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# The immunological impact of lung ventilation during cardiopulmonary bypass

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# DECLARATION

I declare that this thesis is my own work and that contribution from others is duly acