 2005; Park et al., 2009; Sato et al., 1997; Tebbs et al., 1992). The importance of neutrophil ROS production in *S. aureus* clearance, which is the most common bacteria isolated in DFD, is exemplified by the enhanced susceptibility to frequent *S. aureus* infections in chronic granulomatous disease (CGD) (Buvelot et al., 2017; Roos, 2016). Those with CGD have loss of function mutations within the neutrophil NADPH oxidase complex and have impaired generation of ROS (Buvelot et al., 2017; Roos, 2016). Investigating further how intracellular ROS production is impacted in people with DFD will be key in understanding neutrophil function is this patient group. The use of 2',7'-dichlorodihydrofluorescein (DCF) as a marker of intracellular ROS production was selected to detect ROS as it has been widely used and validated in previous work (Bass et al., 1983; McCloskey and Salo, 2000). Neutrophils were pre-incubated for 1 hour in media of varying glucose concentration prior to infection. A one-hour pre-incubation was an arbitrary length time routinely used in the laboratory group to assess changes to neutrophils *in vitro*.

3.2.2.1 *Optimisation of the multiplicity of infection of S. aureus used to induce neutrophil intracellular ROS production*

To optimise assay conditions, ROS production induced by *S. aureus* (SH1000) at different MOIs was investigated (MOI 1, 5, 10). SH1000 is a laboratory reference strain of *S. aureus* widely used in the literature to study host pathogen interactions. SH1000 is derived from the reference strain 8325-4 (Horsburgh et al., 2002). SH1000 possesses key *S. aureus* virulence regulator genes including agr, sarA and sae (Horsburgh et al., 2002). For co-culture with neutrophils, SH1000 was used in the log phase of growth, as *S. aureus* toxins are produced in the later phases of growth, which can lyse neutrophils (Novick, 2003; Bronner et al., 2004; Yang et al., 2019). Neutrophils were gated based on FSC and SSC profiles (Figure 11A) and ROS production was quantified using the geometric mean of the blue 530 nm fluorescent peak (Figure 11B). A co-culture time of 30 minutes was chosen, as preliminary experiments demonstrated higher levels of cell lysis, indicated by a reduction in the percentage of neutrophils in the gated region, after 60 minutes of co-culture (Figure 11C) compared to 30 (Figure 11D), when using an MOI of 5. These findings were in line with previous research from the group, demonstrating that *S. aureus* (SH1000) lyses neutrophils at the later time point (Sadia Anwar, unpublished work). All MOIs induced ROS production, reaching significance at an MOI of 5 (Figure 11E). There was no difference in ROS production between neutrophils stimulated with an MOI of 5 or 10. An MOI of 5 was chosen for future experiments, as it induced the strongest ROS response in

neutrophils. DPI significantly reduced *S. aureus* mediated ROS generation, although this did not reduce the fluorescence signal to zero.

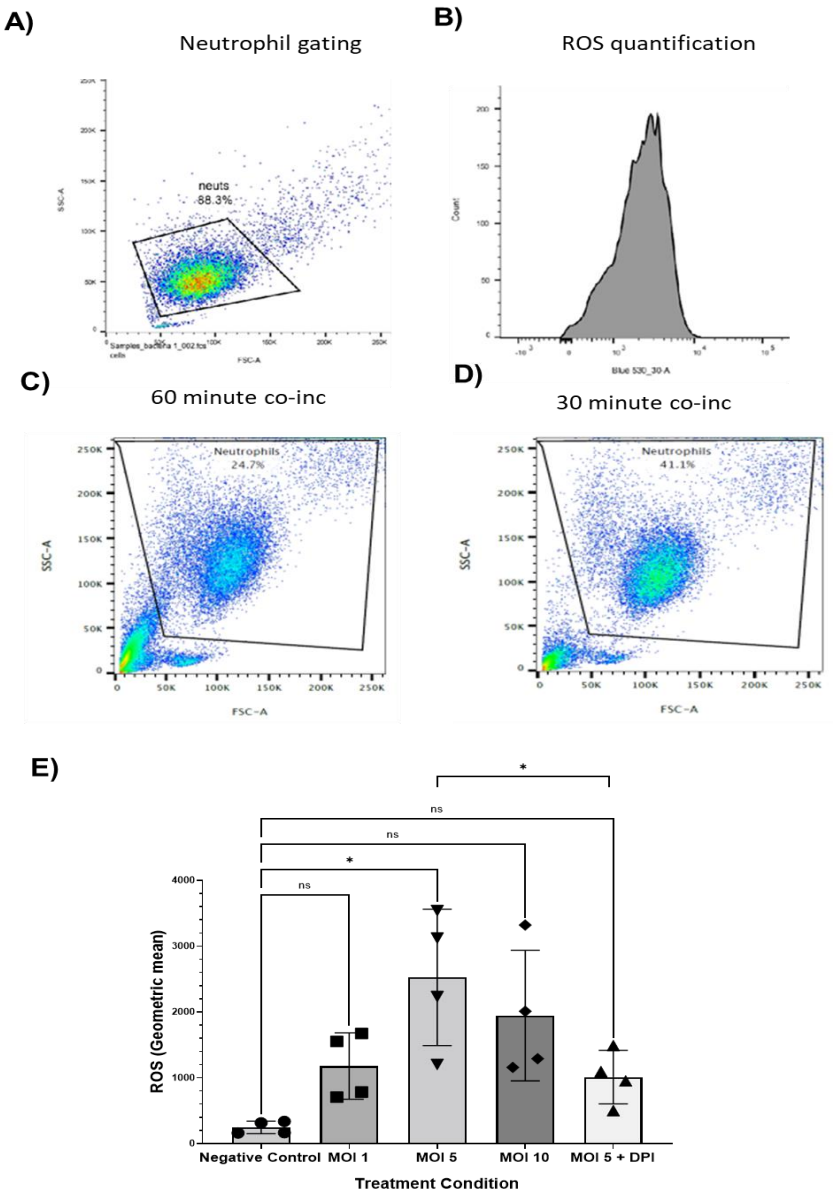


Figure 11-Optimising the detection of neutrophil intracellular ROS production in response to *S. aureus*.

Neutrophils (2.5 x 10^5 cells/well) from healthy donors were co-incubated with *S. aureus* at an MOI of 1, 5 and 10 for 30 or 60 minutes and ROS production was analysed by flow cytometry. DCF was used to detect intracellular ROS production in neutrophils induced by *S. aureus* (SH1000). The BD™ LSRII flow cytometer and the FLOWJO analysis software were used. The 488 nm argon blue laser and 530 nm filter were employed to detect intracellular fluorescence. Ten thousand neutrophils were analysed per sample. A) Neutrophils were gated based on FSC and SSC. B) The geometric mean of the blue 530 peak was used to quantify the amount of ROS production in neutrophils. C) There was a lower percentage of neutrophils in the gated region after incubating neutrophils for 60 minutes with an MOI 5 of *S. aureus* compared to 30 minutes (D) (n=1). E) There was a significant increase in ROS production using an MOI of 5 of bacteria, after a 30-minute incubation, which was inhibitable by DPI. The mean values of duplicate samples of n=4 is shown. The error bars display SD. Statistical analysis was by repeated measures one-way ANOVA with a Dunnett's post-test. Unstimulated neutrophils were used as the negative control. A Bonferroni's post-test was used to compare the MOI 5 bacteria condition with an MOI 5 + DPI. Significance asterisks represent *p< 0.05 and (ns) denotes not significant.

3.2.2.2 *ROS production in varying concentrations of glucose*

To test whether changes in glucose concentration in the cell culture media modified ROS production in response to *S. aureus* (SH1000, MOI 5), the assay was repeated using low, standard or high glucose media. Neutrophils were incubated in the different glucose containing media for 1 hour, prior to infection. The geometric mean of the DCF fluorescent peak was compared between neutrophils incubated in different glucose containing media and there was no significant difference (Figure 12).

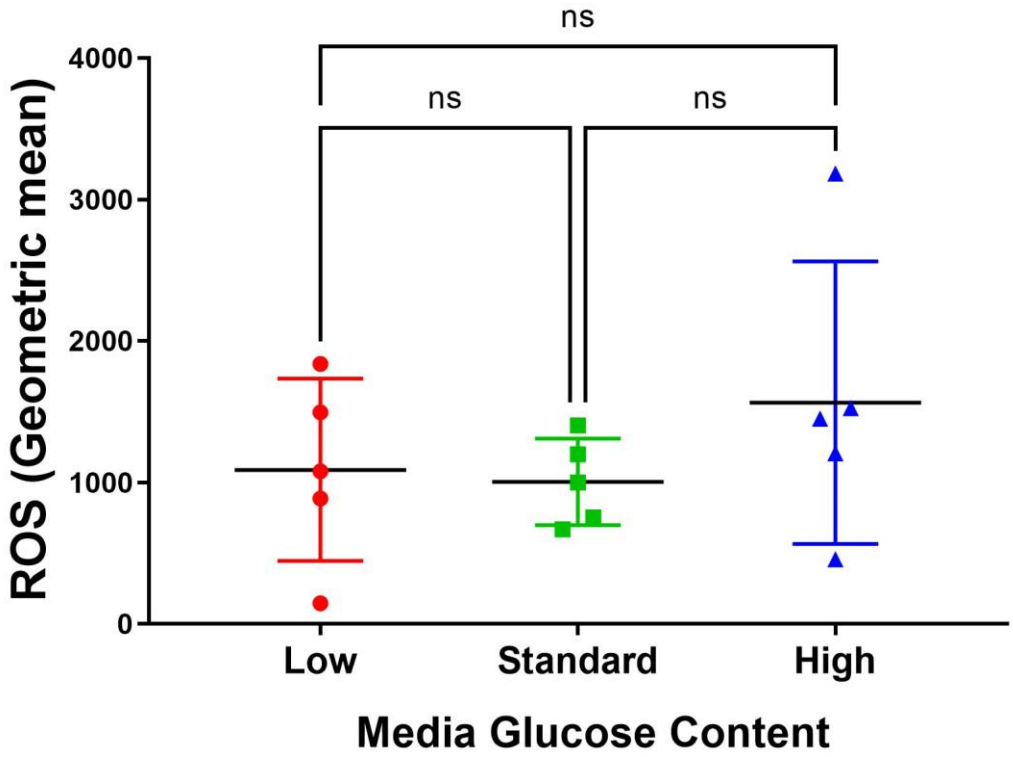


Figure 12-Neutrophil ROS production in media containing different concentrations of glucose.

Neutrophils (2.5 x 10^5 cells/well) from healthy donors were cultured in media containing low glucose [0.3 mM] (red), standard glucose [11 mM] (green), or high glucose [20 mM] (blue) for 1 hour prior to addition of *S. aureus* (SH1000) at an MOI 5. Neutrophils and bacteria were co- incubated for 30 minutes. DCF was used to detect intracellular ROS production. Samples were measured using flow cytometry. The BD™ LSRII flow cytometer, and the FLOWJO analysis software were used. The 488 nm blue laser and 530 nm filter were employed to detect intracellular fluorescence. Ten thousand neutrophils were analysed per sample and duplicate samples were used for each condition. There was no significant difference in the geometric mean of the DCF fluorescent peak from neutrophils cultured in different concentrations of glucose. The mean values of duplicate samples of an n=5 repeats is shown. The error bars display SD. Statistical analysis was by repeated measures one-way ANOVA, with Tukey's post- test and (ns) denotes not significant.

3.2.3 Neutrophil phagocytosis of *S. aureus* in different glucose concentrations

Neutrophil phagocytosis has been explored in many previous studies of diabetes. The weight of evidence suggests phagocytosis is reduced in diabetes, although there are some conflicting studies (Frydrych et al., 2018; Huang et al., 2019; Scully et al., 2017). Opposing results are also demonstrated in the limited research that has focused on neutrophil phagocytosis specifically in those with DFD (Park et al., 2009; Peck et al., 2001). To further characterise the neutrophil phenotype in people with DFD, a phagocytosis assay for use in the patient study was optimised using GFP-labelled *S. aureus* (SH1000) (Boldock et al., 2018). An MOI of 5 *S. aureus* and a 30-minute co-culture was used in these experiments, which was based on the optimisation assays used to detect ROS (section 3.2.2.1). Immediately prior to analysis, cells were pelleted and washed to remove extracellular bacteria and 0.2% trypan blue was added. Trypan blue is a cell impermeable dye which absorbs light at the wavelength emitted by GFP, and is routinely used in flow cytometry to quench extracellular fluorescence (Busetto et al., 2004; Pils et al., 2005). To test the impact of trypan blue on the phagocytosis assay results, a direct comparison was made between neutrophils incubated with an MOI of 5 of GFP-labelled *S. aureus* with and without trypan blue addition prior to analysis. The geometric mean of the GFP positive peak was used to quantify the amount of *S. aureus* ingested by neutrophils, and the percentage of neutrophils positive for GFP-labelled *S. aureus* were compared. The splitter tool on the FLOWJO analysis software was used analyse the percentage of neutrophils positive or negative for fluorescence (530 nm) (Figure 13A). Trypan blue had no impact on the phagocytosis assays results, with regards to both the amount of fluorescence generated by neutrophils (Figure 13B) or the percentage number of neutrophils positive for GFP (Figure 13C). This indicates there was not a high number of extracellular bacteria present in the samples. I decided to continue to use trypan blue in the protocol for the patient samples, as the possibility of reduced phagocytosis in neutrophils from patients with diabetes may mean higher volumes of extracellular bacteria in the assay. Phagocytosis was then quantified in the same way after neutrophils were incubated in varying concentrations of glucose (low, standard, or high) for 1 hour, prior to infection with *S. aureus*. These data demonstrated there was no significant difference in either the percentage number of GFP-positive neutrophils or the geometric mean of the GFP positive peak between the media conditions (Figure 13 D-E). This shows that the number of neutrophils phagocytosing *S. aureus* and the amount of *S. aureus* phagocytosed were not affected by the glucose concentration of the media.

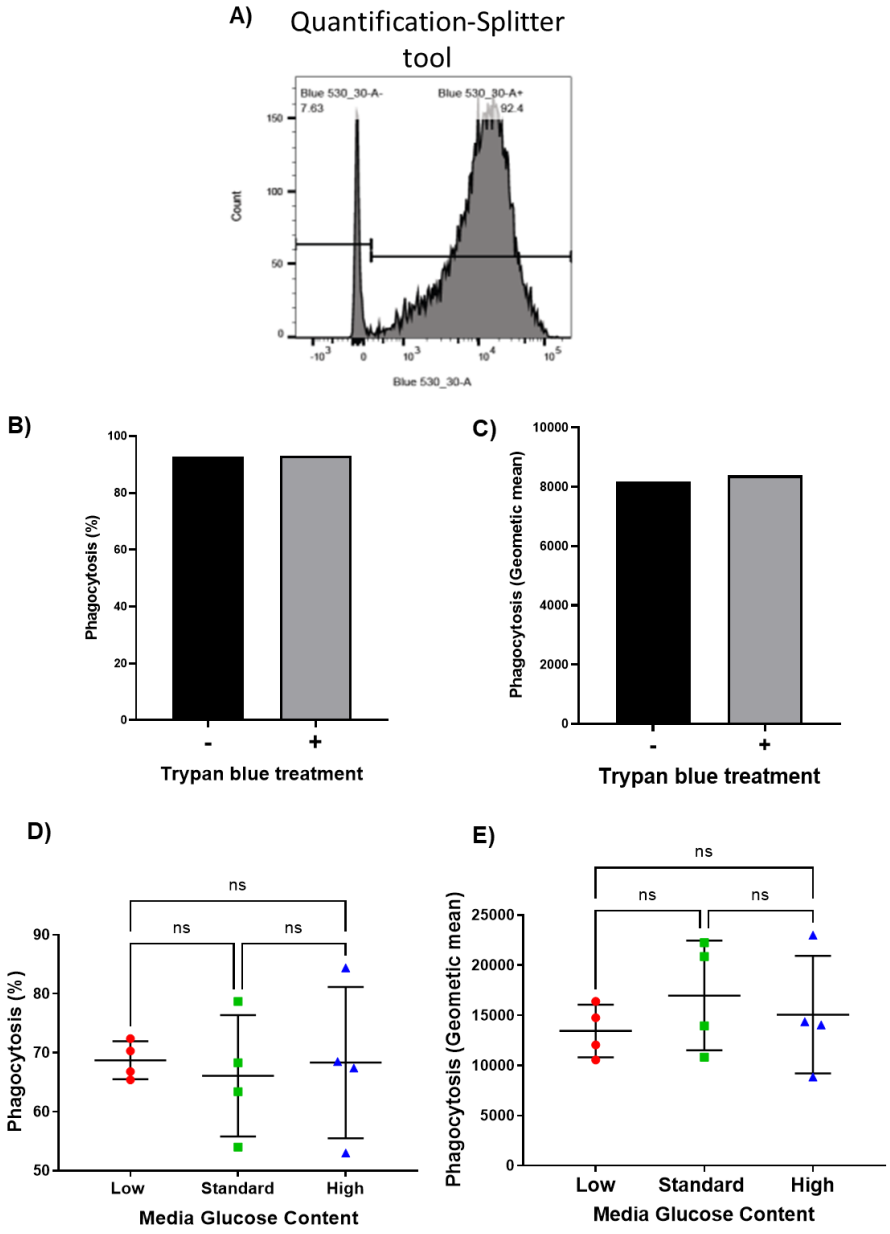


Figure 13-Neutrophil phagocytosis of *S. aureus* in varying concentrations of glucose.

GFP-labelled *S. aureus* (SH1000) of an MOI of 5 was added to neutrophils from healthy donors that were pre-incubated for 1 hour in media containing low [0.3 mM] (red), standard [11 mM] (green) or high [20 mM] (blue) glucose and incubated for 30 minutes. Neutrophils were washed and 0.2 % trypan blue was added prior to analysis using flow cytometry. The BD™ LSRII flow cytometer, and FLOWJO analysis software were used. The 488 nm blue laser and 530 nm filter were used. Ten thousand neutrophils were analysed per sample and duplicate samples were used for each condition. Neutrophils were gated based on forward scatter and side scatter. A) The splitter tool in the FLOWJO analysis software was used to distinguish between the positive and negative fluorescence peaks. B-C) Quenching extracellular fluorescence with trypan blue did not impact the number of neutrophils positive for GFP or the amount of fluorescence in the sample. Data represents an n=1. D-E) There was no significant difference in the percentage number of neutrophils phagocytosing bacteria (D) or the amount of *S. aureus* phagocytosed (E) (n=4). The error bars display SD. Statistical analysis was by repeated measures one-way ANOVA with a Tukey's post-test and (ns) demonstrates not significant.

3.2.4 Intracellular killing assay

Previous research demonstrated impaired killing of bacteria by neutrophils *in vitro* from both people with diabetes and those with DFD (Oncul et al., 2007; Wilson and Reeves, 1986). Reduced bacterial killing was associated with increased bacterial burdens and poor infection resolution in rodent models of diabetes (Hanses et al., 2011; Park et al., 2009). Neutrophil intracellular killing of *S. aureus* (SH1000, MOI 5) was explored in varying concentrations of glucose. This assay is also known as the 'Gentamicin protection assay' and is widely used in the literature to assess neutrophil intracellular killing of pathogens (Gresham et al., 2000; Vaudaux and Waldvogel, 1979; von Köckritz-Blickwede et al., 2008). Neutrophils were incubated in low, standard, or high media for 1 hour prior to infection. After a co-incubation period of 30 minutes to allow neutrophil phagocytosis, gentamicin was added to kill extracellular bacteria. Neutrophils were lysed and the number of intracellular bacteria released was quantified (CFU/ml) and this was compared to the number of viable bacteria recovered from inside neutrophils after 30 and 120 minutes. A reduction in the number of intracellular bacteria over time represents pathogen killing by neutrophils. These data demonstrated a significant decrease in the number of viable *S. aureus* recovered from inside neutrophils 30 minutes and 120 minutes after phagocytosis (0-minute timepoint) (Figure 14). However, intracellular killing was not impacted by the concentration of glucose in the media.

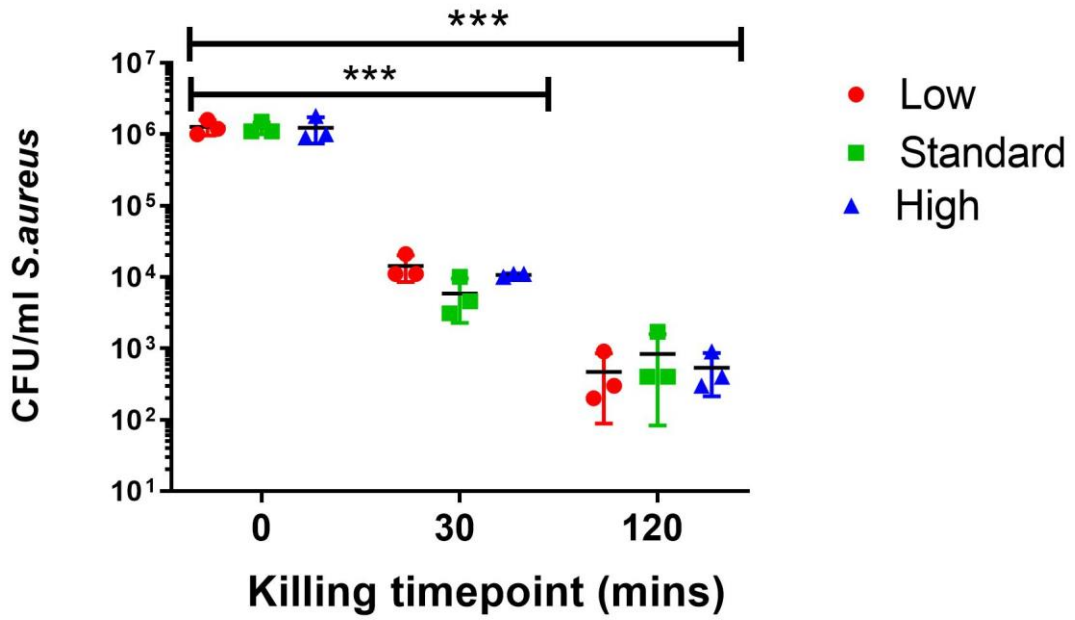


Figure 14-Intracellular killing of *S. aureus* by neutrophils in varying concentrations of glucose.

S. aureus (SH1000) of an MOI 5 was added to neutrophils (2.5 x 10^5) from healthy donors that were pre-incubated for 1 hour in media containing low glucose [0.3 mM] (red) standard glucose [11 mM] (green), or high glucose [20 mM] (blue). Phagocytosis occurred for 30 minutes prior to killing of extracellular bacteria using gentamicin [40 µg/ml]. After 0, 30 or 120 minutes of further incubation neutrophils were lysed to release intracellular bacteria using alkali water. The number of viable bacteria were quantified using the Miles & Misra technique. There was a significant decrease in *S. aureus* between 0-120 minutes post-phagocytosis. The glucose concentration in the media did not impact the killing. The mean values of duplicate samples of an n= 3 are shown. The error bars display SD. Statistical analysis was by a two-way ANOVA with a Tukey's post-test. Significance asterisks represent ***p<0.0001 and highlight changes between the timepoints.

3.2.5 Inducing and detecting neutrophil extracellular traps induced by PMA

Previous literatures demonstrates that NET formation is the most widely studied neutrophil function in the context of DFD, and NETs are emerging as critical mediators of diabetic complications (Fadini et al., 2016; Park et al., 2009; Peck et al., 2001; Wong et al., 2015; Yang et al., 2019). Increased NET formation, which has been linked to poor wound healing and chronicity of infection, is consistently demonstrated in rodent models of diabetes and in patients with DFD (Fadini et al., 2016; Park et al., 2009; Wong et al., 2015; Yang et al., 2019). Furthermore, NET formation in healthy donor neutrophils was augmented when cells were cultured in high glucose media *in vitro* (Menegazzo et al., 2015; Rodríguez-Espinosa et al., 2015). Previous research has successfully employed different therapeutic approaches to reduce NET formation in wounded diabetic rodents, including PKC and PAD4 inhibitors, as well as hydrogen sulphide (Das et al., 2018; Wong et al., 2015; Yang et al., 2019). The previous evidence suggests that NETs are an amenable target for improving neutrophil responses in diabetes,

therefore a NET assay using different glucose concentrations was optimised for use in the patient study. PMA was used as it is a potent NET stimulator that is widely used in the literature, providing a robust signal for comparison between patients and healthy controls (Brinkmann et al., 2004; Rodríguez-Espinosa et al., 2015; Wong et al., 2015; Yang et al., 2019). NETosis was quantified by calculating the DNA area, which involved analysing fixed DAPI-stained cell preparations using fluorescent microscopy. The average area of each image positive for DNA is used as a surrogate marker to quantify NETs (Methods section 2.2.9.2) and was in line with similar image analysis methods used in the literature (Halverson et al., 2015; Rebernick et al., 2018). This technique was chosen as it allowed visualisation of DNA NET structures, which would be useful when comparing patient and control samples. Also, it used fixed slides, meaning image analysis could be conducted on subsequent days from sampling, which would be useful when conducting multiple assays on the same day for the patient study.

3.2.5.1 *Optimising concentration of PMA to stimulate NETosis*

Investigating NETosis was a new experimental procedure not previously used in our research group. Optimisation of a reliable assay of NETosis was required before commencing the patient study. The concentration of PMA to induce NETosis was investigated first. PMA was used at increasing concentrations [10 nM, 25 nM or 100 nM] and the DNA area was quantified. There was a significant increase in NETosis using PMA at 100 nM compared to the media only control (Figure 15A). There was an upward trend in NETosis at 25 nM PMA, but this did not reach significance. The vehicle control, DMSO, did not cause an increase in DNA area. PMA induces NOX-dependent NETosis, therefore DPI was used to ensure this pathway was activated and that NETosis could be therapeutically modified (Fuchs et al., 2007). DPI [10 uM] significantly reduced the DNA area and thereby the amount of NETosis in cells stimulated with 25 nM PMA. Representative images demonstrate unstimulated neutrophil nuclear morphology (Figure 15B) and the characteristic nuclear morphology of PMA induced NETs, with nuclear decondensation and web-like structures of DNA (Figure 15C) (de Bont et al., 2018; Neubert et al., 2018). Both spread and diffuse NET morphologies were visualised in line with the previous literature (Gray et al., 2018; Hakkim et al., 2011). Twenty-five nanomolar was selected as the concentration of PMA for use in subsequent assays of NETosis, as it provided an upward trend in NETosis, but was not as high as 100 nM, therefore allowing possible increases in NETosis to be detected in the patient study.

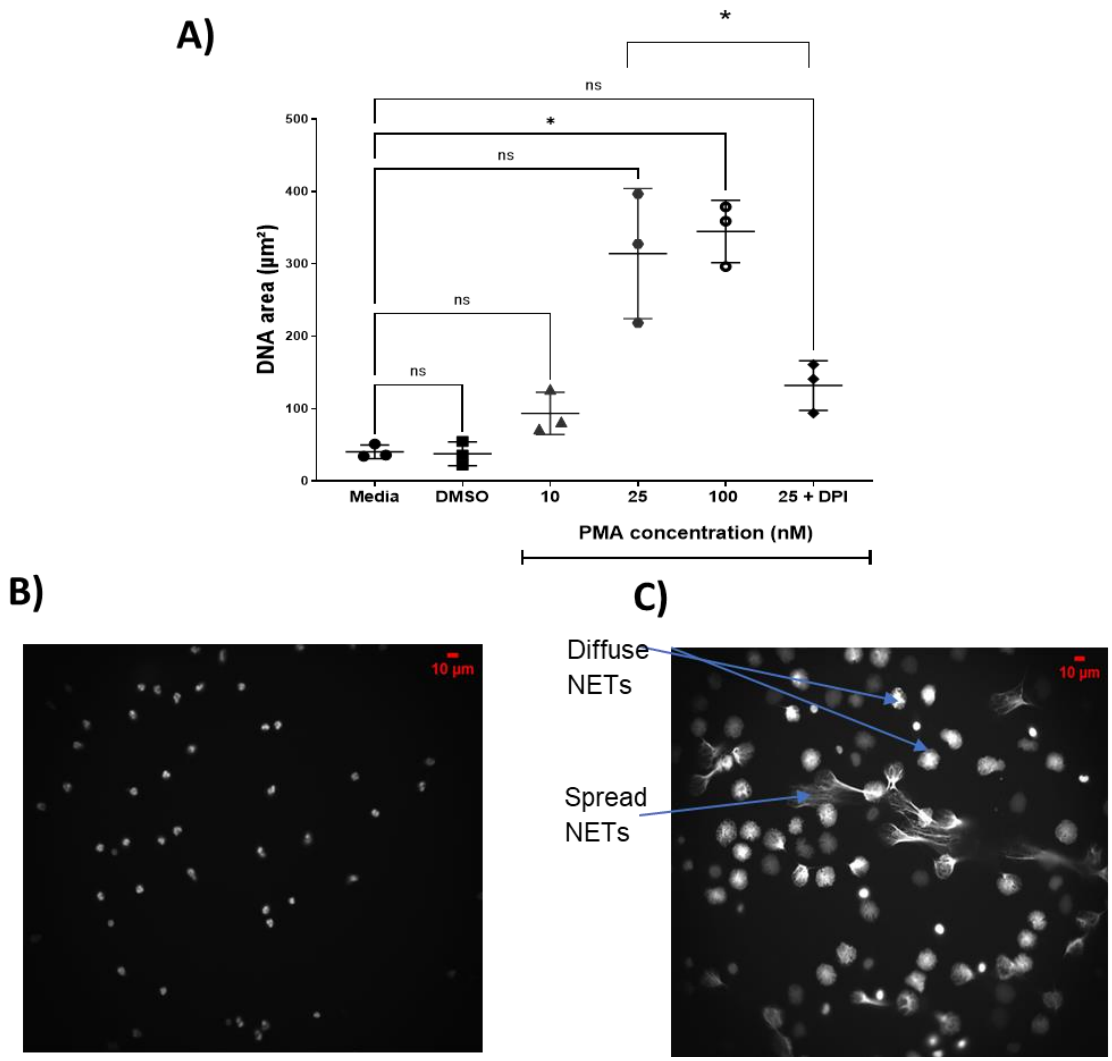


Figure 15-Optimising the concentration of PMA to stimulate NETosis.

Neutrophils (1 x 10^6 cells) from healthy donors were seeded onto 0.01% poly-l-lysine coated coverslips and stimulated for 3 hours with either DMSO (vehicle control), 10 nM, 25 nM (+/- DPI), or 100 nM PMA. Media only without PMA was used as the negative control. Cells were fixed using 4% PFA for 15 minutes and then washed with PBS before mounting onto microscope slides using DAPI containing ProLong Gold Antifade Mountant ™. Slides were visualised using a Nikon Widefield microscope with the 395-455 nm wavelength filter set for DAPI detection. The 40x oil immersion objective lens was used. Quantification of DNA area was conducted using the FIJI image analysis software. Between 3-5 images per condition were analysed, including approximately 200 cells across duplicate samples. A) There was a significant increase in NETosis in cells stimulated with 100 nM compared to the media control. Adding DPI significantly reduced NETosis when cells were stimulated with 25 nM PMA. The mean values of a n= 3 are shown. The error bars display SD. Statistical analysis was by repeated measures one-way ANOVA, with Dunnett's post-test with media only used as the control. A Bonferroni's post- test was used for comparing neutrophils stimulated with 25 nM PMA ± DPI. B) The nuclei of unstimulated neutrophils were small and retained a multi-lobed morphology. C) Diffuse and spread NETs were visible after stimulation with 25 nM PMA. Significance asterisks represent * $p < 0.05$ and (ns) denotes not significant. Scale bar equals 10 µm.

3.2.5.2 *Validation of PMA induced NETs using an anti-myeloperoxidase antibody*

The formation of NETs in response to PMA was validated using immunocytochemistry for DNA and NET components, which is a widely used methodology and considered the gold standard for identifying NETs (Buhr and Köckritz-Blickwede, 2020; Jiang et al., 2017). The abundant NET associated protein- MPO was used for detection and DNA was stained with DAPI as before (Metzler et al., 2011; Petretto et al., 2019). Immunocytochemistry images of unstimulated and stimulated neutrophils are shown in Figure 16. In unstimulated neutrophils the distinct cell compartments are visible, with the nucleus staining blue and the cytoplasm staining red for the presence of MPO. In response to PMA, characteristic cell morphology is seen (Neubert et al., 2018), where cells undergoing NETosis lose the multi-lobulated nuclear structure, becoming MPO positive, and strand-like networks of DNA, also positive for MPO are seen extruding from the cell body, which is indicated by white arrows. The co-localisation of DNA (blue), with anti-MPO (red) is characteristic of a NET (Neubert et al., 2018). There was no MPO signal detected in the IgG control samples, confirming the absence of non-specific staining.

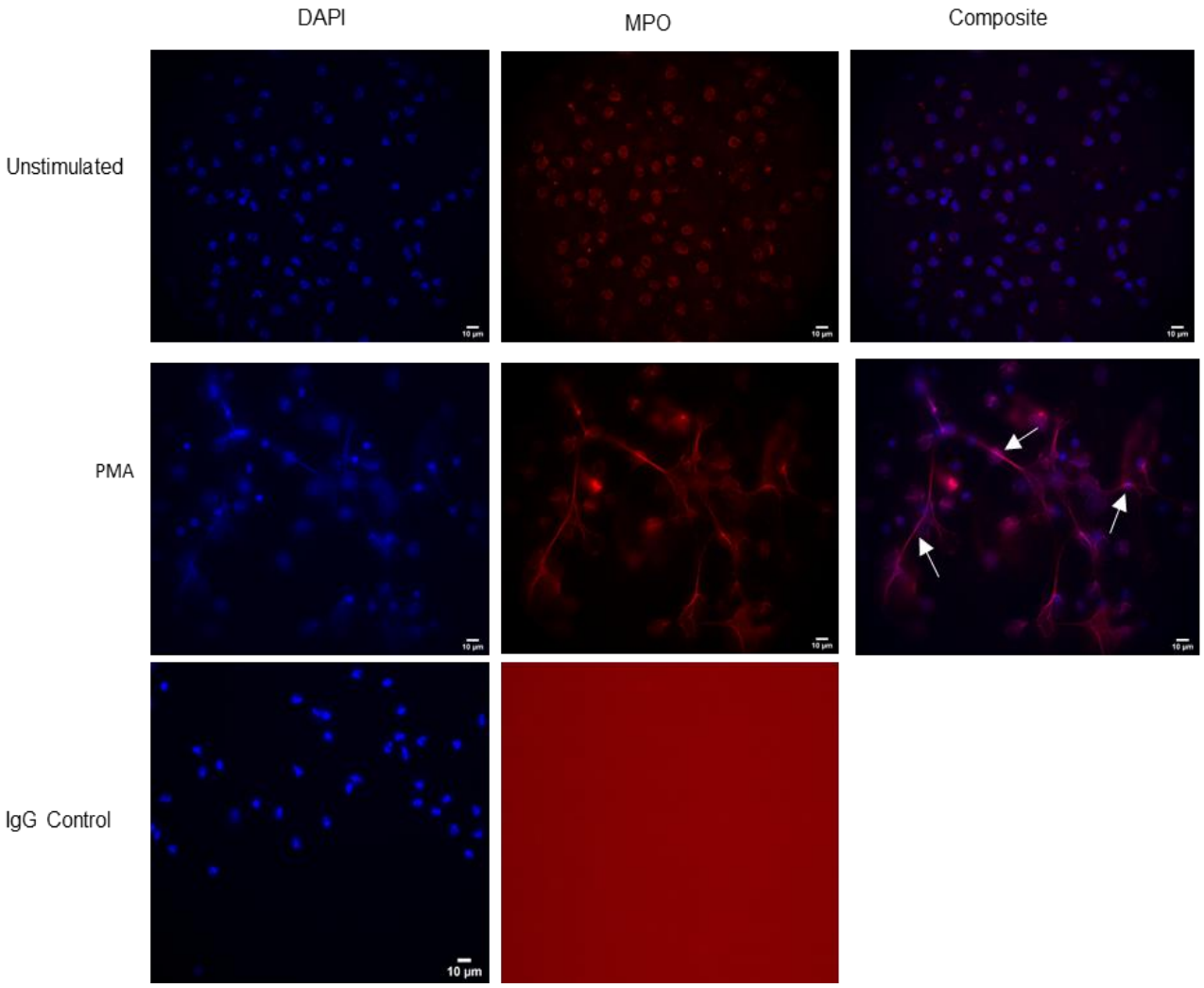


Figure 16-Immunocytochemistry of PMA induced NETs.

Neutrophils (5 x 10^5) were seeded into IBIDI™ 8 well chamber slides and stimulated with 25 nM PMA for 3 hours. Cell culture media was removed, and wells were fixed with 4% PFA for 15 minutes. Wells were blocked and permeabilised with blocking buffer (5% BSA, 5% normal goat serum and 0.1% saponin) for 1 hour. Primary rabbit anti human myeloperoxidase (MPO) antibody (A0398) or rabbit IgG control antibody was added (1:500 dilution) for 90 minutes. Slides were washed before adding the goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor® 594 (ab150088) for 45 minutes. Wells were washed as before prior to adding ProLong™ Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI). Slides were imaged using the Nikon Widefield fluorescent microscope, using the 60x oil immersion objective lens and the DAPI (excitation/emission 395/455 nm) and Texas red filter sets (excitation/emission 555/605 nm). Image analysis was conducted using the FIJI image analysis software. DAPI (blue) and MPO (red) channels were merged to produce composite images. Scale bar represents 10 µm. The co-localisation of DNA and MPO is considered a NET (white arrows).

3.2.5.3 *NET formation in response to different glucose concentrations in the media*

NETosis is upregulated in high glucose conditions *in vitro* (Menegazzo et al., 2015; Rodríguez-Espinosa et al., 2015). In support of this, Figure 17A demonstrates a significant increase in NETosis in neutrophils cultured in high glucose [20 mM] compared to no glucose, when stimulated with PMA. To ensure that increases in NETosis were not directly due to changes in osmolarity of the cell culture conditions, high glucose without PMA was included in the assay and there was no NETosis in this condition. Example images show large increases in PMA-induced NET structures in neutrophils cultured in high compared to low glucose (Figure 17B-C).

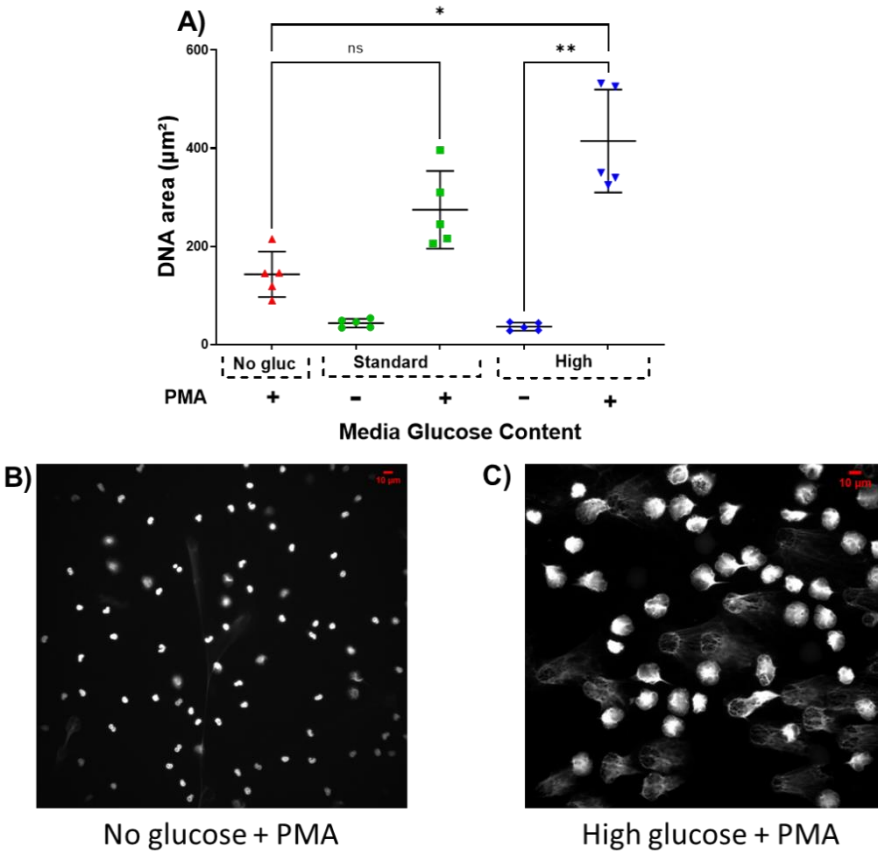


Figure 17-Culturing neutrophils in high glucose increases NETosis when stimulated with PMA.

Neutrophils (1 x 10^6 cells) from healthy donors were seeded onto 0.01% poly-l-lysine coated coverslips and stimulated for 3 hours with 25 nM PMA in media containing either no glucose (red) standard glucose [11 mM] (green), or high glucose ±PMA [20 mM] (blue). Unstimulated neutrophils in standard media were the negative control. Cells were fixed using 4% PFA for 15 minutes and then washed with PBS before mounting onto microscope slides using DAPI containing ProLong Gold Antifade Mountant ™. Slides were visualised using a Nikon Widefield microscope with the 395-455 nm wavelength filter set for DAPI detection. The 40x oil immersion objective lens was used. Quantification of DNA area was conducted using the FIJI image analysis software. Between 3-5 images were analysed per condition across duplicate slides. Scale bar equals 10 µm. A) There was a significant increase in NETosis in cells cultured in high glucose compared to no glucose when stimulated with PMA. High glucose concentrations did not induce NETosis without PMA. The mean values of a n= 5 are shown. The error bars display SD. Statistical analysis was by repeated measures one-way ANOVA, with a Bonferroni's post-test and comparisons are shown on the graph. B) Cells stimulated with PMA and cultured in low glucose containing media display a small amount of NETosis. C) Cells stimulated with PMA and cultured in high glucose containing media display a large amount of NETosis. Significance asterisks represent *p<0.05 & **p<0.01 and (ns) denotes not significant.

3.2.6 Inducing and detecting neutrophil extracellular traps in response to *S. aureus*

People with diabetes are pre-disposed to *S. aureus* infection and *S. aureus* is frequently isolated from people with DFD. *S. aureus* has previously shown to induce NETosis by a non-lytic mechanism termed 'vital NETosis', in contrast to the lytic NETosis pathway induced by PMA (Pilsczek et al., 2010; Yipp et al., 2012). To explore both NETosis pathways in neutrophils isolated from patients with diabetes, *S. aureus* induced NET formation was investigated. In a pilot experiment overnight cultures of *S. aureus* (SH1000) were used to induce NETs as *S. aureus* toxins, produced in the later phases of growth, are involved in the induction of NET formation (Bronner et al., 2004; Novick, 2003). In particular, leukotoxins and phenol soluble modulins have been demonstrated to mediate NET formation in response to *S. aureus* (Björnsdottir et al., 2017; Mazzoleni et al., 2021). An MOI of 10 and a co-incubation period of 2 hours were used, in line with previous literature (Hoppenbrouwers et al., 2017; Wan et al., 2017). The morphology of *S. aureus* induced NETs, defined by the co-localisation of DNA and MPO, were distinct from PMA treated cells demonstrated previously (Figure 18A). The neutrophil nuclei remained small with only minor NET-like protrusions visible. There was not a visibly robust NET response in neutrophils, therefore I investigated whether established stimulators of neutrophils, including LPS [100 ng/ml], granulocyte-macrophage colony-stimulating factor (GM-CSF) [20 ng/ml] or Formyl-Methionyl-Leucyl-Phenylalanine (FMLP) [1 µM] primed neutrophils to undergo greater NETosis upon subsequent incubation with *S. aureus*. The co-incubation time of *S. aureus* and neutrophils was also extended to 3 hours. There was no visible increase in the number of NETs made by neutrophils in response to *S. aureus* after treatment with priming agents (Figure 18B). There was very little, if any, NET-like protrusions visible. Data represents n=1. Based on these findings quantification of NET formation using DNA area would not be suitable for neutrophils treated with *S. aureus* due to the small changes in nuclear morphology in comparison to PMA-induced NETs and the low numbers of neutrophils visibly undergoing NETosis.

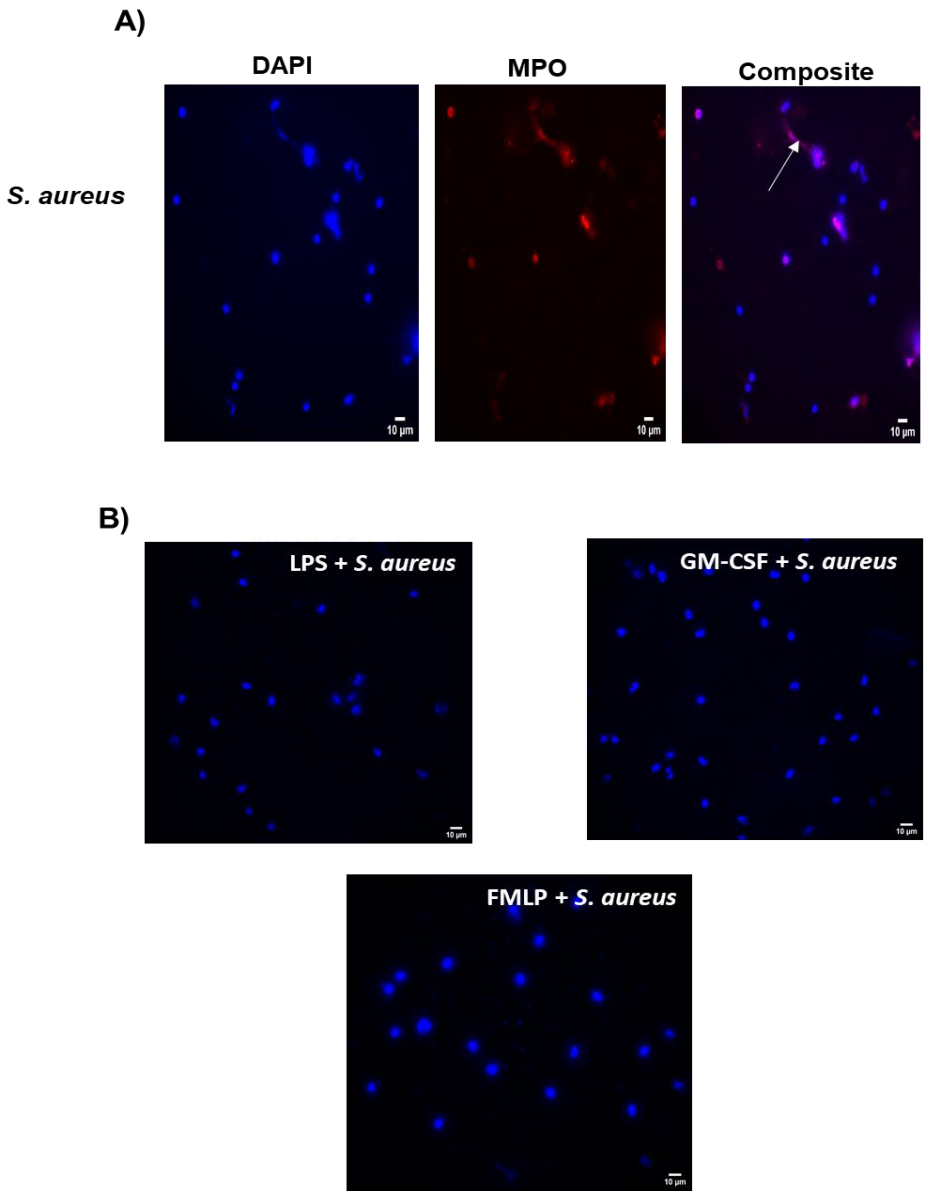


Figure 18-*S. aureus* induced NET formation.

A) Neutrophils (5 x 10^5) were seeded into IBIDI™ 8 well chamber slides and stimulated with overnight cultures of *S. aureus* (SH1000) at an MOI 10 for 2 hours. Cell culture media was removed, and wells were fixed with 4% PFA for 15 minutes. Wells were then blocked and permeabilised with blocking buffer (5% BSA, 5% normal goat serum and 0.1% saponin) for 1 hour. Primary rabbit anti-human myeloperoxidase (MPO) antibody (A0398) was then added (1:500 dilution) for 90 minutes. Slides were washed before adding the goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor® 594 (ab150088) for 45 minutes. Wells were washed as before prior to adding ProLong™ Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI). White arrows indicate neutrophil extracellular traps. Images are representative of an n=1. B) Neutrophils (1 x 10^6) were seeded onto 0.01% poly-l-lysine coated coverslips and stimulated for 1 hour with either GM-CSF [20 ng/ml], LPS [100 ng/ml) or FMLP [1 µM], prior to adding an MOI 10 of overnight cultures of *S. aureus* (SH1000). Bacteria and neutrophils were co-incubated for 3 hours, before fixing as before and mounting onto a microscope slide with ProLong™ Gold Antifade Mountant with DAPI. Images are representative of an n=3. Slides were imaged using the Nikon Widefield fluorescent microscope, using the 60x (A) or 40x (B) oil immersion objective lens and the DAPI (excitation/emission 395/455 nm) and Texas red filter sets (excitation/emission 555/605 nm) were used where required. Image analysis was conducted using the FIJI image analysis software. DAPI (blue) and MPO (red) channels were merged to produce composite images. Images present n=1. Scale bar represents 10 µm.

3.2.6.1 *Quantifying S. aureus induced NET formation using the SYTOX™ Green assay*

SYTOX™ Green is a cell impermeable DNA binding dye that is used to stain extracellular DNA and is widely used as a surrogate marker for NET formation (Locke et al., 2020; Zuo et al., 2020). This method does not rely on cell morphology and has been used to quantify *S. aureus* induced NETosis previously (Halverson et al., 2015; Pilsczek et al., 2010). Also, due to the low number of NETs in response to *S. aureus* visualised previously using the SH1000 strain I investigated whether the more virulent methicillin-resistant USA300 *S. aureus* strain, (JE2) induced more NETosis. *S. aureus* strain JE2 is able to produce the Panton-Valentine leucocidin (PVL), which was previously demonstrated to mediate NET formation *in vitro* (Mazzoleni et al., 2021; Münzenmayer et al., 2016). Additionally, *S. aureus* is well equipped at inducing other modes of neutrophil cell lysis, including necrosis and necroptosis (Yang et al., 2019). To determine whether non-NETotic cell death was being quantified a sensor histidine kinase (SaeS) mutant strain of JE2 (USA300 SaeS::Tn bursa aurealis EryR, LinR), generated from the Nebraska transposon mutant library was used. SaeRS is a two-component regulator, which mediates release of proteases, leucocidins, hemolysins and proteases and this mutant strain was previously demonstrated to not cause neutrophil cell lysis *in vitro* (Liu et al., 2016; Yang et al., 2019). An MOI of 5 of each bacteria strain was added to neutrophils for 2 hours, before analysing the extracellular fluorescence. There was no significant increase in SYTOX™ Green output when using any of the *S. aureus* strains compared to unstimulated cells, with only the PMA positive control showing a significant change (Figure 19A). However, there was an upward trend in fluorescence in the *S. aureus* conditions compared to the unstimulated neutrophils. There was no difference in SYTOX™ Green quantification between the SH1000, JE2 or *saeS* mutant strain. Next, I investigated increasing the MOI of bacteria from 5 to 10 for the SH1000 and JE2 strain, to understand whether a higher bacterial load impacted NET formation. There was a significant increase in SYTOX™ Green staining when using an MOI of 10 of the SH1000 strain compared to unstimulated cells (Figure 19B). Increasing the MOI was without effect on the SYTOX™ green values for the JE2 strain. After quantifying the SYTOX™ Green values, the wells were fixed and imaged. There were no NET-like strands of DNA present in the wells (Figure 19C). These results showed only a weak induction of NET formation in response to *S. aureus*

using two different techniques, and thus for the purposes of the patient studies planned, I elected not to include them in the patient study.

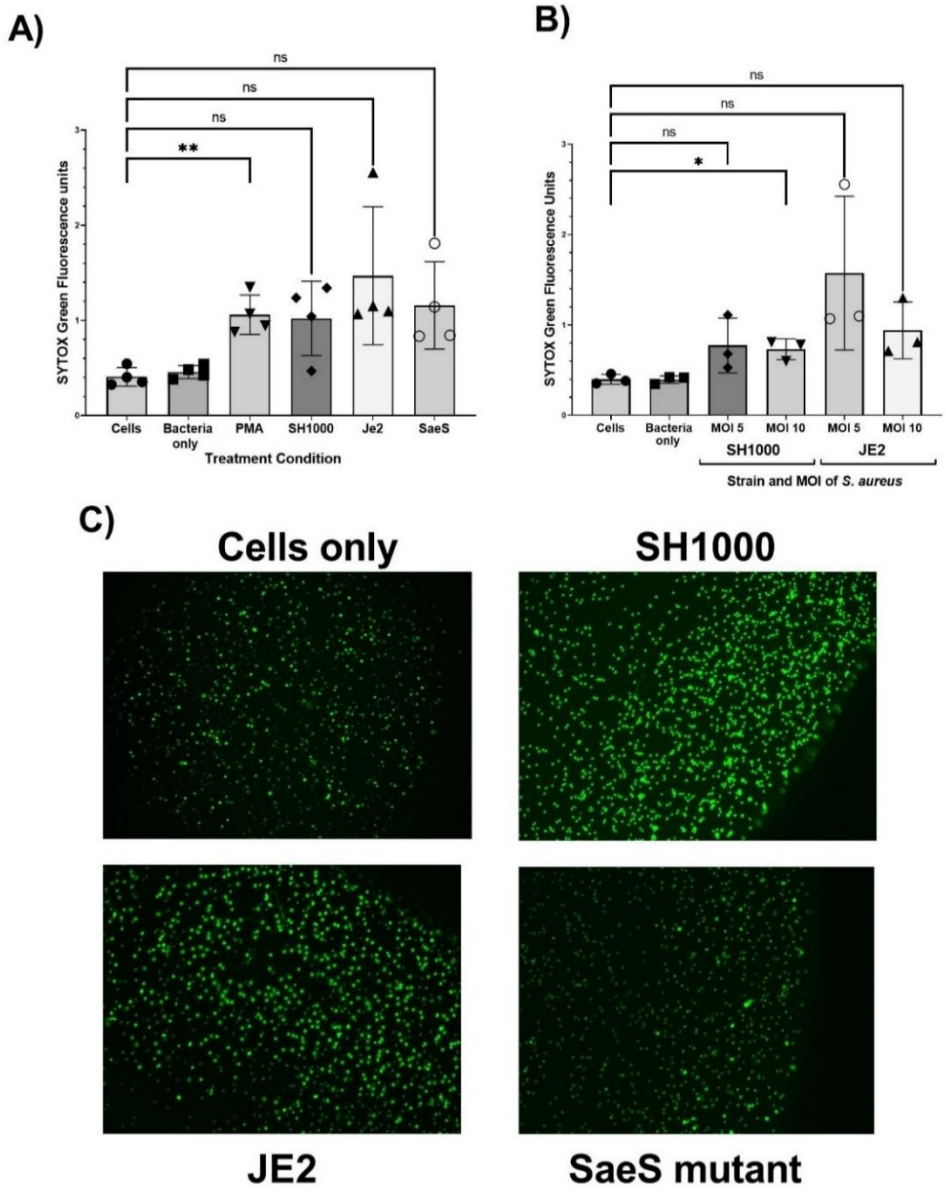


Figure 19-Quantifying NETosis induced by different strains of *S. aureus* using SYTOX Green.

Neutrophils (5×10^4) were stimulated with an MOI of 5 (A-B) or 10 (B) of *S. aureus* strain SH1000, JE2 or *SaeS* mutant for 2 hours. PMA [100 nM] was used as the positive control. SYTOX™ Green was added to all wells (555 nM) and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Excitation/emission 490/537 nm was used. A) There was no significant difference in NET formation in *S. aureus* treated cells compared to unstimulated neutrophils or between the *S. aureus* strains. B) There was a significant increase in NET formation when using an MOI of 10 of *S. aureus* strain SH1000 compared to unstimulated cells. The median values of quadruplicate wells are shown. These data represent an n=4 (A) and n=3 (B). C) After analysis, 96 well plates were fixed with 4% PFA for 15 minutes and imaged using the NIKON Widefield fluorescence microscope. The 10x objective lens and FITC filter set (excitation/emission 470/525 nm) was used. Images were processed using the FIJI image analysis software. Statistical analysis was by one-way ANOVA with a Dunnett's post-test and cells only was used as the control. Error bars display SD. Significance asterisks represent *p<0.05 and (ns) denotes not significant.

3.3 Patient study investigating neutrophil function in people with diabetic foot disease

The data presented are of an n=2 and therefore no statistical analysis could be completed or any conclusions on neutrophil function in those with DFD compared to age-matched controls drawn. For this thesis limited observations were made on these data. Neutrophil apoptosis appeared higher after stimulation with LPS in those with DFD (Figure 20A). The percentage of neutrophils undergoing apoptosis was lower for the two patients at 22 hours in unstimulated cells when cultured in standard and high glucose (Figure 20B). ROS production in response to *S. aureus* (SH1000) was similar across the media conditions in patients with DFD compared to healthy controls (Figure 21). There was no obvious increase in ROS production in the two patients at baseline. Large differences in both the number of neutrophils phagocytosing *S. aureus* (Figure 22A) and the number of bacteria being ingested were demonstrated for the two patients with DFD (Figure 22B). NETosis in response to PMA was similar between patients and controls (Figure 23). The clinical data collected for the two patients is not presented here, as with n=2 it would not help the interpretation of the data, and low n non-aggregated clinical data increases theoretical risks of accidental deanonymisation.

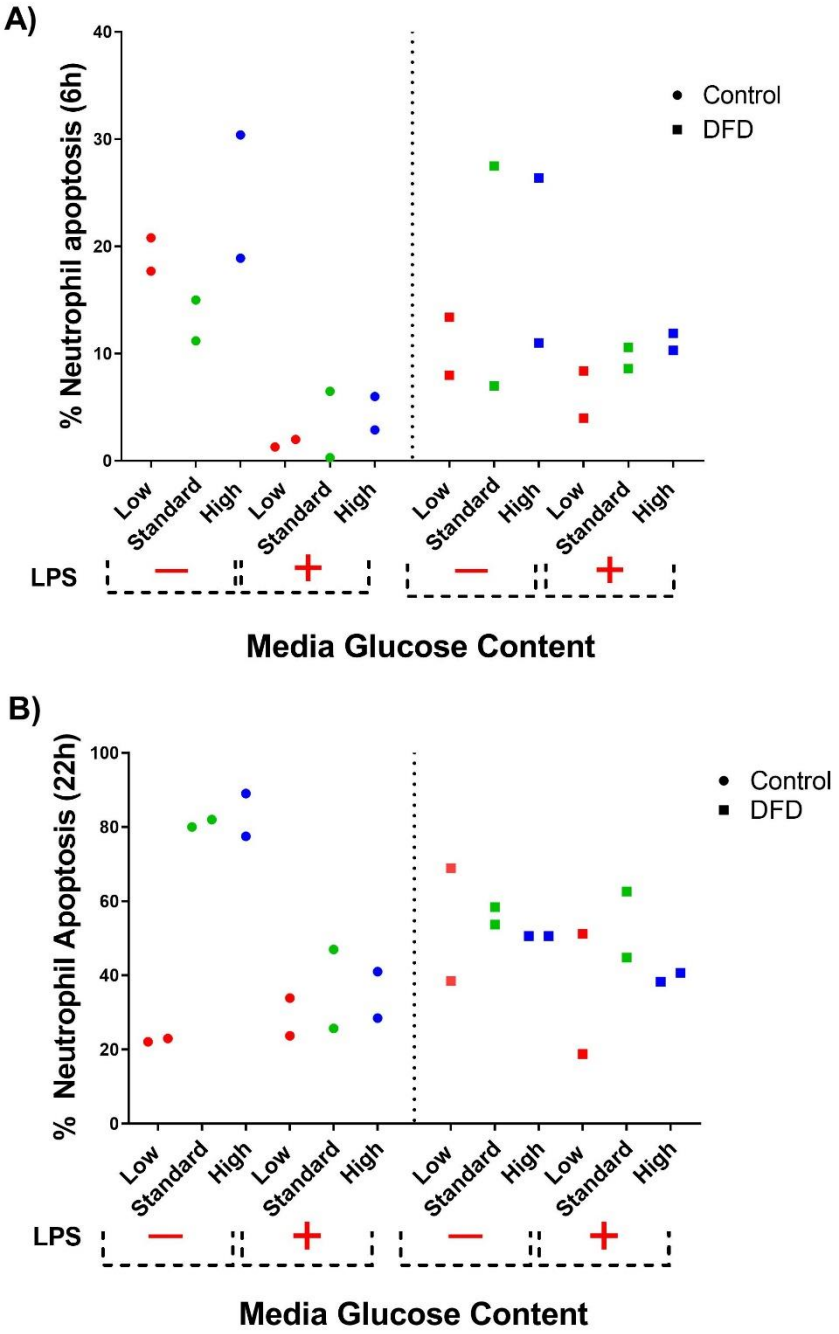


Figure 20-Apoptosis in neutrophils from patients with diabetic foot disease.

Neutrophils (2.5 x 10^5 cells/well) isolated from healthy controls (circle) or patients with diabetic foot disease (DFD) (square) were cultured for either 6 (A) or 22 (B) hours in media containing low [0.3 mM] (red), standard [11 mM] (green) or high [20 mM] (blue) concentrations of glucose. The effect of LPS [100 ng/ml] on apoptosis was compared in each of the media conditions. A total of 300 cells were counted in each condition and the % apoptosis was calculated. Mean values from duplicate samples of an n=2 is shown.

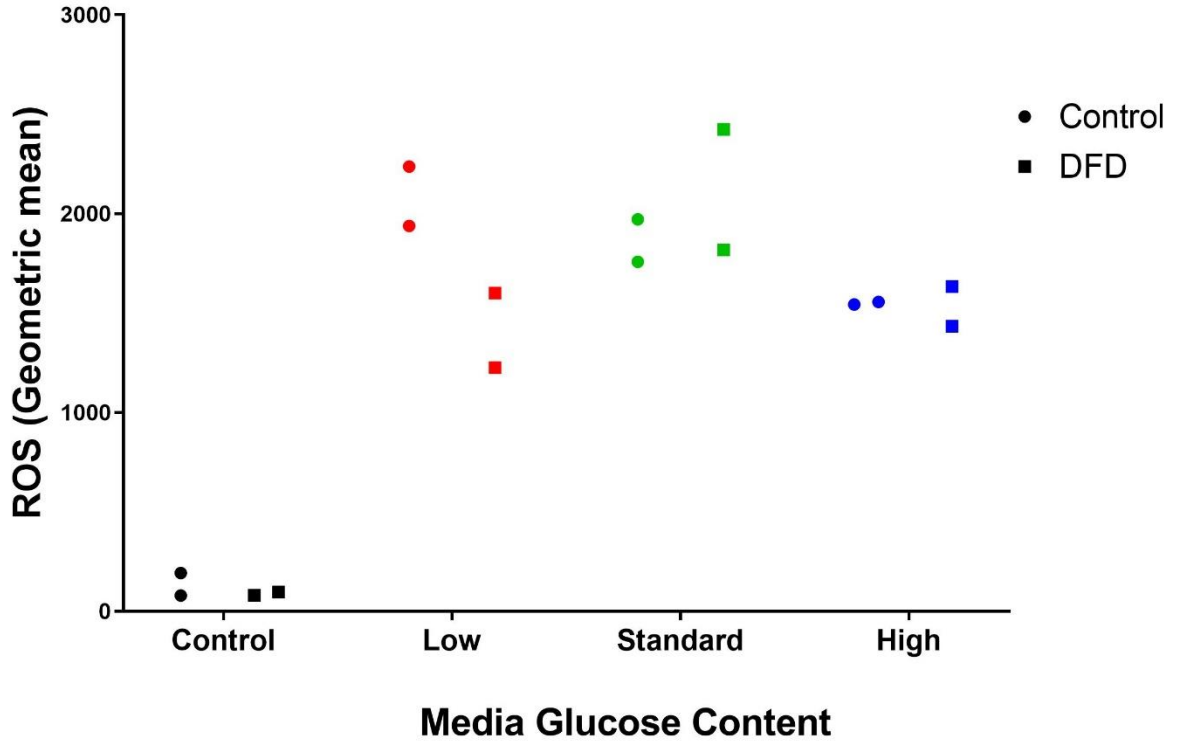


Figure 21-Intracellular ROS production by neutrophils from patients with diabetic foot disease.

Neutrophils (2.5 x 10^5 cells/well) isolated from healthy controls (circle) or patients with diabetic foot disease (DFD) (square) were cultured in media containing low glucose [0.3 mM] (red), standard glucose [11 mM] (green), or high glucose [20 mM] (blue) for 1 hour prior to addition of *S. aureus* (SH1000) at an MOI 5. DCF was used to detect intracellular ROS production. Media only was used as the negative control. Neutrophils and bacteria were co-incubated for 30 minutes. Samples were analysed using flow cytometry. Neutrophils were gated based on the forward and side scatter. The BD™ LSRII flow cytometer, and the BD FACSDiva™ software were used for analysis. The 488 nm blue laser and 530 nm filter were employed to detect intracellular fluorescence. Ten thousand neutrophils were analysed per sample and duplicate samples were run per condition (means plotted). Mean values from duplicate samples of an n=2 is shown.

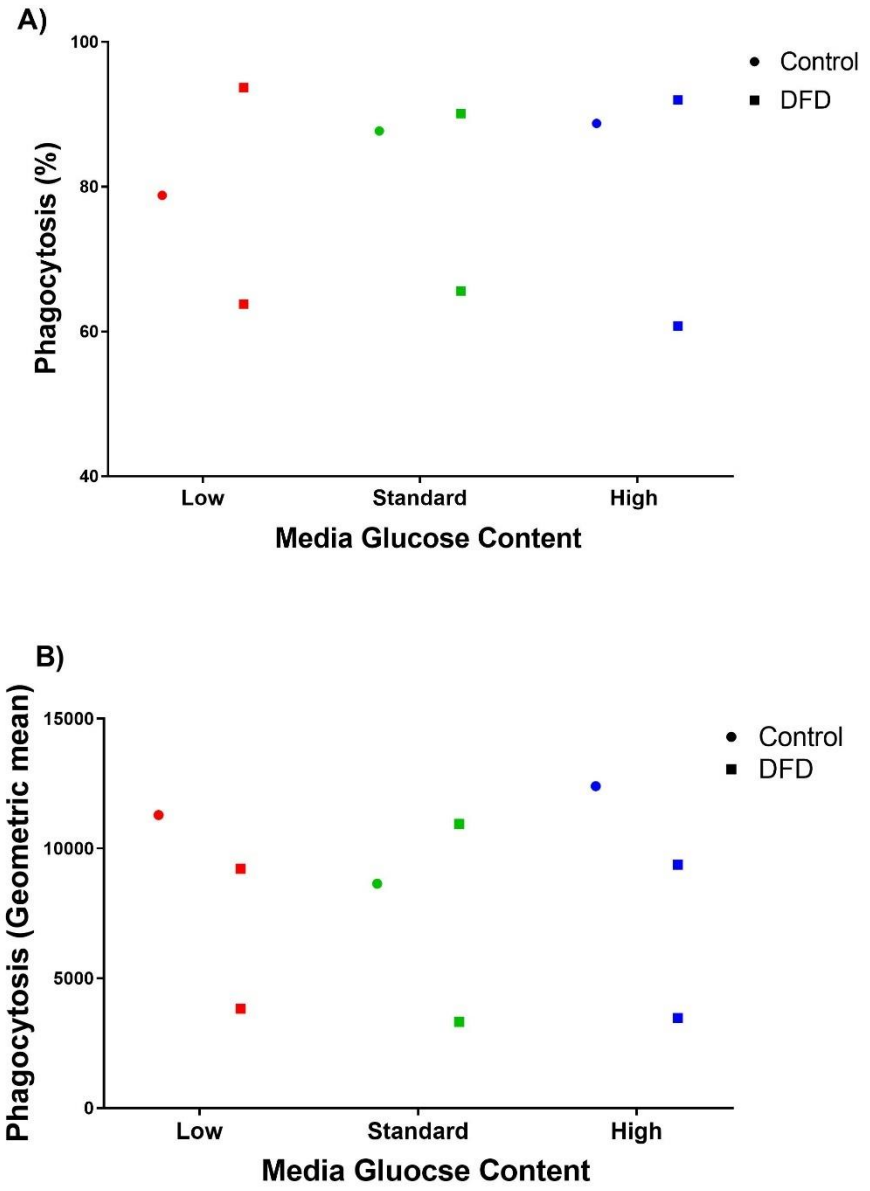


Figure 22-Phagocytosis of *S. aureus* by neutrophils from patients with diabetic foot disease.

Neutrophils (2.5 x 10^5 cells/well) isolated from healthy controls (circle) or patients with diabetic foot disease (DFD) (square) were cultured in media containing low glucose [0.3 mM] (red), standard glucose [11 mM] (green), or high glucose [20 mM] (blue) for 1 hour. GFP-labelled *S. aureus* (SH1000) of an MOI 5 were added to neutrophils and they were co-incubated for 30 minutes. Neutrophils were washed and extracellular fluorescence quenched using 0.2% trypan blue prior to analysis using flow cytometry. The BD™ LSRII flow cytometer, and the BD FACSDiva™ software were used for analysis. The 488 nm blue laser and 530 nm filter were employed to detect intracellular fluorescence. Ten thousand neutrophils were analysed per sample. Neutrophils were gated based on forward and side scatter. A) These data show the percentage number of neutrophils which had phagocytosed *S. aureus* in different glucose containing media. The mean values of duplicate samples of an n=2 for patients with DFD and an n=1 for healthy controls is demonstrated.

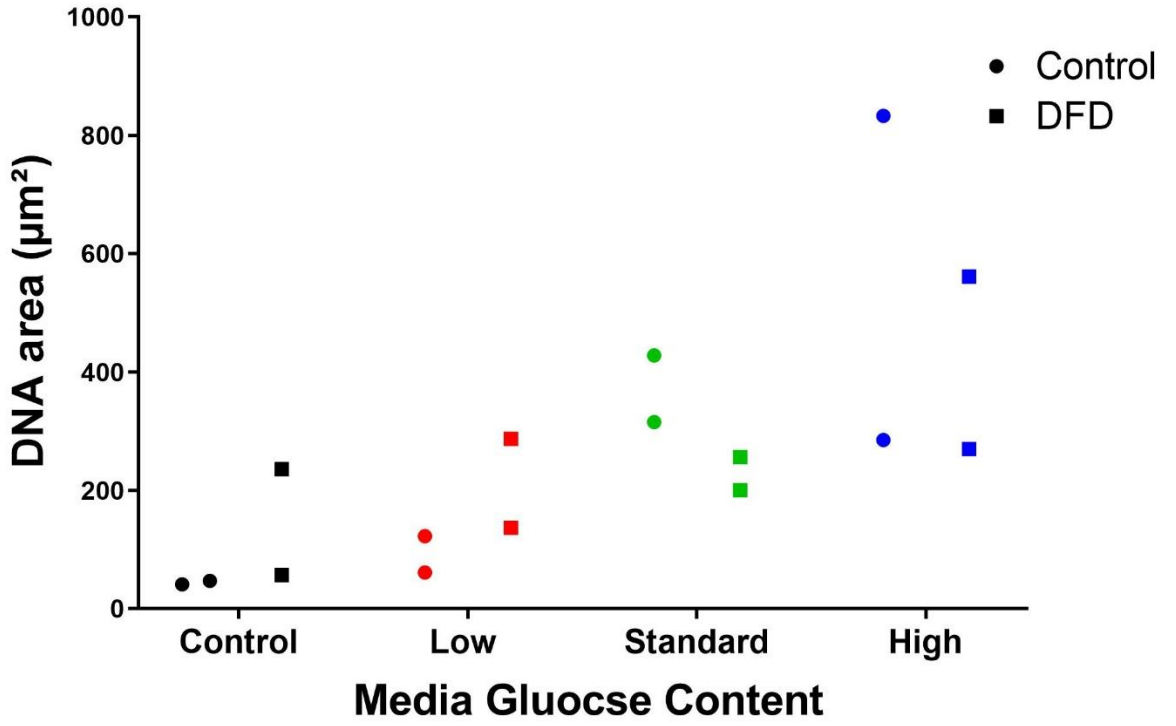


Figure 23-NETosis by neutrophils from patients with DFD induced by PMA.

Neutrophils (2.5 x 10^6 cells) isolated from healthy controls (circle) or patients with diabetic foot disease (DFD) were seeded onto 0.01% poly-l-lysine coated coverslips and stimulated for 3 hours with 25 nM PMA in media containing either low glucose [0.3 mM] (red) standard glucose [11 mM] (green), or high glucose [20 mM] (blue). Unstimulated neutrophils in standard media were the negative control (black). Cells were fixed using 4% PFA for 15 minutes and then washed with PBS before mounting onto microscope slides using DAPI containing ProLong™ Gold Antifade Mountant. Slides were visualised using a Nikon Widefield microscope with the 395-455 nm wavelength filter set for DAPI detection. The 40x oil immersion objective lens was used. Quantification of DNA area was conducted using the FIJI image analysis software. Five images were analysed per condition across duplicate slides. Fields of view for imaging and analysis were selected at random by an independent person. These data represent an n=2.

3.4 Discussion

3.4.1 Investigating neutrophil function in people with DFD

Neutrophil function has been studied in the context of diabetes over many decades, yet no targeted therapy to restore neutrophil action has been found. Multiple neutrophil effector mechanisms have been shown to be dysregulated in diabetes including enhanced NET formation and extracellular ROS production and decreased phagocytosis, chemotaxis and apoptosis (Alexiewicz et al., 1995; Delamaire et al., 1997; Hand et al., 2007; Ihm et al., 1997; Manosudprasit et al., 2017; Wong et al., 2015). Through extensive review of the literature, it was found that neutrophil function in people with DFD was relatively understudied, despite this group of patients being pre-disposed to chronic infection. The majority of previous literature in the context of DFD has focused on enhanced NETosis in the pathology of poor wound healing (Fadini et al., 2016; Wong et al., 2015; Yang et al., 2020). The neutrophil phenotype in DFD was yet to be fully defined. To address this knowledge gap, a pilot phenotyping study of neutrophil function in people with DFD in different concentrations of glucose was set up. A collection of assays of neutrophil function were optimised and designed to be conducted simultaneously. Devising the patient study was a dynamic process that involved many discussions regarding participant selection, choice of healthy controls and inclusion/exclusion criteria, which were critical in framing the aims of the project.

3.4.2 The impacts of glucose on neutrophil function *ex vivo*

Neutrophil cell death pathways were modified by transient *in vitro* changes in the cell culture media when using cells from healthy donors, with NETosis increased in high glucose (20 mM) and apoptosis decreased in low glucose media (0.3 mM). PMA-induced NETosis is dependent on glucose availability and multiple studies demonstrate enhanced NETosis in high glucose concentrations (Joshi et al., 2020; Menegazzo et al., 2015; Rodríguez-Espinosa et al., 2015; Wang et al., 2019). Glucose stimulates activation of PKC, which mediates ROS production and NET formation (Giacco and Brownlee, 2010; Wang et al., 2019). However, ROS production in response to infection with *S. aureus* was not increased in high glucose in this study. This contrasts with the findings by Wang et al. (2019) who showed increased neutrophil ROS production in high glucose media (25 mM) 15-120 minutes after incubation, when using cells from healthy donors (Wang et al., 2019). DCF was also to used detect ROS generation, therefore differences in experimental technique are unlikely to account for these findings. However, in the study by Wang et al. (2019), neutrophils were not stimulated with *S. aureus*, with the increase in ROS production resulting from the elevated glucose concentration in the media only. *S. aureus* is a strong inducer of neutrophil ROS (Anderson et al., 2008), therefore this may have masked any subtle changes in ROS production resulting from varying glucose concentration in my assay.

The impacts of low glucose conditions on neutrophil function *in vitro* have not been widely explored previously. In this chapter it was demonstrated that culturing neutrophils in low glucose significantly reduced constitutive apoptosis after 6 hours, but not after 22 hours. Neutrophil apoptosis is reduced in hypoxic environments, such as sites of inflammation and abscesses, which are characteristically low in glucose (Taylor and Colgan, 2017; Walmsley et al., 2005). Neutrophils were thought to rely exclusively on glycolysis for energy (Warburg metabolism), however recent research has demonstrated the metabolic plasticity of neutrophils (Warburg, 1956; Kumar and Dikshit, 2019; Sadiku et al., 2021). Neutrophils utilise glycogen stores formed via gluconeogenesis, glycogenesis and glutaminolysis to meet energy demands (Sadiku et al. 2021). Investigation of how metabolic changes impact neutrophil survival could identify novel therapeutic targets to improve infection resolution. Previous work in our laboratory group demonstrated that experimental hypoglycaemia in healthy volunteers activated the innate immune response and increased neutrophil counts *in vivo* (Iqbal et al., 2019). This links with the reduction in apoptosis in low glucose observed in this chapter, supporting the pro-inflammatory effects of hypoglycaemia on neutrophil function. However a previous study demonstrated that low glucose conditions increased neutrophil apoptosis *ex vivo* after 24 hours (Healy et al., 2002). Also, neutrophils isolated from people with glycogen storage disease type 1b, which is characterised by persistent hypoglycaemia, showed increased apoptosis compared to healthy controls (Kuijpers et al., 2003). Neutrophil phagocytosis was unchanged in varying concentrations of glucose, which supports previous literature (Wilson and Reeves, 1986). Interestingly, phagocytosis of *S. aureus* by granulocytes was decreased in an early study which exposed cells to intermittent hyperglycaemia (Van Oss, 1971). This was conducted by seeding cells on coverslips and exposing them to a concentrated glucose solution for 5 seconds, every 2 minutes for 2 hours. Although this methodology would not be suitable for the phenotyping study in this chapter, it raises the issue of using static concentrations of glucose in the experiments. Fluctuating blood glucose levels are a feature of diabetes and the impacts of oscillating blood glucose levels on neutrophil function have not been investigated in detail before.

3.4.3 Limitations and future work

Completion of the patient study investigating the neutrophil phenotype in patients with DFD would be important, as it would provide novel data regarding neutrophil function in the context of chronic infections. Completion of the pilot study could identify aberrant neutrophil effector mechanisms, which could be explored as therapeutic targets to improve the host immune response to infection. Also, this research could provide the basis to design larger more tightly controlled follow-up studies, based on the results. A key limitation of the *in vitro* investigations of neutrophil function is that they studied the effects of short-term changes in glucose upon cell function only. Hyperglycaemia impacts

both circulating cells and the stem cell niche in the bone marrow in diabetes (Fadini et al., 2014). Also neutrophils display 'metabolic memory', whereby altered phenotypes are maintained once blood glucose levels have been normalised, supporting there could be longer term changes to the neutrophil in diabetes (Carestia et al., 2016; Ceriello et al., 2009; Corgnali et al., 2008). Prior to the COVID-19 pandemic I planned and designed a collaborative project with Dr Joby Cole (Dept. Infection, Immunity & Cardiovascular Disease, University of Sheffield), to investigate epigenetic histone modifications in peripheral blood neutrophils from people with DFD. Epigenetic changes are defined as alterations in chromatin structure and gene expression that occur without modifications to the DNA sequence (Gibney and Nolan, 2010). DNA is packaged around histone proteins, forming chromatin. Histones regulate gene expression by controlling the condensation of chromatin, altering which genes are accessible for gene transcription (Eberharter and Becker, 2002). Histone acetylation is a permissive change in gene expression, whereas methylation is largely repressive (Eberharter and Becker, 2002). Hyperglycaemia causes epigenetic reprogramming of cells in diabetes. Murine macrophages were epigenetically reprogrammed by histone post translational modifications (PTMs) to confer a pro-inflammatory phenotype in a model of T2D (Gallagher et al., 2015). There was a reduction in methylation at the *IL-12* gene promoter (H3K27me3), with increased levels of this cytokine detected in mice, which was associated with poor wound healing in response to sterile injury (Gallagher et al., 2015). Research using human participants demonstrates increased acetylation (H3K9Ac) of gene promoters for pro-inflammatory genes involved in the NF-κB signalling pathway in lymphocytes and monocytes isolated from patients with T1D undergoing conventional versus intensive blood glucose controlling therapy (Miao et al., 2014). The investigation of epigenetic changes in neutrophils isolated from people with diabetes has not been explored previously. Nonetheless, neutrophils undergo epigenetic remodelling in disease and neutrophils isolated from patients with anti-neutrophil cytoplasmic autoantibody-associated vasculitis demonstrated decreased methylation (H3K9me2) at gene promotors for MPO and proteinase 3 (Yang et al. 2016). The hypothesis for this work was that neutrophils in DFD were epigenetically reprogrammed to have a pro-inflammatory gene signature. To complete this work, I planned to extract histones from neutrophils from people with DFD and then in collaboration with Dr Cole analyse histone PTMs using mass spectrometry. Differences in histone PTMs in cells isolated from patients of controls, if any, would be investigated using Chromatin Immunoprecipitation Sequencing (ChiP-Seq) to identify the genetic regions modified.

4 Bioinformatics project- Is transcription important in NETosis?

4.1 Introduction

Modifying neutrophil function in disease is an overarching aim of my PhD and whether gene transcription is required for NETosis is disputed and remains an important question that the changes in circumstances driven by the COVID-19 pandemic gave me a chance to explore and address. NETosis is pathological in a range of diseases and further investigation of the underlying mechanisms of this pathway could present new therapeutic strategies.

4.1.1 Project background

I specifically wanted to explore important controversies in the field driven by work of Khan & Palaniyar (2017), who demonstrated that gene transcription is needed for NETosis to occur. They showed that neutrophils, in response to prototypical NET inducers PMA or ionophore A23187, transcribe genes at distinct and overlapping loci, which is required for chromatin decondensation in NETosis (Khan and Palaniyar, 2017). They identified approximately 200 genes upregulated in NETosis (Log fold change ≥ 1.5) and this was referred to as 'transcriptional firing'. PMA and A23187 are prototypical NET inducers which stimulate NOX-dependent (PMA) or NOX-independent (A23187) NETosis (Sollberger et al., 2018). Importantly they showed that NETosis was inhibited by the transcription inhibitor, actinomycin D, but was unaffected by translation inhibitor cycloheximide (Khan and Palaniyar, 2017). Whether the genes upregulated in the transcriptional firing process are unique to a NETosis transcriptional mechanism, or are required for NETosis, is not known. A subsequent study from the same research group demonstrated that anthracyclines, which are anti-tumour therapeutics, also inhibited PMA and A23187-mediated NETosis *in vitro* (Khan et al., 2019). Anthracyclines intercalate with DNA, preventing gene transcription and DNA replication in dividing cells (Shandilya et al., 2020). Khan et al. (2019) suggested that inhibition of NETosis by anthracyclines provides further support for the importance of transcriptional firing in the mechanism of NETosis (Khan et al., 2019). It's important to note that the results demonstrated by Khan and Palaniyar (2017) are an anomaly in comparison to other studies in the field, but they raised important questions. Previous research showed that NETosis induced with a range of stimuli, including PMA and A23187, was not inhibited by actinomycin D and proceeded unaffected by the transcription inhibitor (Kenny et al., 2017; Sollberger et al., 2016; Tatsiy and McDonald, 2018). Due to the empirical evidence demonstrating that NETosis is not impacted by actinomycin D, it is generally accepted that transcription is not required for NETosis. However, to my knowledge, the discrepancy in the literature regarding the importance of gene transcription in NETosis, has not been formally explored before. I therefore sought to examine the work of Khan and Palaniyar in more detail. I considered that the transcriptional firing mechanism proposed by Khan and

Palaniyar would be to some degree supported if the pattern of genes activated also showed some unique links to NETosis.

4.1.2 Hypothesis and aims

To complete this project, a bioinformatics approach was used to explore whether the genes most upregulated in response to PMA and A23187, as reported by Khan and Palaniyar (2017), which will now be referred to as the 'transcriptional firing gene set', were similar or distinct to neutrophil gene transcription in response to other pro-inflammatory stimuli. I hypothesised that a high level of similarity in genes upregulated in response to other known inducers of NETosis, could represent a specific NETosis gene transcription programme. Alternatively, if these genes were frequently upregulated in response to multiple different stimuli, not known to induce NETosis, this would suggest pro-inflammatory gene activation, rather than a specific NETosis transcription programme, and would make it less likely that such transcriptional firing underpinned NETosis. Furthermore, diabetes is one of several diseases that is associated with a pro-NETotic phenotype and I aimed to investigate if the genes identified in the transcriptional firing gene set were upregulated in people with T2D compared to healthy controls (Fadini et al., 2016; Wong et al., 2015). To complete this bioinformatics project, the following aims were set:

1) Identify the genes most upregulated in response to NETosis stimulants PMA and A23187, to define the transcriptional firing gene set.
2) Explore the function of the genes upregulated in the transcriptional firing gene set to determine whether these genes were known to be associated with the mechanism of NETosis.
3) Determine whether the genes identified in aim 1 were also upregulated in datasets stimulating neutrophils with a range of pro-inflammatory mediators.
4) Determine whether the genes in aim 1 were upregulated in people with T2D.

4.2 Methods

4.2.1 Acquisition of datasets

Neutrophil transcriptomic data was retrieved using the Gene Expression Omnibus (GEO) Datasets online tool https://www.ncbi.nlm.nih.gov/gds/ and by literature searching. Searches were filtered to include only *Homo Sapiens,* and the keyword was 'neutrophil'. As this was an exploratory project there were no inclusion/exclusion criteria and studies were selected to include the activation of neutrophils with a range of different stimuli. The datasets interrogated in the project and how neutrophils were stimulated in each study are described in Table 5.

Table 5- Neutrophil transcriptomic data sets interrogated

Accession Number	Study Title	Experiment type	Method of neutrophil stimulation analysed in this project	Citation
GSE80489	Transcriptional Firing helps to drive NETosis	Human transcriptome 2 (HTA 2) Affymetrix gene-chips	PMA (25 nM), A23187 (4 µM) or DMSO (negative control) for 30 and 60 minutes (n=3)	(Khan and Palaniyar, 2017)
GSE126758	Upon microbial challenge, human neutrophils undergo rapid changes in nuclear architecture and chromatin folding to orchestrate an immediate inflammatory gene program	RNA sequencing (Illumina HiSeq 2500)	*E. coli* strain K1 (MOI 5) for 3 hours v.s unstimulated (n=5)	(Denholtz et al., 2020)
GSE39889	Mycobacterium abscessus Induces a Limited Pattern of Neutrophil Activation That Promotes Pathogen Survival	Affymetrix HG-U133A Plus 2.0 microarrays	Clinical strain of *S. aureus* (MOI 10) treated for 2 hours v.s unstimulated (n=4)	(Malcolm et al., 2013)
GSE33939	Sub lytic concentrations of Staphylococcus aureus Panton-Valentine leucocidin alter human PMN gene expression and enhance bactericidal capacity	Affymetrix GeneChip Human Genome U133 Plus 2.0 Array.	PVL subunits (LukF-PV and LukS-PV) (1 nM) purified from culture supernatants of a USA300 hlgABC deletion strain. Treatment for 180 minutes v.s unstimulated (n=3)	(Graves et al., 2012)
GSE94923	NR4A orphan nuclear receptor family members, NR4A2 and NR4A3, regulate neutrophil number and survival	Affymetrix GeneChip Human Genome U133 Plus 2.0 Array.	GM-CSF (100 U/mL), hypoxia (3 kilopascal), PKA agonist- N6/8-AHA (1 mM), LPS (1 µg/ml) for 4 hours, compared to unstimulated control (n=5)	(Prince et al., 2017)
N/A	Abnormal Peripheral Neutrophil Transcriptome in Newly Diagnosed Type 2 Diabetes Patients	RNA sequencing (BGISEQ-500 platform)	Baseline gene expression in patients with type 2 diabetes compared to healthy controls (n=8)	(Lin et al., 2020)

Protein Kinase A (PKA), **N6/8-AHA** (8-AHA-cAMP and N6-MB-cAMP)

4.2.1 Identification of the transcriptional firing gene set and interrogation of datasets

Datasets that had a Gene Expression Omnibus series (GSE) accession number were analysed using the GEO2R web-based platform, which is an online tool using an R script to compare gene expression between different groups in a dataset (https://www.ncbi.nlm.nih.gov/geo/geo2r). Prior to the analysis of each dataset, the normalisation of the data were checked. This was conducted by using the GEO2R platform to generate a box plot to visualise value distribution for the samples in a dataset. This was a quality control step, as data with a similar median across sample groups is indicative of normalised data that can be used to compare gene expression between samples. An example boxplot generated for dataset GSE80489 is shown in Figure 24. Each bar represents an individual sample within the dataset. Similar median centred values were found for all samples indicating that these data were suitable for cross comparison.

To define the transcriptional firing gene set the study by Khan and Palaniyar (2017) (GSE80489) was interrogated. The top 25 genes (an arbitrary number selected to show the key genes and generate a manageable comparison set) upregulated in response to PMA or A23187 compared to the DMSO control were identified and these genes are described in results section 4.3.1. The function of the genes in the transcriptional firing gene set were defined using the Human Gene Database Genecards®- https://www.genecards.org/. The GeneOntology (GO) Panther Classification system http://pantherdb.org/ was used to explore the biological processes and pathways for these genes (Mi et al., 2021). The expression of the genes identified in the transcriptional firing gene set were then interrogated in the datasets outlined in Table 5. The log fold change (LogFC) was used to compare differential gene expression and LogFC of ≥1.5 was used as an arbitrary cut-off to demonstrate a potentially important change in gene expression. Adjusted p values were not used to compare differential gene expression as the abundance of transcript was not important for this project, rather, it was the differential upregulation of specific genes which were compared. For datasets without a GSE accession number or where GEO2R analysis was not supported, differential gene expression was interrogated manually using available processed supplementary data and normalisation was confirmed by referring to the methodology section in the original research article.

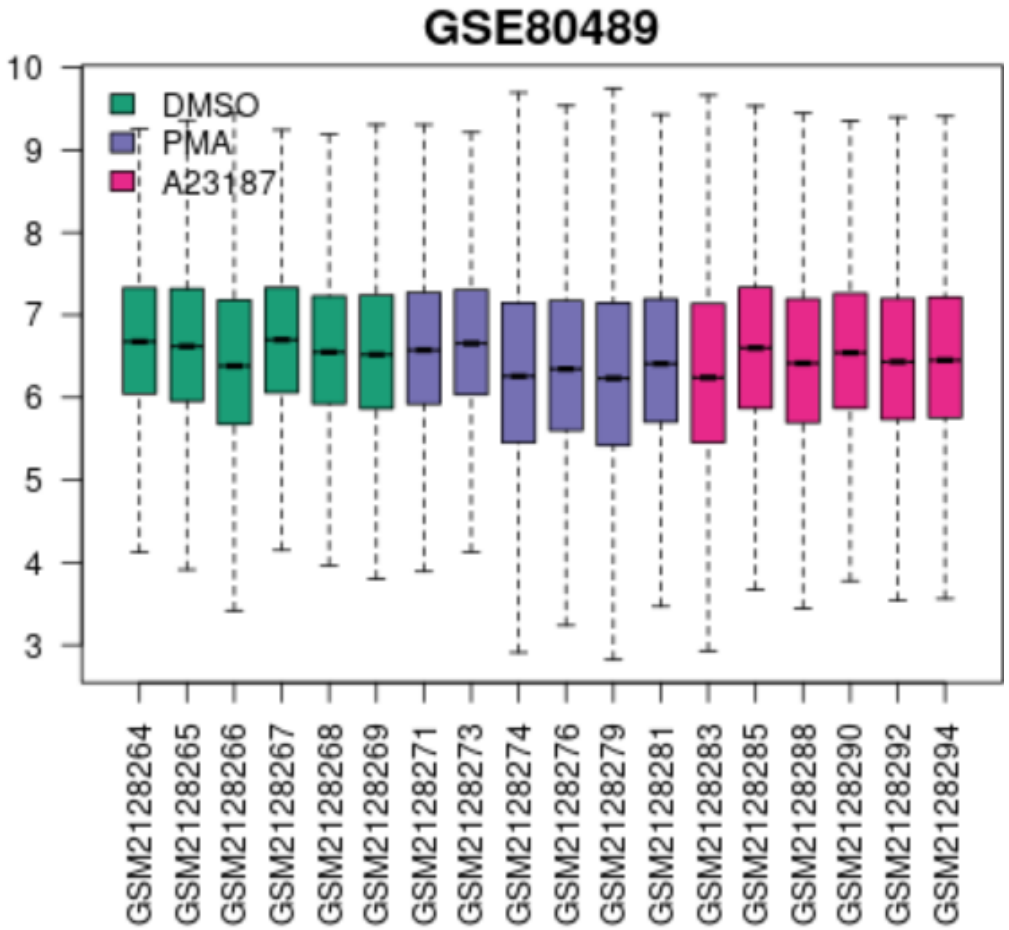


Figure 24-Value distribution box plot for dataset GSE80489.
A value distribution box plot was generated using the GEO2R online too for dataset GSE80489. Each bar represents an individual sample in the dataset (Green=DMSO, Purple= PMA, Pink=A23187). Data represents n=6. The median values were similar between samples, which is indicative of normalised data that can be used to investigate differential gene expression.

4.3 Results

4.3.1 Identifying the transcriptional firing gene set

To identify the most upregulated genes in response to PMA and A23187, the dataset generated in the transcriptional firing study (GSE80489) was interrogated (Khan and Palaniyar, 2017). Gene expression data for neutrophils isolated from 3 healthy donors stimulated for 30 and 60-minutes with either PMA (25 nM) or A23187 (4 µM) compared to the DMSO control were used to identify the top 25 genes upregulated in response either stimulus. For these 50 genes (top 25 in response to PMA or A23187), 10 were common to both stimuli. This therefore yielded 40 unique genes for subsequent interrogation in the other datasets, these genes are referred to as the transcriptional firing gene set in this project (Figure 25A). The 10 overlapping genes, which were highly upregulated in response to both PMA and A23187 were *EGR1, HCAR2, OLR1, CD69, FOSB, FOS, OSM, GLA, DDX3Y*. The LogFC values for the transcriptional firing gene set are shown in Figure 25B. The top 4 most upregulated genes were *EGR1, FOSB, NR4A3* and *OLR1* with LogFC values of 3.74, 2.8, 2.78 and 2.71 respectively. These genes were

highly upregulated in both PMA and A23871 treated neutrophils. When genes were common to both PMA and A23187 stimulation, the highest LogFC was presented. Known functions of the 40 genes are displayed in Table 6. Many of the genes were associated with the regulation and activation of neutrophil transcription (*EGR1, EGR3, NFIL3, PURB, CCNL1, SRSF3, NR4A2 & NR4A3*) and encoded for transcription factors (*DDIT3, FOS, FOSB*). Several chemokine genes were also upregulated (*CCL3L3, CCL2, CCL3, CCL4L1* and *CCRL2*). Genes involved in cell and lipid metabolism were upregulated (*HCAR2, HCAR3, OLR1, GLA & LIPN*). Functions of other genes included apoptosis (*PMAIP1*) and antiviral immune cell signalling (*RSAD2*).

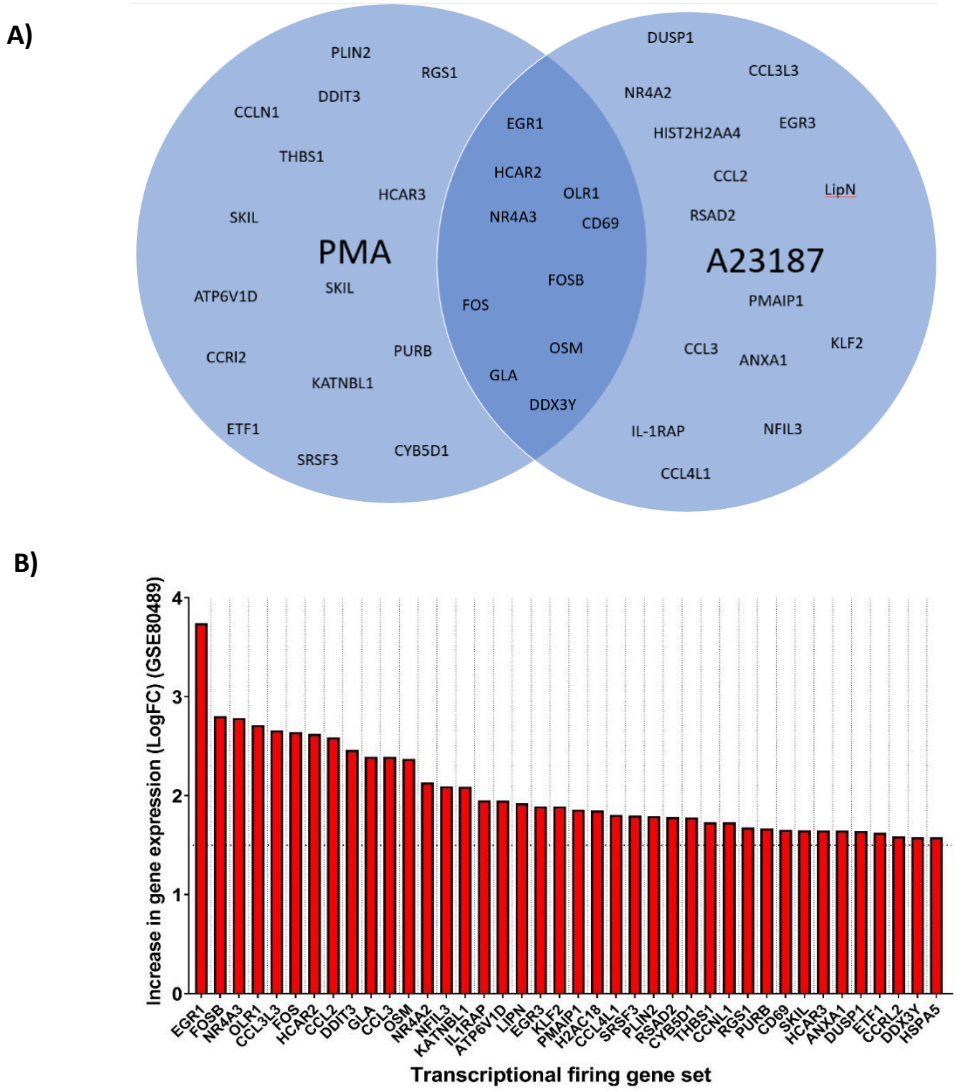


Figure 25-Identification of the genes in the transcriptional firing gene set.
Data set GSE80489 was interrogated using the GEO2R online tool to identify the top 25 upregulated genes in neutrophils stimulated with either PMA (25 nM) or A23187 (4 µM) for 60 minutes. Log fold change (LogFC) was used to compare gene expression compared to the DMSO control. A) There were 10 genes upregulated in both PMA and A23187 treated cells, yielding 40 unique genes to explore in subsequent datasets. These 40 genes represent the transcriptional firing gene set. B) The LogFC is demonstrated for the transcriptional firing gene set.

Table 6- Function of the genes identified in the transcriptional firing gene set

Gene	Function	Gene	Function
EGR1	Transcriptional regulator	**PMAIP1**	Apoptosis
FOSB	Transcription factor subunit	**H2AC18**	Histone
NR4A3	Transcriptional activator	**CCL4L1**	Chemokine
OLR1	Low density lipoprotein receptor	**SRSF3**	Splicing Factor
CCL3L3	Chemokine	**PLIN2**	Associated with lipid globule surface membrane
FOS	Transcription factor subunit	**RSAD2**	Anti-viral immune signalling
HCAR2	Hydrocarboxylic acid receptor	**CYB5D1**	Heme binding
CCL2	Chemokine	**THBS1**	Adhesive glycoprotein (cell-cell and cell-matrix interactions)
DDIT3	Transcription Factor	**CCNL1**	Pre-mRNA splicing
GLA	Hydrolysis of lipids	**RGS1**	Regulator of G-protein signalling
CCL3	Chemokine	**PURB**	Controls DNA replication and transcription
OSM	Growth and cytokine regulator	**CD69**	Lymphocyte marker
NR4A2	Transcriptional regulator	**SKIL**	Regulates cell growth and differentiation
NFIL3	Transcriptional regulator	**HCAR3**	Hydrocarboxylic acid receptor
KATNBL1	Regulates microtubule severing	**ANXA1**	Regulates inflammation
DUSP1	Phosphatase- MAP kinase signalling	**ETF1**	Translation
IL1RAP	Interleukin 1 receptor accessory protein	**CCRL2**	Chemokine Receptor
ATP6V1D	Component of vacuolar ATPase, mediating acidification of intracellular organelles.	**DDX3Y**	RNA Helicase
LIPN	Lipase	**HSAP5**	Folding and assembly of proteins
EGR3	Transcriptional Regulator	**KLF2**	Transcription Factor

4.3.2 Gene Ontology analysis for the transcriptional firing gene set

The GO of the transcriptional firing gene set was investigated to determine the biological processes and pathways for the genes. The complete set of GOs for biological processes are shown in Figure 26A, which included 17 categories including localisation, locomotion, growth and adhesion. The highest proportion of genes – 26/40 - were assigned to 'cellular process', with the next highest process being 'biological regulation' (23/40) and then 'response to stimulus' (14/40). There were genes involved in biological processes distinct from inflammation and the immune response, including 'reproduction; and 'growth'. GO pathway analysis was then conducted and a diverse range of pathways were identified (Figure 26B). The highest number of genes were involved in the chemokine and cytokine signalling pathway (6/40 genes), followed by Gonadotropin-releasing hormone receptor pathway (5/40 genes) and the apoptosis signalling pathway (3/40). Several pathways were identified which only had a single gene associated with it, such as 'interleukin signalling' (1/40) and 'T cell activation' (1/40). It's important to note that a GO pathway for 'NETosis' or 'neutrophil extracellular trap formation' does not exist on the Panther classification system.

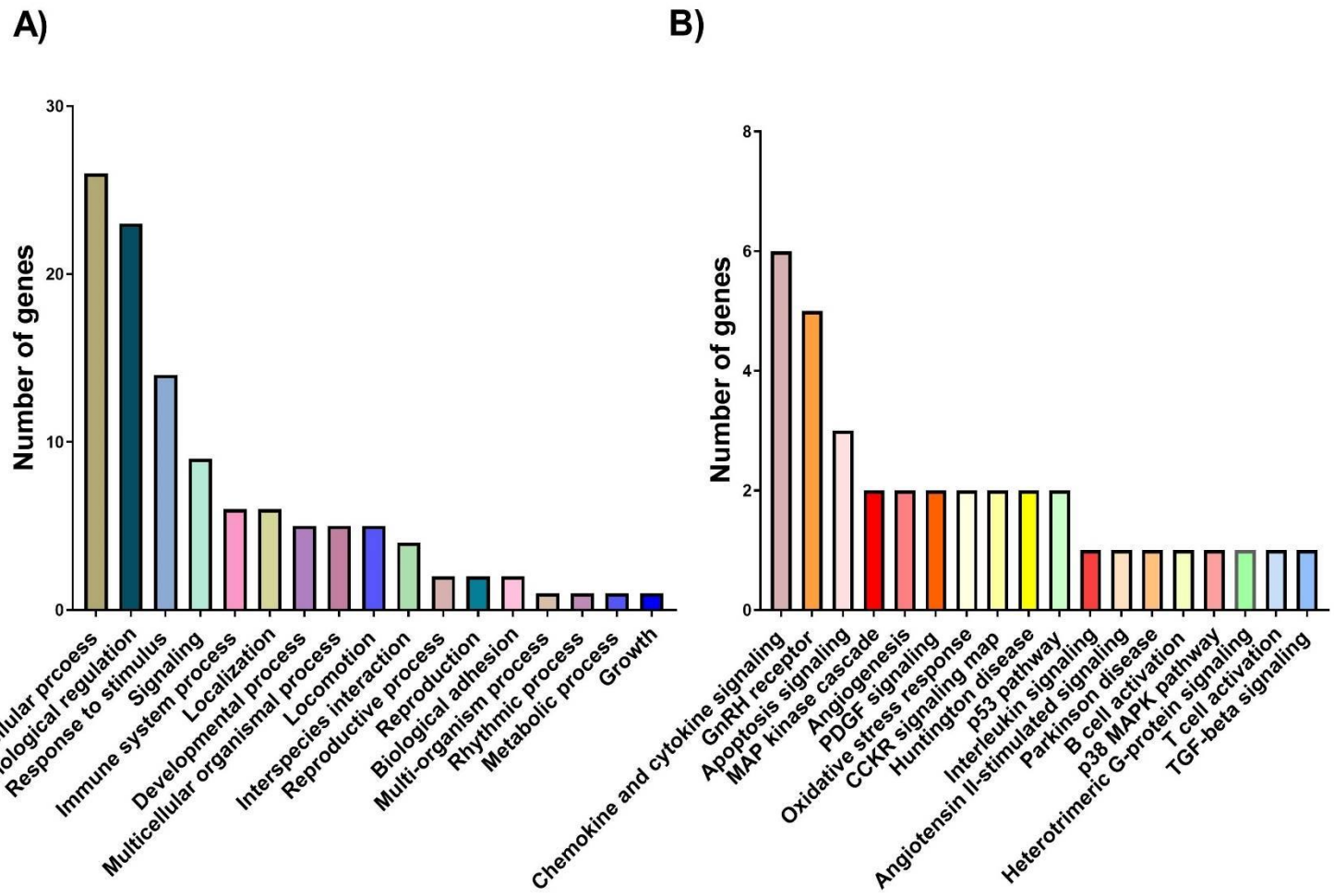


Figure 26-Gene Ontology analysis for the transcriptional firing gene set (GSE80489)

The Gene Ontology (GO) Panther Classification system was used to identify the biological process ontology (A) and pathway ontology (B) for the genes in the transcriptional firing gene set.

4.3.3 Interrogation of the transcriptional firing gene set in publicly available transcriptomic datasets using bacteria or bacterial products to stimulate neutrophils

4.3.3.1 *Rationale for selecting the studies to interrogate*

Gene transcription of the transcriptional firing gene set was explored in datasets which used bacteria or bacterial products to stimulate neutrophils. The datasets described below were chosen as numerous studies have shown that specific bacteria can induce NETosis, and that NETosis may be part of the immune response controlling bacterial infection. The datasets interrogated used the following stimuli:

- *E. coli* (MOI 5) for 3 hours (Denholtz et al., 2020) (GSE126758)
- LPS (1 µg/ml) for 4 hours (Prince et al., 2017) (GSE94923)
- *S. aureus* (MOI 10) for 2 hours (Malcolm et al., 2013) (GSE39889)
- Sub-lytic concentrations of PVL (1 nM) for 3 hours (Graves et al., 2012) (GSE33939)

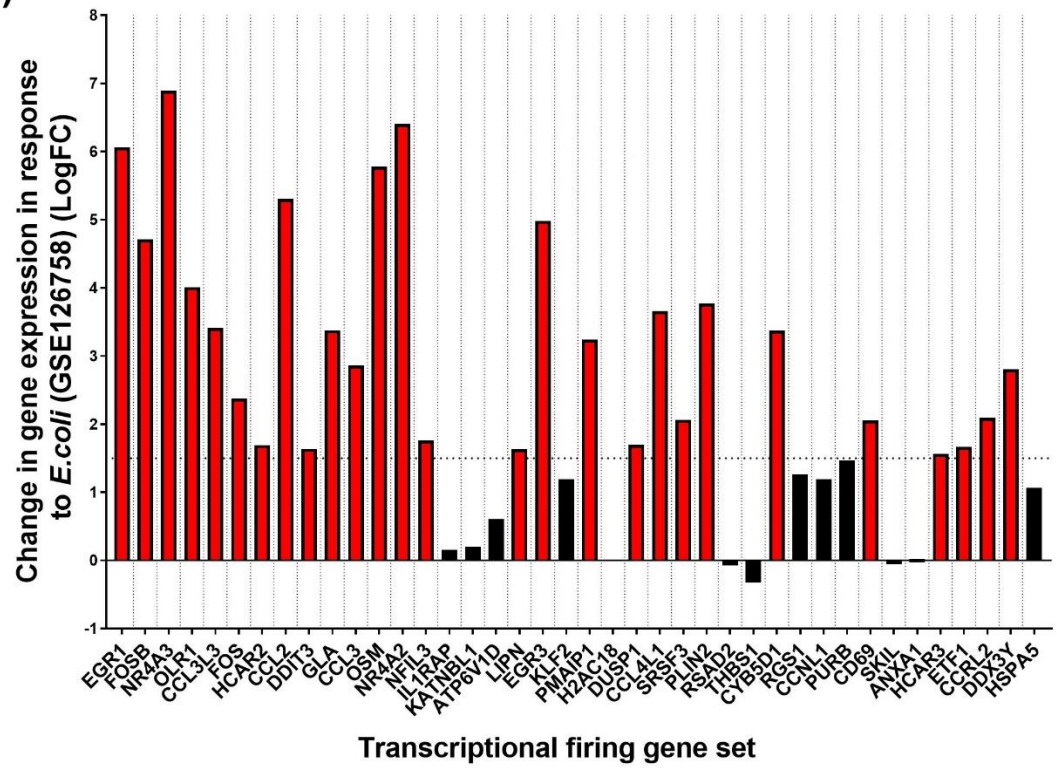
The datasets interrogated were not from studies investigating NETosis. However, the MOI of bacteria/concentration of bacterial-derived products and the length of the neutrophil incubation periods coincide with previous studies showing that NETs were generated under similar conditions (Joshi et al., 2011; Mazzoleni et al., 2021; Parker et al., 2012). The first dataset interrogated was GSE126758 (Denholtz et al., 2020). I was particularly interested to investigate this dataset as in this study they demonstrated that the neutrophil genetic material is arranged in repressive heterochromatin or permissive euchromatin structures, which regulate which genes are active or inactive for gene transcription (Denholtz et al., 2020). This ensures that pro-inflammatory genes are not constitutively expressed in the cell, which could cause host tissue damage. They demonstrated that neutrophil genetic material rapidly undergoes chromatin remodelling to transcribe pro-inflammatory genes when stimulated with PMA or *E. coli* (Denholtz et al., 2020). These data demonstrate that neutrophils are transcriptionally active when stimulated and I was interested to investigate if the transcriptional firing gene set was upregulated in response to *E. coli* in this study. Next, if the transcriptional firing gene set was upregulated in LPS stimulated neutrophils (GSE94923) was investigated (Prince et al., 2017). LPS is a component of the outer membrane of Gram-negative bacteria, which is often used to induce NETosis *in vitro* at concentrations usually at 5-25 µg/ml (Arroyo et al., 2019; Khan et al., 2017). In the study by Prince et al. (2017), they investigated neutrophil transcription in response to pro-survival stimulants, including LPS. LPS can induce both apoptosis and NETosis, which is dependent on the concentration used (Khan et al., 2017). Prince et al. (2017) used a 1 µg/ml concentration of LPS, which has been shown to induce NETosis previously, although it is lower than used in many studies to induce NETosis, but a concentration of 1 µg/ml was higher than that classically used to prolong neutrophil survival *in vitro* (Dick et al., 2009; Sabroe et al., 2002). Gram-

positive bacteria also induce NETosis, which can occur by a ROS-independent process (Pilsczek et al., 2010). In the study by Malcolm et al. (2013) (GSE39889), they investigated the neutrophil response to *Mycobacterium abscessus* and used *S. aureus* as a positive control organism, as *S. aureus* is a potent activator of neutrophils. Lastly, gene transcription in response to sub-lytic concentrations of PVL (GSE33939) was explored (Graves et al., 2012). In this study, PVL primed neutrophil ROS production and pro-inflammatory cytokine generation. In a previous study, a similar concentration of PVL induced a ROS-independent mechanism of NETosis, therefore I wanted to investigate if gene transcription of the transcriptional firing gene set was upregulated in response to PVL, and if this transcriptional profile was similar to when using whole *S. aureus* (Mazzoleni et al., 2021).

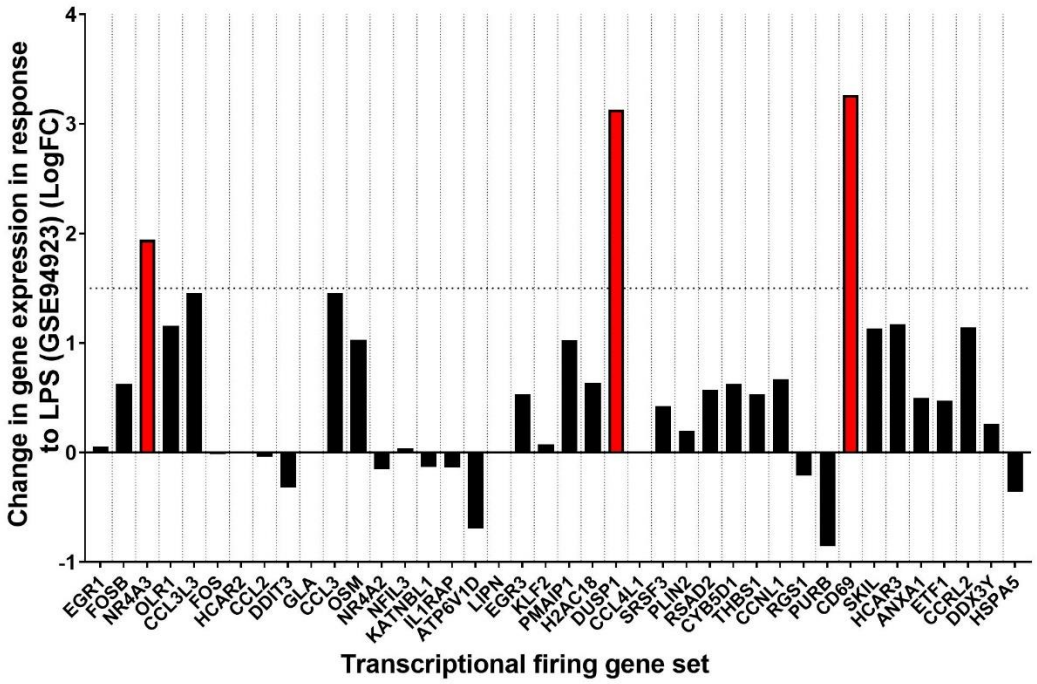
4.3.3.2 *Neutrophil transcription of the transcriptional firing gene set in response to bacteria or bacterial products*

Genes with a LogFC of ≥1.5 were deemed to have undergone a potentially important change in expression compared to the unstimulated control and are indicated by red bars in Figure 27. There were a high number of the genes in the transcriptional firing gene set that were also upregulated in response to *E. coli* (Figure 27A), with 27/40 genes having a LogFC of ≥1.5. Genes involved in transcription regulation including *EGR1*, *EGR3*, *NR4A3* and *NR4A2* were highly upregulated, in addition to chemokine genes *CCL2* and *CCL3L3*. Genes involved in cytokine signalling (*FOSB* & *OSM)* were also highly upregulated in response to *E. coli*. No genes were highly downregulated in response to *E. coli*. However, LPS-stimulated neutrophils upregulated only a small number of the genes investigated (Figure 27B), with only 3/40 genes having a LogFC of ≥1.5. These were *NR4A3*, *DUSP1* and *CD69*. In response to *S. aureus*, 21/40 of the genes in the transcriptional firing gene set had a LogFC of ≥1.5 (Figure 27C), which was similar to the transcriptional profile of *E. coli* treated neutrophils. Genes involved in neutrophil transcription regulation and chemokine signalling were also highly upregulated in response to *S. aureus.* In response to PVL, 14/40 of the genes were also upregulated in this dataset (Figure 27D). The transcriptional regulation genes *EGR1, EGR3, FOSB, NR4A3* and *NR4A2* were upregulated in response to PVL. *NR4A3* was the only gene upregulated across all the datasets in Figure 4 and *FOSB, GLA, OSM, NR4A2, EGR3, EGR1, SRSF3, CD69* and *ETF1* were upregulated in ¾ of the datasets.

A)



B)



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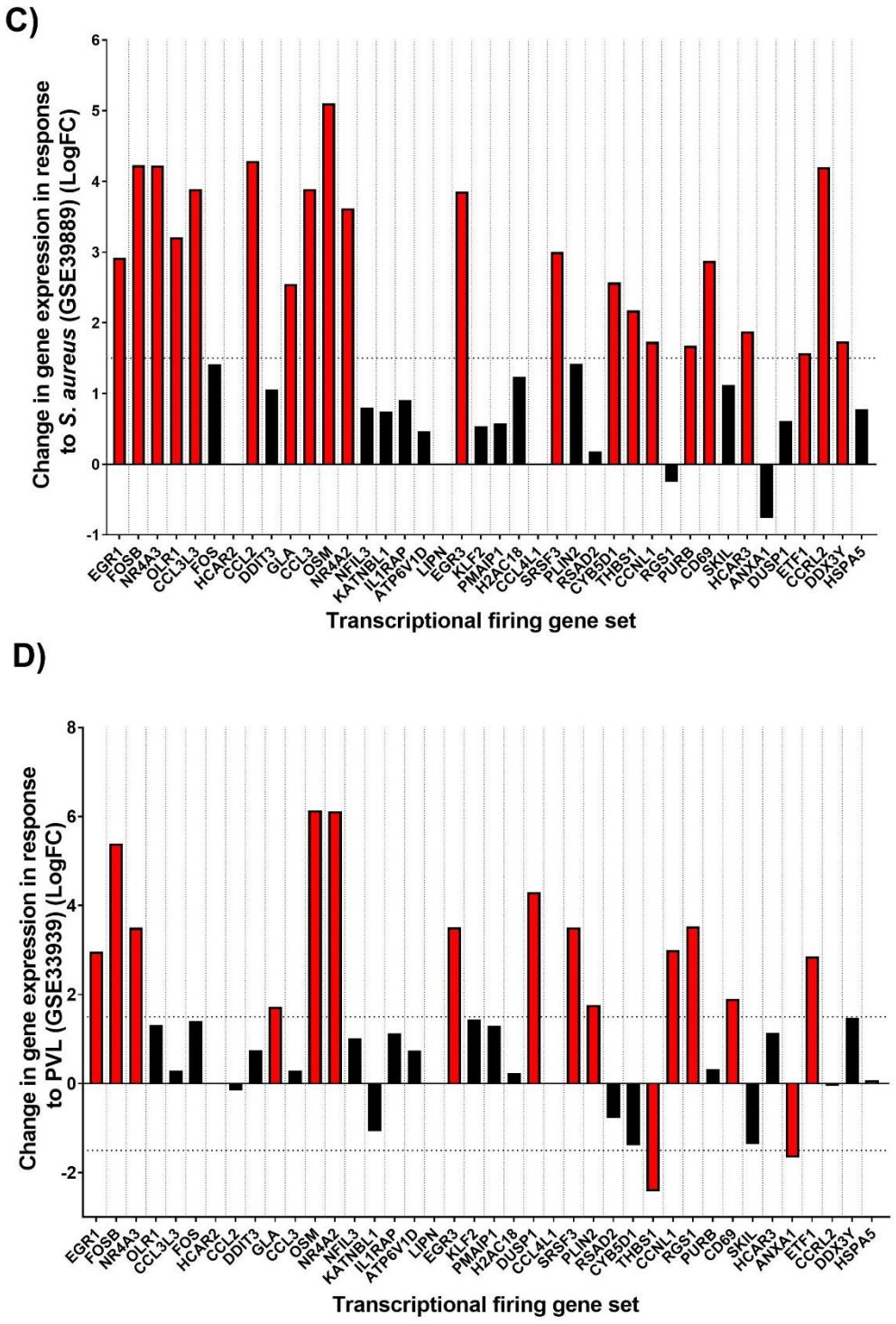
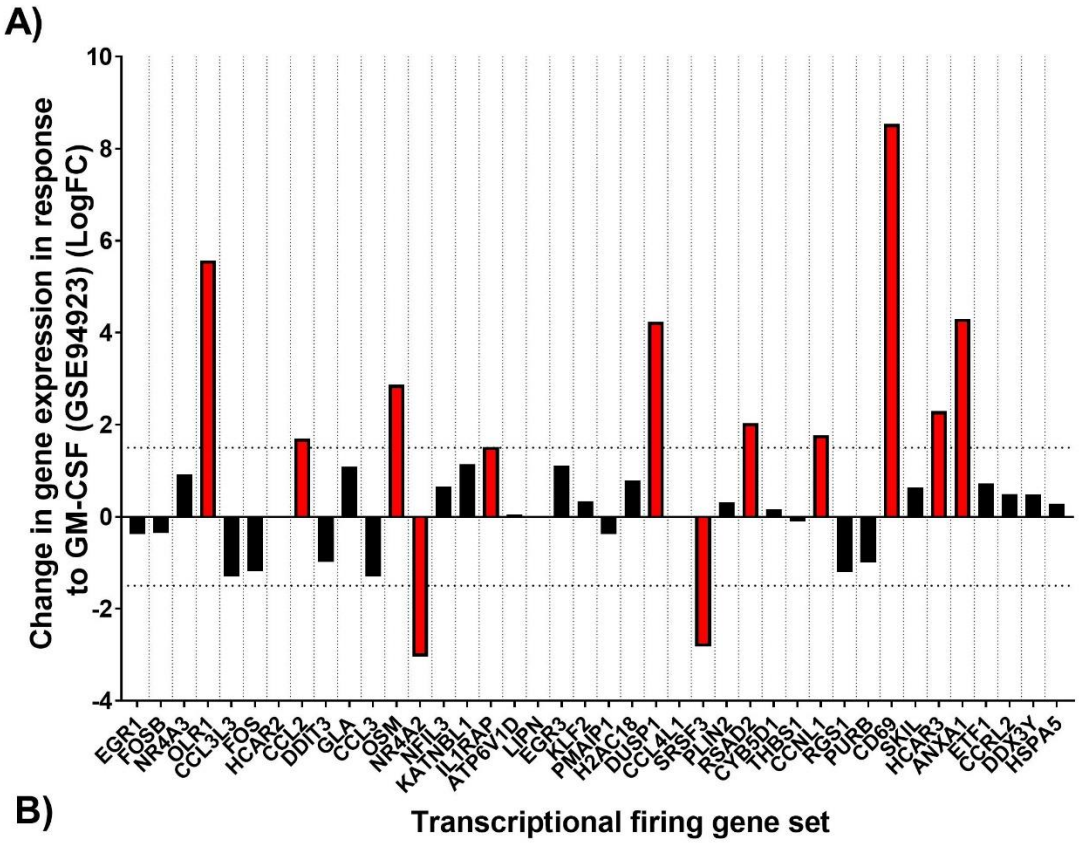


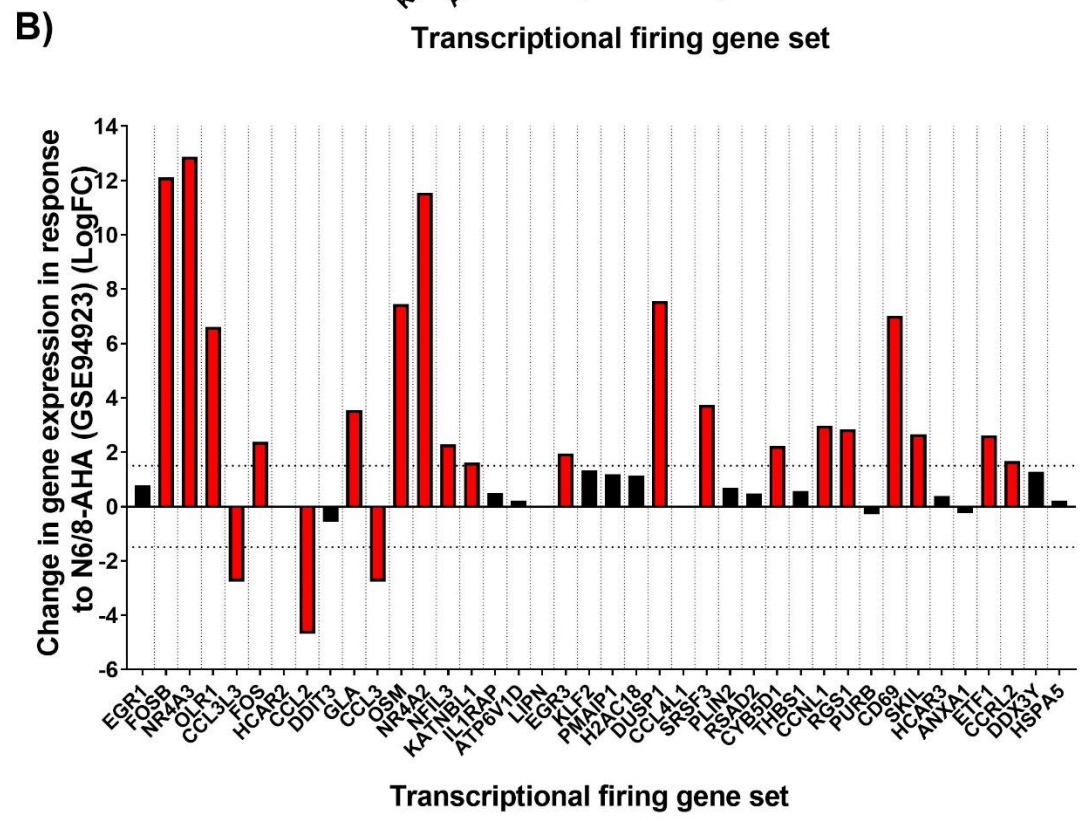
Figure 27-Transcription of the transcriptional firing gene set in neutrophils stimulated with bacteria and bacterial products.

The transcription of the transcriptional firing gene set was investigated in transcriptomic datasets which stimulated neutrophils with bacteria or bacterial products. Neutrophils were stimulated with either (A) *E. coli* (MOI 5) (3 hours), (B) LPS [1 µg/ml] (4 hours), (C) *S. aureus* (MOI 10) (2 hours) or (D) Panton-Valentine leucocidin (PVL) [1 nM] (3 hours). Differences in gene expression were compared to the unstimulated control used in the associated dataset. The Log fold change (LogFC) was plotted for each gene where available. Bars highlighted in red represent a LogFC of ≥1.5.

4.3.4 Interrogation of the transcriptional firing gene set using publicly available transcriptomic datasets using non-bacterial stimulants to activate neutrophils

Transcription of the transcriptional firing gene set was investigated in neutrophils stimulated with GM-CSF [100 U/mL], PKA agonist N6/8-AHA [1 mM], or hypoxia [3 kPa] for 4 hours (Prince et al., 2017). The aim of the study by Prince et al. (2017) was to investigate neutrophil transcription in response to pro-survival stimuli. Interestingly, in a different study when neutrophils were stimulated for a similar length of time, with 1 nM GM-CSF, which is lower than the concentration used by Prince et al. (2017), GM-CSF induced NETosis (Tatsiy and McDonald, 2018). GM-CSF can also prime neutrophils to undergo NETosis, when stimulating for a shorter length of time (Yousefi et al., 2009). Neutrophils stimulated with GM-CSF demonstrated upregulation in 10/40 of the genes investigated, with *CD69* showing the greatest increase (Figure 28A). *EGR1, EGR3, NR4A3*, *NR4A2* and *FOSB,* were not upregulated in response to GM-CSF. As a comparison to the other datasets investigated, transcription in response to neutrophil stimulants not known induce NETosis was investigated. The PKA agonist N6/8-AHA, which has no known role in NETosis, demonstrated upregulation of 19/40 of the genes in the transcriptional firing gene set (Figure 28B). *FOSB, GLA, SRSF3, NR4A3, NR4A2, CD69* and *ETF1* were upregulated, which was consistent with the gene transcription identified in datasets using NET inducing bacterial stimuli to activate neutrophils. Hypoxia prolongs neutrophil survival and is also not known to induce NETosis. Conflicting literature exists regarding whether NETosis is reduced in hypoxia (Lodge et al., 2020). Hypoxia did not show upregulation of any of the genes in the transcriptional firing gene set (Figure 28C).





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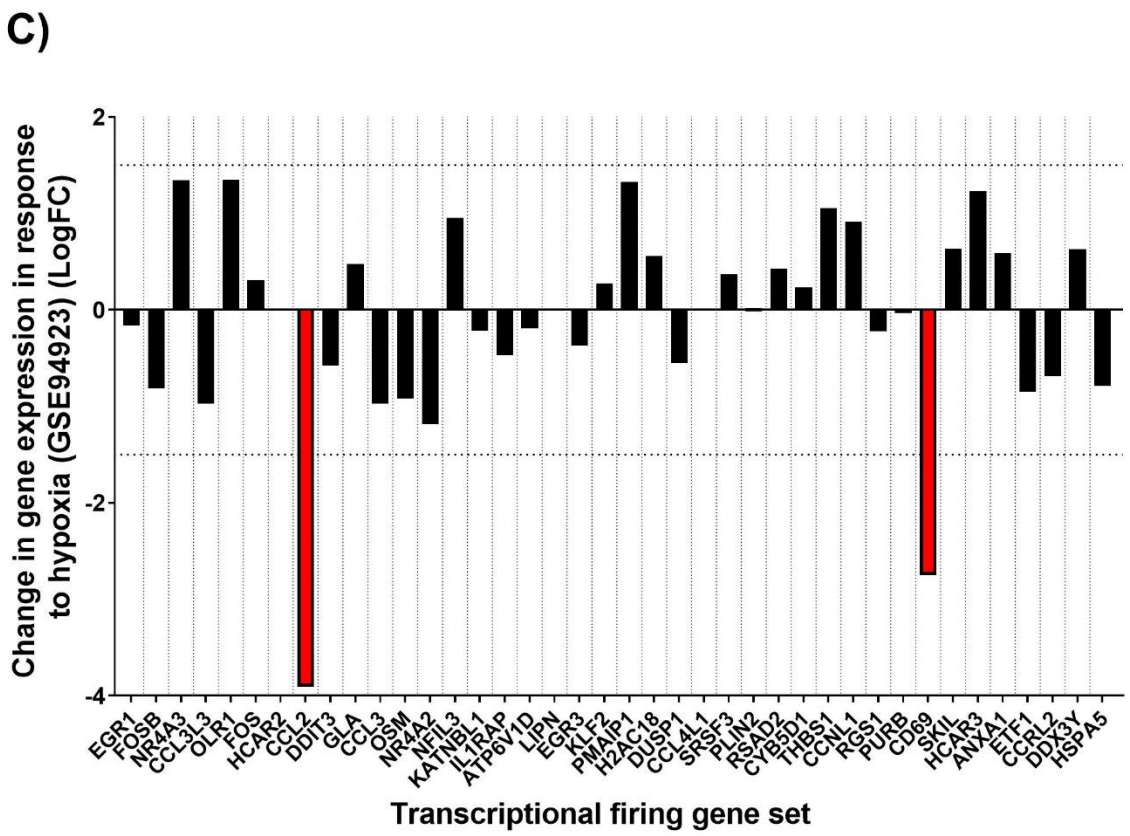


Figure 28-Transcription of the transcriptional firing gene set in neutrophils stimulated with pro-inflammatory agents.

The transcription of the genes in the transcriptional firing gene set were investigated in transcriptomic data sets which had stimulated neutrophils with pro-inflammatory agents. Neutrophils were stimulated with (A) GM-CSF [100 U/mL], (B) PKA agonist- NH6/8-AHA [1mM] or (C) hypoxia (3 kPa) for 4 hours. Differences in gene expression were compared to the unstimulated control used in the associated dataset The log fold change (LogFC) was plotted for each gene where available. Bars highlighted in red represent a ≥1.5 fold change.

4.3.5 Interrogation of the transcriptional firing gene set in a publicly available transcriptomic study of neutrophils from people with type 2 diabetes

Previous literature demonstrates there is a pro-NETotic phenotype in diabetes (Fadini et al., 2016; Wong et al., 2015), therefore whether the transcriptional firing gene set was more highly expressed in neutrophils isolated from people with T2D compared to healthy controls was explored (Figure 29) (Lin et al., 2020). In the Lin et al. (2020) study they investigated the neutrophil transcriptome in unstimulated peripheral blood neutrophils from patients with T2D (n=5) and age and sex-matched healthy controls. The study found that neutrophils from people with T2D had increased expression of pro-inflammatory genes associated with leukocyte activation, including genes for chemokine receptors (*CXCR1* & *CXCR2*) and adhesion molecules P and L-selectin (*SELP & SELL*). There was only 1 gene of the 40 examined that was upregulated (*CCL2*) in this dataset and nearly all others investigated

were downregulated in T2D, including *FOSB, NR4A3, OSM, OLR1* and chemokine-associated genes *CCL3* and *CCL4L1*. Over half the genes in the transcriptional firing gene set were not available to interrogate in this dataset, which are represented by blank spaces on the bar graph. There were no data on *EGR1* and *EGR3* gene expression, which were commonly upregulated across the other datasets already described.

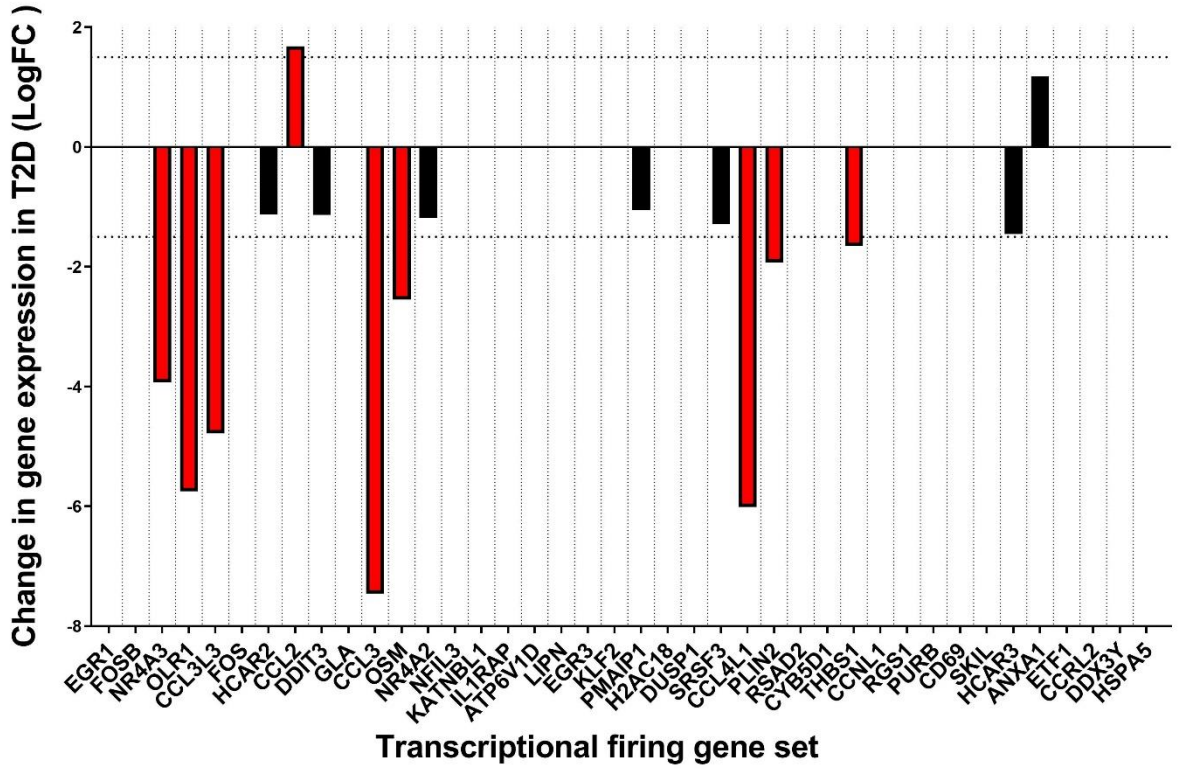


Figure 29-Transcription of the transcriptional firing gene set in neutrophils isolated from people with type 2 diabetes.

The transcription of the transcriptional firing gene set was investigated in unstimulated peripheral blood neutrophils from people with type 2 diabetes (T2D) compared to healthy controls. The Log fold change (LogFC) was plotted for each gene where available. Bars highlighted in red represent a ≥1.5 fold change.

4.4 Discussion

The rationale for this bioinformatics project was to examine the conflicting literature in the field regarding the requirement for gene transcription in NETosis. Khan and Palaniyar (2017) showed that transcription in NETosis occurred across multiple loci and was hypothesised to drive the chromatin decondensation necessary for NET formation. NETosis was shown to be inhibitable by the transcription inhibitor actinomycin D and this process was termed 'transcriptional firing' (Khan and Palaniyar, 2017). However, conflicting studies demonstrate that NETosis proceeds in the presence of transcription inhibitor actinomycin D, meaning transcription is not required for NETosis, which is widely accepted in the field (Kenny et al., 2017; Sollberger et al., 2016; Tatsiy and McDonald, 2018).

Despite the general understanding that transcription is not required for NETosis, this discrepancy in the literature has not been formally explored. To investigate whether there was a unique transcriptional fingerprint specific to NETosis, the top 40 genes upregulated in response to the prototypical NET inducers PMA and ionophore A23187 were identified, which was named the 'transcriptional firing gene set' in this thesis. The gene expression pattern of these genes in response to classical NETosis activators, other pro-inflammatory stimuli, and stimuli associated with regulation of neutrophil function but not NETosis was investigated.

4.4.1 Function of the genes identified in the transcriptional firing gene set

The function of the genes in the transcriptional firing gene set were not associated with the known mechanism of NETosis. These genes predominantly consisted of ubiquitous immediate-early genes, encoding transcription regulators and factors including *EGR1, EGR3, FOS, FOSB, NR4A3, NR4A2. EGR1* encodes for the early growth response protein 1 (EGR-1) transcription regulator, which has diverse roles. EGR-1 controls cell differentiation, proliferation and wound repair in a variety of cell types (Gashler and Sukhatme, 1995; Havis and Duprez, 2020; Thiel and Cibelli, 2002; Zhang et al., 2019). In neutrophils, EGR-1 is constitutively expressed and regulates expression of key pro-inflammatory genes including IL-1β and TGFβ-1 (Cullen et al., 2010). *EGR3* encodes for the ubiquitous transcription regulator, early growth response protein-3 (EGR-3). The role of EGR-3 has not been widely explored in neutrophils but it has shown to regulate IL-1β production, similarly to EGR-1 (Kenyon et al., 2017). The *FOS* and *FOSB* genes encode for transcription factor sub-units c-Fos and FosB. These transcription factor subunits form dimeric structures with Jun proteins to form the activator protein-1 (AP-1) transcription factor (Bahrami and Drabløs, 2016). AP-1 protein mediates gene transcription in multiple contexts including cancer, neuronal plasticity, and in cell apoptosis and survival (Bahrami and Drabløs, 2016). In neutrophils, AP-1 regulates nitric oxide production and IL-8 production (Filep et al., 2005; Ratajczak-Wrona et al., 2013). The Nuclear receptor 4A (NR4A) subfamily are part of the nuclear receptor superfamily of transcription regulators (Chen et al., 2020). *NR4A2* and *NR4A3* encode for two NR4A transcriptional regulator proteins. These proteins are ubiquitously involved in a range of cell processes throughout the body including in cancer, neurological and metabolic disease, autophagy, and inflammation (Chen et al., 2020). The roles of NR4A2 and NR4A3 have not been widely explored in neutrophils previously, but they did positively regulate neutrophil survival and development in limited previous research (Prince et al., 2017). The transcription of some of the genes identified could play a role in the mechanism of NETosis, such as the histone coding gene *H2AC18*, with histones playing a role in chromatin decondensation in NETosis (Wang et al., 2009). The *ANXA1* gene, which encodes the calcium-dependent phospholipid-binding protein annexin A1 (ANXA1), was upregulated in the transcriptional firing gene set. ANXA1 is anti-inflammatory mediator and proteomic studies

demonstrate that ANXA1 is present on extruded NETs. However, as NETosis occurs in the presence of translation inhibitor cycloheximide, the translation of any of the upregulated transcripts, were not required for NETosis (Khan and Palaniyar, 2017; Sollberger et al., 2016). The identification that the genes in the transcriptional firing gene set predominantly consisted of global transcriptional factors and regulators, which are involved in a wide range of biological and cellular processes, provides evidence to support the hypothesis that the genes upregulated in NETosis were not part of a specific NETosis transcriptional program and instead could represent a global inflammatory response.

4.4.2 Neutrophil expression of the transcriptional firing gene set in response to other pro-inflammatory stimuli

Once the functions of the genes in the transcriptional firing gene set were identified, I anticipated there would be some similarity in the upregulation of these genes in response to other pro-inflammatory stimuli, as many of the genes were global transcription regulators. An important finding in this project is that there was a high number of the genes in the transcriptional firing gene set that were also upregulated in *E. coli* treated neutrophils, when using experimental conditions known to stimulate NETosis. This was particularly important as this dataset was generated as part of the Denholtz at al. (2020 study), which explored neutrophil chromatin remodelling. They demonstrated that neutrophils remodel their chromatin structure, to specifically transcribe pro-inflammatory genes when activated. They used chromosome conformation capture (HiC) and demonstrated that the gene expression identified in response to *E. coli* or PMA was a result of chromatin remodelling, which occurred via the formation of chromatin loops linking gene enhancers to inflammatory gene promoters (Denholtz et al., 2020). This study could explain why there is gene transcription in neutrophils stimulated with NET-inducing agents. It is likely that gene transcription occurs in response to the pro-inflammatory stimuli used to induce NETs, which occurs independently and is not required for the NETosis mechanism. This could explain why most genes were global regulators of inflammation. It is possible that the decondensation of DNA in NETosis promotes gene transcription, rather than gene transcription being required for NETosis. In the transcriptional firing paper, Khan and Palaniyar (2017) found that the degree of chromatin decondensation was associated with a higher amount of gene transcription, which could support that transcription occurs secondary to, or is promoted by NETosis. Further data supporting that the transcriptional firing gene was not specific to NETosis was that many of the genes examined (19/40) were also upregulated in response to PKA agonist N6/8-AHA. PKA signalling is important in neutrophil chemotaxis and cell survival and PKA activation has been shown to inhibit NETosis induced by PMA previously (Prince et al., 2017; Shishikura et al., 2016). An interesting observation in this project was that the genes in the transcriptional firing gene set were mostly downregulated in unstimulated peripheral blood neutrophils isolated from people with T2D compared to healthy controls (Lin et al., 2020). Aberrant neutrophil function is

demonstrated in diabetes and global transcription regulators and chemokine genes were downregulated in diabetes. *NR4A3* was downregulated which is interesting in the context of diabetes as NR4A3 is involved in the regulation of fuel utilisation for the body and *NR4A3* knockdown mice were shown to develop glucose intolerance (Yang et al., 2020; Zhang et al., 2020). Whether *NR4A3* plays a role in neutrophil cell metabolism and if downregulation of NR4A3 affects the neutrophil phenotype in diabetes is not known and further exploration of the role of NR4A3 in neutrophils would be of value. The importance of *NR4A3* in the neutrophil response to inflammation is supported by the finding that it was repeatedly upregulated in response to a range pro-inflammatory of stimuli investigated in this study.

4.4.2.1 *Limitations and future work*

This project was designed to be an exploratory investigation of neutrophil gene expression in NETosis. A key limitation of this work is that definitive conclusions cannot be drawn from these data, however they have provided interesting discussion points regarding the necessity of transcription in NETosis. To confirm that the genes identified in the transcriptional firing gene set were not required for NETosis, future work could include the use of genetic knockdowns of the key genes identified, to establish if NETosis could still occur in mutants. These experiments could be conducted by using the gene editing technology, CRISPR/Cas9. In brief, this technology uses a Cas9 enzyme and a guide RNA to cause double stranded breaks in the target gene, with mutation prone DNA repair resulting in gene silencing (Ran et al., 2013). A zebrafish model would be used for these experiments, as zebrafish neutrophils undergo NETosis and a CRISPR/Cas9 technology specifically targeting neutrophil gene expression in zebrafish was developed (Isles et al., 2021; Wang et al., 2021). Specifically editing neutrophils would be an advantage as many of the genes in the transcriptional firing gene set were transcription regulators that are ubiquitously expressed, therefore genetic knockdowns may cause off-target defects in the zebrafish and be lethal.

4.4.3 Conclusion

To conclude, Khan and Palaniyar have argued that transcriptional firing without translation is a necessary pre-requisite for NETosis. The analysis here links the genes involved in this transcriptional firing to other stimuli that can also be involved in NETosis, but also shows that these genes are activated by stimuli that do not induce the NETosis response. Whilst it is possible that transcription helps activate chromatin decondensation and promotes NETosis, other studies have not supported a role for transcription in NETosis. The analysis here does not support a specific gene activation profile associated with NETosis, and overall, it seems most likely that transcriptional firing is not essential for NETosis but is a general part of neutrophil responses to the strong activation induced by stimuli such as PMA. I surmise that transcription occurs in response to pro-inflammatory stimuli independently of

NETosis and it is possible that the unwinding of DNA in NETosis may further drive gene transcription. Based on my findings targeting gene transcription would therefore not be a viable therapeutic strategy to inhibit NETosis.

5 Investigating NETosis in Hospitalised Patients with COVID-19

Emerging evidence suggests NET formation contributes to lung damage and thrombosis in COVID-19 (Middleton et al., 2020; Veras et al., 2020). NETs are also implicated in the development of ARDS, which is a severe complication of COVID-19 (Yang et al., 2021). Markers of NETs (cell-free DNA and DNA-myeloperoxidase complexes) are elevated in sera of COVID-19 patients and an increased neutrophil-lymphocyte (NLR) ratio is a marker of severe COVID-19 disease (Jimeno et al., 2021; Middleton et al., 2020; Zuo et al., 2020). Furthermore, SARS-CoV-2 directly induces NETosis *in vitro* and NETs are found at increased amounts in the lungs of deceased COVID-19 patients (Radermecker et al., 2020; Veras et al., 2020). This results chapter will detail how neutrophil function and NETosis was investigated in two independent patient studies, using neutrophils isolated from hospitalised COVID-19 patients. The rationale and design of each study will be explained in the relevant sections.

The hypothesis for the work in this chapter was: **'NETosis is increased in COVID-19 and represents a pathway that could be targeted to improve inflammation and lung damage in this disease'**

To address this hypothesis, the following aims were set:

1) Contribute to the national 'Superiority trial of protease inhibition in COVID-19' (STOP-COVID) clinical trial, to understand if inhibiting neutrophil serine proteases (NSPs), reduces NETs and their activity and improves clinical outcomes in COVID-19 patients.
2) Complete my own study investigating NETosis as part of the UK COVID Immune Consortium (UK-CIC), using neutrophils from hospitalised COVID-19 patients to explore if NETosis is increased in comparison to healthy controls and if this pathway can be modified using experimental inhibitors *in vitro*.

5.1 STOP-COVID clinical trial

5.1.1 Study background

STOP-COVID was a national phase III placebo-controlled clinical trial, led by the University of Dundee (UoD), investigating the selective dipeptidyl peptidase 1 (DPP1) (cathepsin C) inhibitor, brensocatib (INS1007) (NCT04817332). DPP-1 activates NSPs in maturing neutrophils in the bone marrow (Pham et al., 2004). NE is an NSP that plays an important role in inflammation and tissue damage in lung disorders including cystic fibrosis, bronchiectasis, and COPD (Voynow and Shinbashi, 2021). NE mediates chromatin decondensation and pore formation in NETosis and is also highly abundant on extruded NETs (Chen et al., 2020; Papayannopoulos et al., 2010; Urban et al., 2009). Those with a genetic mutation in the DPP-1 gene (*CTSC*) develop the rare disease, Papillon-Lefèvre syndrome (Sreeramulu et al., 2015). This syndrome is characterised by thickening of the skin (palmoplantar keratosis), increased susceptibility to infections and severe periodontitis, resulting in premature tooth

loss (Sreeramulu et al., 2015). Those with Papillon-Lefèvre syndrome generate less NETs *ex vivo*, providing further rationale to investigate NETosis after brensocatib treatment (Roberts et al., 2016). NE is also released from activated neutrophils via degranulation (Weiss, 1989). NE is associated with lung damage in COVID-19 and levels of NE are increased in patients (Akgun et al., 2020; Guéant et al., 2021; Ng et al., 2021). A previous large phase 2 clinical trial of brensocatib was conducted by the team at the UoD, in patients with non-cystic fibrosis bronchiectasis (Chalmers et al., 2020). Brensocatib treatment significantly reduced the activity of NE in the lung, the frequency of exacerbations and improved clinical outcomes in patients (Chalmers et al., 2020). There was no difference in severe adverse events between the trial groups (Chalmers et al., 2020). For the STOP-COVID clinical trial hospitalised COVID-19 patients were recruited and randomised to receive either brensocatib (25 mg) or placebo, once daily for 28 days. Healthy controls were not enrolled in this trial. In Sheffield, recruitment for the trial was led by Dr Roger Thompson and the clinical research facility staff at the Royal Hallamshire Hospital (RHH). Full details regarding patient recruitment methodology and ethics permissions are detailed in section 2.3.1.

5.1.2 STOP-COVID – assays of neutrophil function

Sheffield was the only satellite site conducting *in vitro* assays of neutrophil function for the STOP-COVID trial, and I worked with Rebecca Hull, a third year PhD student, to set up the assays in Sheffield for use in the study. Neutrophils were isolated from peripheral whole blood by negative magnetic selection. This method was chosen as it provided highly pure neutrophils and it used a rapid protocol that was suitable for high-throughput isolations of patient samples in the category 3 containment facility. Assays of NETosis, phagocytosis, and neutrophil cell surface marker expression were conducted on day 1 of treatment initiation, and day 29 when treatment ended. Assays of neutrophil function were completed on day 15 if the patients were still hospitalised, this was predominantly conducted by the team at the UoD and in Sheffield we focused on completing assays on day 1 and 29. Treatment groups were blinded. Raw data were analysed by the team at the UoD. I received permission from the principal investigator of the trial, Professor James Chalmers, to include the neutrophil function assay data generated at both the UoD and the UoS in this thesis. These assays were considered supportive to the clinical trial and were not listed as primary or secondary trial outcomes; therefore, power calculations were not conducted. The processed data for both sites was received from the UoD in a series of spreadsheets, which I combined and presented into GraphPad Prism and conducted the statistical analyses. In the following figures in this section data generated in Sheffield will be presented using purple symbols and data generated in Dundee will be represented by green symbols. Inter-observer comparisons were not conducted between sites, due to the logistical barriers of accessing the same samples. The trial enrolled 406 patients across 14 sites throughout the

UK and assays of neutrophil function were conducted on approximately 118 participants. In Sheffield, 17 patients were recruited, and I completed assays of neutrophil function on 17 sets of patient samples. Data in this chapter will present only the neutrophil function data generated for the trial. Information regarding the clinical efficacy of brensocatib is not included and this thesis will be embargoed until the trial data is published.

5.1.3 STOP-COVID results- *In vitro* NET formation

The NETosis assay was included in the analysis of patient neutrophils recruited to the trial as DPP-1 inhibits the activation of NE, which plays a role in chromatin decondensation and cell lysis in NETosis (Papayannopoulos et al., 2010; Sollberger et al., 2018). In this study two inducers of NETs were used, which were the prototypical NET inducer PMA [100 nM], and the more physiologically relevant NET stimulus, LPS [5 µg/ml]. SYTOX™ green was used to stain extracellular DNA and acted as a surrogate for NET formation in the assay. The fold change in SYTOX™ green fluorescence signal between unstimulated and stimulated neutrophils was used to calculate the NET response. The data were normalised in this way by the team at the UoD. There was no difference in NET generation in response to either LPS, after 1 or 4 hours of stimulation (Figure 30A-B) or in response to PMA after 4 hours of stimulation (Figure 30C), between COVID-19 patients who were receiving brensocatib or placebo. Also, there was no difference in NETosis between the two groups after any length of treatment (day 1, 15 or 29). The data generated in Sheffield (purple) displayed an even spread throughout the entire dataset (green) for nearly all conditions and timepoints analysed. However, the NET response appeared higher in the day 29 Sheffield samples for both brensocatib and placebo treated patients

when neutrophils were stimulated with LPS and read after 4 hours, compared to the data produced in Dundee (Figure 30B).

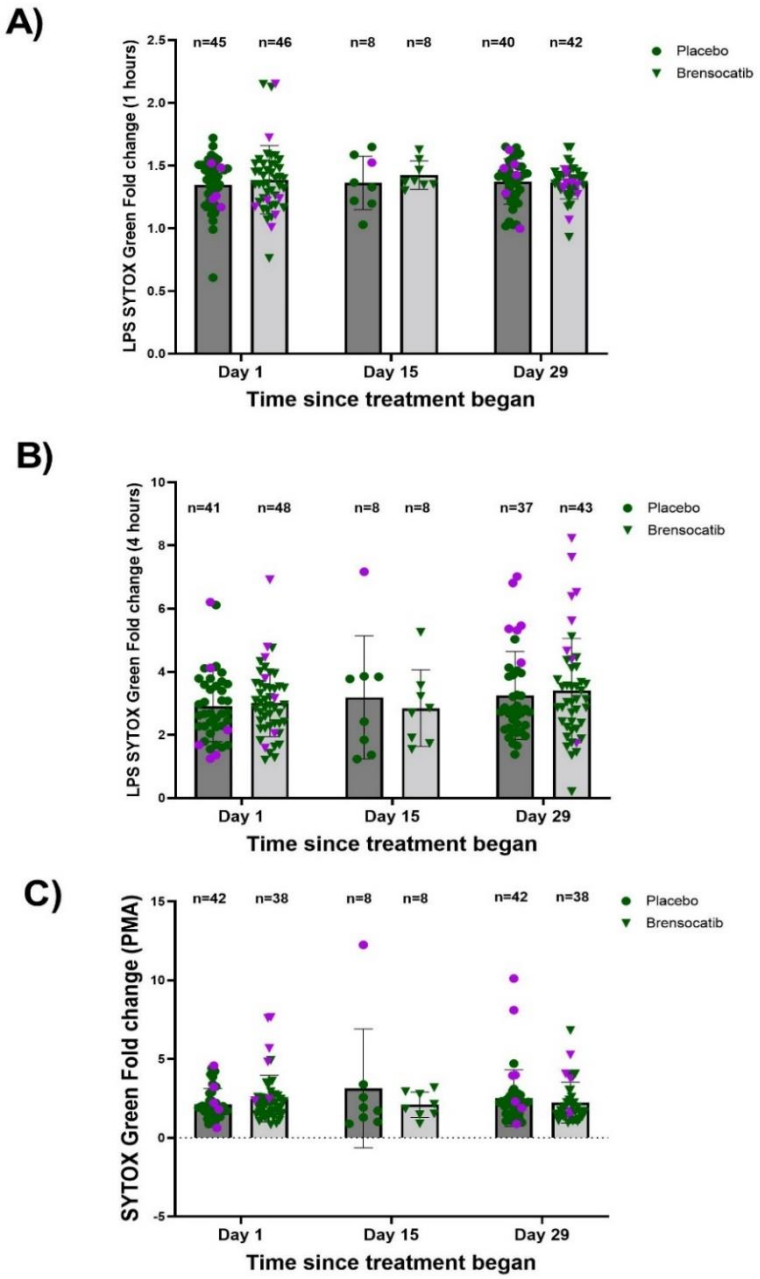


Figure 30-Brensocatib treatment does not inhibit NETosis.

Neutrophils were isolated from peripheral whole blood from hospitalised patients with COVID-19 admitted to Ninewells Hospital, Dundee (green symbols) or the Royal Hallamshire Hospital, Sheffield (purple symbols). Patients were randomised to receive brensocatib (25 mg daily) (triangle) or placebo (circle) and the study was blinded. Sampling was conducted on day 1, 15 and 29 of treatment. Neutrophils (5×10^4) were stimulated with either LPS [5 µg/ml] or PMA [100 nM] for 4 hours. SYTOX™ Green was added to all wells [555 nM] and extracellular DNA (NETs) was quantified using a fluorescent plate reader after 1 hour (A) and 4 hours (B) for LPS treated neutrophils and 4 hours for PMA-treated cells (C). Excitation/emission 490/537 nm was used. There was no significant difference in NETosis between patients receiving brensocatib or placebo during treatment and there was no difference in NETosis between the treatment days analysed. Fold change was calculated by quantifying the difference in raw SYTOX™ green values between unstimulated and stimulated neutrophils. Statistical analysis was by a mixed-effects model with a Tukey's post-test. The number of repeats is demonstrated on the graph for each condition. Error bars represent SD.

5.1.4 STOP-COVID results- Neutrophil phagocytosis of *E. coli*

It was important to determine if brensocatib was detrimental to neutrophil pathogen handling. Bacterial co-infection and secondary bacterial infections can occur in patients with COVID-19, and in one series were identified in 30.5% of COVID-19 patients admitted to critical care (Russell et al., 2021). Neutrophil phagocytosis of *E. coli* was investigated in this trial and previous research demonstrates that *E. coli* was identified in 20% of positive blood cultures from hospitalised COVID-19 patients (Russell et al., 2021). There was no significant difference in the amount of opsonised heat-killed *E. coli* phagocytosed by neutrophils between the two treatment groups, at day 1, 15 or 29 (Figure 31A). Interestingly, in patients receiving either placebo or brensocatib there was a significant increase in the percentage of neutrophils phagocytosing *E. coli* at day 29 of treatment compared to day 1 (Figure 31B). The data generated in Sheffield (purple) displayed an even spread throughout the entire dataset (green) for all conditions in this assay.

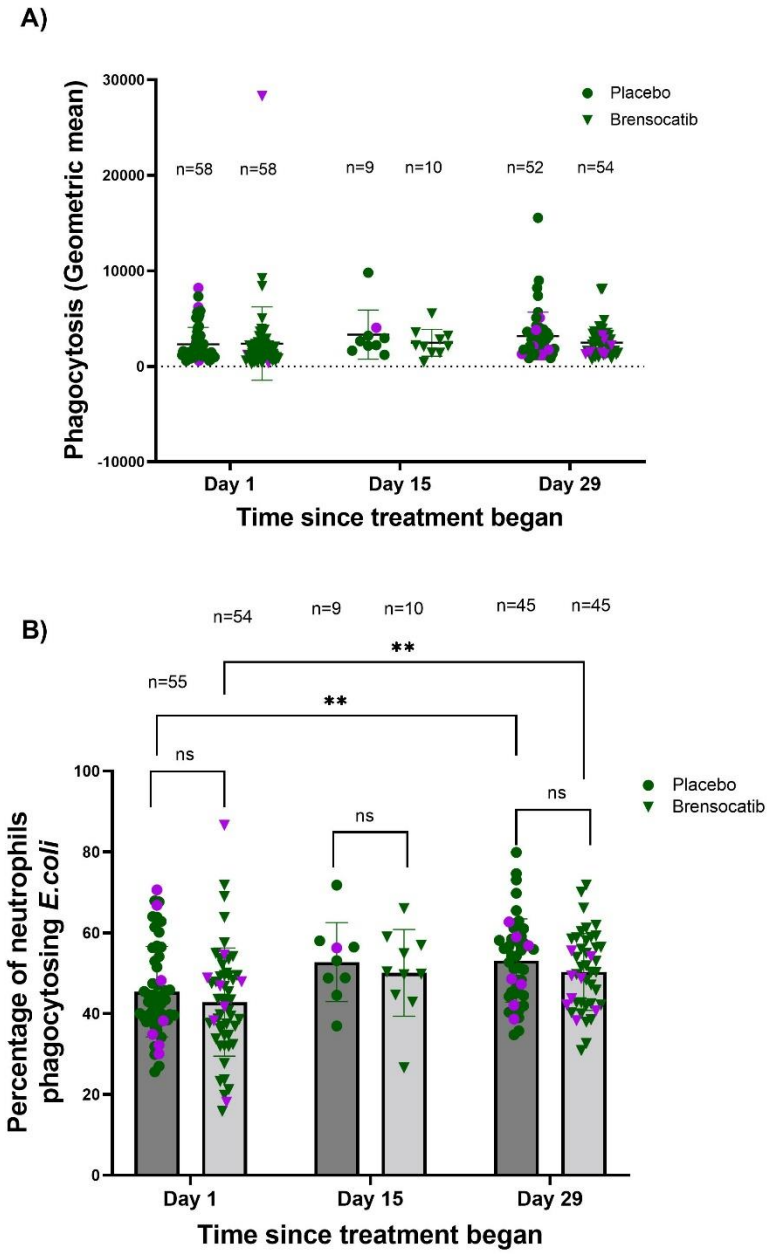


Figure 31-Brensocatib does not impact phagocytosis of *E. coli.*

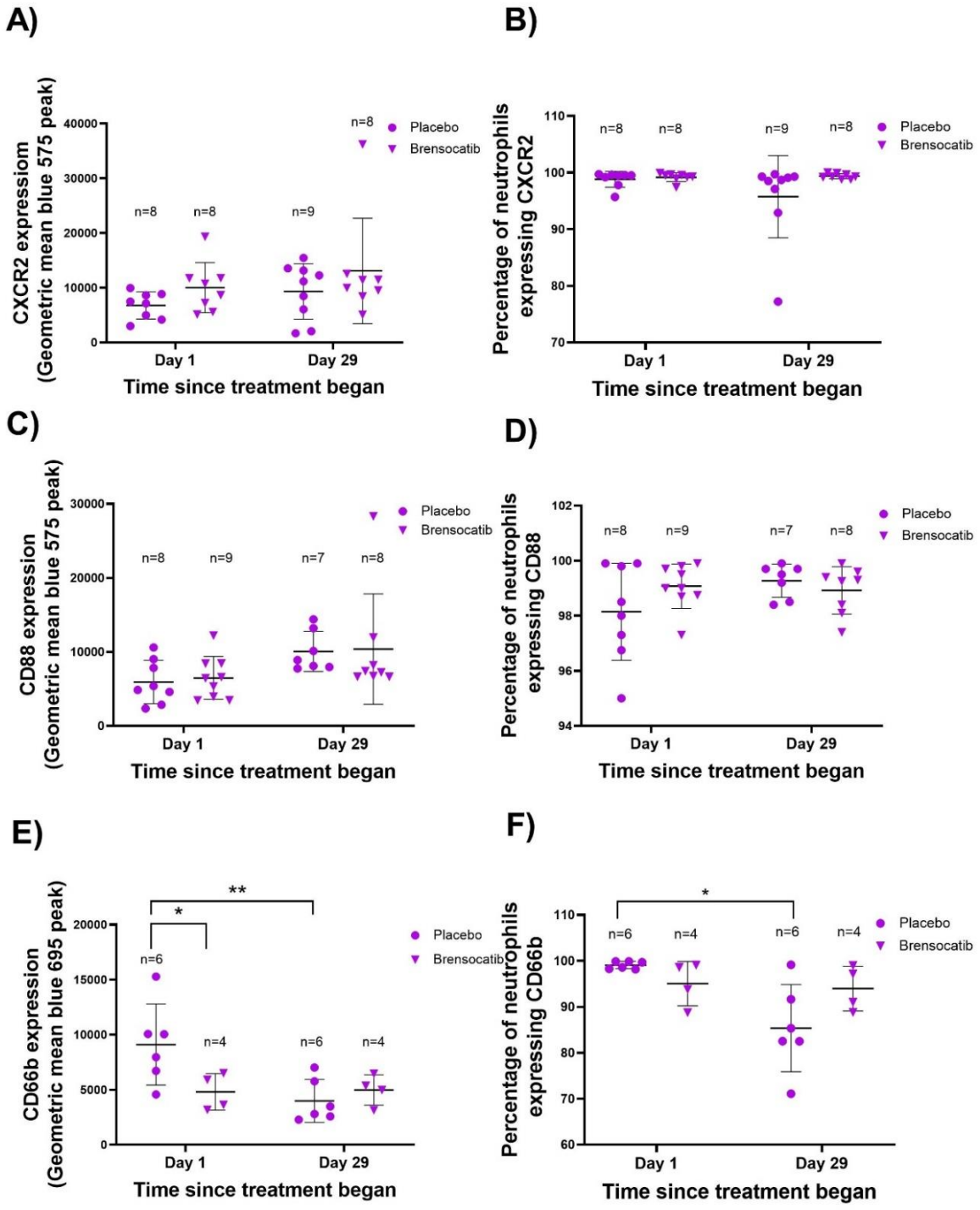
Neutrophils were isolated from peripheral whole blood from hospitalised patients with COVID-19 admitted to Ninewells Hospital, Dundee (green), or the Royal Hallamshire Hospital, Sheffield (purple). Patients were randomised to receive brensocatib (25 mg daily) (triangle) or placebo (circle) and the study was blinded. Sampling was conducted on day 1, 15 and 29 of the trial. Opsonised FITC-labelled heat-killed *E. coli* at an MOI of 10 were added to neutrophils (0.5×10^6) and incubated for 30 minutes. Cells were washed twice and fixed in 4% PFA for 90 minutes. Cells were washed again and resuspended in 500 µl 2% BSA. The BD™ LSRII flow cytometer was used to analyse the samples. The 488 nm argon blue laser and 530 nm filter were employed. Neutrophils were gated using forward and side scatter and 10,000 events were analysed per sample. Raw data files were analysed by the UoD, by gating the neutrophils and quantifying the geometric mean for the blue 530 nm peak (A) and the percentage of neutrophils positive for fluorescence (B). There was no effect of brensocatib treatment on neutrophil phagocytosis. There was a significant increase in the percentage of neutrophils phagocytosing bacteria at day 29 compared to day 1 in both the placebo and brensocatib treatment groups. The number of repeats is demonstrated on the graph. Statistical analysis was by a mixed-effect analysis and a Tukey's post-test when comparing timepoints and a Bonferroni's post-test when comparing treatment groups Significance asterisks represent **p< 0.01 and (ns) denotes not significant.

5.1.5 STOP-COVID results- Neutrophil cell surface marker expression

Neutrophil cell surface marker expression was explored to determine if brensocatib altered the neutrophil phenotype in COVID-19 patients and to determine the potential wider impacts of brensocatib on neutrophil function. Five key cell surface markers were explored, which were CD182 (CXCR2), CD11b, CD66b, CD63 and CD88. CXCR2 is an important chemokine receptor, which signals in response to cytokines including CXCL8, which is elevated in COVID-19 (Sabroe et al.,1997; Del Valle et al., 2020). CXCR2 mediates neutrophil recruitment, and CXCR2 signalling is associated with inflammation in diseases such as COPD and asthma (Pease and Sabroe, 2002; Stadtmann and Zarbock, 2012). Previously the team at the UoD conducted a clinical study investigating the effects of CXCR2 antagonist daniraxin on NETosis in patients with COPD (Keir et al., 2020). However, danirixin did not reduce NETs in the sputum of patients. Due to the role of neutrophils in COVID-19-associated ARDS (section 1.3), CXCR2 antagonists are suggested to be potential therapeutic strategies in COVID-19 (Koenig et al., 2020). CD11b, is a member of the β-integrin family of neutrophil adhesion proteins, which are located in secondary granules and are expressed on the cell surface when neutrophils are stimulated with chemotactic factors (Hughes et al., 1992). CD11b is used along with CD66b as neutrophil activation markers (Skubitz et al., 1996; Weirich et al., 1998). CD63 is a marker of degranulation of azurophil granules and was included to investigate if inactive NE impacted neutrophil degranulation (Kuijpers et al., 1991). Expression of the receptor for the complement protein C5a (CD88) was investigated. C5a is an anaphylatoxin and stimulates increased chemotaxis, ROS production, degranulation, and expression of adhesion molecules in neutrophils (Haynes et al., 2000; Denk et al., 2017; Wood et al., 2018). In a previous study elevated expression of CD88 was found on myeloid cells isolated from COVID-19 patients and the impacts of C5a are associated with lung damage and hyperinflammation in COVID-19 (Carvelli et al., 2020).

The raw flow cytometry data generated in Sheffield did not integrate with the data generated at the UoD. There were large differences in raw fluorescent values for labelled neutrophils generated between sites, which may be due to the different analysers used or the sub-set of patients studied, therefore only the data generated in Sheffield is presented. Flow cytometry was not conducted on day 15 samples in Sheffield. Firm conclusions cannot be drawn from the following results, as it represents only a small sub-set of the entire dataset. The geometric mean of the fluorescence peak, which indicates the degree of expression of the markers investigated and the percentage number of neutrophils positive for the markers are displayed. There was no significant difference in CXCR2 (Figure 32A-B), CD88 (Figure 32C-D), or CD11b (Figure 32G-H) expression in neutrophils isolated from patients receiving brensocatib or placebo at day 1 or day 29 of the trial. Patients receiving brensocatib expressed significantly less CD66b compared to the placebo group on day 1 of treatment, but there

was no difference between the groups at day 29 (Figure 32E-F). However, there was significantly less CD66b expression at day 29 for neutrophils from placebo treated patients. The percentage number of neutrophils expressing CD66b also decreased in the placebo group at day 29. A significant reduction in CD63 expression, analysed by both the geometric mean and % positive neutrophils, were shown in the placebo group between day 1 and day 29 (Figure 32I-J).



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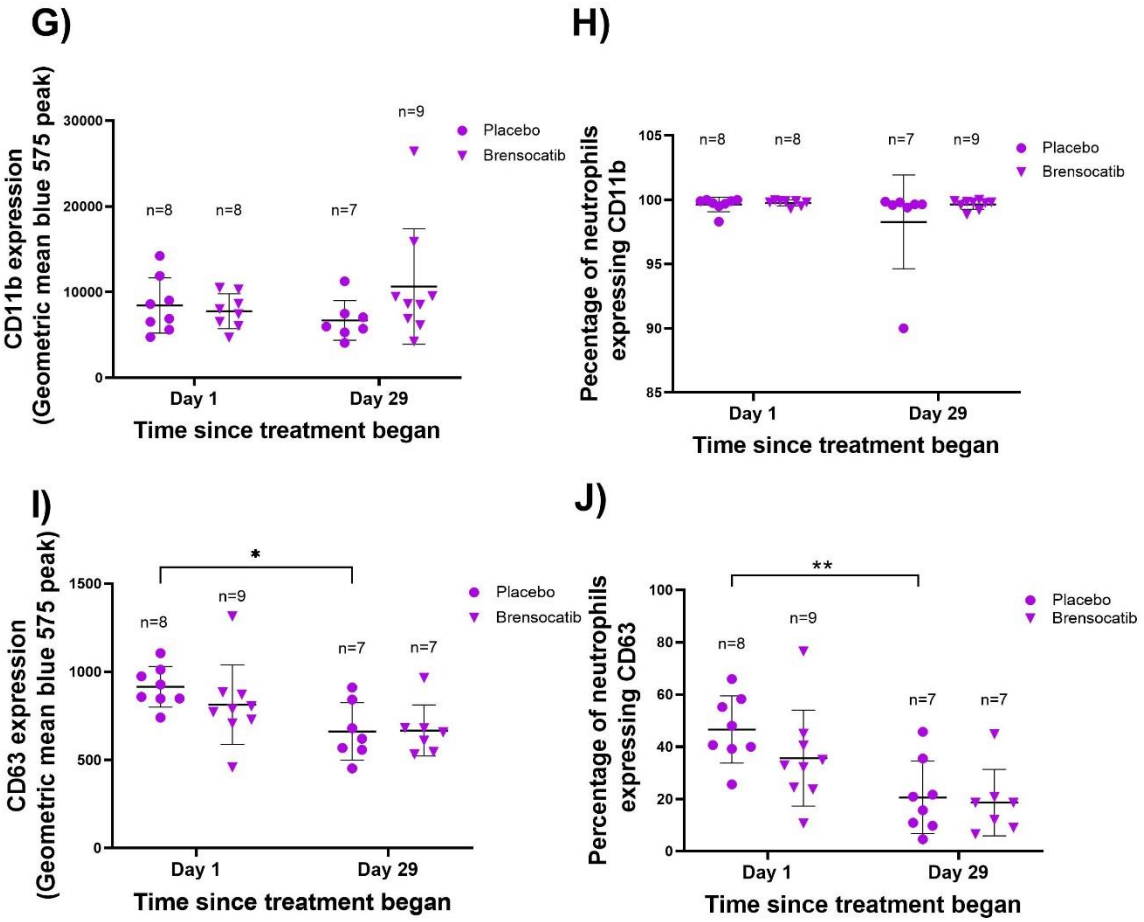


Figure 32-Impact of brensocatib on neutrophil surface marker expression.

Neutrophils were isolated from peripheral whole blood from hospitalised patients with COVID-19 admitted to the Royal Hallamshire Hospital, Sheffield. Patients were randomised to receive brensocatib (25 mg daily) (triangle) or placebo (circle) and the study was blinded. Sampling was conducted on day 1 and 29 of the trial. Neutrophils (0.5 x 10^6) were labelled with fluorescent antibodies for the following cell surface markers PE-CD182 (CXCR2) (A-B), PE-CD88 (C-D), PerCP-Cy5.5-CD66b (E-F), PE-CD11b (G-H) and PE-CD63 (I-J). The BD™ LSRII flow cytometer and the FLOWJO analysis software were used. The 488 nm argon blue laser and the 575 nm or 695 nm (E-F) filter were employed. Ten thousand neutrophils were analysed per sample. The geometric mean and the % of neutrophils positive for the cell surface marker was reported. Using the geometric mean there was significant decrease in CD66b expression in the brensocatib treatment compared to placebo group at day 1 of treatment and between day 1 and day 29 in the placebo group. There was a significant decrease in the percentage of neutrophils expressing CD66b between day 1 and day 29 in the placebo group. There was a significant decrease in the geometric mean and the percentage of neutrophils expressing CD63 at day 1 and day 29 in the placebo group. The number of patient samples analysed is displayed above each bar. Statistical analysis was by a mixed-effect model with a Bonferroni's post-test. Statistical analysis was conducted to compare cell surface marker expression between day 1 and day 29 and between placebo and brensocatob at each timepoint. Significance asterisks represent *<0.05 & **<0.01.

5.2 UK-CIC Study- Investigating NETosis in hospitalised patients with COVID-19 and how this can be therapeutically modified *in vitro*

5.2.1 Rationale and design of the UK-CIC study

Neutrophils and NETs contribute to lung damage and thrombosis in COVID-19, which can lead to the development of ARDS (section 1.3.2.3) (Arcanjo et al., 2020; Borges et al., 2020). Despite a highly effective vaccine programme in the UK, hospitalisation and deaths from COVID-19 occur. There is an unmet need for better treatments for COVID-19 to support the vaccine programme, provide treatment for those who get severe disease despite vaccines or who have not been vaccinated, and to provide additional therapies when SARS-Cov-2 variants emerge which escape current vaccines. This project was adapted to investigate NETosis in COVID-19 and how we could modulate this pathway *in vitro*. This was important to investigate to provide important understanding to the field of immunopathology in COVID-19 and to identify potentially new therapeutic strategies to target NETosis in this disease. Some of the work in the chapter is written as the form of a manuscript, which has been accepted at the ERJ Open research.

To complete this study, hospitalised patients with COVID-19, who were not part of the STOP-COVID clinical trial, were recruited by Dr Joby Cole and the clinical research facility staff at the RHH, within 1-3 days of hospital admission. The ethical permissions allowing this work and further details of the recruitment procedure are explained in Methods section 2.3.2.1. Between 5-10 ml of blood was collected from each patient, which was part of a wider blood draw for other research activities conducted by the wider Sheffield UK-CIC team. Samples from 39 COVID-19 patients, who were admitted to the RHH, Sheffield, between November 2020 and June 2021 were analysed. Experiments were repeated on a small number (n=7) of patients at a follow up time point 3-4 months post-acute sampling. This was conducted to explore whether there were any longer-term changes in the NET phenotype in recovered COVID-19 patients. Anonymised clinical data was collected for all patients. NETosis was also investigated in 9 healthy controls. The healthy controls were predominantly young colleagues within the department as many people were working remotely at this time. To make better comparisons to COVID-19 patients' wider recruitment of healthy controls, both in number and the recruitment of controls with advanced age and underlying co-morbidities would have been of value in this study. However, this was not possible due to the coronavirus lockdown restrictions, which were in place throughout much of the time when conducting this study. The vaccination status of the participants in the trial was not collected, however due to the timespan of the patient recruitment (November 2020-June 2021), it is unlikely that many patients were fully vaccinated against SARS-CoV-2. Linking with the previous PhD work, it was investigated if those with diabetes and COVID-19 produced more NETs than those with COVID-19 without this co-morbidity. This was especially relevant given the evidence for increased NET formation occurring in infections in people with diabetes, and

evidence of diabetes as a major predictor of worse outcomes in COVID-19 infection (Menegazzo et al., 2015; Wong et al., 2015; Wu et al., 2020).

To investigate the hypothesis stated in section 5, detailed aims of this study were as follows:

1) Compare the NET response in neutrophils from COVID-19 patients and healthy controls *in vitro.*
2) Explore whether NETosis could be inhibited *in vitro* using experimental inhibitors.
3) Investigate the NET response in COVID-19 patients over time by analysing samples from patients at the acute stage of infection and at follow up.
4) Determine if those with diabetes and COVID-19 produce more NETs than those without diabetes.

5.2.2 Design and optimisation of the NETosis assay used in the UK-CIC study

To investigate NETosis, neutrophils were isolated from peripheral whole blood using negative magnetic selection and NET formation was investigated in response to either LPS [5 µg/ml] or PMA [100 nM]. The incubation time of neutrophils with the NET stimuli were modified for this study. In the STOP-COVID clinical trial, readings were taken after 1 and 4 hours of incubation. In this study NETosis was analysed after a 3 hour incubation with PMA or LPS. This was because in the STOP-COVID trial, there were only low levels of NETs generated in response to LPS after 1 hour. Also, a 1-hour pre-incubation step in these experiments was included to investigate NETosis inhibitors.

5.2.2.1 *Choosing potential inhibitors of NET formation to test in the study*

Preceding and in parallel with my work, other studies have also investigated targeting NETosis in COVID-19, which are detailed in section 1.3.2.4. To advance the field potential inhibitors of NETosis were tested *in vitro*, which had not been explored in the context of NETosis in COVID-19 previously. The inhibitory effects of dexamethasone, cl-amidine and ruboxistaurin were explored and the rationale for these decisions are explained in the sections below.

5.2.2.1.1 Dexamethasone

Dexamethasone was the first therapy demonstrated to reduce COVID-19-associated mortality and was licensed for use in treating hospitalised COVID-19 patients who required supplemental oxygen in September 2020 (The RECOVERY Collaborative, 2020). Dexamethasone is a potent anti-inflammatory steroid that can affect neutrophil function. Dexamethasone reduced neutrophil recruitment, ROS generation, and pro-inflammatory cytokine production (IL-8, IL-6, and IL-1β) previously (Dandona et al., 1999; Langereis et al., 2011; Mianji et al., 1996; Zentay et al., 1999). Also, steroids can prolong neutrophil survival and increase circulating numbers of white blood cells (Liles et al., 1995; Mishler and Emerson, 1977; Nakagawa et al., 1998). However, the impacts of dexamethasone on NETosis have

been variable between studies. Dexamethasone reduced NETosis in response to *S. aureus* using human neutrophils *in vitro* (Wan et al., 2017), however this contrasts with opposing literature that found dexamethasone was without effect on PMA and TNF-α induced NETosis *in vitro* (Lapponi et al., 2013). Dexamethasone also reduced NETosis in a murine model of fungal keratitis (Fan et al., 2020). The effects of dexamethasone on NETosis in COVID-19 has not been explored and this research aimed to address this gap in the literature.

5.2.2.1.2 Ruboxistaurin

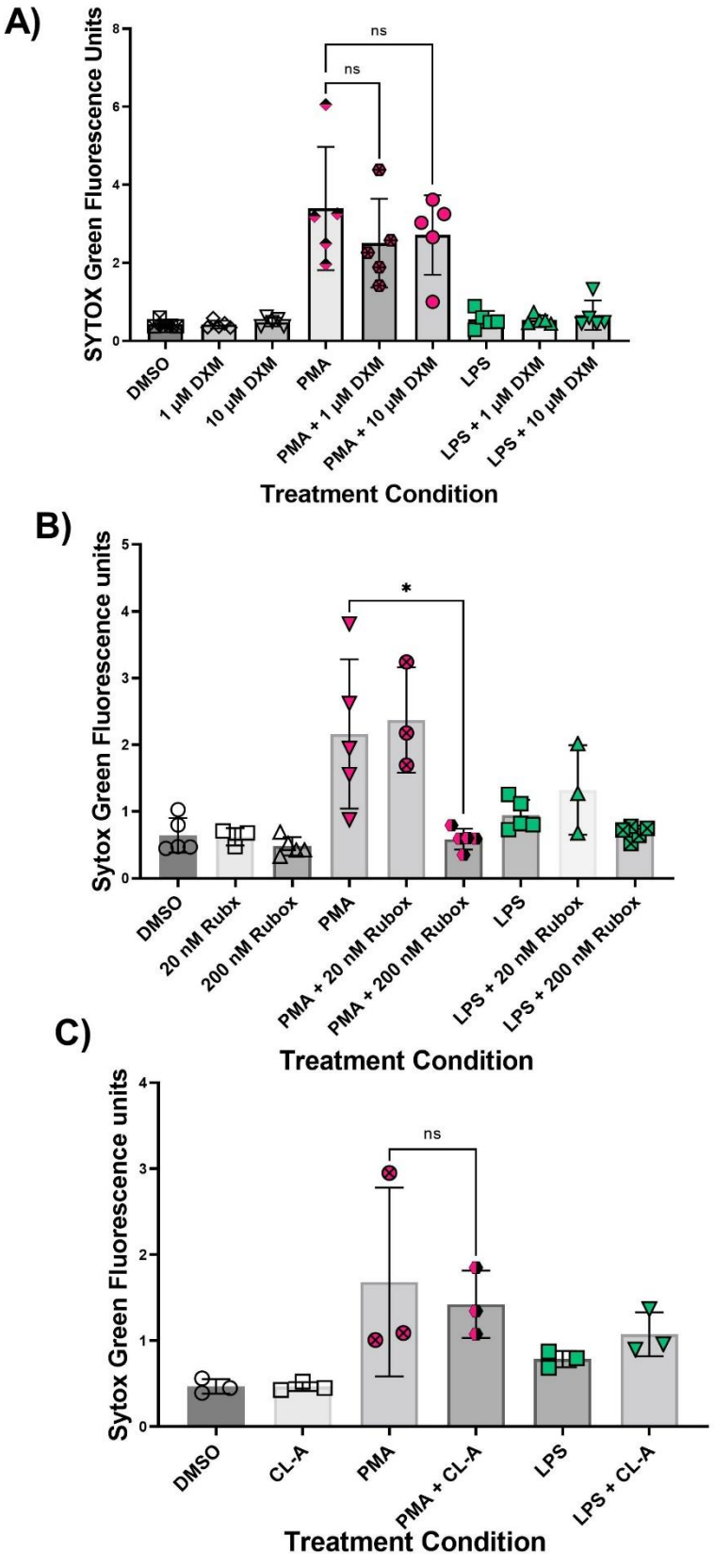
Ruboxistaurin, a specific PKC-β inhibitor, was another inhibitor investigated in the study. PKC-β is an isoform of the PKC family, which are a group of key enzymes that phosphorylate proteins at serine/threonine residues (Cosentino-Gomes et al., 2012). They are ubiquitous signalling enzymes present throughout the body (Cosentino-Gomes et al., 2012). A key role of PKC in neutrophils is the activation of the $p47^{phox}$ subunit of the NADPH oxidase complex, which is required for ROS generation (Cosentino-Gomes et al., 2012; Fontayne et al., 2002). I discovered the pre-existing literature on ruboxistaurin whilst exploring neutrophil function in DFD for the previous patient study. Ruboxistaurin was explored in the treatment of vascular complications of diabetes including retinopathy, neuropathy and cardiovascular sequalae (Aiello, 2005; Bansal et al., 2013; Connelly et al., 2009). Importantly, ruboxistaurin was demonstrated to be well tolerated in patients with diabetes in multiple previous clinical trials (phases 1-3) (Aiello et al., 2006; Casellini et al., 2007; PKC-DRS Study Group, 2005). However, a lack of efficacy in the treatment of diabetes complications prevented the development of ruboxistaurin for clinical use (Javey et al., 2010). In the context of neutrophil function, Gray et al. (2013) were the first to demonstrate that PKC, and specifically the PKC- β isoform, were critical in mediating ROS-dependent NETosis (Gray et al., 2013). Furthermore, inhibiting PKC-β using ruboxistaurin prevented ROS-dependent NETosis *in vitro* (Gray et al., 2013). Subsequently, Das et al. (2018) showed that administration of ruboxistaurin in a diabetic murine model of wound healing reduced NET formation and stimulated angiogenesis *in vivo* (Das et al., 2018). Based on the clinical safety of ruboxistaurin and the efficacy of ruboxistaurin to inhibit NETosis in healthy neutrophils this inhibitor was used in the study.

5.2.2.1.3 Cl-amidine

Cl-amidine is an experimental pan-peptidylarginine deiminase inhibitor. Peptidylarginine deiminase 4 (PAD4) citrullinates histones, aiding chromatin decondensation in NETosis (Leshner et al., 2012; Li et al., 2010; Wang et al., 2009). Cl-amidine previously reduced PMA induced NET formation *in vitro* and lowered inflammation and organ damage in rodent models challenged with LPS (Kusunoki et al., 2016; Siddiqui et al., 2021; Wang et al., 2020). Also, cl-amidine inhibited SARS-CoV-2-induced NET formation *in vitro,* supporting its potential usefulness in COVID-19 (Veras et al., 2020).

5.2.2.1.4 Optimising the concentration of experimental inhibitors of NETosis to use in the UK-CIC study

Using healthy donor neutrophils, the concentrations of dexamethasone, cl-amidine and ruboxistaurin used to inhibit NETosis were optimised, prior to conducting the patient study. The selection of the concentrations of inhibitors was based on previous *in vitro* studies which successfully used these compounds to reduce NETosis (Gray et al., 2013; Veras et al., 2020; Wan et al., 2017). LPS induced only a small amount of NETosis in neutrophils isolated from healthy donors, therefore the effects of the inhibitors on LPS-induced NETosis could not reliably be explored (Figure 33A-C). None of the inhibitors increased the background SYTOX™ green values in unstimulated neutrophils. For PMA-stimulated neutrophils, neither dexamethasone nor cl-amidine modulated NETosis (Figure 33A & C). However, there was a significant reduction in PMA-induced NETosis in response to 200 nM of ruboxistaurin, but not by 20 nM ruboxistaurin (Figure 33B). These findings were supported by fixing the cells and imaging the wells of the 96- well plates after completing the SYTOX™ green assay (Figure 33D). There were visibly less NET-like strands of DNA and cell death in cell treated with PMA and 200 nM ruboxistaurin compared to PMA alone. Cells treated with 20 nM ruboxistaurin had a similar morphology to the cells treated with PMA only. NET-like structures are highlighted with white arrows. The highest concentration of dexamethasone (10 µM) was selected for use in the patient study, as despite not having efficacy in healthy control neutrophils, there could be a different effect when using patient neutrophils.



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D)

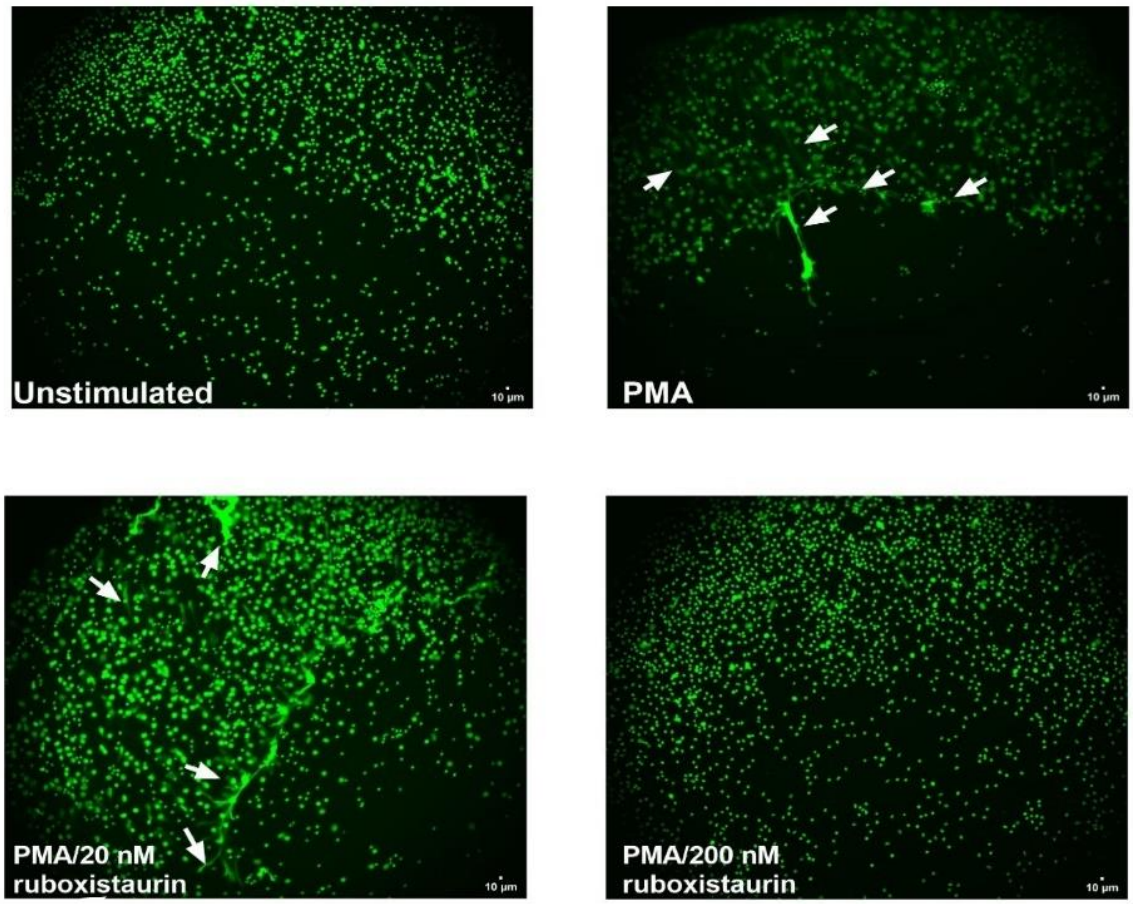

Figure 33-Testing potential NETosis inhibitors on neutrophils from healthy donors.

Neutrophils (5×10^4) isolated from healthy donors were pre-incubated with dexamethasone (DXM) [1 µM & 10 µM], ruboxistaurin (Rubox) [20 nM & 200 nM] or Cl-amidine (CL-A) [200 µM] for 1 hour. Neutrophils were stimulated with either PMA [100 nM] or LPS [5 µg/ml] for a further 3 hours. SYTOX™ Green was added to all wells [555 nM] and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Excitation/emission 490/537 nm was used. The median values of quadruplicate wells are shown. A) DXM was without effect on NETosis. B) There was a significant reduction in NETosis in PMA-stimulated neutrophils treated with 200 nM ruboxistaurin. C) Cl-amidine was without effect on NETosis. D) After analysis, 96-well plates were fixed with 4% PFA for 15 minutes and imaged using the NIKON Widefield fluorescence microscope. The 10x objective lens and FITC filter set (excitation/emission 470/525 nm) was used. Images were processed using the FIJI image analysis software. Unstimulated neutrophils demonstrated a small round structure. Visibly less NET-like strands of extracellular DNA and cell death were visible in the presence of 200 nM ruboxistaurin for PMA-stimulated neutrophils compared to PMA-stimulated neutrophils. White arrows indicate NETs. Data demonstrates n=3-5. Statistical analysis was by a one-way ANOVA with a Bonferroni's post-test and compared PMA with PMA + treatment. Asterisks represent *$p<0.05$ and (ns) denotes not significant. Error bars display SD. Scale bar represents 10 µm.

5.2.3 Manuscript demonstrating the key findings from the UK-CIC study of NETosis in COVID-19 patients- 'Enhanced neutrophil extracellular trap formation in COVID-19 is inhibited by the PKC inhibitor ruboxistaurin'

The research conducted as part of the UK-CIC consortium, exploring NETosis in hospitalised patients with COVID-19 was accepted for publication at ERJ Open research. The ERJ Open research is a fully open access journal, and this article is presented under the creative commons attribution license. In the manuscript presented in section 5.2.3.2, the ethics and methods section were removed where information is already presented in the materials and methods of this thesis.

5.2.3.1 *Statement of author contributions*

I designed and conducted all the neutrophil isolations and NETosis experiments presented in the manuscript, with guidance and support from my PhD supervisors- Lynne Prince and Ian Sabroe. I analysed all the raw data and presented it graphically and conducted all statistical analysis. Lynne and I wrote the manuscript together and all authors edited and revised drafts. Joby Cole recruited and consented patients, with the ethics and governance provided by Allan Lawrie. Anonymised clinical data was collected by Joby Cole, Chenghao Huang and Jacob Whatmore. Access to patient samples, intellectual guidance and support was provided by leading members of the UK-CIC consortium Alison Condliffe, Endre Kiss-Toth and Roger Thompson. Intellectual input on the use of ruboxistaurin, which was previously used in clinical trials for diabetes, was provided by our clinical collaborator Ahmed Iqbal. Joanne McKenzie, Kirsty Bradley and Rebecca Hull conducted the epithelial cell damage assays and cell viability analysis and Rebecca Hull also conducted killing assays. I designed the epithelial cell damage assays and analysed the raw data.

5.2.3.2 *Manuscript*

Title: Enhanced neutrophil extracellular trap formation in COVID-19 is inhibited by the PKC inhibitor ruboxistaurin.

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Keywords: Neutrophil, COVID-19, NETosis, Ruboxistaurin

Abstract

Background. Neutrophil extracellular traps (NETs) are web-like DNA and protein lattices which are expelled by neutrophils to trap and kill pathogens, but which cause significant damage to the host tissue. NETs have emerged as critical mediators of lung damage, inflammation and thrombosis in COVID-19 and other diseases, but there are no therapeutics to prevent or reduce NETs that are available to patients.

Methods. Neutrophils were isolated from healthy volunteers (n=9) and hospitalised patients with COVID-19 at the acute stage (n=39) and again at 3-4 months post-acute sampling (n=7). NETosis was measured by SYTOX green assays.

Results. Here, we show that neutrophils isolated from hospitalised patients with COVID-19 produce significantly more NETs in response to LPS compared to cells from healthy control subjects. A subset of patients were captured at follow-up clinics (3-4 month post-acute sampling) and while LPS-induced NET formation is significantly lower at this time point, it remains elevated compared to healthy controls. LPS- and PMA-induced NETs were significantly inhibited by the protein kinase C (PKC) inhibitor ruboxistaurin. Ruboxistaurin-mediated inhibition of NETs in healthy neutrophils reduces NET-induced epithelial cell death.

Conclusion. Our findings suggest ruboxistaurin could reduce proinflammatory and tissue-damaging consequences of neutrophils during disease, and since it has completed phase III trials for other indications without safety concerns, it is a promising and novel therapeutic strategy for COVID-19.

Introduction

Excessive inflammation is characteristic of severe COVID-19 disease. Neutrophils are recruited to the lungs in response to SARS-CoV-2 infection and are a principal cause of tissue damage and ongoing inflammation (Chiang et al., 2020). Neutrophil activation at the alveolar space is thought to contribute to the development of acute respiratory distress syndrome (ARDS) in COVID-19 as well in other lung infections (Zhu et al., 2018; Potey et al., 2019; Narasaraju et al., 2020). Here, neutrophils perform antimicrobial effector functions including production of reactive oxygen species (ROS), degranulation of cytotoxic proteins and release of NETs via NETosis. NETs are extracellular DNA lattices coated in histones and antimicrobial proteins including cathepsins and myeloperoxidase (MPO). NETs are antimicrobial, but they also cause significant host tissue damage and exacerbate inflammation in multiple acute and chronic diseases, including those of the lung (Uddin et al., 2019). NET production is increased during COVID-19, with NETs identified in plasma, lung autopsy samples from deceased patients with COVID-19 and in bronchoalveolar lavage fluid (BALF) (Middleton et al., 2020; Veras et al., 2020; Borella et al., 2021; Huckriede et al., 2021; Ng et al., 2021; Ouwendijk et al., 2021). Furthermore, SARS-CoV-2 directly induces NETosis *in vitro,* via a ROS-dependent mechanism, and circulating markers of NETosis (including cell free DNA and NE) are associated with increased COVID-19 severity (Arcanjo et al. 2020; Middelton et al., 2020; Zuo et al., 2020 Ng et al., 2021). NETs are highly pro-thrombotic *in vivo*, aggregating with platelets and the activated endothelium in COVID-19 to form microthrombi, which occlude the vasculature and further perpetuate inflammation (Middleton et al., 2020) Furthermore, SARS-CoV-2 induced NETs induce epithelial cell death, driving the catastrophic damage to the airway epithelium that is associated with severe disease (Veras et al., 2020). This growing evidence indicates that inhibiting NET formation is an important and viable therapeutic strategy. Here we show for the first time that NETs are elevated in response to LPS from neutrophils isolated from hospitalised patients with COVID-19 and that the orally active PKC inhibitor, ruboxistaurin (LY-333531), is a potent inhibitor of NETosis in this cohort. Since ruboxistaurin has completed phase III trials for other indications and is safe in humans, we believe it could be quick to enter the clinic as a new drug for COVID-19.

Material and methods (amended to only include those methods not included in thesis section 2)

Human bronchial epithelial cell culture. Human bronchial epithelial cells (HBEC3-KT) were grown in a humidified incubator at 37 ◦C, 5% CO2. Cells were maintained in basal growth medium; Keratinocyte-SFM (1X) with L-glutamine (Gibco, UK), supplemented with bovine pituitary extract, epidermal growth factor, and Gentamicin sulfate-Amphotericin – 1000 (GA-1000) (Lonza, Switzerland). Cells were passaged twice weekly when at 70-80% confluency and used for experiments between passage 12 and passage 22.

Cell viability assay. Neutrophils (2.5 x $10^{\wedge 6}$) were seeded in microcentrifuge tubes and stimulated to induce NET formation with PMA ± ruboxistaurin as described above. Cells were spun at 2500 *g* for 5 minutes and the cell-free supernatants (SPN) were removed and stored at -80◦C until required. HBEC3-KT cells were seeded into a 24-well plate at 1.2 x $10^{\wedge 6}$ per plate and grown overnight to reach 90-100% confluency, before overnight incubation in basal media with depleted growth factors. HBEC3-KT cells were incubated with neutrophil SPNs at 1:2 dilution ± ruboxistaurin [200 nM] for 24 hours. CellTiter-Glo® was used as a measure of cell viability. Spent media was removed and pre-prepared CellTiter-Glo® reagent added at a 1:2 dilution with basal medium to the tissue culture plate. The plate was incubated (with shaking) at room temperature for 2 minutes then for an additional 10 minutes at room temperature (without shaking). Samples were added in duplicate to a white opaque 96–well plate (Costar) and luminescence determined using a fluorescent plate reader at 480 nm.

Results

Neutrophils isolated from patients with COVID-19 generate more NETs in response to LPS. Neutrophils were isolated from venous blood from healthy volunteers (healthy controls), or patients hospitalised following a positive PCR test for SARS-CoV-2 (n=39). Of the 39 COVID-19 patients recruited to the study, 38 required supplemental O_2, 32 received dexamethasone, 3 were subsequently admitted to intensive care, and 2 died (Table 1). Neutrophils were treated with LPS [5 µg/ml] or phorbol myristate acetate (PMA) [100 nM], which induce NADPH oxidase- and PKC-dependent NETosis (Fuchs et al., 2007; Gray et al., 2013; Khan et al., 2017; Arroyo et al., 2019). PMA was chosen because this is the prototypical and most commonly used NET-inducer, as well as being profoundly effective at inducing NETs. A limitation of using PMA is that it is a chemical stimulant and considered by many as not physiologically relevant. To overcome this, we also induced NETs with LPS, which is a naturally occurring bacterially-derived molecule. Although LPS is not directly associated with viral infection *per* se, it does model the additive effect of secondary bacterial infections, which are not uncommon in COVID-19 and which, via the effect on NETosis, may add to the inflammatory pathology seen in this disease. NET formation was measured by SYTOX™ Green staining of extracellular DNA (Zuo et al., 2020; Gray et al., 2013). Compared with healthy control subjects, neutrophils from people with acute COVID-19 generated significantly more NETs in response to LPS and a similar amount of NETs in response to PMA (Figure 1). Three patients were admitted to the intensive care unit (ITU) during our study (indicated as open red squares, Figure 1) and generated among the highest SYTOX™ green values following PMA treatment. The increased capacity of neutrophils to undergo LPS-induced NETosis during the acute stage of COVID-19 adds to existing data suggesting this could be a key element of the dysregulated and deleterious inflammatory response in COVID-19.

Table 1. All COVID-19 patient characteristics

Demographics	
Total number of participants	39
Age in years: mean ± stdev	57.4±12.3
Age in years: range	29-83
Female: number (percent)	12 (30.8%)
Male: number (percent)	27 (69.2%)
Clinical data	
Days following symptom onset of neutrophil sampling: mean ± stdev	12.9±7
Days following symptom onset of neutrophil sampling: range	4-43
Length of stay in hospital (days): mean±stdev	11±13.2
WHO symptom severity score: mode	1
Required supplemental O2: number (percent)	38 (97.4%)
Receiving dexamethasone: number (percent)	32 (82.1%)

Receiving tocilizumab: number (percent)	1 (2.6%)
Admitted to ITU: number (percent)	3 (7.7%)
Deaths: number (percent)	2 (5.1%)
Neutrophil Count (x 10^9/L): mean±stdev	5.6±2.3
CRP (mg/L): mean±stdev	49±42.6
Platelet count (x 10^9/L): mean±stdev	280±103
Comorbidities: number (percent)	
None	6 (15.4%)
Diabetes (incl. pre-diabetes)	14 (35.8%)
Hypertension	11 (28.2%)
Asthma	8 (20.5%)
Cancer	6 (15.4%)
Cardiovascular Disease	6 (15.4%)
Obesity	4 (10.3%)
Kidney Disease	2 (5.1%)
COPD	2 (5.1%)
Bronchiectasis	1 (2.6%)

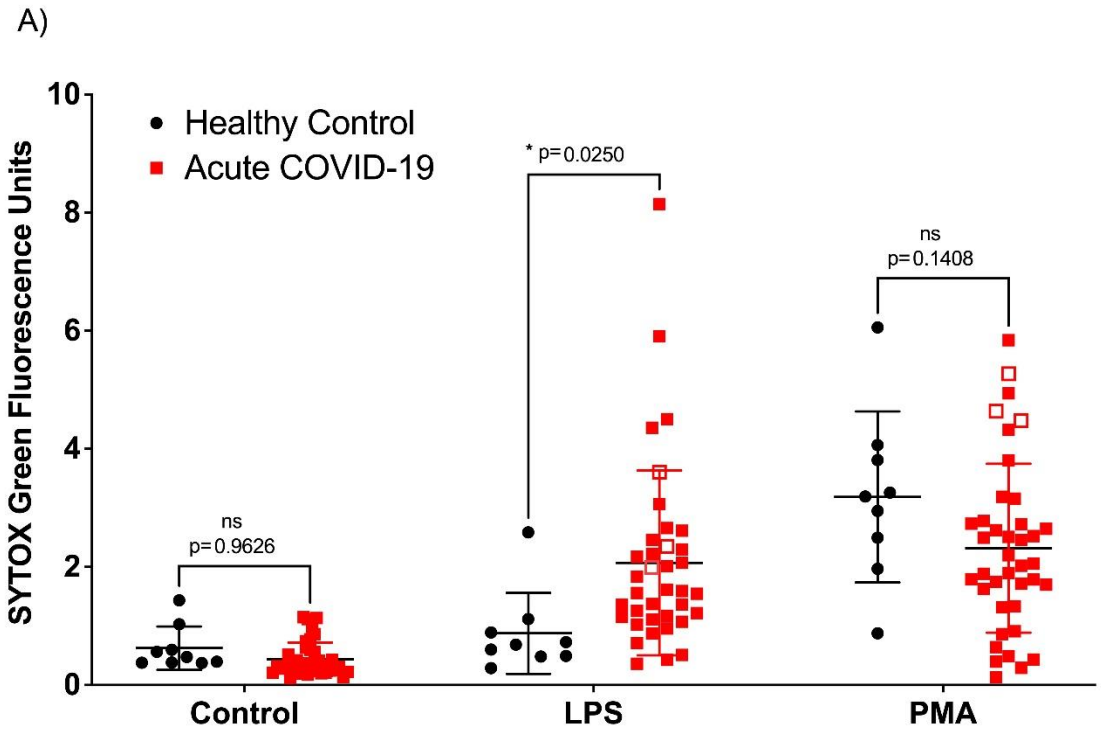


Figure 1. LPS-stimulated NET release is significantly elevated in acute COVID-19 patients.

Neutrophils isolated from peripheral whole blood from healthy control subjects (black circles, n=9) or hospitalised patients with COVID-19 (red square, n=37 LPS, n=39 PMA), were stimulated for 3 hours with either LPS [5 µg/ml] or PMA [100 nM]. Open red squares indicate patients (n=3) who were admitted to ITU during the study. SYTOX green was added, and extracellular DNA release (NETs) was quantified using a fluorescent plate reader. A significant increase in NET formation was shown in acute COVID-19 patients in response to LPS but not in response to PMA. Statistical analysis used a mixed-effects model with a Šidák post-test. Error bars represent standard deviation.

Dexamethasone does not modify elevated NETosis in hospitalised patients with COVID-19. Dexamethasone was the first therapy demonstrated to reduce COVID-19 associated mortality and was licensed for use in treating hospitalised COVID-19 patients requiring supplemental oxygen in September 2020 (The Recovery Collaborative, 2021). Dexamethasone is an anti-inflammatory drug and has previously shown to reduce neutrophil recruitment and NETosis both *in vitro* and *in vivo* murine models (Wan et al., 2017; Fan et al., 2020). We examined whether pre-treatment with dexamethasone [10 µM] for 1 hour prior to stimulation with LPS or PMA, as before, impacted the NETosis response from COVID-19 neutrophils. There was no significant effect of dexamethasone on either LPS- or PMA-induced NETosis (Figure 2 A & B). However, 82% of COVID-19 patients in the study were receiving dexamethasone at time of sampling, meaning neutrophils analysed could have been previously exposed to the drug *in vivo*. The experiment was therefore repeated with healthy donor neutrophils, which were naïve to dexamethasone. While the LPS-induced NET response was low, as is typically seen with this concentration in healthy neutrophils, there was no impact of dexamethasone on either LPS- or PMA-induced NET formation using healthy donor neutrophils (Figure 2C).

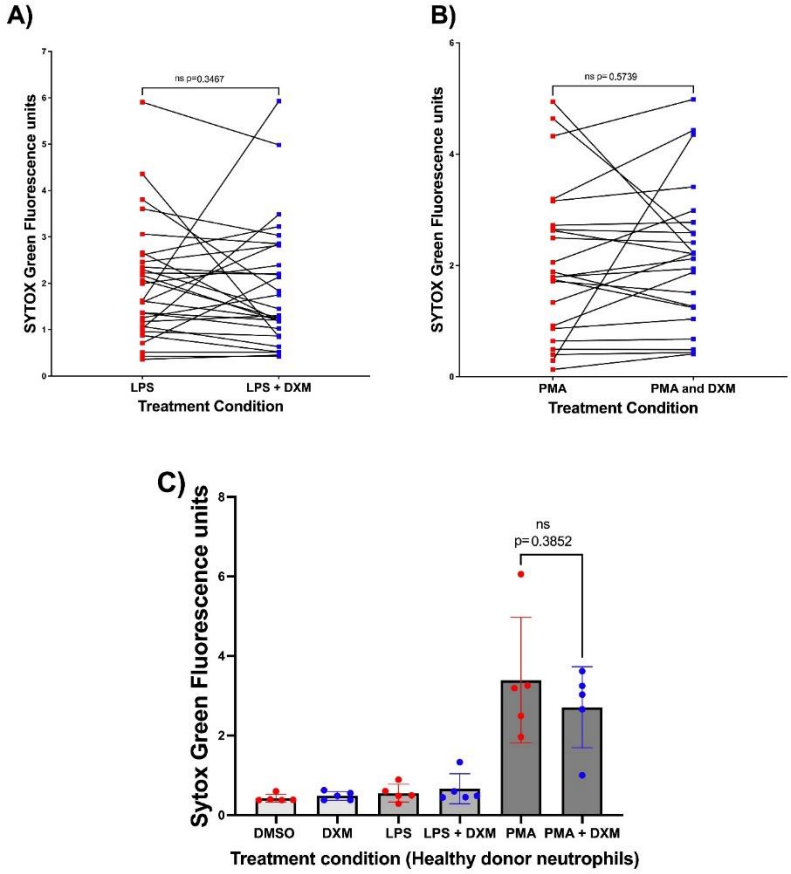


Figure 2. Dexamethasone does not impact NETosis in neutrophils isolated from hospitalised COVID-19 patients or healthy donors.

Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 (A-B) were pre-incubated with dexamethasone (DXM) [10 µM], for 1 hour (blue squares). Neutrophils were stimulated with LPS [5 µg/ml] (A, n=31) or PMA [100 nM] (B, n=23) for a further 3 hours (red squares). SYTOX Green was added, and extracellular DNA (NETs) was quantified using a fluorescent plate reader. C) The assay was repeated using healthy donor neutrophils (red circles) treated with dexamethasone as before (blue circles) (C, n=5). Data set A) was normally distributed and a paired t-test was conducted. Data set B) was not normally distributed and a Wilcoxon matched pairs signed rank test was conducted. A one-way ANOVA with a Bonferroni's selected pairs post-test was completed for panel C where PMA alone was compared with PMA + DXM. Error bars represent standard deviation.

The orally active inhibitor of PKC, ruboxistaurin, inhibits LPS-induced *ex vivo* NET formation in COVID-19. NETosis can occur via ROS-dependent mechanisms, and we set out to determine whether this was the case in the context of COVID-19 (Rochael et al., 2015; Muraro et al., 2018; Arcanjo et al., 2020). We show both PMA- (Figure 3A) and LPS- (Figure 3B) induced NET formation in neutrophils from people with acute COVID-19 is significantly reduced by the NADPH oxidase inhibitor, diphenyleneiodonium (DPI). PKC is a key signalling component of ROS-dependent NET formation (Gray et al., 2013). Ruboxistaurin is an effective inhibitor of PKC-β, has completed phase III clinical trials for diabetic retinopathy and is well-tolerated by patients (Aiello et al., 2006). We show for the first time that ruboxistaurin is a potent inhibitor of NET formation in COVID-19 neutrophils, significantly reducing both LPS- (Figure 4A) and PMA- (Figure 4B) induced NETs. During NETosis, neutrophils release DNA which is decorated with antimicrobial components including myeloperoxidase (MPO) (Brinkmann et al., 2004). We confirmed biochemically and morphologically that neutrophils from patients with COVID-19 generate MPO-positive NETs in response to PMA and LPS and that fewer NETs are visualised in the presence of ruboxistaurin (Figure 4C). To understand whether components of SARS-CoV-2 could directly induce NET formation that is amenable to inhibition by ruboxistaurin, we incubated neutrophils from healthy subjects (to exclude the possibility that neutrophils had previously been exposed to viral proteins *in vivo*) with purified SARS-CoV-2 nucleocapsid and spike proteins. Neither antigen induced NETs alone, nor in the presence of LPS, supporting the observation that SARS-CoV-2-mediated NETosis is dependent on viral replication (Supplemental Figure 1) (Arcanjo et al., 2020; Veras et al., 2020).

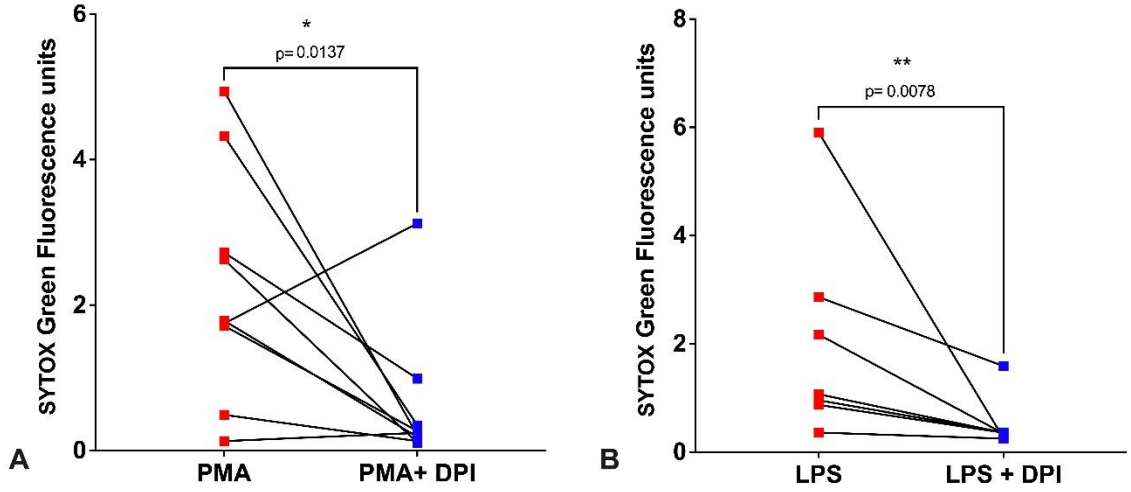


Figure 3. The ROS inhibitor DPI significantly reduces both PMA- and LPS-stimulated NET formation in neutrophils from acute COVID-19 patients.

Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 were pre-incubated with ROS inhibitor, DPI (10 µM), for 1 hour (blue squares). Neutrophils were stimulated with PMA [100 nM] (A, n=9) or LPS [5 µg/ml] (B, n=7) for a further 3 hours (red squares). SYTOX Green was added and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Statistical analysis was performed by one-tailed Wilcoxon matched-pairs signed rank test and significance values are as indicated.

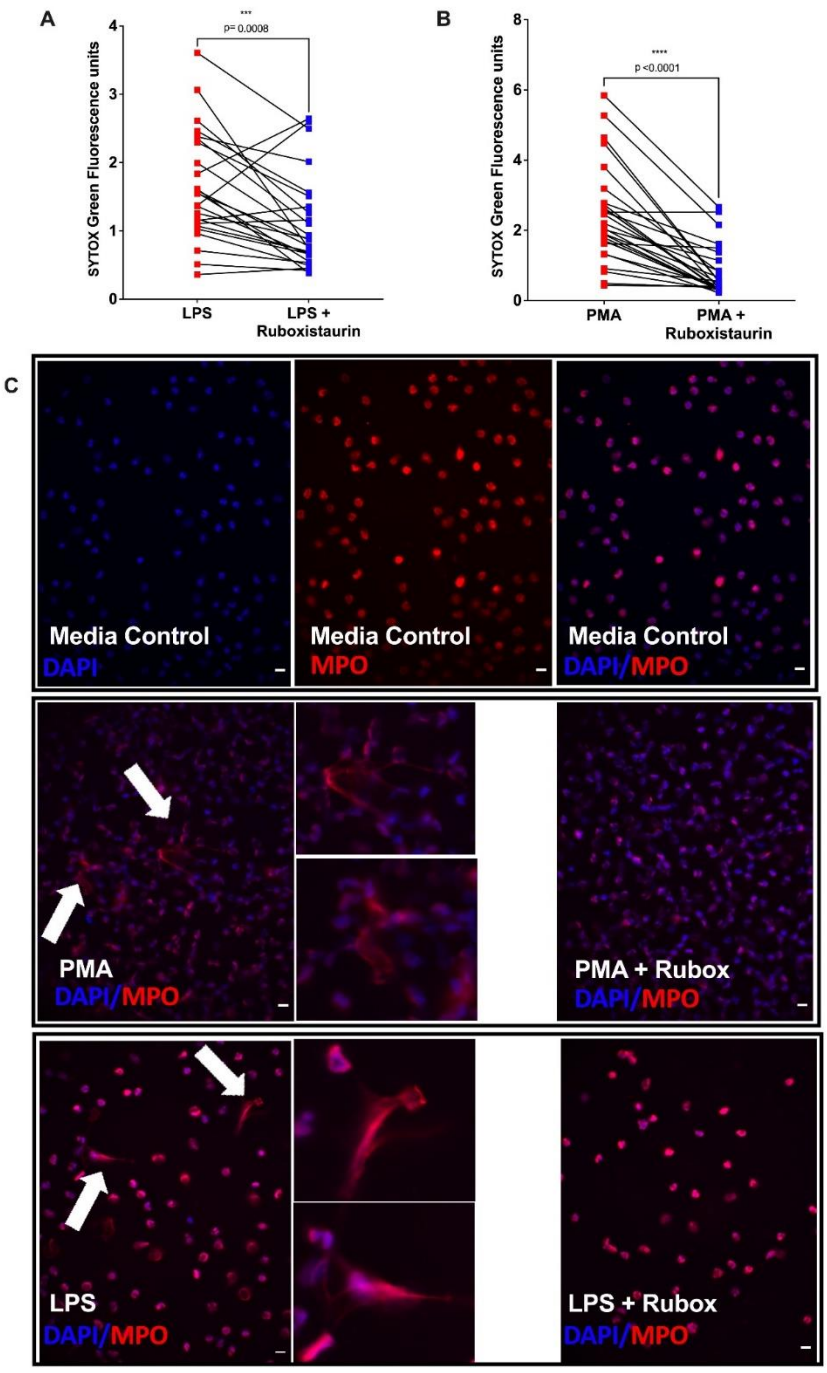


Figure 4. Ruboxistaurin significantly reduces both LPS- and PMA-stimulated NET formation in neutrophils from acute COVID-19 patients.

Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 (A n=26, B n=28) were preincubated with ruboxistaurin [200 nM] for 1 hour (blue squares). Neutrophils were stimulated with LPS [5 µg/ml] or PMA [100 nM] for 3 hours (red squares). SYTOX green was added and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Statistical analysis was performed by Wilcoxon matched-pairs signed rank test (A, B) and significance values are as indicated. (C) COVID-19 patient derived neutrophils were seeded in IBIDI™ chamber wells and stimulated as described for panels A-B, plus media control. Neutrophils were stained for myeloperoxidase (MPO) and detected using Alexafluor 597 fluorochrome (red). DNA was visualised with ProLong™ Gold Antifade Mountant with DAPI (blue). Cells were viewed by fluorescence microscopy (40x magnification) and images are representative of 3 independent experiments. Fields of view were selected at random. Arrows indicate NETs (zoomed images show NET morphology). Scale bar = 10 µm.

Ruboxistaurin inhibits NET mediated human airway epithelial cell death. NETs directly induce epithelial cell damage (Saffarzadel et al., 2012; Veras et al., 2020). Here we show that supernatants (SPNs) from PMA-treated neutrophils isolated from healthy volunteers induce death of human bronchial epithelial (HBEC3-KT) cells, which is significantly reduced by ruboxistaurin (Figure 5A). Rounding up and detachment of the monolayer was visible in HBEC3-KT cells cultured with SPNs from PMA-treated neutrophils, which was reduced in the presence of ruboxistaurin (Figure 5B). To determine whether ruboxistaurin was having a direct effect on epithelial cells, we incubated cells with media or ruboxistaurin and added SPNs from PMA-treated neutrophils. Ruboxistaurin does not reduce epithelial cell death, suggesting its protective effect is via neutrophils and the reduction in NET formation (Figure 5C). Secondary infections are not uncommon in COVID-19, therefore it is important not to compromise neutrophil microbicidal functions. To this end, we measured killing of the human pathogen *Staphylococcus aureus* by COVID-19 neutrophils and show ruboxistaurin had no effect on the ability of neutrophils to kill S. *aureus* (data not shown).

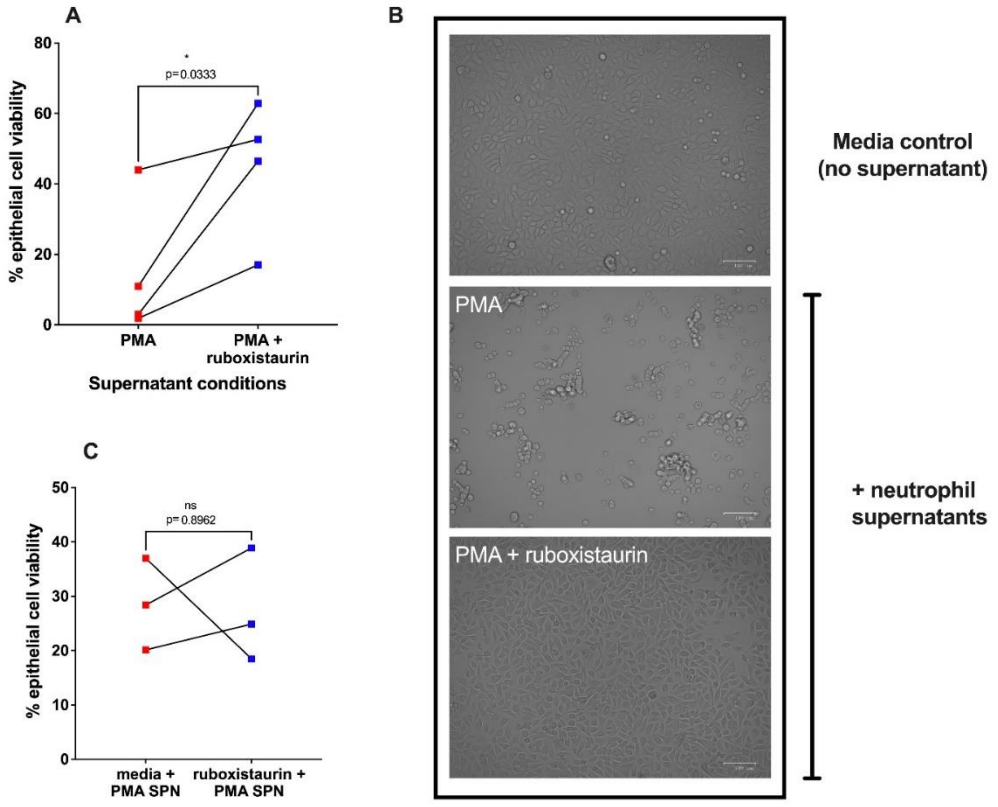


Figure 5. Ruboxistaurin reduces neutrophil supernatant-induced epithelial cell damage. Cell free SPNs from neutrophils isolated from healthy donors stimulated with PMA [100 nM] ± ruboxistaurin [200 nM] were added to confluent human bronchial epithelial cells (HBEC3-KT) at a 1:2 dilution (A). After 24 hours HBEC3-KT cell viability was assessed using CellTitre Glo® (n=4). B) Cell monolayers were imaged using the Zoe fluorescent cell imager, using the brightfield setting and 20x objective lens. Images are representative of 4 donors and fields of view were selected at random. Scale bar represents 100 µm. C) Epithelial cells were incubated with either media (red squares) or ruboxistaurin [200 nM] (blue squares) plus SPNs from neutrophils isolated from healthy donors stimulated with PMA [100 nM] for 24 hours. Cell viability was assessed as above (n=3). Statistical analysis was performed by Student's t-test and significance values are as indicated.

Elevated NETosis in acute COVID-19 patients reduces over time but remains higher than in healthy controls. Neutrophils have been shown to be reprogrammed during COVID-19 and we aimed to investigate whether the pro-NET phenotype observed during the acute stage persisted after infection (Aschenbrenner et al., 2021). To do this we studied a subset of 7 individuals at follow-up clinics held 3-4 months following acute sampling (Table 2). LPS-induced NETosis was significantly reduced at the follow-up time point (Figure 6A) indicating a reduction in the pro-NETosis phenotype in this population. PMA-induced NETosis did not differ between the acute and follow-up time points (Figure 6B). This is not unexpected since PMA is a potent inducer of NETs in healthy cells. In comparison to NETosis in healthy neutrophils however, (previously shown in Figure 1A) LPS-induced NET formation (Figure 6C) but not PMA-induced NET formation (Figure 6D) remained significantly elevated in neutrophils isolated at the follow-up time point.

Table 2- Follow-up patient characteristics

Demographics	
Total number of participants	7
Age in years: mean ± stdev	57.2±13.3
Age in years: range	29-70
Female: number (percent)	2 (28.5%)
Male: number (percent)	5 (71.5%)
WHO symptom severity score: mode	1
Comorbidities number (percent)	
None	2 (28.6%)
Diabetes (incl. pre-diabetes)	1 (14.2%)
Asthma	1 (14.2%)
Cardiovascular Disease	1 (14.2%)
Cancer	1 (14.2%)
Obesity	1 (14.2%)

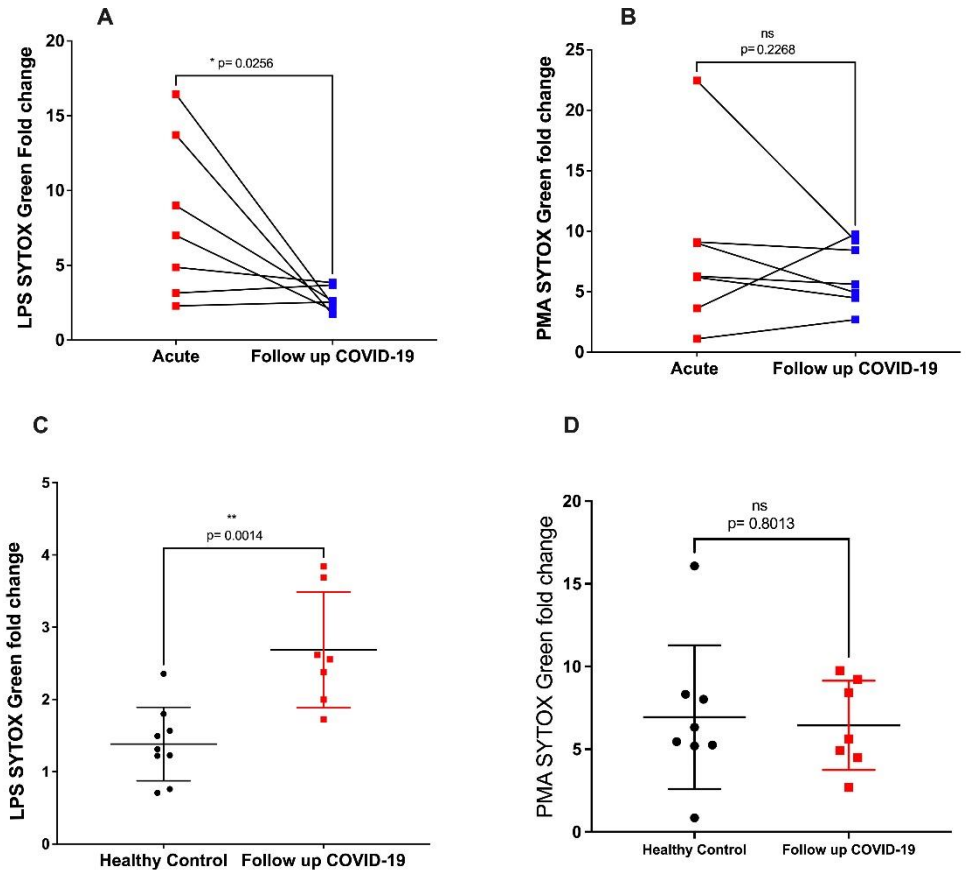


Figure 6. LPS-induced NETosis is reduced in follow-up COVID-19 patients but remains significantly higher than in healthy controls.

Seven previously hospitalised patients with COVID-19, who were part of the acute COVID-19 cohort in Figure 1A, returned to a follow up clinic 3-4 months post-acute sampling. Neutrophils were stimulated as previously described with LPS [5 ug/ml] (A) or PMA [100 nM] (B) and NET formation was quantified using SYTOX green. To show linked data from individual patients at acute and follow up time points, fold data were expressed by calculating fold change to DMSO control. Lines link values from the same patient (n=7). There was a significant reduction in LPS induced NET formation at the follow up time point but no difference in PMA stimulated neutrophils. Follow up data were also compared to healthy control data (n=9), these control samples being also used in Figure 1A, in response to LPS (C) or PMA (D). LPS induced NETs were significantly higher in follow-up COVID-19 patients compared to healthy controls but there was no difference in PMA stimulated neutrophils. Statistical analysis was performed by a one-tailed paired Student's t-test (A,B) and a two-tailed unpaired Student's t-test (C,D) and significance values are as indicated.

Discussion

Our findings show neutrophils isolated from patients with acute COVID-19 undergo significantly more LPS-induced NETosis than healthy control cells. Although LPS-induced NET formation significantly reduces in COVID-19 patients over time, levels at follow-up time points remained higher than in healthy control cells. We are the first to show that LPS-induced NETosis can be inhibited by ruboxistaurin *in vitro*, indicating a role for PKC-β in this pathway. This finding not only supports the importance of the PKC-β signalling pathway in neutrophils in COVID-19, but also reveals a potential therapeutic strategy for this disease.

Middleton *et al* demonstrated elevated baseline NET levels in neutrophils isolated from COVID-19 patients, which were not further increased by PMA (Middleton et al., 2020). In contrast, we do not show elevated baseline (unstimulated) NET formation, which may reflect differences in disease severity, patient demographics, activation during the isolation procedure, or sensitivity of the NET assay. Neutrophils from COVID-19 patients in our study robustly responded to PMA and generated NETs to levels comparable to healthy control cells. Interestingly, individuals with some of the greatest PMA-induced NET responses went on to require ITU support. Since this was a very small sub-group (n=3) more work is required to determine whether there is an association here.

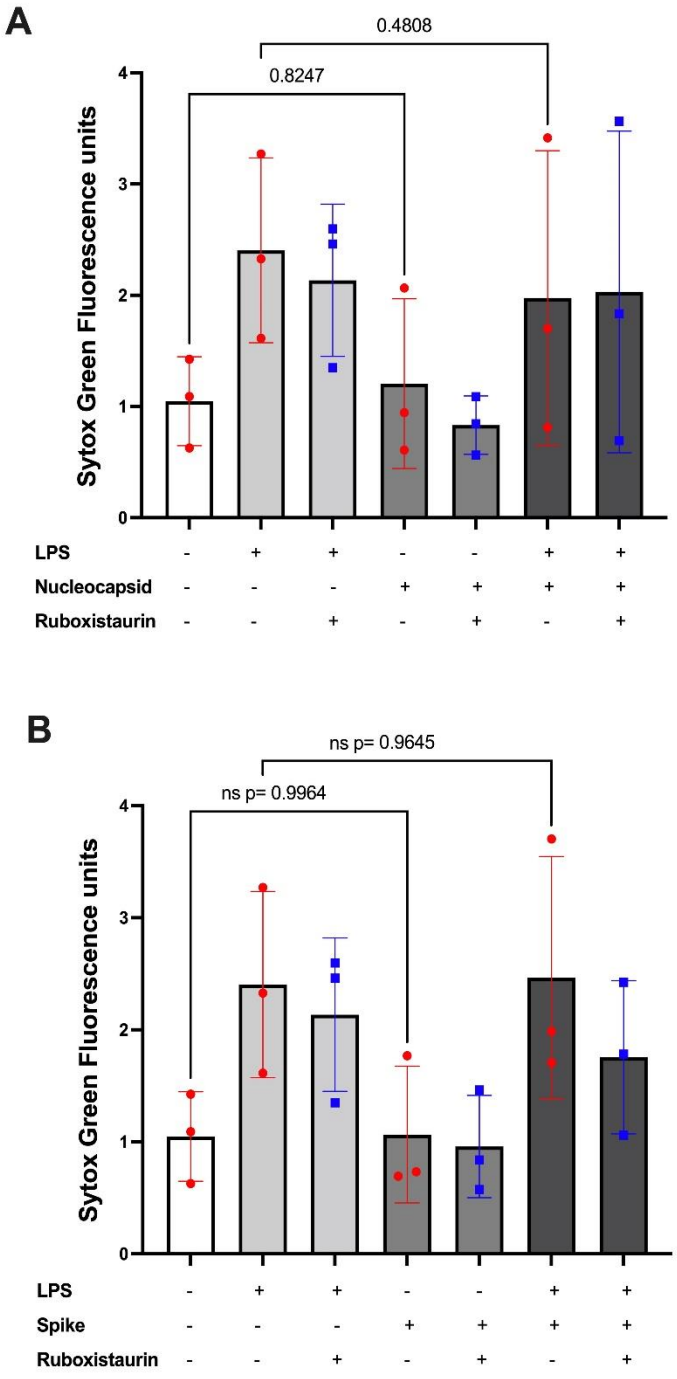
Whilst others have also shown increased NETosis in people with COVID-19 in response to PMA, a potent PKC activator, we are the first to show an increase in NETs in response to LPS, a receptor-driven neutrophil stimulator and typically less potent inducer of NETosis. Increased sensitivity to LPS-induced NETosis has implications in the case of secondary infections, and shows neutrophils are primed to increased NET formation to this, and therefore potentially other, proinflammatory stimuli (Vaillancourt et al., 2020). The mechanism by which neutrophils from people with COVID-19 are more sensitive to undergoing NETosis is unclear. SARS-CoV-2 directly triggers NET formation (Veras et al., 2020) as does sera from COVID-19 patients (Zuo et al., 2020). In keeping with the work of others who show live, but not inactivated SARS-CoV-2 induces NETs (Arcanjo et al., 2020; Veras et al., 2020), we demonstrated that purified viral antigens did not induce NET formation. Furthermore, increased NET formation in isolated neutrophils *ex vivo* suggests this is not as a result of direct SARS-CoV-2 exposure and is more likely due to the neutrophils being in an activated and primed state and therefore being inherently more sensitive to NET stimuli. This is supported by other studies which describe neutrophil 'hyperactivation' in COVID-19, whereby neutrophils are transcriptionally reprogrammed and which is a predictor of severe disease (Parackova et al., 2020; Meizlish et al., 2021; Reyes et al., 2021). Furthermore, circulating neutrophils from critically ill COVID-19 patients have exaggerated ROS production which may contribute to increased NET production (Masso-Silva et al., 2021). Although significantly less than at the acute stage of infection, LPS-induced NET formation remained higher in the subset of individuals who were re-sampled after 3-4 months, compared to healthy controls. This may suggest a pro-NETotic phenotype continues beyond the period of active SARS-CoV2 infection, or could reflect pre-existing patient co-morbidities in which increased NETosis is observed.

LPS is a weak inducer of NETs in healthy neutrophils compared with PMA, which in part explains why we do not see differences in PMA-induced NETs when comparing healthy control cells with neutrophils isolated from people with COVID-19 (regardless of the time point). It is possible that upregulation of the TLR4 receptor and/or downstream signalling components in COVID-19 is responsible for the increased sensitivity to LPS-induced (but not PMA-induced) NETs, as seen in monocytes (Sohn et al., 2020). SARS-Cov-2 spike protein activates TLR4 in neutrophil-like cells *in vitro*, and therefore has the potential to cause priming to subsequent exposure to LPS in circulating neutrophils, although whether sufficiently high levels of spike exist in the blood to allow this to occur is unknown (Zhao et al., 2021).

A limitation of our study is around the demographics of the healthy control subjects compared to the patient cohort, the latter of whom are older, have more comorbidities and are receiving medications that may impact on neutrophil function (including dexamethasone). However, studying patients at 3-4 months post-acute sampling means participants serve as appropriate age- and comorbidity-matched controls and allow us to understand differences in neutrophil function at the acute stage of the disease.

Vaccination weakens the link between infection and critical illness, but vaccine breakthroughs are seen, particularly in the case of viral variants, such as the Omicron variant, which will continue to emerge (Hacisuleyman et al., 2021). It is therefore critical that we develop alternative and complementary strategies to prevent severe COVID-19 disease, and the innate immune response is an ideal target for this. Since NETs are known drivers of pathology in several diseases including, not limited to COVID-19, targeting NETosis is a logical therapeutic strategy for the future. A growing number of studies describe increased levels of NETosis in disease, as well as the deleterious role of NETs in driving inflammation, thrombosis and disease severity, but few have offered a solution. Our study indicates that ruboxistaurin could reduce NET formation and ultimately diminish airway inflammation and other events including microvascular thrombosis and is a novel and promising therapeutic strategy for COVID-19 (Middleton et al., 2020; Veras et al., 2020). Furthermore, our preliminary data demonstrates reducing NET formation with ruboxistaurin protects airway epithelial cells *in vitro*. Maintaining airway epithelial integrity could provide protection against secondary bacterial infection, which is important since secondary infection is a predictor of death in COVID-19 patients (Vareille et al., 2011; Shafran et al., 2021). Targeting NETosis in COVID-19 is a strategy shared by others in the field. Therapies inhibiting NET-associated protease activity (NCT04817332) and targeting the breakdown of NETs with DNases are also currently in clinical trials for COVID-19 (NCT04359654). However, ruboxistaurin has the advantage that it prevents NET formation by circulating neutrophils, rather than either modifying or disrupting NETs once they have been formed. Ruboxistaurin has been demonstrated to reduce NET formation in an *in vivo* mouse model and an *in vitro* study of healthy neutrophils, suggesting it has promise in targeting NETs in disease (Gray et al., 2013; Das et al., 2018). Since phase 3 trials for diabetic retinopathy show ruboxistaurin is a well-tolerated inhibitor of PKC, we believe it could be relatively quick to translate to the clinic, providing a novel therapeutic pathway to treat neutrophil mediated immunopathology in COVID-19 (Aiello et al., 2006).

Supplemental Figure



Supplemental Figure 1. SARS-CoV-2 antigens do not induce NET formation in neutrophils from healthy donors. SARS-CoV-2 spike protein was produced by Dr Martin Nicklin (University of Sheffield) based on the protocol and plasmid from Stadlbauer *et al* (Current Prot Microbiol 2020 57 e100), and nucleocapsid protein by Professor Jon Sayers as per Colton *et al (Wellcome Open Research, 2022).* Neutrophils isolated from peripheral whole blood from healthy donors were preincubated with media (red circles) or ruboxistaurin [200 nM] (blue squares) for 1 hour and then stimulated with LPS [5 µg/ml], nucleocapsid protein [200 nM] (A), or spike protein [10 nM] (B) for a further 3 hours (n=3). SYTOX Green was added and extracellular DNA (NETs) was quantified using a fluorescent plate reader. A one-way ANOVA with a Bonferroni's selected pairs post-test was performed, with comparisons as indicated. Error bars represent standard deviation.

5.2.4 Correlations of patient clinical phenotypes with the NET response

5.2.4.1 *Patient Characteristics*

The findings presented in the manuscript demonstrated increased NET formation in neutrophils studied *ex vivo* from people with COVID-19, so it was therefore important to determine whether NETs correlated with surrogate markers of disease severity. Clinical data was collected for all patients at the end of the study, by medical Students, Chenghao Huang and Jacob Whatmore (UoS), who also anonymised these data. Key patient characteristics including age, gender, and co-morbidities are described in Table 1 of the manuscript (section 5.2.3.2). In brief, the mean age of the patients was 57.4 years, and they were predominantly male (69.2%). The average time of neutrophil sampling since symptom onset was 12.9 days and the average length of hospital stay was 11 days. Most patients were receiving supplemental oxygen therapy (97.4%) and dexamethasone (82.1%). None of the patients were currently receiving treatment in the ITU at the time of blood sampling, however 3 patients were subsequently admitted to ITU, and 2 patients died. The patients in this study were classified as 'low' severity (score of 1) based on the world health organisation (WHO) symptom severity score at the time they were sampled. Patients also had a range of co-morbidities with the most common being diabetes in 14/39 patients.

5.2.4.2 *Choosing patient clinical parameters to correlate with the NETosis response*

A selection of patient characteristics to correlate with the NETosis response were chosen. These were exploratory, hypothesis-generating evaluations that were not powered and therefore robust conclusions cannot be drawn from these data. However, due to COVID-19 being a novel disease, where understanding of immunopathology is still advancing, it was still worthwhile to investigate these correlations. A limited number of correlations were conducted, whilst being aware of issues regarding increased likelihood of false positive results, which can occur with indiscriminate and excessive correlative analysis (Forstmeier et al., 2017). The rationale of which clinical parameters were explored are described in the numbered list below.

1. Diabetes status- A key aim of the study was to determine if COVID-19 patients with diabetes produced more NETs than patients without diabetes. This investigation linked with my previous PhD research, investigating neutrophil function in diabetes. NETosis is increased in people with diabetes and those with diabetes are at enhanced risk of severe COVID-19 disease and death, therefore whether NETosis was particularly high in this sub-group of patients was explored (Barron et al., 2020; Huang et al., 2020; Wong et al., 2015).
2. HbA1C- HbA1C is a measure of long-standing blood glucose levels. This parameter was included as it links with the investigation of NETosis in diabetes, with HbA1C used as indicator of diabetes control. Hyperglycaemia was demonstrated to be a predictor of poor prognosis

and mortality in COVID-19 patients (Carrasco-Sánchez et al., 2020; Coppelli et al., 2020; Menegazzo et al., 2015; Wang et al., 2020).

3. Highest oxygen requirement- The highest oxygen requirement that patients received whilst admitted to hospital was used as a marker of COVID-19 disease severity. Hypoxemia was independently associated with COVID-19 mortality in previous research (Xie et al., 2020).
4. Age- Neutrophil function changes with age, and those with advanced age are at higher risk of severe COVID-19 disease and mortality (Bajaj et al., 2021; Cox et al., 2020; Wenisch et al., 2000). However, NETosis is reduced in older adults (Hazeldine et al., 2014). The age range of the patients in this study was large (29-83 years), therefore if participants with a younger age was associated with increased NETosis was explored.
5. Time of neutrophil sampling since symptom onset- Blood samples were taken for analysis between 1-3 days after hospital admission, however patients had experienced symptoms for a varying length of time (4-43 days). To investigate if NETosis was enhanced in patients either at the start of symptoms onset or if it was increased after a prolonged period this parameter was correlated with the NETosis response. This was important to investigate as it could inform when to target NETosis in the disease.
6. C-reactive protein (CRP)- CRP is an acute phase inflammatory protein primarily produced by the liver (Sproston and Ashworth, 2018). CRP is a clinical marker of inflammation and routine blood tests quantifying levels of CRP were conducted for the patients in the study (Sproston and Ashworth, 2018). CRP is elevated in COVID-19 and was a predictor of COVID-19 severity and mortality (Smilowitz et al., 2021; Stringer et al., 2021). CRP has previously shown to correlate with cell-free DNA, a marker of NETosis, in the serum of COVID-19 patients (Zuo et al., 2020).
7. Neutrophil- lymphocyte ratio (NLR)- NLR is the ratio of neutrophils to lymphocytes in the blood and is clinical a biomarker for inflammation. NLR is widely cited to be a predicative marker of COVID-19 disease severity and mortality (Liu et al., 2020; Zeng et al., 2021).
8. Neutrophil Count- High neutrophil counts are a characteristic feature of severe COVID-19 disease (Qin et al., 2020; Wang et al., 2020). Neutrophil count correlated with cell-free DNA and DNA-MPO complex, which are markers of NETs, in the serum from COVID-19 patients previously (Zuo et al., 2020).
9. Platelet Count- Activated platelets can induce NETosis and NETs form aggregates with platelets, contributing to clot formation in the lungs in COVID-19 (Carestia et al., 2016; Middleton et al., 2020). Due to the association of platelets and NETs in immunothrombosis these parameters were correlated.

5.2.4.3 *Results from the association of clinical parameters with NETosis*

To investigate the association between patient clinical characteristics and NETosis the raw SYTOX™ green fluorescence values generated in response to either LPS or PMA and the fold change in NET response was compared. Calculation of fold change was based on the difference in SYTOX™ green fluorescence units between stimulated neutrophils and the DMSO control. There were 14/39 patients who had either type 2 diabetes, type 1 diabetes or pre-diabetes and these were categorised as the 'diabetes' group. There was no significant difference in NET formation between patients with or without diabetes, either at baseline or after stimulation with LPS or PMA (Figure 34A-B).

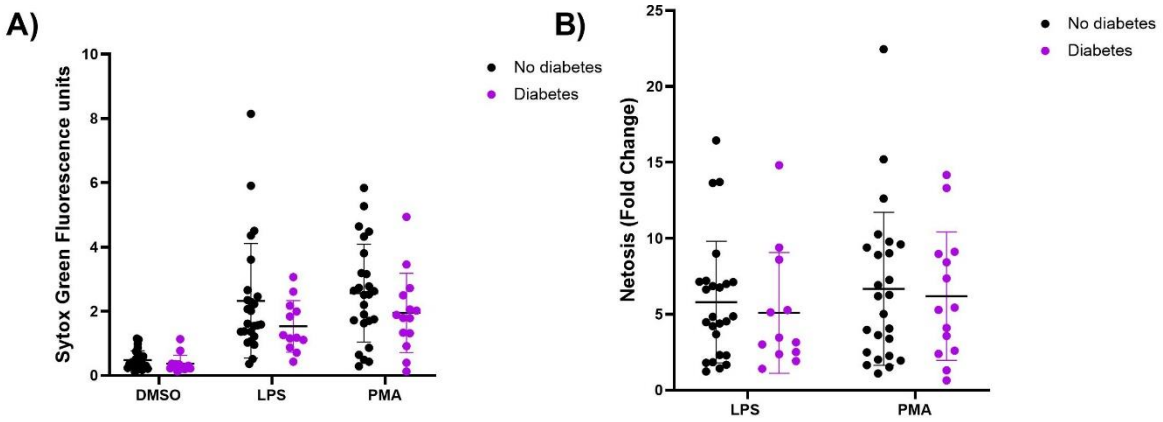


Figure 34-NET formation in patients with COVID-19 and diabetes.

Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 were stimulated with either LPS [5 µg/ml] or PMA [100 nM] for 3 hours. SYTOX™ Green was added to all wells [555 nM] and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Excitation/emission 490/537 nm was used. A) Anonymised clinical data was collected for all patients and SYTOX™ Green values were stratified based on whether patients had type 2 diabetes, type 1 diabetes, and pre-diabetes (purple circle) or no diabetes (black circle). B) The analysis was repeated using the fold change in NETosis. Fold change as calculated based on the difference in SYTOX™ green values between stimulated neutrophils and the DMSO control. There was no significant difference in NET formation at baseline or induced by LPS or PMA between the no diabetes and diabetes group. These data represent n= 25 for patients without diabetes (LPS & PMA) and n=14 for patients with diabetes after PMA stimulation and an n= 12 for patients with diabetes after LPS stimulation. Statistical analysis was by Mixed effects model with a Bonferroni's post-test and compared neutrophils at baseline (DMSO) to neutrophils stimulated with LPS or PMA in patients with or without diabetes. Error bars represent SD.

Next, the NETosis response was stratified based on age, and 55 was used as a cut off between the two groups. This age was selected as COVID-19 mortality rates significantly increase in persons aged over 55 compared to younger individuals (Yanez et al., 2020), and also due to the spread of ages in the patients in the study this would allow a similar number of patients in each group for comparison. There was no significant difference in the NET response between patients based on age (Figure 35A-B).

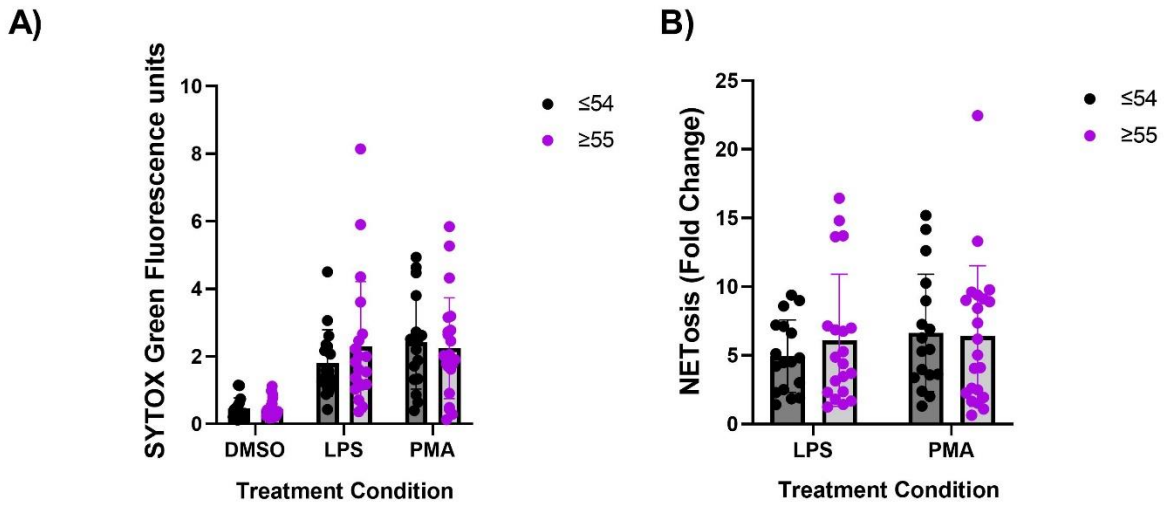


Figure 35-NETosis in patients over 55 years old.

Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 were stimulated with either LPS [5 µg/ml] or PMA [100 nM] for 3 hours. SYTOX™ Green was added to all wells [555 nM] and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Excitation/emission 490/537 nm was used. A) Anonymised clinical data was collected for all patients and SYTOX™ Green values were stratified based on whether patients were aged ≤54 years (black circle) or ≥55 years (purple circle). B) The analysis was repeated using the fold change in NETosis. Fold change as calculated based on the difference in SYTOX™ green values between stimulated neutrophils and the DMSO control. There was no significant difference in NET formation at baseline or induced by LPS or PMA the two age groups. These data represent n= 17 for patients aged ≤54 years and n=22 for patients aged ≥55 years after DMSO or PMA treatment and n= 20 for patients aged ≥55 years after LPS stimulation. Statistical analysis was by a mixed effects model with a Bonferroni's post-test and compared neutrophils at baseline (DMSO) to neutrophils stimulated with LPS or PMA in patients aged ≤54 or ≥55. Error bars represent SD.

Also, the NET response was stratified based on the highest oxygen requirement administered whilst patients were in hospital. There was no difference in the amount of NETosis between patients requiring low (0-4), medium (5-10) or high (11-15) volumes of oxygen (litres), when using the raw SYTOX green fluorescence units, but there was a significant increase in the fold change in NET response in PMA-stimulated neutrophils from patients receiving 5-10 litres of oxygen compared to 0-4 litres of oxygen (Figure 36A-B).

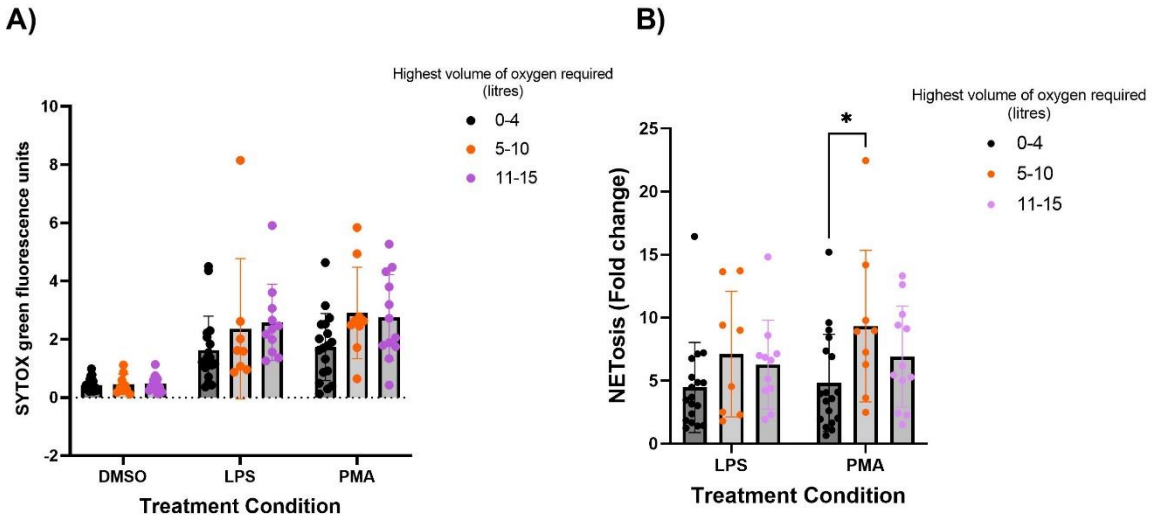


Figure 36-NETosis response in patients requiring different volumes of oxygen therapy.
Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 were stimulated with either LPS [5 µg/ml] or PMA [100 nM] for 3 hours. SYTOX™ Green was added to all wells [555 nM] and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Excitation/emission 490/537 nm was used. Anonymised clinical data was collected for all patients and the raw SYTOX™ Green fluorescent units were stratified based on the highest volume of oxygen that patients required whilst hospitalised. The categories were 0-4 (black), 5-10 (orange), 11-15 (purple) and the units were litres of oxygen. The analysis was repeated using the fold change in NETosis. Fold change was calculated based on the difference in SYTOX™ green values between stimulated neutrophils and the DMSO control. A) There was no significant difference in NET formation at baseline or induced by LPS or PMA when using the raw SYTOX™ units. B)There was a significant increase in PMA-induced NETosis calculated by fold change in patients receiving 5-10 litres of oxygen compared to patients receiving 0-4 litres. These data represent n= 18 (0-4 litres), n=8 & 9 (5-10 litres) and n=11 & 12 (11-15 litres). Statistical analysis was by a mixed effects model with a Bonferroni's post-test and compared neutrophils at baseline (DMSO) to neutrophils stimulated with LPS or PMA in patients receiving different volumes of oxygen. Error bars represent SD.

For clinical parameters with continuous variables, correlations were conducted with the NETosis response, as before. Correlations were made between NETosis and HbA1c (Figure 37A-D), time since symptom onset (Figure 37E-H), CRP (Figure 37I-L), NLR (Figure 37M-P), neutrophil count (Figure 37Q-T) and platelet count (Figure 37U-X). There were no correlations between NETosis in response to either LPS or PMA (raw SYTOX™ green values or fold change in NETosis) with any of the clinical parameters explored.

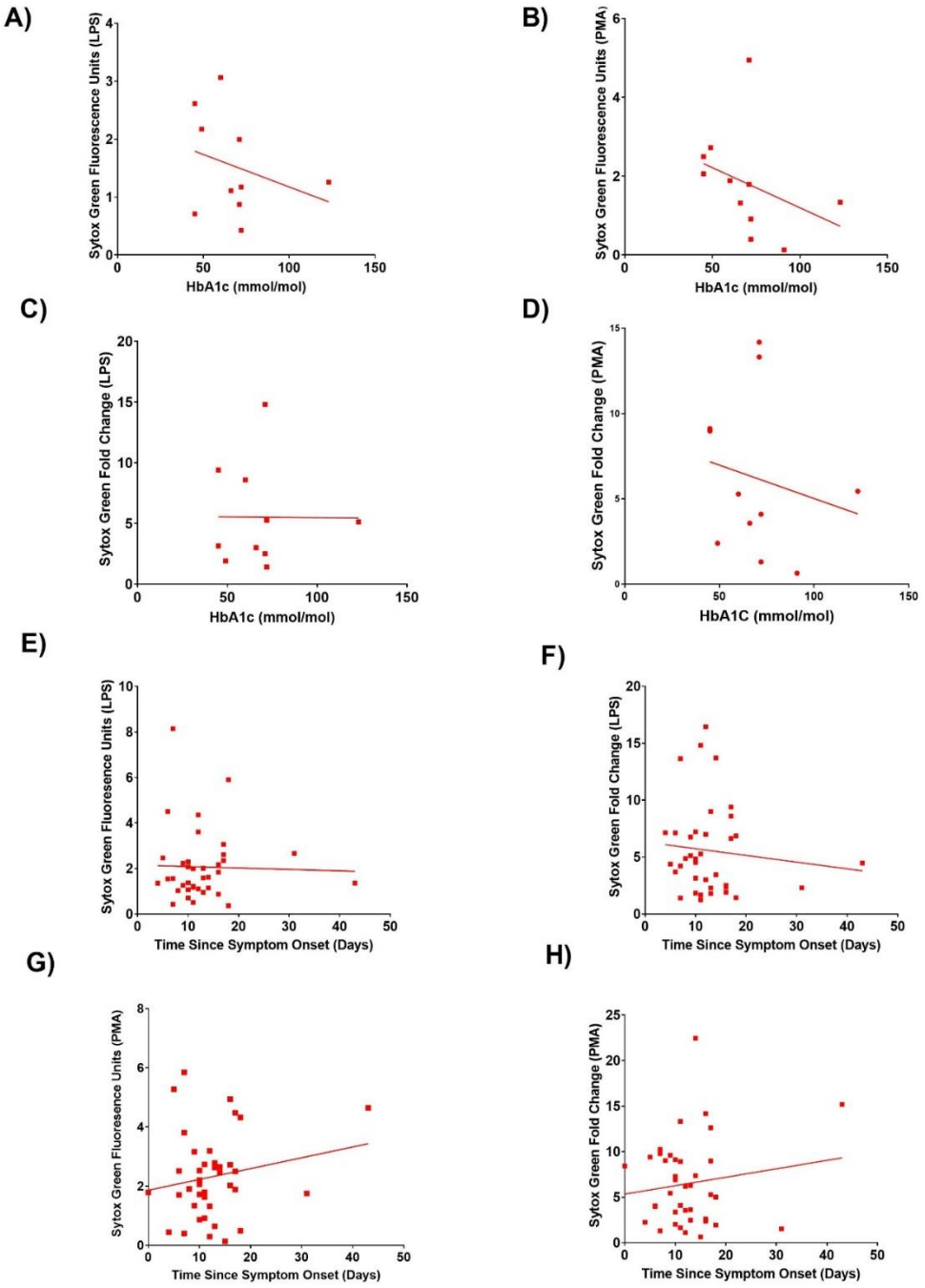


***Figure continued overleaf**

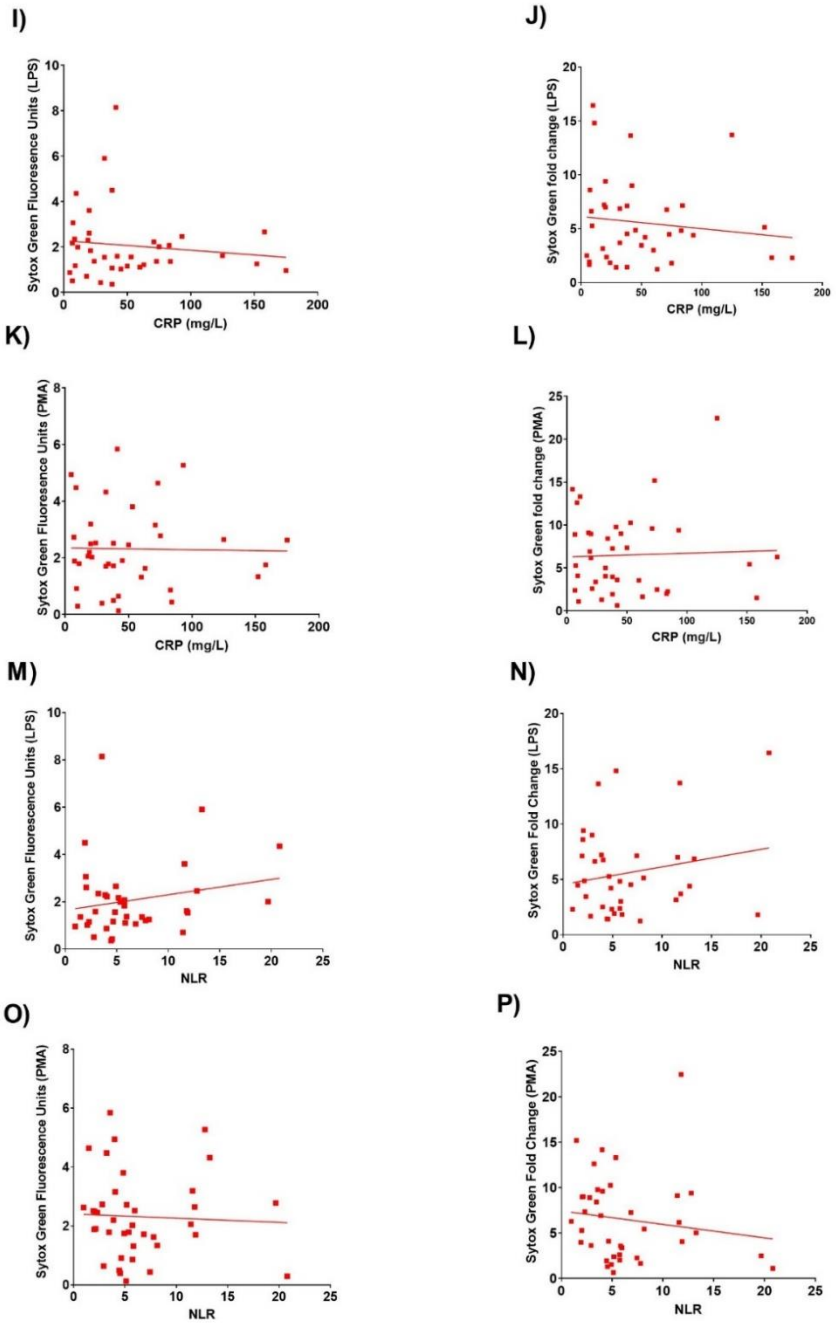


***Figure continued overleaf**

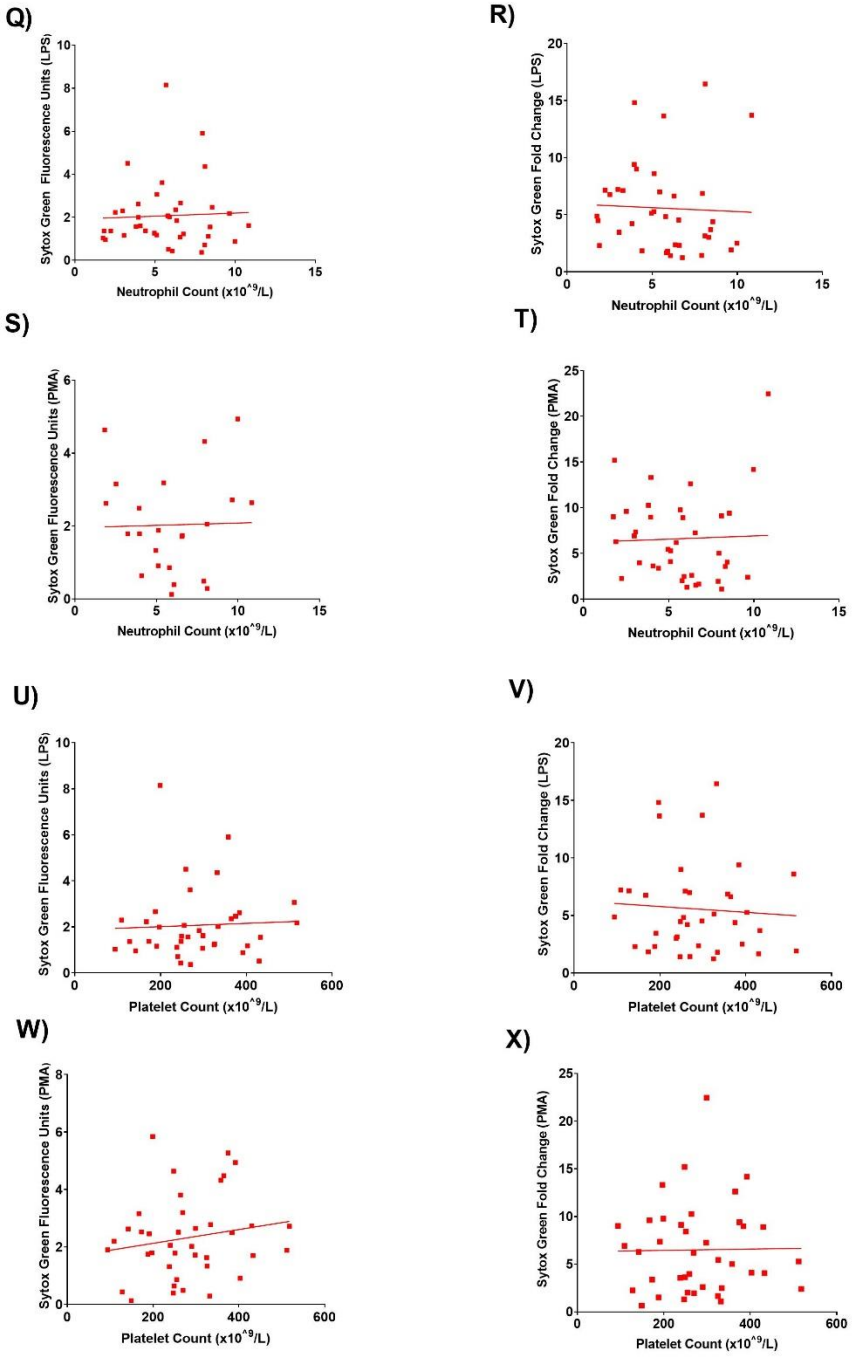


Figure 37-Correlating clinical characteristics of COVID-19 patients with *in vitro* NET formation.
Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 were stimulated with either LPS [5 µg/ml] or PMA [100 nM] for 3 hours. SYTOX™ green was added to all wells (555 nM) and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Excitation/emission 490/537 nm was used. Anonymised clinical data was collected for patients with COVID-19. The raw SYTOX™ green values after LPS or PMA stimulation and the fold change in NET formation was correlated with carefully selected patient characteristics. Fold change was quantified by calculating the difference in the SYTOX™ green values between stimulated neutrophils and the DMSO control. Correlations were conducted between NETosis and **(A-D)** HbA1c (mmol/L), **(E-H)** time since symptom onset (days), **(I-L)** C-reactive protein (CRP) (mg/L). **(M-P)** neutrophil lymphocyte ratio (NLR) (**Q-T)** neutrophil count (x10^9/L), **(U-X)** platelet count (x10^9/L). Data represents n= 10 (LPS) & n= 11 (PMA) for panels **A-D**. All other panels represent n= 37 (LPS) & n=39 (PMA). Lines depict linear regression. The correlation co-efficient and p-value were calculated using Pearson correlation. There were no significant correlations.

5.2.5 Cl-amidine does not inhibit NETosis

In the manuscript it was demonstrated that ruboxistaurin inhibited NETosis. However, there was no significant reduction in NET formation in response to either LPS [5 µg/ml] or PMA [100 nM] in the presence of cl-amidine [200 µM] (Figure 38A-B). These data were not included in the manuscript.

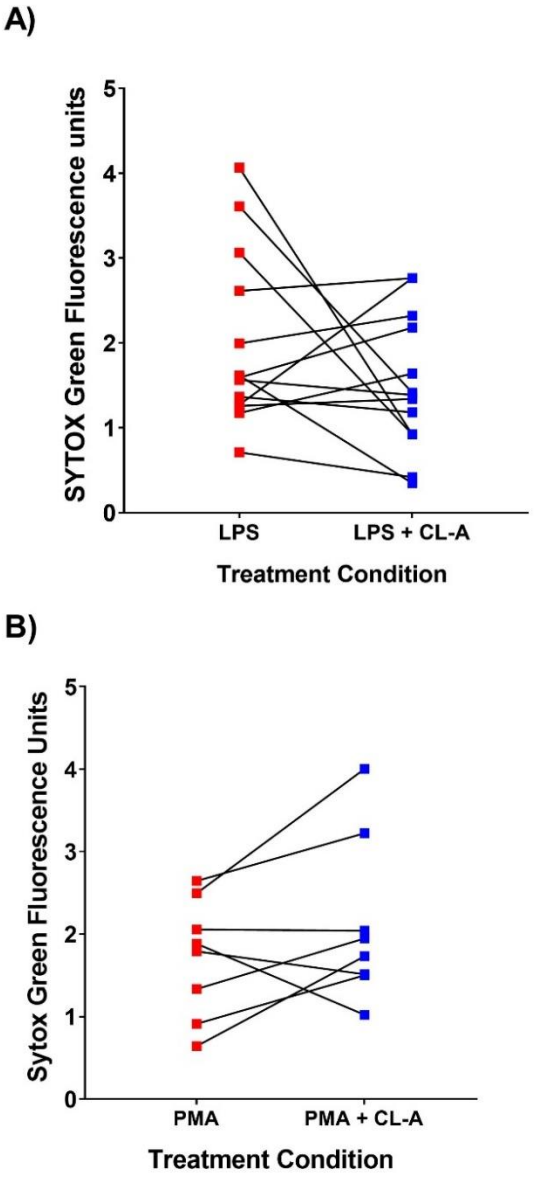


Figure 38-Impact of Cl-amidine on NETosis using COVID-19 patient neutrophils

Neutrophils isolated from peripheral whole blood from hospitalised patients with COVID-19 were pre-incubated with Cl-amidine (CL-A) [200 µM], for 1 hour (blue). Neutrophils were stimulated with either LPS [5 µg/ml] (A) or PMA [100 nM] (B) for a further 3 hours (red). SYTOX™ Green was added to all wells [555 nM] and extracellular DNA (NETs) was quantified using a fluorescent plate reader. Excitation/emission 490/537 nm was used. There was no significant difference in SYTOX™ Green fluorescence in the presence of CL-A. The median values of quadruplicate wells are shown. Lines link individual patients. These data represent n= 13 (A), and n=8 (B). Statistical analysis was by paired t-test.

5.2.6 Impacts of ruboxistaurin on IL-6 production in neutrophils from COVID-19 patients

5.2.6.1 *Rationale for exploring the effects of ruboxistaurin on neutrophil IL-6 gene expression*

PKC-β can regulate gene transcription by phosphorylation of histones (Lim et al., 2015). PKC-β is involved in the activation of NF-κβ and subsequent inflammatory cytokine production, including transcription of TNFα and IL-6, in response to TLR4 and TLR2 activation in neutrophils (Asehnoune et al., 2005). Inhibition of PKC-β using ruboxistaurin decreased the amounts of IL-6 in intestinal cells in a murine model of ischemia and reperfusion injury (Chen et al., 2014). The impacts of ruboxistaurin on IL-6 production in neutrophils has not been explored previously. IL-6 is important in inflammatory signalling mediating COVID-19 disease and IL-6 induces NET formation; therefore, the effect of ruboxistaurin to modify IL-6 gene expression in response to LPS from neutrophils isolated from hospitalised COVID-19 patients was investigated (Joshi et al., 2011; The RECOVERY Collaborative, 2021). Due to the impact of ruboxistaurin on inhibition of NETosis at 3-hours (section 5.2.3.2), gene expression was measured by qPCR at the same time point. These experiments provide only preliminary data, as only n=4 patient samples were analysed. There were challenges in obtaining enough cells from each patient to complete these experiments, as only 5-10 ml of blood per patient was collected.

5.2.6.2 *qPCR results*

Firstly, the specificity of the IL-6 primers was investigated by assessing the qPCR melt-curve. A uniform melt curve, with a single peak was demonstrated for IL-6, demonstrating there was no non-specific binding of the primers or self-annealing (Figure 39A). Next, IL-6 gene expression in unstimulated neutrophils from COVID-19 patients was investigated. This investigation was conducted to determine whether neutrophils from COVID-19 patients contribute to the high levels of IL-6 found in this disease at baseline (Del Valle et al., 2020). There was only a small amount of IL-6 gene amplification in unstimulated neutrophils. The amplification of this gene was detected on average at 35 cycles, with a Ct value for one sample nearly reaching 40 cycles, which is the qPCR endpoint (Figure 39B). Gene amplification detected between 35-40 qPCR cycles typically indicates only a very low amount of RNA is present. Next, the difference in IL-6 gene expression between unstimulated neutrophils and neutrophils stimulated with LPS [5 µg/ml] for 3 hours was explored. LPS stimulates IL-6 gene expression in neutrophils via TLR4 activation of the NF-κβ transcription factor, therefore this analysis was conducted as a quality control step to ensure changes in IL-6 gene expression in neutrophils from COVID-19 patients could be detected (Euler et al., 1998; Marie Moresco et al., 2011; Riedemann et al., 2004). There was a significant increase in IL-6 gene expression in neutrophils stimulated with LPS, supporting previous literature (Figure 39C) (Zimmermann et al., 2015). Statistical analysis was conducted using the ΔCt values, which quantifies the difference in qPCR cycle number between the

gene of interest (IL-6) and the 'housekeeping' gene, which was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The impact of ruboxistaurin on IL-6 expression was compared and there was no significant difference between the IL-6 ΔCt values in the presence of ruboxistaurin in LPS stimulated neutrophils from COVID-19 patients (Figure 39D).

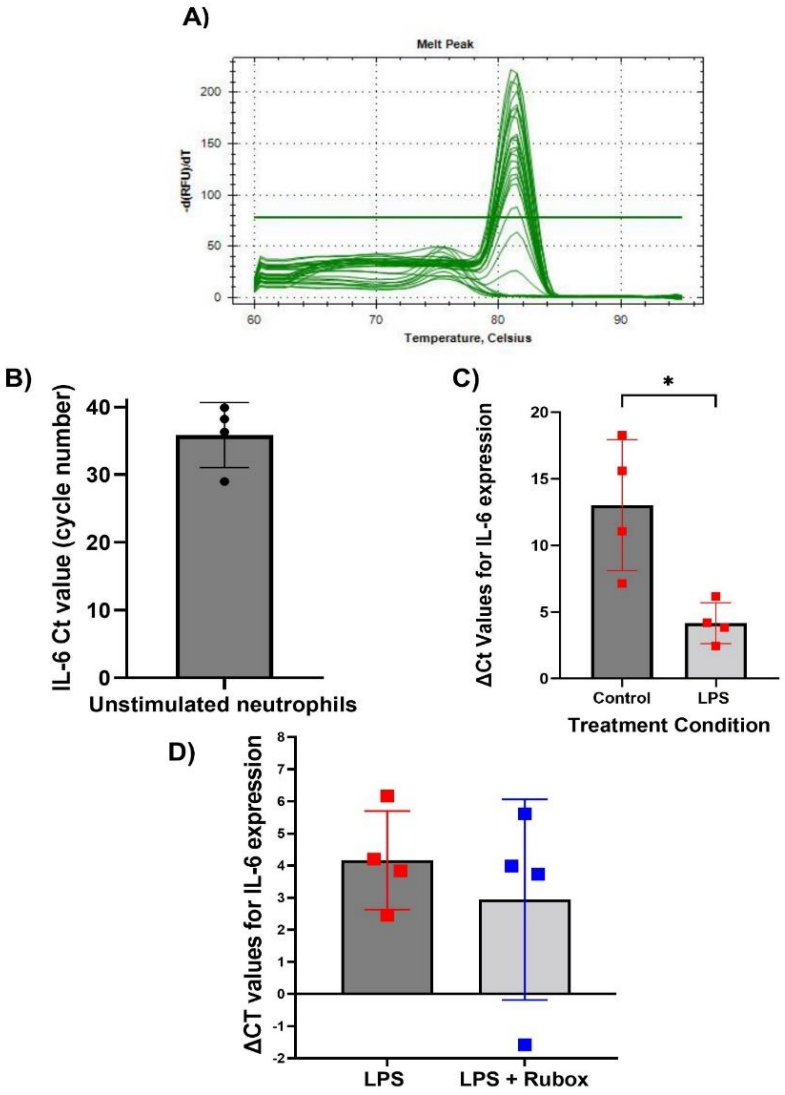


Figure 39-Ruboxistaurin does not impact IL-6 gene expression.

Neutrophils (5 x 10^6) isolated from peripheral whole blood from hospitalised patients with COVID-19 were pre-incubated with ruboxistaurin (Rubox) [200 nM], for 1 hour prior to stimulation, with LPS [5 µg/ml] for 3 hours. Cells were centrifuged (300 *g*, 5 minutes), supernatants removed, and the cell pellet was resuspended in 1 ml of Trizol before storing at -80°C until required. Neutrophil RNA was extracted using the RNeasy Micro Kit (Qiagen) and the TURBO DNase-free™ kit as per the manufacturer's instructions. cDNA was synthesised and RT-qPCR was conducted using SYBR Green Precision®PLUS master mix. The housekeeper gene GAPDH was used. A) A single peak was identified for the IL-6 primer melt curve. B) IL-6 gene amplification occurred at high qPCR cycle numbers (Ct value) in unstimulated neutrophils C) There was a significant increase in IL-6 gene expression in LPS stimulated neutrophils, using the ΔCt values for statistical analysis. D) The ΔCt values for IL-6 gene expression were compared between LPS stimulated neutrophils ± Rubox and there was no significant difference in gene expression. Data represents mean values of triplicate wells. Four patients were analysed across two independent experiments. Statistical analysis was conducted using a paired t-test. Errors bars represent SD. Significance asterisks represent *p< 0.05.

5.2.7 Impacts of ruboxistaurin on neutrophil recruitment to the zebrafish tail fish injury site

The previous experiments in this chapter relied on assessing the impacts of ruboxistaurin on neutrophils *ex vivo.* To further examine the potential for ruboxistaurin as a treatment for aberrant NETosis, *in vivo* data would be required to demonstrate safety and efficacy of the drug in this context. Zebrafish are a well-established *in vivo* model of the innate immune system and neutrophil function and zebrafish neutrophils undergo NETosis in response to a range of stimuli (Isles et al., 2021; Renshaw et al., 2006). Furthermore, zebrafish express PKC-β and as this model was available locally it provided a valuable opportunity to test ruboxistaurin *in vivo* (Williams et al., 2011). Unfortunately, there was not time to explore the effects of ruboxistaurin on NETosis using zebrafish neutrophils *in vivo,* as this required extensive use of specialised reporter fish lines and live imaging microscopy (Isles et al., 2021). Instead, a preliminary investigation exploring the effects of ruboxistaurin on neutrophil recruitment to the tailfin injury site was completed. Tailfin injury is an established model to investigate the zebrafish neutrophil response to inflammation and injury (Isles et al., 2021; Mathias et al., 2006; Renshaw et al., 2006). Although, not directly related to NETosis this experiment was important, as ruboxistaurin has not been used in a zebrafish model previously, therefore it would determine if ruboxistaurin was tolerated by the fish and if this system could be useful for future *in vivo* work.

Transgenic zebrafish larvae (*Danio rerio*) (TgBAC(mpx:EGFP)i114), which have GFP inserted within the neutrophil specific MPO gene promotor, were used (Renshaw et al., 2006). Tail fin transection involves removing the tail fin immediately posterior to the circulatory loop using a scalpel, which was conducted on zebrafish larvae 3 days post fertilisation (d.p.f), by Amy Lewis (Isles et al., 2021; Renshaw et al., 2006). Zebrafish were incubated with either DMSO (0.5%), 200 nM ruboxistaurin or 100 µM ruboxistaurin. DMSO at a concentration of 0.5% is the highest concentration of DMSO that can be tolerated by zebrafish. Ruboxistaurin at a concentration of 200 nM was used as this matched the concentration used to inhibit NETosis in human neutrophils *in vitro* and a concentration of 100 µM ruboxistaurin was trialled as it was the highest concentration of the drug that could be tolerated in the fish without exceeding the 0.5% DMSO limit. After 4 hours and 24 hours fluorescently-labelled neutrophils that had migrated to the tail fin injury site were counted using fluorescent microscopy (Figure 40A). There was a significant decrease in the number of neutrophils at the injury site in larvae treated with 100 µM ruboxistaurin, but not 200 nM, after 4 hours, when compared to the DMSO control (Figure 40B). After 24 hours, there was no significant difference in neutrophil recruitment in the presence of either concentration of ruboxistaurin compared to the DMSO control (Figure 40C). Whole body neutrophils counts were conducted on uninjured zebrafish larvae 3 d.p.f after incubation in 100 µM for 4 hours. This was conducted to determine if the reduction in neutrophils at the tail fin injury site was due to a reduction in the total number of neutrophils in the zebrafish. There was no

difference in the whole-body neutrophil number between zebrafish larvae cultured in either DMSO or 100 µM ruboxistaurin (Figure 40D).

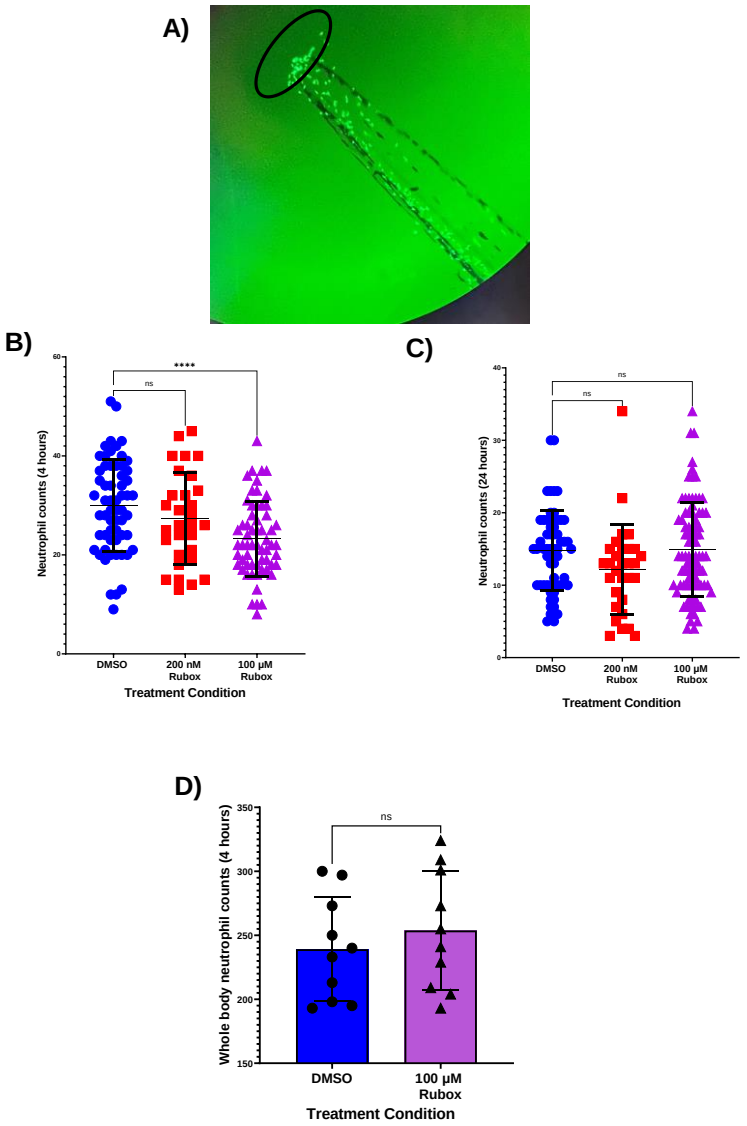


Figure 40-Ruboxistaurin reduces the number neutrophils at the zebrafish tail fin injury site.

Zebrafish larvae (*Danio rerio*) (TgBAC(mpx: EGFP)i114) at 3 days post fertilisation (d.p.f.) were anaesthetised with 4% tricaine in E3 media containing either DMSO [0.5 %] (blue circle) or ruboxistaurin (Rubox) [200 nM or 100 µM] (red square and purple triangle). Tail fin transection was conducted by Amy Lewis, by slicing with a scalpel immediately posterior to the circulatory loop. A) After 4- or 24-hours neutrophils were counted at the tail fin injury site. Imaging used a Leica MDG41 stereo microscope with Leica EL6000 fluorescence light source and an 80x objective lens. The black circle highlights the region of cell counting at the tail fin injury. B) There was a significant decrease in the number of neutrophils recruited to the tail fin injury site in the presence of 100 µM ruboxistaurin after 4 hours. B) There was no difference in neutrophil recruitment to the tail fin injury site after 24 hours. D) Whole body neutrophil counts were conducted in uninjured zebrafish larvae 3 d.p.f incubated in either DMSO or 100 µM ruboxistaurin. There was no significant difference in whole body neutrophil counts. (B-C) Data represents n=2 for DMSO and 100 µM ruboxistaurin condition, using 60 larvae and n=1 for 200 nM ruboxistaurin condition using 30 larvae. D) These data represent n=1 using 15 larvae. Statistical analysis was by a one-way ANOVA with a Dunnett's post-test (B-C), using DMSO as the control and an unpaired t-test was conducted for panel (D). Significance asterisks represent ****P< 0.0001 and (ns) denotes not significant. Error bars represent SD.

5.3 Discussion

5.3.1 LPS induced NETosis is increased in patients in hospitalised patients with COVID-19

In the manuscript, 'Enhanced neutrophil extracellular trap formation in COVID-19 is inhibited by the PKC inhibitor ruboxistaurin' (section 5.2.3.2), we discussed the findings of elevated NETosis in response to LPS in hospitalised patients with COVID-19 and how this was inhibitable by the PKC-β inhibitor ruboxistaurin. This finding provides further evidence of hyperactivation of neutrophils in this disease. Neutrophils in the circulation are quiescent and become primed when they encounter a pro-inflammatory stimulus such as TNFα (Vogt et al., 2018). Primed neutrophils generate a much greater neutrophil response, including increased ROS production and degranulation, on subsequent activation (Vogt et al., 2018). The increased NET response to LPS when using neutrophils from COVID-19 patients *in vitro,* could signify priming of neutrophil TLR4 signalling in COVID-19, which is the key pro-inflammatory neutrophil signalling pathway induced by LPS (Fan et al., 2020; Hoshino et al., 1999). TLR4 signalling mediates key neutrophil effector mechanisms including apoptosis, ROS production and pro-inflammatory cytokine generation (Prince et al., 2011; Sabroe et al., 2002; Sabroe et al. 2003). TLR4 signalling occurs by two main pathways, which are mediated by signalling adaptor proteins (El-Zayat et al., 2019; Parker et al. 2007; Sabroe et al. 2003). These pathways are the myeloid differentiation primary-response protein (MyD88)- dependent pathway and the TIR domain-contain adaptor protein inducing interferon-β (TRIF) pathway (El-Zayat et al., 2019; Parker et al. 2007; Sabroe et al. 2003). TLR4 signalling initiates ROS production, which is required for NOX-dependent NETosis, via interleukin-1 receptor-associated kinase-4 (IRAK-4), which is a downstream signalling molecule in the MyD88-dependent pathway (Pacquelet et al., 2007; Parker et al. 2007; Sabroe et al. 2003). SARS-CoV-2 spike protein binds to TLR4 (Zhao et al., 2021) and this could provide a potential mechanism for the increased NETosis found in COVID-19. TLR4 signalling can also be initiated by other damage associated molecular patterns (DAMPs), such as heat shock proteins and alarmins (Land, 2021; Wheeler et al., 2009). These are released from virally infected cells and may further prime neutrophils to undergo NETosis (Land, 2021; Wheeler et al., 2009). TLR4 signalling has emerged as a key pathway contributing to the cytokine storm in COVID-19 by activation of NF-κB and TLR4 antagonists are under investigation for reducing inflammation in COVID-19 (Kaushik et al., 2021; Sohn et al., 2020). The data in this chapter suggests that TLR4 signalling is also potentially important in NETosis in COVID-19. LPS induces NOX-dependent lytic NETosis via c-Jun N-terminal kinase (JNK) signalling, which acts as a molecular switch determining the cell-fate between apoptosis and NETosis (Khan et al., 2017). NETosis occurs in response to higher concentration of LPS than those traditionally used to activate neutrophils (> 1μg/ml) (Dick et al., 2009; Khan et al., 2017). I demonstrated that PAD4 was not critical in the elevated LPS-stimulated NETosis pathway in COVID-19, as cl-amidine, a pan-PAD enzyme inhibitor, did not reduce NETosis in either COVID-19 patient neutrophils or healthy donor cells. These data support

previous literature, demonstrating the LPS-induced NETosis can proceed in PAD-deficient murine neutrophils.

Another reason for elevated NETosis in COVID-19 could be due to an increased proportion of low density neutrophils (LDNs) found in these patients (Carissimo et al., 2020; Morrissey et al., 2021). LDNs are a subset of neutrophils with an 'immature' band-like nuclear structure, which have a pro-inflammatory phenotype (Morrissey et al., 2021; Tay et al., 2020). In one recent study, LDNs were found to represent 35% of the neutrophil population in patients with severe COVID-19 admitted to ITU (Morrissey et al., 2021). LDNs undergo increased NET formation and are associated with COVID-19 disease severity (Carissimo et al., 2020; Morrissey et al., 2021). If there was more time and access to patient blood samples, determining whether the elevated NETosis found in this study was due to an increased amount of LDNs, would have been useful. To conduct these experiments, LDNs would be separated from mature neutrophils using histopaque-1077 (Sagiv et al., 2015). Then, the NETosis assay would be conducted on both sets of neutrophils simultaneously to determine if the LDNs produced more NETs than mature neutrophils. Whole blood would be stained for neutrophil cell surface markers for mature and LDN sub-types to determine the percentage of cells making up the LDN compartment, using flow cytometry. Staining for cell surface marker expression would use a fluorescently labelled CD66b antibody, to detect all neutrophils in the blood and then staining for CD16, which is a cell surface marker of immature neutrophils (Morrissey et al., 2021). This would determine the percentage number of granulocytes that were LDNs, which would be important to determine if NET formation from this cell type could have biologically meaningful effects.

At the end of the study anonymised clinical data was provided for all patients by clinical collaborators within the UK-CIC at the UoS. Exploratory analysis to determine whether there was an association between patient demographics and the elevated NETosis response was completed. These data could be used to conduct power calculations to inform future research. A range of patient characteristics were carefully selected to correlate with the NETosis response. An aim of this study was to investigate whether those with diabetes and COVID-19 produced more NETs than those without diabetes. This hypothesis was based on the previous PhD work conducted before the pandemic, where I had studied the previous literature surrounding the pro-NETotic phenotype in diabetes (Fadini et al., 2016; Menegazzo et al., 2015; Wong et al., 2015). Those with diabetes are at an enhanced risk of severe COVID-19 disease and 14/39 patients had T1D, T2D or pre-diabetes. However, there was no difference in NETosis in COVID-19 patients with and without diabetes. Those with diabetes can have a range of complications including cardiovascular disease, nephropathy and obesity which could contribute to the susceptibility to severe COVID-19 disease (Apicella et al., 2020). Markers of NETosis (cell-free DNA and DNA-MPO complex) in the sera of COVID-19 patients, were shown to correlate with platelet count,

neutrophil count and CRP in a previous study of hospitalised patients with COVID-19 (Zuo et al., 2020). However, there was no correlation between the *in vitro* NETosis response and those same clinical parameters in this study. A reason for this discrepancy could be due to difference in disease severity between the two studies. There were no patients mechanically ventilated in my study, however 32% of patients received mechanical ventilation in the study by Zuo et al. (2020). Mechanical ventilation induced NETosis in a rodent model of ventilator-induced lung injury, which could account for this difference (Li and Pan, 2016; Yildiz et al., 2015). Interestingly, ventilator-induced lung injury was dependent on TLR4 signalling in this model, supporting the importance of this signalling pathway in NETosis (Li and Pan, 2016). The highest amount of oxygen required was the only patient demographic, which had an association with elevated NETosis. Those receiving 5-10 litres of oxygen produced significantly more NETs in response to PMA, compared to patients receiving 0-4 litres. However, there was no difference in patients receiving 11-15 litres. This is interesting as respiratory failure is a serious complication in severe COVID-19 disease, and oxygen requirement is a marker of disease severity (Turcotte et al., 2020; Xie et al., 2020). However, the reason why elevated NETosis was associated with the mid-range maximal oxygen volume and not 11-15 litres is unknown, also as this was only seen in response to neutrophil stimulation with PMA and not LPS these findings should not be over interpretated.

5.3.2 Ruboxistaurin is a potential therapeutic for targeting NETs in COVID-19

Elevated LPS induced NETosis was significantly reduced by inhibition of ROS production, using DPI, supporting previous research (Khan et al., 2017). These data supported the rationale to use a specific PKC-β inhibitor, ruboxistaurin to inhibit LPS induced NETosis, which we demonstrated to be a successful strategy in modulating this pathway *in vitro*. The main role of PKC-β in neutrophils is phosphorylation of the $p47^{phox}$ subunit of the NADPH oxidase complex, activating its formation at the membrane and production of ROS (Cosentino-Gomes et al., 2012; Fontayne et al., 2002). PKC-β has shown to be important for mediating neutrophil ROS production and ROS-dependent NETosis (Gray et al., 2013). PKC-β is activated in response to TLR4 signalling, supporting the use of ruboxistaurin to inhibit LPS-mediated NETosis (Asehnoune et al., 2005). Ruboxistaurin is a highly selective competitive inhibitor of the ATP binding site for the PKC-β isoform, with a half maximal inhibitory concentration 50 (IC_{50}) of 4.7 nM (PKC-βI) and 5.4 nM (PKC-βII) (Jirousek et al., 1996). There was at least a 50-fold increase in the IC_{50} of ruboxistaurin needed to inhibit to other PKC isoforms (Jirousek et al., 1996). Importantly, ruboxistaurin is highly selective for the PKC family of enzymes. The activity of ruboxistaurin has been tested in a panel of kinases previously. Activity towards other kinases required a 300-2000 fold increase in the IC_{50} to partially inhibit kinases including PKA, src tyrosine kinase and AMP-activated kinase (Jirousek et al., 1996; Komander et al., 2004). This is not unexpected as PKC

inhibitors are known to have off-target effects at high concentrations, which is why the development of PKC inhibitors with high potency, such as ruboxistaurin, have been important in developing potential new therapeutic inhibitors (Davies et al., 2000). The safety of ruboxistaurin in patients has been established in phase three clinical trials for diabetic retinopathy and inhibiting NETosis would be a new area in which to trial this drug (Aiello et al., 2006; PKC-DRS Study Group, 2005). Using ruboxistaurin at 200 nM to inhibit NETosis is in line with a previous study using human neutrophils *in vitro* (Gray et al., 2013). Also, previous pharmacokinetic studies demonstrate that the maximum serum concentration (Cmax) of ruboxistaurin after a single dose of 32 mg in healthy human volunteers (n=233) reached an average concentration of 86.2 nmol/L, with a range of 36.4-199 nmol/L (European Medicines Agency, 2007). This means that the concentration used to inhibit NETosis *in vitro* was not excessively higher than that present in the serum after oral dosing of the drug, providing support that ruboxistaurin may be useful for inhibiting NETosis *in vivo*. Also other isoforms of PKC are not inhibited by ruboxistaurin at this concentration (Jirousek et al., 1996). Therapies targeting PKC in neutrophils to modify inflammation in COVID-19 has not been explored previously, despite transcriptomic analysis demonstrating that the gene encoding PKC (*PRKC*) was elevated in severe COVID-19 patients (Aschenbrenner et al., 2021). The data in this chapter demonstrates the potential use of ruboxistaurin to modify NETosis in COVID-19.

To explore the wider impacts of ruboxistaurin on neutrophil function the impact of ruboxistaurin treatment on *IL-6* expression by neutrophils from COVID-19 patients stimulated with LPS was investigated. IL-6 is key pleiotropic cytokine, which plays a central role in the host immune response to infection and in the cytokine storm displayed in severe COVID-19 (Hirano, 2021; Chi Zhang et al., 2020). In response to TLR4 and TLR2 activation in neutrophils, PKC-β was shown activate NF-κB causing subsequent inflammatory cytokine production, including expression of IL-6 (Asehnoune et al., 2005). However, ruboxistaurin was without effect on IL-6 production in COVID-19 neutrophils. The reason for this finding is unknown and would require further experimentation to explore whether this was due to the timepoint post-stimulation with LPS that was investigated (3 hours), the use of a high concentration of LPS to induce NETosis or the concentration of ruboxistaurin used. Further experiments would be required to conclude if PKC-β was important for IL-6 production.

5.3.2.1 *Using zebrafish to investigate the impacts of ruboxistaurin on neutrophils in vivo*

To develop a therapy for potential future use in patients, it must undergo rigorous experimentation and testing. The impacts of ruboxistaurin on neutrophils in an *in vivo* zebrafish tail injury model were examined, as a next step to further explore ruboxistaurin for treating aberrant NETosis, and to build upon the *in vitro* data generated. This was a first exploratory step to determine if ruboxistaurin could

be tolerated by zebrafish *in vivo* and if ruboxistaurin impacted the neutrophil response to injury. Ruboxistaurin at a concentration of 100 µM significantly reduced recruitment of neutrophils to the tail injury site after 4 hours and it was not lethal to the fish after 24 hours. This high concentration was selected as it represented the upper limit of solvent that the zebrafish could tolerate. PKC-β activates protein kinase D, which is required for actin rearrangement in neutrophil chemotaxis (Xu et al., 2015). However, at these high concentrations ruboxistaurin may be having off-target effects, therefore the reduction in chemotaxis may not be due to PKC-β inhibition. There was no impact of ruboxistaurin at 200 nM on neutrophil recruitment *in vivo*, which could be due to the differences in drug concentration needed to inhibit chemotaxis compared to NETosis and the effects on potency due to absorption and distribution in an *in vivo* model. To my knowledge, these are the first data using zebrafish as a model to study the effects of ruboxistaurin on cell function *in vivo*, with most previous studies using rodents (Das et al., 2018; Koya et al., 2000). Zebrafish neutrophils undergo NETosis in response to a range of stimuli, therefore these data confirm that the utilisation of this model could be effective for further exploration of NET inhibition by ruboxistaurin in future work (Isles et al., 2021).

5.3.3 Targeting neutrophil serine proteases in COVID-19 does not reduce NETosis

Preliminary clinical data from the STOP-COVID clinical trial demonstrates that brensocatib treatment did not improve clinical outcomes (https://stop-covid19.org.uk/results/) [Accessed 20th January 2022]. Full trial data are not yet publicly available. COVID-19 patients receiving brensocatib generated a similar amount of NETs to patients receiving the placebo drug *in vitro*. These data show that preventing the activation of NSPs does not inhibit the ability of neutrophils to generate NETs, which does not support the generally accepted mechanism of NETosis. However, recent research shows that NSPs were not required for NETosis, challenging the current understanding of the NETosis pathway (Kasperkiewicz et al., 2020). Preliminary data from the trial found that circulating levels of NSPs were significantly lower in the brensocatib treatment group (https://stop-covid19.org.uk/results/) [Accessed 20th January 2022]. These data could demonstrate that inactivating NSPs, which are abundant NET proteins, is not an effective therapeutic strategy for NET-mediated pathologies. It is possible that aiming to inhibit the impacts of NETs once they have already formed is not effective at reducing inflammation and preventing the formation of NETs would be a better approach. NETs cause host tissue damage, pro-inflammatory cytokine production, and activate platelets, which can in turn induce more NETosis, causing a positive feedback loop (Apel et al., 2021; Kahlenberg et al., 2013; Yaqinuddin, 2020; Zucoloto and Jenne, 2019). Seeking to modulate the NET once it is formed may not be as useful as preventing its production. Also, NSPs are not the only cytotoxic proteins found on NETs; MPO and histones are also highly abundant and mediate tissue damage and inflammation (Saffarzadeh et al., 2012; Silk et al., 2017). Furthermore, there was no difference in NET formation

between day 1 and day 29 of the trial, which is interesting in the context of the findings from the UK-CIC study where there was a significant decrease in NET formation 3-4 months post-acute sampling. This could indicate that there are longer lasting effects on NET formation after acute COVID-19 disease, which could last between 1-3 months post disease. These data support the rationale for future work to investigate the NET phenotype in the context of long COVID. Whether elevated NETosis plays a role in long COVID is unknown, and this would be a very important area to explore, as there are no treatments for the long term sequalae of COVID-19.

The wider impacts of brensocatib treatment on neutrophil activation were explored by investigating neutrophil cell surface marker expression. These data were preliminary and cannot be used to draw definitive conclusions from the trial, as only those data generated in Sheffield are included in these analyses. Nonetheless, brensocatib did not impact neutrophil activation in this limited dataset, which corroborates with the lack of change in NETosis. However, these data demonstrated interesting observations regarding the change in neutrophil activation over time in COVID-19. There was a significant decrease in neutrophils isolated from patients in the placebo group for neutrophil activation marker CD66b and marker of degranulation CD63 at day 29 compared to day 1. These data support the pro-inflammatory and hyperactivated neutrophil gene signature in acute COVID-19 (Aschenbrenner et al., 2021; Parackova et al., 2020).

When targeting neutrophils in COVID-19, it was important to determine the wider implications of treatment on neutrophil effector functions, as secondary bacterial infections are found in 12-35% COVID-19 patients (Russell et al., 2021; Shafran et al., 2021). Brensocatib treatment did not impact neutrophil phagocytosis. Interestingly, in both the placebo and treatment groups there was a significant increase in phagocytosis between day 1 and day 29, suggesting that neutrophil pathogen handling is compromised in patients with COVID-19. In-depth functional neutrophil phenotyping studies of patients with COVID-19 are yet to be published, however a study currently in pre-print also demonstrated reduced neutrophil phagocytosis and elevated NETosis in hospitalised patients with COVID-19 *in vitro*, supporting the findings of these data in this chapter (Belchamber et al., 2021).

5.3.4 Study Limitations and future work

The key limitation about the work is regarding the potential effectiveness of ruboxistaurin to target NETosis in COVID-19. Cell based *in vitro* experiments are often the first step in a long process required to develop a new treatment, therefore the findings in this chapter demonstrate only a preliminary insight into the potential use of ruboxistaurin to target NETosis in COVID-19 and other lung diseases. To address this limitation future work should investigate NETosis inhibition in a rodent model of SARS-CoV-2 infection and acute lung injury. These investigations would provide proof-of-concept data regarding the safety and efficacy of ruboxistaurin. An advantage of the potential future use of

ruboxistaurin is that it has undergone rigorous clinical safety testing in animals and humans previously, meaning important information regarding drug absorption and metabolism is already known. The safety of ruboxistaurin has been examined in 11 placebo controlled clinical trials and 22 clinical pharmacology studies and was concluded to be safe and well-tolerated for use in patients (McGill et al., 2006). However, ruboxistaurin has not been trialled in the context of acute respiratory lung disease, therefore safety testing in this sub-group patients would be needed. There were some adverse drug reactions detected at a significantly higher amounts in ruboxistaurin treated patients compared to placebo in previous trials which included; dyspepsia (4.3%), skin discolouration (0.5%), elevated creatinine phosphokinase (1%) and superficial thrombosis (0.4%), although these reactions were noted to not preclude the future use of ruboxistaurin in patients (McGill et al., 2006).

Furthermore, I have investigated the impacts of ruboxistaurin predominantly in the context of aberrant NETosis. A key issue with the use of kinase inhibitors is that they are ubiquitous signalling enzymes playing a role in a number of cell mechanisms, throughout multiple different cell types in the body (Kolczynska et al., 2020). The impacts of ruboxistaurin on a range of neutrophil functions are needed to explore the potential effects of ruboxistaurin on other pathways. In the manuscript (section 5.2.3.2) Rebecca Hull demonstrated that ruboxistaurin did not impact intracellular killing of *S. aureus* by neutrophils from COVID-19 patients, however this was only a small n and warrants additional research.

To conclude, in this chapter I have demonstrated that NETosis is elevated in response to LPS in hospitalised patients with COVID-19 in the acute stage of infection. This elevated NETosis response may indicate neutrophil priming to undergo activation by TLR4 stimulation in COVID-19, which is interesting as the SARS-CoV-2 spike protein has shown to interact with this receptor. The results from my study and those of the STOP-COVID clinical trial demonstrate that neutrophil function is altered in COVID-19 disease. However, targeting NSP activation did not reduce NET formation or improve clinical outcomes in patients. These data suggest that preventing NET formation may be a better therapeutic strategy to target NETosis, and we show that ruboxistaurin inhibits elevated LPS induced NETosis in COVID-19. To take this research forward future work should explore if ruboxistaurin can inhibit NETosis mediated by SARS-CoV-2, which will be critical in defining whether ruboxistaurin will be a future useful treatment for COVID-19.

6 Discussion

6.1 Summary of findings

This thesis aimed to understand how neutrophil function is altered in acute and chronic inflammation and to identify aberrant cell death pathways that could be targeted with existing therapeutics to improve the host immune response to infection. Initially, neutrophil function in the context of DFD was investigated, due to the prevalence of chronic infections in this cohort and based on the existing literature demonstrating that neutrophil function is changed in people with diabetes (Das et al., 2018; Dowey et al., 2021). The neutrophil phenotype in people with DFD has not been fully defined. In optimisation experiments for the study it was demonstrated that the neutrophil cell death pathways NETosis and apoptosis, were altered by transient changes in glucose concentration in the cell culture media, when using cells from healthy donors. NETosis was increased in high glucose conditions, supporting previous research (Rodríguez-Espinosa et al., 2015). NETosis is associated with the immunopathology in a range of diseases including diabetes, COPD, cystic fibrosis, and rheumatoid arthritis. After the suspension of the diabetes patient study, the rest of this thesis focused on NETosis and how it could be modulated *in vitro,* as NETosis is a dysfunctional pathway common to many diseases. The mechanism of NETosis is not fully elucidated and conflicting literature exists regarding the role of transcription to facilitate NETosis. A transcriptomics project was conducted to address this, as identifying a unique NETosis 'transcriptional fingerprint' could identify new therapeutic strategies to inhibits this pathway. However, the genes upregulated in NETosis were predominantly global transcriptional regulators (*EGR1*, *EGR3*, *NR4A3*), that were also upregulated in response to pro-inflammatory agents not associated with induction of NETosis. Based on these findings I concluded that targeting gene transcription would not be a useful therapeutic strategy to modify NETosis. In the final part of the thesis, NETosis was investigated in hospitalised patients with COVID-19 as part of the UK-CIC. NETs play a role in tissue damage and thrombosis, contributing to the development of ARDS in COVID-19 (Hazeldine and Lord, 2021). NETosis was significantly elevated in patients compared to healthy controls in response to LPS *ex vivo*, which was higher at the acute stage of infection compared to 3-4 months later. Furthermore, elevated NETosis was significantly reduced by the PKC-β inhibitor ruboxistaurin, but dexamethasone was without effect on NETosis. I also conducted assays of neutrophil function for the national STOP-COVID clinical trial, and in combination with the data generated by the team at the UoD I showed that inactivation of NSPs using brensocatib did not impact neutrophil function, including NETosis. However, these data showed temporal changes in neutrophil function in COVID-19 patients between day 1 and day 29 of the trial, with neutrophil activation marker CD66b and marker of degranulation CD63 reduced in the placebo group at day 29.

6.2 Implications of these findings for the targeting of neutrophilic inflammation in disease

The work in this thesis showed for the first time that NETosis in response to LPS was significantly increased in patients with COVID-19, which was inhibited by ruboxistaurin *ex vivo*. These findings could have important implications in the future development of therapies to target aberrant NETosis. Despite an effective vaccine programme in the UK, hospitalisations and death still occur due to COVID-19, highlighting the unmet need for better therapies. Targeting PKC-β in neutrophils effectively reduced NET formation, which has not been explored in COVID-19 before and is a novel therapeutic strategy to target inflammation in this disease. The advantage of ruboxistaurin is that it was safe in humans in phase 3 clinical trials for diabetic retinopathy, therefore reducing the likelihood of toxicity being a barrier to the approval of this drug for treating COVID-19. To progress this work and address a key limitation of this research the efficacy of ruboxistaurin should be tested in response to live SARS-CoV-2. This could be done by both assessing the effects of ruboxistaurin to inhibit SARS-CoV-2 mediated NETosis *in vitro* and investigating the efficacy of ruboxistaurin in a rodent model of infection. This would provide important efficacy and safety data required for the development of human trials of ruboxistaurin. Furthermore, aberrant NETosis is not limited to COVID-19 and previous research showed that ruboxistaurin reduced NETs and improved would healing in a rodent model of diabetes (Das et al., 2018). Using ruboxistaurin to target aberrant NETosis may be useful in targeting neutrophilic inflammation in a range of diseases. Elevated LPS induced NETosis in COVID-19, provides evidence for the role of TLR4 hyperactivation in this disease. TLR4 has emerged as an important receptor mediating the cytokine storm in COVID-19 (Aboudounya and Heads, 2021). These findings support the use of TLR4 antagonists in COVID-19, which are suggested to be a novel therapeutic strategy to target inflammation in COVID-19 and have been trialled in other diseases such as sepsis (Aboudounya and Heads, 2021). The results from the STOP-COVID clinical trial showed that inactivation of NSPs using brensocatib did not reduce NETosis *ex vivo* and preliminary trial data showed brensocatib did not improve clinical outcomes in patients (unpublished) (STOP-COVID19, 2021). These findings demonstrate that targeting the formation of NETs may be a more effective therapeutic strategy than modulating the protease activity of the NETs once they have already formed.

6.3 Conclusion

Neutrophilic inflammation is implicated in a wide range of diseases. NETosis has emerged as a key mediator exacerbating inflammation and tissue damage. The work in this thesis shows that NETosis is elevated in COVID-19 and targeting PKC-β using ruboxistaurin is novel therapeutic strategy to reduce inflammation and NETosis in this disease. Targeting the neutrophil response to SARS-CoV-2 infection could reduce the development of severe complications such as ARDS.

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8 Appendix

8.1 Appendix 1- PIS for diabetes study

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The Control of Innate Immunity, Host-Pathogen Interactions, and Leukocyte Function in Disease: Information Sheet

You are invited to take part in a research study. Before you decide whether or not you wish to take part it is important for you to understand why the study is being done and what it will involve if you agree to take part. Please read the following information carefully. Discuss it with your friends and relatives if you wish. Ask us if there is anything you don't understand or if you would like more information. You will be given as much time as you want to make a decision.

What is the purpose of the study?

We want to find out how some inflammatory diseases are caused, in particular how the white blood cells that can both cause and fight disease are actually controlled. These studies will have significant future benefit for a wide range of patients, e.g. those suffering from asthma, heart disease, diabetes, or arthritis, as we may help to develop new treatments for troublesome human diseases. We are looking at both the genetic and cellular ways that these cells are controlled. We have made various discoveries about how the immune system works, and want to see if work we have done in people without any illnesses also applies to people who have one or more illnesses.

Why have I been invited?

You will have been invited to take part in this study either because you have an illness that we want to study, or because you are healthy and of a similar age group to the people with illnesses, and can act as a reference (also called a control) in comparison. We need people with a variety of illnesses to give us understand what is different between people without illnesses and people who have illnesses. To understand how the immune system is different in people with illnesses we need to take blood and study it in the laboratory. We are asking if you would be prepared to donate blood

for research, as part of a pool of donors to help us with a range of projects as needed. If you feel you would not like to take part for any reason, or you are worried that you may not be eligible, then you do not have to proceed with the study, nor do you have to give a reason for why you don't want to take part. If you have any questions about taking part, please ask the doctor who suggested you might be interested.

Who is organising the study?

Doctors and scientists in the University of Sheffield and Sheffield Teaching Hospitals NHS Foundation Trust. Research carried out using these samples may be funded by the pharmaceutical industry or other organisations.

The study has been reviewed and approved by the Cambridge South Research Ethics Committee.

What will happen to me if I take part?

You will be given time to carefully consider if you wish to take part in this study, and to ask any further questions that you might have.

If you agreed to take part, we would contact you to arrange for you to come to the Royal Hallamshire Hospital to give blood. We will ask you some screening questions to confirm your current health. If you have been chosen for the study because you are in good health, we will not look at your clinical records. If you have an illness, we are asking for permission for one of the lead doctors involved in this study (or an existing member of the team caring for you) to collect information about you, to make sure that we know all that is necessary to help us work out what the results we get might mean. In particular, we would be collecting data such as your age, your illnesses, their severity, how long you have had them, and any treatment you might be taking. We would collect this information both from yourself and your hospital clinical records. We will not be collecting results from your GP records.

We would keep the information we learn about you confidential and private. We would use it to help us understand the results we get, so the research team which includes laboratory researchers may learn what sort of illness you have and other details about you, and would keep this information confidential. Any results that we make public will be entirely anonymous.

On the day of study, you will be asked to give a blood sample of between 5 ml and 150 ml in volume (about 35 – 75 ml is typical). The maximum volume (150 ml) is about a quarter of what you might give if you were giving to the blood transfusion service. The blood would be taken either in our University of Sheffield phlebotomy suite located on K floor of the Royal Hallamshire Hospital or the Clinical Research Facility (CRF) located on O floor of the hospital, either by a doctor or by another member of the laboratory team who is qualified to take blood. You would be likely to meet some of the researchers who will be using your blood, and who would be happy to tell you exactly what they plan to do with your blood if you would like to know.

We will use your blood to purify the white blood cells and/or the fluids it contains, and to then see how they work. Sometimes it will be useful to study the genes that control the function of these white blood cells, and so we will also sometimes separate your genetic material (DNA) from all of the other constituents of the blood cells. Your blood and DNA will not be used for any other purposes and will be destroyed at the end of the study. The white blood cells will only be kept for a maximum of six weeks. The samples will be tested in a range of ways that look at how the immune system functions, for example seeing how blood cells kill bacteria, or control immune responses to viruses. The work will help us understand the way that responses to infection are controlled and why this

may go wrong in people with illnesses. Samples will be studied in the research laboratories in the University of Sheffield, and will be stored anonymously in these laboratories.

We sometimes want to look to see if illness changes the immune response by affecting DNA. Also, if any individual we study has an unusual immune response, we might want to look at the DNA to understand why this is. We do not expect to ever find DNA information that would be important for you or your family, and would not expect to tell you of the results of any DNA work we do.

We may ask you to give blood on another occasion. We would like to build up a pool of blood donors who can give us blood from time to time. The exact number of donations would depend on you. Repeated donations are entirely voluntary and if you only wish to give blood once, that is fine. We will not take more than 500 ml every 12 months, which is about half of the amount you might give if you were going to the transfusion service. If you decide after one or more donations that you would like to be taken off the donor list just tell us and we won't ask again. After three years we will take you off the list anyway.

Samples will be studied in our own laboratories, and might also be studied in other locations (for example, if we are working on a project in collaboration with another research lab or the pharmaceutical industry). We would only share samples as part of this study, and not for research outside the scope of that which is described here. Any information about you (such as your illness and treatments) would be shared in a strictly anonymous way, so that no features that could identify you would be shared.

What will happen to the DNA and information resulting from the study?

DNA, or any other sample derived from your blood and stored for analysis, will be destroyed after 5 years. We do not expect that this study will change your own care, but may help develop new treatments for others in the future. Accordingly, we will not be able to return any individual results about your own cells or DNA to you.There is a chance that this study could lead to a commercially valuable discovery and that we may seek a patent for this. A patent is a monopoly right to the exclusive use of an invention, such as a new test for diagnosis or a new drug treatment. This means that if a pharmaceutical company were to develop a test useful in the treatment of inflammatory diseases, or a new treatment, as a result of this research and took out a patent, they would have the sole right to sell this treatment for the duration of the patent. You would not have any financial gain from such a patent. Any financial gain that we might have would be used to help support research and treatment of disease.

What are the possible risks of taking part?

The commonest risk of taking part in the study is that of bruising at the site used for taking blood. This is uncommon. All blood samples will be taken by trained staff. Some donors occasionally feel faint, and we are able to deal with that easily if it is a problem. Fainting or serious bruising is extremely rare, though it can occur.

Do I have to take part?

No, your taking part in this study is entirely voluntary. If you would prefer not to take part you do not have to give a reason.

Are there any financial expenses available for this study?

We may be able to refund travel expenses for our participants and will advise you if this is possible at the time. We will try to schedule visits to give blood at times that you might be coming to the hospital anyway, but this may not be possible. If it would be difficult for you to take part because of travel costs, please make sure you tell us this.

What if something goes wrong?

If you have any cause to complain about any aspect of the way you have been approached or treated during the course of the study you should contact the Principal Investigator, Professor Ian Sabroe (address provided at the top of this letter, direct telephone 0114 271 3243). Alternatively the normal National Health Service (via letter to The Medical Director, Sheffield Teaching Hospitals NHS Foundation Trust, 8 Beech Hill Road, Sheffield S10 2SB) or the normal University complaints mechanisms are available (via letter to University of Sheffield Registrar and Secretary, Firth Court, Western Bank, Sheffield. S10 2TN).

Confidentiality - who will see my records and know about my taking part?

The information collected about you during the course of the research will be kept confidential. No names will be mentioned in any reports of the study and care will be taken so that individuals cannot be identified from details in reports of the results of the study. There will be no way in which anyone will be able to identify you from any publications or reports arising from the study.

As described above, members of the research team will be able to look at your clinical records to get relevant information needed for the research. This information will be kept confidential.

The Sheffield Teaching Hospitals NHS Foundation Trust (STHNFT) is the sponsor for this study based in the UK. STHNFT will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. STHNFT will keep identifiable information about you until the study finishes. STHNFT will then archive the study anonymously for a minimum of 5 years. In practice, information about your health that is taken will be kept in the secure project file for as long as the project is active and for up to 10 years after that, in case of any queries over the scientific processes and analysis of results.

Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. We can destroy stored material on request. To withdraw from the study, please contact the principle investigator, Ian Sabroe, through the address above. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information at https://www.sheffield.ac.uk/govern/data-protection All information collected during this study will be kept confidential. However, authorised representatives from the STHNFT research office or UK regulatory authorities might perform an audit of the study and review study data and your medical records. The only other people in STHNFT who will have access to information that identifies you will be people who need to contact you for the study or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name or contact details.

What will happen to the results of the study?

The results of these studies will be presented at national and international Immunology and Medical scientific conferences and will be put forward for publication in medical and scientific journals. They may also form part of educational reports or qualifications (e.g. for PhD students). If you would be interested in hearing about our results, please let us know and we will update you with what we find.

What if I have any other concerns?

If you have any problems, concerns, complaints or other questions about this study, you should preferably contact the investigator, Professor Ian Sabroe (address provided at the top of this letter, direct telephone 0114 271 3243). Alternatively you may contact Sheffield Teaching Hospitals NHS Foundation Trust or Sheffield University, via the addresses listed under the heading 'What if anything goes wrong?' above

You can keep this information sheet and will be given a copy of the signed consent form to keep.

Thank you for taking the time to consider entering this study.

8.2 Appendix 2- NHS research passport

Sheffield Teaching Hospitals NHS
NHS Foundation Trust
Research Department

Letter of access for researchers who do not require an honorary research contract

07 February 2019

Rebecca Dowey
University of Sheffield
L Floor
Medical School
Beech Hill Road
Sheffield
S10 2RX

Dear Rebecca

STH ref: STH20579
Study title: The Control of Innate Immunity, Host-Pathogen Interactions, and Leukocyte Function in Disease
Chief Investigator: Ian Sabroe
Principal Investigator: Ian Sabroe

Letter of access for research
This letter confirms your right of access to conduct research through Sheffield Teaching Hospitals NHS Foundation Trust for the purpose and on the terms and conditions set out below. This right of access commences on **07 February 2019** and ends on **06 February 2022** unless terminated earlier in accordance with the clauses below.

You have a right of access to conduct such research as confirmed in writing in the letter of permission for research from this NHS organisation. Please note that you cannot start the research until the Principal Investigator for the research project has received a letter from us giving permission to conduct the project.

The information supplied about your role in research at Sheffield Teaching Hospitals NHS Foundation Trust has been reviewed and you do not require an honorary research contract with this NHS organisation. We are satisfied that such pre-engagement checks as we consider necessary have been carried out.

Status
You are considered to be a legal visitor to Sheffield Teaching Hospitals NHS Foundation Trust premises. You are not entitled to any form of payment or access to other benefits provided by this NHS organisation to employees and this letter does not give rise to any other relationship between you and this NHS organisation, in particular that of an employee.

Reporting Arrangements
While undertaking research through Sheffield Teaching Hospitals NHS Foundation Trust you will remain accountable to your place of study **University of Sheffield** but you are required to follow the reasonable instructions of **Ian Sabroe** in this NHS organisation or those given on his or her behalf in relation to the terms of this right of access.

Legal Claims
Where any third party claim is made, whether or not legal proceedings are issued, arising out of or in connection with your right of access, you are required to co-operate fully with any investigation by this NHS organisation in connection with any such claim and to give all such assistance as may reasonably be required regarding the conduct of any legal proceedings.

Policies and Procedures
You must act in accordance with Sheffield Teaching Hospitals NHS Foundation Trust policies and procedures, which are available to you upon request, and the Research Governance Framework.

8.3 Appendix 3 - Participant with Diabetes Case Report Form

Subject Name:

Date of Birth:

Pre-screen in diabetes clinic:

Please circle yes or no if the participant meets the following inclusion criteria:

- 1) Diabetes (any type) + foot infection from an active ulcer
  Yes No
- 2) BMI < 35 kg/m2
- **Yes** No
- 3) Non-smoker or < 5 pack yr hx not having smoked in the last 5 years
  Yes No
- 4) No significant contraindication to venesection (principally anaemia)
  Yes No
- 5) Age between 50-75 year old inclusive
  Yes No

If yes to all please circle that the individual has the received patient information sheet and is happy to be contacted via a telephone to follow up regarding participation **Yes** No

Contact telephone number:

Telephone Screening:

Participant wishes to proceed with screening for the study **Yes** No

Screening conducted by:

Date of telephone screen:

<u>Telephone Screening Questions</u>

- Age:
- Sex:
- Height:
- Weight: *Note: Please calculate BMI:*

If BMI > 35 Kg, exclude from study (please complete screening section and store case report form in the site file)

<u>Inclusion- Please circle as appropriate</u>

Age between 50-75 year old inclusive **Yes** No

A diagnosis of diabetes **Yes** No

Active diabetic foot ulceration infected. Defined by a break in the skin below the ankle plus with evidence of infection defined by one or more of the following signs: malodour, erythema, swelling, discharge **Yes** No

<u>Exclusion-If the participant answers yes to any of the following questions, please EXCLUDE from study:</u>

Admission to hospital with sepsis or a serious infection from any cause in the last 6 weeks

Yes **No**

Current use of oral steroids or other immunusuppresants including macrolide antibiotics

Yes **No**

Recent (last 3 months) use of oral steroids Yes **No**

An established diagnosis of COPD Yes **No**

Non-smoker and if an ex-smoker to have stopped 5 years ago with a maximum 5 pack year history Yes **No**

Any significant comorbidities that in the opinion of the investigator would be associated with substantial changes in neutrophil function?

Yes **No**

Lists of declared comorbidities and investigator comments in box below

Confirmation of eligibility for the study

Is the participant suitable for enrolment based on the inclusion/ exclusion criteria?

Yes **No**

Is the participant happy to be contacted to arrange visit for venesection

Yes **No**

Arrangement of venesection

Date of telephone call to arrange venesection:

Name of individual contacting participant:

Subject wishes to attend CRF for blood sample:

Date and time of venesection:

CONSENT

Consent form completed prior to venesection **Yes** No

Date and time of written consent

Patient Clinical information

Hospital number:

Type of diabetes:

Type 1 diabetes ☐

Type 2 diabetes ☐

Undetermined ☐

Duration of diabetes:

Last recorded HbA1c:

Date:

Value:

List any comorbidities the patient may have in the box below e.g high blood pressure, osteoarthritis

List names and doses of diabetes medications:

Drug name	Dose	Frequency

If on any medication other than diabetes medication (including antibiotics) list in the table.

Name of drug	Dose	Route

If currently not on antibiotics detail antimicrobial history for the last 3 months

Name of antibiotic	Dose	Date stopped

Last available microbiology culture results from ulcer(s) site:

Ulcer site	Organism	Date of culture

If applicable:

Staphylococcus aureus positive culture site	Date of culture	Antibiotic sensitivity

Duration of diabetic foot ulceration:

NB: if a healed ulcer has re-ulcerated date from when re-ulcerated

NB: if ulcers on both feet record dates for both

Please note any additional relevant investigator notes not identified in the list above or where space is insufficient.

INVESTIGATOR'S POST RECRUITMENT CHECKLIST

Please tick box:

Record subjects entry into the study on the subject ID log ☐

Complete GP letter informing GP of subject's entry into study ☐

GP Address:

INVESTIGATOR'S DECLARATION

By signing below, I declare that the information presented in this Case Record Form accurately reflects the medical records, including results of tests and evaluations performed on the dates specified.

Name of person completing form (capitals)

Signature of person completing form

Date (dd/mm/yyyy)

8.4 Appendix 4- Healthy Control Case Report Form:

Participant name:

Contact details (telephone or email):

Date participant received the participant information sheet:

Please tick that the participant has confirmed they wish to be screened for the study

Yes No

Screening

Date of screening:

Screening conducted by:

Please answer the following questions:

- Participant Age:
- DOB
- Height:
- Weight:

Note: Please calculate BMI:

If BMI > 35 Kg, exclude from study (please complete screening section and store case report form in the site file)

Exclusion- If yes to any of the questions below please exclude from study:

Have Type 1, type 2 or pre-diabetes

Yes **No**

Admission to hospital with sepsis or a serious infection from any cause in the last 6 weeks

Yes **No**

Current or recent (last 3 months) use of oral steroids or other immunusppresants including macrolide antibiotics e.g erthromycins Yes **No**

An established diagnosis of COPD Yes **No**

Currently smoke (or ex-smoker who has smoked within the last five years with a >5 pack year history) **Yes** No I

Inclusion

Age between 50- 75 years Yes **No**

Participant considered 'healthy' based on the exclusion criteria above Yes **No**

Please tick that the participant is happy for venesection to arranged

Yes **No**

Is the subject currently taking any medication? If yes, please list

Yes No

Does the subject have any declared comorbidities e.g high blood pressure? If yes, please detail below

Yes No

Arrangement of venesection

Venesection arranged by:

Date and time of venesection:

CONSENT

Consent form completed prior or venesection **Yes** No

Date and time of written consent	

8.5 Appendix 5- Letter to participant GP

Faculty of Medicine Dentistry & Health
Professor Dame Pamela J Shaw
Vice President & Head of Faculty

Professor Ian Sabroe
Department of Infection, Immunity & Cardiovascular Disease (IICD)
Medical School
Beech Hill Road
Sheffield S10 2RX

Telephone: +44 (0) 114 271 3243
Telephone: +44 (0) 114 215 9508 (Secretary)
Fax: +44 (0) 114 271 1863
Email: i.sabroe@sheffield.ac.uk
www.sheffield.ac.uk/iicd

Date:

Dear…

Your patient
NAME

NHS NUMBER

ADDRESS

Has kindly agreed to take part in a research study, **The Control of Innate Immunity, Host-Pathogen Interactions, and Leukocyte Function in Disease.**

This study has been approved by the Research Ethics Committee, REF XXXXXXXX, and by the sponsor (Sheffield Teaching Hospitals NHS Foundation Trust), REF STH20579. I am the principal investigator, acting on behalf of a team of reseachers.

The study involves taking blood from people with a variety of illnesses to see how the immune system works. The study does not require any change to treatment. We will take a maximum of 500 ml blood every 12 months, in one or more donations to be agreed between the research team and the subject.

This study therefore does not expose your patient to any significant risks, or require any action on your behalf. However, if you have any concerns about the study or your patient's participation, please contact me at the address/numbers above.

Yours sincerely

Professor Ian Sabroe

8.6 Appendix 6- STOP-COVID PIS

STOP-COVID19

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Participant Information Sheet

Trial title

STOP-COVID19: **S**uperiority **T**rial **O**f **P**rotease inhibition in **COVID-19**

Trial Researcher

Dr Roger Thompson and Prof Alison Condliffe

We're inviting you to take part in a research trial

Before you choose whether or not to take part, we want you to understand why we're doing the trial. We also want to tell you what it will involve if you agree to take part. Please take time to read this information carefully. You can ask us any questions you have and talk to other people about it if you want. We'll do our best to answer your questions and give you any information you ask for. You don't have to decide straight away but, we would like to start the trial treatment as soon as possible.

Why are we doing this trial?

About 8 out of 10 people who get COVID-19 get better without going to hospital. Most patients admitted to hospital with COVID-19 get better, but most need oxygen and some need help to breathe (put on a ventilator) before they get better. However, a small number of people don't get better.

There are only a couple of drugs at the moment which we know will definitely help people with COVID-19. A company in the USA, Insmed Inc., have developed a drug called Brensocatib (INS1007) which we think might help people with COVID-19. The drug isn't licenced for doctors to prescribe yet but the company has done trials using the drug. The trials gave it to healthy people, to see if it was safe, and to people with a lung condition called bronchiectasis, to see if it helped control their symptoms (bronchiectasis is a lung condition which causes frequent chest infections and inflamation in the air passages). Brensocatib reduced inflammation in the lungs in people with bronchiectasis. We think that Brensocatib may help people with COVID-19 in a similar way. We want to know if taking

Brensocatib shortens your time in hospital. We also want to find out if you are less likely to need oxygen for a long period or put on a ventilator and if you're more likely to recover.

What is being tested?

Each participant will get one Brensocatib tablets or one dummy tablet (placebo) every day for 28 days.

Whether you get the Brensocatib tablets or the dummy tablet will be decided randomly (like tossing a coin but using a computer). Neither you nor your trial team will be able to decide if you get Brensocatib or the dummy tablet. To take part in the trial you will need to be happy to either take the Brensocatib tablets or the dummy tablets.

Why have I been asked?

We're asking you to take part as you have been admitted to hospital as you may have COVID-19. A total of 300 participants with COVID-19 at different hospitals in the UK will take part in the trial.

Do I have to take part?

No. Taking part in this trial or not is entirely up to you. If you choose to take part you can stop the trial at any time. You don't have to give a reason for not taking part or for stopping and the medical care you get and your relationship with the medical or nursing staff looking after you won't be affected.

What will happen to me if I take part?

We'll check your medical notes to see if you're able to take part. We'll check what the nursing and medical staff looking after you have recorded about your condition and will look at the results of tests that you've had for example blood tests, chest x-ray and CT scan. We'll also look at what medications you are already taking.

A member of the research team will speak to you about the trial and answer any questions you have. If you want to take part we'll ask you to fill out and sign a consent form. This will confirm that you understand what the trial means for you and that you agree to take part.

Will I need to have any other tests? If you haven't had a blood test to check your kidneys, liver or blood count (haemoglobin) in the last 3 days we will take a blood test to check this. We will also check your blood pressure, pulse, temperature and oxygen levels from your finger if you have not had these recorded in the last 24 hours. We expect that you'll have had these done already by the nurses or doctors looking after you, and if you have, we won't do them again.

Then we'll let you know if you're suitable to take part in the trial or not.

We'll use a computer to find out what bottle of tablets you have been allocated. Your doctors and nurses will not know if the bottle contains Brensocatib tablets or dummy tablets. The nurse looking after you will give you one of the trial tablets every day along with any other medications that you have been prescribed while you're in hospital. You'll take the trial tablets for 28 days.

We'll check your medical notes every day while you are in hospital to see how you are getting on. We will record these details for a maximum of 29 days.

If COVID-19 makes you very unwell during the trial and you can't to take anything by mouth you may have a tube put in by the clinical team which is passed through your nose and down into your stomach (this is called a nasogastric or NG tube). We won't put a NG tube in just for the trial but if you have one the nurse giving you your medications will dissolve the trial tablet in water and give it to you through your NG tube. We'll continue to look at your medical notes and collect your details for 29 days as described above.

If you leave hospital before the end of the 29 days we'll give you the trial tablets to take home to finish. We'll phone you a maximum of 4 times (on the 3rd, 5th, 8th and 15th day after you started the trial tablets). We will ask you how you are getting on, if you have been unwell for any reason since leaving hospital and if any of your usual medications have been changed. When you leave hospital we will give you a diary to fill in to write down if you have any new symptoms or feel unwell and if you have any changes to your prescribed medications. We'll ask you about his when we phone you. We'll also give you a phone number so that you can call us if you need to.

We'll ask to see you on the 29th day after you started to take your tablets. This visit will be either in the hospital or at your home. We'll arrange a taxi or pay for travel expenses if you come to the hospital. At this visit we'll check your blood pressure, pulse and temperature and oxygen levels from your finger. We will also take blood samples. We'll also ask how you have been getting on like we'll do when we phone you.

In total we'll take four extra blood samples. These samples will be obtained at the same time as any clinical samples are being taken to monitor your condition. These samples will be used by the scientists at the University of Sheffield and the University of Dundee for research into how your body responds to COVID19 and the trial drugs.

When we collect the details about you and your condition we save these on a computer but we'll not save you name or other personal details which will be able to identify you.

We'll keep your contact details separately so if you leave hospital we can phone you.

Will taking part in the trial affect my usual care?

No, you'll get all the usual care by the nurses and doctors looking after you.

What will happen when the trial finishes?

You won't continue to get the trial medication when the trial finishes.

What are the possible benefits of taking part?

The trial may not immediately benefit you, but if the results of the trial are good this may improve how we treat people with COVID-19.

What are the possible disadvantages and risks of taking part?

Brensocatib is an un-licensed medicine but it's already been used in clinical trials. Trials with healthy people and those with lung conditions (with over 250 people involved) showed that the medicine was generally well tolerated by people in the trial.

The most common side effects reported were cough, increased phlegm, headache and breathlessness. These are common symptoms for people with lung conditions and they were also reported frequently by people taking the placebo tablets.

Two side that effects we know are possible with this medicine are thickening of the skin and inflammation of the gums both of which happen rarely. We'll ask you regularly if you've had any problems with skin or teeth during the trial.

When you're in hospital you will be closely monitored by the clinical team. we'll ask the clinical team to let us know if they have any worries about you taking the trial tablets. We'll also check your medical records frequently and we'll report any concerns to the trial doctors and your clinical team. If you leave hospital before the end of the 29 days we'll phone you as we said above.

Contraceptive advice

If you're a woman who could get pregnant and you are sexually active you must be willing to have a pregnancy test before starting the trial. You must be willing to use a birth control method which is medically approved while you take the trial tablets and for 30 days after you take the last tablet.

If you're a man and are sexually active with a woman who could get pregnant you must be willing to use a birth control method which is medically approved.

Medically approved birth control methods:

Combined Oral Contraceptive Pill

Intrauterine device – ‘coil’

Male condom

Injected, patch or implant contraceptive

Male partner vasectomy - sterilisation

Who is organising and funding this research?

This trial is being sponsored by the University of Dundee and NHS Tayside. It is being funded by Insmed Inc. The trial is being organised by Professor James Chalmers, University of Dundee.

What will happen with the information you collect about me?

Identifiable information (for example: your name, hospital number and telephone number) and the information we collect about you during the trial (trial information) will be stored by your local research team. Only specified members of the research team can see this information.

Any personal information which could identify you will be encoded by the research team so that your details will be anonymous. This means that your name or anything linked to your name (for example, your hospital number) won’t be used. Your information will be stored securely on databases managed by the University of Dundee with access restricted. Some members of the data management team may also have access to your identifiable information to manage your information and maintain the database.

Your information will be kept securely for 25 years after the end of the trial. After 25 years it will be destroyed. This is a legal requirement for trials using medication.If you’d like us to inform you about future trials that you might be interested in taking part in we’ll ask you to agree by signing the optional section of the consent form to allow us to hold your contact details.

We’ll ask your permission to tell your GP that you’re taking part in this trial.

Information which identifies you personally won’t be published or shared.

We may share your trial information with other researchers but any information which identifies you will be removed before we share it.

The Data Protection Privacy Notice section gives more information about this.

What if something goes wrong?

If you're concerned about taking part in the trial you have the right to discuss your concern with a researcher involved in carrying out the trial or a doctor involved in your care.

If you have a complaint about your participation in the trial, first of all you should talk to a researcher involved in the trial. You can also make a formal complaint. You can make a complaint to a senior member of the research team or to the Complaints Officer for Sheffield Teaching Hospitals NHS Foundation Trust at sth.pals@nhs.net
or on 0114 271 2400

If you think you have come to harm due to taking part in the trial there aren't any automatic arrangements to get financial compensation. You might have the right to make a claim for compensation. If you wish to make a claim, you should think about getting independent legal advice but you might have to pay for your legal costs.

Insurance

The University of Dundee and Tayside Health Board are Co-Sponsoring the trial. The University of Dundee has a policy of professional negligence clinical trial insurance which gives legal liability cover and no fault compensation for accidental injury. Tayside Health Board is a member of the NHS Scotland Clinical Negligence and Other Risks Insurance Scheme (CNORIS) which gives legal liability cover of NHS Tayside for this trial.

As the trial involves University of Dundee staff carrying out clinical research on NHS Tayside patients, these staff hold honorary contracts with Tayside Health Board. This means they will be covered under Tayside's membership of the CNORIS scheme.

Other Scottish Health Boards are participating as trial sites and they are also members of CNORIS. This will cover their liability for carrying out the trial.

NHS Health Trusts in England are taking part as trial sites and they have membership of a scheme like CNORIS from the NHS Litigation Authority (NLA).

If you apply for health, life, travel or income protection insurance you may be asked questions about your health. These questions might include questions about any medical conditions you have or have had in the past. We don't expect that taking part in the trial will adversely affect your ability to buy insurance. Some insurers may use this information to limit the amount of cover, apply exclusions or increase the cost of insurance. Your insurer may take in to account any medical conditions you have, including any which are diagnosed as part of a research trial, when deciding whether to offer insurance to you.

Who has reviewed this trial?

This trial has been reviewed and approved by Scotland "A" Research Ethics Committee who are responsible for reviewing research which is carried out in humans. The Research Ethics committee doesn't have any objections to this trial going ahead.

Detail how patients and the public been involved in the trial

The Edinburgh Clinical Research Facility – Covid19 Patient Public Involvement Advisory Group have helped to write the information that we give to you.

Data Protection Privacy Notice

How will we use information about you?

We'll need to use information from you and from your medical records for this research trial.

This information will include your initials, NHS number, name and contact details. Staff will use this information to do the research or to check your records to make sure that the research is being done properly.

People who don't need to know who you are won't be able to see your name or contact details. Your data will have a code number instead.

We'll keep all information about you safe and secure.

Once we've finished the trial, we'll keep some of the data so we can check the results. We'll write our reports in a way that no-one can work out that you took part in the study.

What are your choices about how your information is used?

- You can stop being part of the trial at any time, without giving a reason, but we'll keep information about you that we already have.
- If you choose to stop taking part in the study, we'd like to continue collecting information about your health from your hospital records. If you don't want this to happen, tell us and we'll stop.
- We need to manage your records in specific ways for the research to be reliable. This means that we won't be able to let you see or change the data we hold about you.

Where can you find out more about how your information is used?

You can find out more about how we use your information at:

- www.hra.nhs.uk/information-about-patients/
- http://www.ahspartnership.org.uk/tasc/for-the-public/how-we-use-your-information
- https://www.dundee.ac.uk/information-governance/dataprotection/

- http://www.nhstayside.scot.nhs.uk/YourRights/PROD_298457/index.htm
- or by contacting Research Governance, Tayside Medical Science Centre (TASC), 01382 383900 email tascgovernance@dundee.ac.uk

Contact details for further information.

PI – Dr Roger Thompson r.thompson@sheffield.ac.uk

Lead research nurse - Janet Middle – 01142713339

Thank you for taking time to read this information and for considering taking part in this trial.

If you'd like more information or want to ask questions about the trial please contact the trial team using the contact details above.

You can contact us Monday – Friday between 09:00-17:00.

Outside of those hours, if you need advice you can contact your out of hours GP service/NHS24 via 111.

8.7 Appendix 7- PIS UK-CIC study

The University Of Sheffield.

Sheffield Clinical Research Facility
Royal Hallamshire Hospital
O Floor Glossop Road
Sheffield S10 2JF
Telephone: +44 (0)114 271 3339
Fax: +44 (0)114 226 8993
www.sheffield.crf.nihr.ac.uk

The Sheffield Teaching Hospitals Observational Study of Patients with Pulmonary Hypertension, Cardiovascular and other Respiratory Disease (STH-ObS): Participant Information Sheet – COVID-19

STH-ObS is a research study that has been established within Sheffield Teaching Hospitals (STH) since 2008. A central element of the study is a Biobank, a facility for the safe and secure long term storage of samples from patients with pulmonary hypertension, and other respiratory disease, cardiovascular disease and connective tissue disease, and a database to store information about these samples and other tests you undergo. We also collect samples from healthy volunteers for comparison. The aim of this research is to increase our understanding of the causes of these conditions, how they cause symptoms and illness, and help us to develop new ways to diagnose, treat, and prevent diseases. In some cases we may ask if you wish to participate in a study to collect activity or health and symptom related data via your mobile device (e.g. iPhone) or fill in some questionnaires. With your permission, data will be collected to help researchers understand patterns in your activity and information about your cardiovascular health. We may also notify you of other linked research projects, taking part in these are completely up to you.

Why have we asked you to take part?

You are being asked to take part because you have suspected or confirmed COVID-19, have had suspected or confirmed COVID-19 in the past, or are deemed to be at risk of severe COVID-19. We wish to obtain samples and data from these groups of patients for research. We wish to obtain samples and data from those who have had, are suspected to have or currently have COVID-19 for research. Samples will be used to study the coronavirus, its effects on the body and your immune system including looking in detail at the ways in which your body may have responded to, and built up defences against, the COVID-19 virus in order to try and protect you from being infected in the future. Your blood sample may also be used to develop tests, and set reference standards for blood tests, and to make products, including commercial products.

What are we asking you to do?

1. We would like to ask your permission to obtain the data from tests you will have, or have had as part of your routine clinical care and from other research studies you may have been involved in. All data that we collect will be stored in a secure database for use in research.

2. We may ask you to give blood, saliva, nasal and throat swab samples to help our research, we may also ask for urine samples.

3. Some patients may have another illness compatible with COVID-19 in the future. If this happens, we would like to ask permission to obtain a swab from your nose or throat at the time.

4. There are no fixed timescales for you participating in the study. We will aim to collect samples and data from you each time you return to the hospital for a clinical follow-up visit, for as long as you are willing to be involved. If you are not attending hospital clinics, we may contact you to arrange specific research visits.

5. We will ask if you are willing to be approached for additional research tests (described briefly below), more detail will be provided if we get in touch to ask you this but you do not have to do these test if you do not want to.

6. We may contact you ask you for additional consent to participate in studies linked to this research, this is optional.

What will happen if you agree to participate?

The first thing you will be asked to do is give your written consent by signing the form attached to this information sheet. Please keep this information sheet to remind you of what you were asked to do. Once you have agreed to take part, the following will happen:

DATA COLLECTION

- We will ask your permission to obtain data that is collected during your normal clinical care and other research projects that you have been involved in (where ethical approval allows this). This will include information about your condition, other diseases, previous assessments and treatments, test results, and images (such as X-ray, CT or MRI scans). The research team will collect this data from your medical records, and store it in a secure research database at the Royal Hallamshire Hospital. We will use this data in research.
- We would like you to give permission for regulatory authorities or officials from STH or University of Sheffield to have access to your medical notes and any data we collect for monitoring purposes.

BLOOD SAMPLES

- We may collect blood samples from you over the course of your illness, during your recovery and into the future, this is to understand more about your body's defences against COVID-19 and whether there is any long term effects of the COVID-19 virus. Wherever possible, if you are coming into hospital for clinical follow-up, we will collect blood for research at these visits. This will be like any other blood donation and should not harm you. You are free to refuse to give a repeat sample and your clinical care will not be affected. If you are not coming into hospital routinely, we may contact you to ask you to come in to donate blood. You are free to say no to this at any time, this will not affect your involvement in other parts of the study.
- No more than 100ml (6 tablespoons) of blood will be taken for research at any one time and no more than 100ml (6 tablespoons) will be taken for research in any 24 hours. This will be in addition to any blood that you have to give for clinical purposes.

SALIVA SAMPLES

- We may collect a sample of saliva from your mouth using a swab each time you have a follow-up or research visit.

NOSE OR THROAT SWABS

- We may ask you for a nose or throat swab at your visits. For example, if in future if you have another illness related to COVID-19 we will ask to take another swab to test for the virus.

URINE SAMPLES

- We may collect urine samples while you are in hospital and may request additional samples each time you have a clinical follow-up or research visit.

All blood, urine and saliva samples will be stored in the Sheffield Biorepository at the Royal Hallamshire Hospital, which is a secure storage facility licensed by the Human Tissue Authority.

MOBILE AND HOME ACTIVITY DATA (OPTIONAL)

We are interested in determining whether everyday activity data measured by 'smart' devices and sensors are helpful in assessing disease. We are currently working with expert partners including Stanford University, Samsung and colleagues at the University of Sheffield. A member of the STH-Obs research team will inform you about this research taking place at Stanford University, USA called "My Heart Counts" which uses a mobile App. If you are interested in taking part, you can download the app for free onto your iPhone or other mobile device. If you agree to participate in the MyHeartCounts study you will be given a unique MyHeartCounts study identification number. The app will collect data about your levels of daily activity. The STH-Obs research team in Sheffield will then be able to use the MyHeartCounts activity data to link it to the STH-Obs study data and make direct comparisons between

hospital exercise tests and your daily activity. The Stanford University researchers will not have access to any of your clinical data.
We have similar arrangements with PAI Health to obtain heart rate data, Samsung for their ACTIVAGE programme, and investigators at the University of Sheffield for their Aequora programme. Agreement to participate in either of these studies may involve the receipt of smart devices and home sensors which would be installed in your home (with your consent) by authorised specialist installers.

If you agree to participate in home or activity sensing tests, the study team will contact you provide you with further information about these studies and how to get involved.

If you are interested in being contacted please provide your email address on the consent form. This will be stored securely by the STH-ObS study team. No one other than the STH clinical team and the STH-Obs research team will have access to your clinical data.

OPTIONAL ADDITIONAL RESEARCH TESTS

There are several different tests we may want to perform and you can give your permission to have all, some, or none of these performed. Your clinical care will not be affected in any way. If you agree to be contacted for this purpose, we will send you an additional information sheet with more details of these tests if there is an opportunity to take part in STH-ObS sub-studies involving these. These optional additional tests may be:

- Exercise Tests
- Magnetic Resonance Imaging
- Breath analysis
- Mobile and Home Activity Data
- Health-related questionnaires
- Interviews

If you give your permission to take part in these additional tests the study team will provide you with detailed further information about what these tests involved.

USE OF SAMPLES AND DATA IN RESEARCH

The samples and data that you donate will be stored indefinitely, for as long as we have the required ethics and research governance approval to undertake this research.

Researchers may request access to the samples and data that we have collected from you and other participants. A Scientific Advisory Board will review applications for samples and data to ensure the research meets appropriate standards. If the request is approved, we will provide the samples and data to the researchers. We may share samples and data with clinical, academic, or commercial institutions, from inside or outside the UK.
No readily identifiable information will be given to the researchers, meaning that you nor other participants cannot be identified from the information given. When the STH-Obs provides samples to researchers they are obliged to only use the samples for the research they said they would do. Researchers will be bound by a Material Transfer Agreement reviewed by the Sponsor R&D Department.

Data Protection Information

The University of Sheffield (TUOS) is the sponsor for this study and will act as the data controller. This means that TUOS is responsible for looking after your information and using it properly. Your rights to access, change or move your information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible.
You can find out more about how we use your information at https://www.sheffield.ac.uk/govern/data-protection
Staff from the Sheffield Clinical Research Facility, as well as research team members from the University of Sheffield, may use your name and contact details to contact you about the study, and make sure that relevant information about the study is recorded for your care, and to oversee the quality

of the study. Individuals from TUOS and regulatory organisations may look at your medical and research records to check the accuracy of the research. The only people in TUOS who will have access to information that identifies you will be people who need to contact you about the study or audit the data collection process. The people who analyse the information will not be able to identify you and will not be able to find out your name, NHS number or contact details. TUOS will keep identifiable information about you for 15 years after the study has finished.

This information will not identify you and will not be combined with other information in a way that could identify you. The information will only be used for the purpose of health and care research, and cannot be used to contact you or to affect your care. It will not be used to make decisions about future services available to you, such as insurance.

What will happen if you say no?

You are free to decline to participate in this research. Your decision will NOT affect the care you receive from the hospital or doctor, now or in the future. If you say no, we will not take samples or data from you other than for normal clinical care. We may ask you to help us understand why you said no – but you do not have to tell us.

What happens if you agree to participate but then wish to withdraw from participation?

You can withdraw from the study at any time by contacting the research team (details given below). We will ask you to sign a "Withdrawal of Consent Form" so we can keep a record of anyone who has withdrawn. If you request that you no longer want any of the samples you have previously donated to be used in research, all samples in remaining in storage will be destroyed. If you wish to withdraw your consent, it is possible that some or all of the samples may have already been used by researchers.

What are the benefits to you?

The samples and information that you give will be treated as gifts that could help research to benefit those affected by disease in the future. It is unlikely you will benefit directly from the research as it usually takes many years for research to produce medical advances. The results of research tests involving you as an individual will NOT be put in your health records or told to you, your relatives or your doctors because the researchers cannot identify who you are. However we will publicise the research findings from the tissue bank as a whole on the websites www.lungsheffield.org, the University of Sheffield website https://medicine.dept.shef.ac.uk/news/index.php/2015/07/21/the-pulmonary-hypertension-blood-biobank/ , and the Donald Heath Research Programme website (https://donaldheath.org/home/research/). Findings will also be publicised at research conferences and via publications in academic journals.

What are the risks to you?

There are NO significant risks to donating samples for this research. There are no more risks of giving blood for research than there are for being a blood donor or giving a routine blood sample.

Your clinical and research teams will take every precaution to prevent researchers from obtaining information that identifies you. The only people who will know your identity are the hospital staff and trained research staff dealing with patient records who are bound by a professional duty to protect your privacy.

Other things you should consider

The samples and information you have gifted will be made available to researchers in the UK or overseas, in universities, hospitals or private companies that do medical research. This research will always be relevant to understanding how the body works, which may help us understand COVID-19 in more detail. You will not receive any personal financial reward for making your gift.

Sometimes samples are used for genetic research about diseases that are passed on in families. However any genetic results about an individual will not be revealed to that individual. Your samples will not be used for research involving animals or reproductive cloning.

What if I have any questions or concerns?

If you have questions about this research, please ask a member of the study team by emailing sth.obs@nhs.net, research nurse, or your consultant:

Local Consultants:

Prof David Kiely, Pulmonary Vascular Disease Unit, M Floor, Royal Hallamshire Hospital, Sheffield, S10 2JF

Tel 0114 271 2132

Dr Thushan de Silva, Senior Clinical Lecturer and Honorary Consultant Physician in Infectious Diseases, University of Sheffield Medical School. Tel 0114 2159532

What if there is a problem or if I wish to make a complaint?
If there is a problem, please contact the study team (details above).
If wish to complain formally, you can write to:
Professor Christopher Newman, Dean of the Medical School, University of Sheffield Medical School, Beech Hill Rd, Sheffield S10 2RX.

If you are harmed during the course of the study and this is due to someone's negligence, you may have grounds for a legal action for compensation against the employing NHS Trust, but you may have to pay your legal costs. The normal NHS complaints mechanisms will still be available to you.

Research Tissue Bank Study ID: _____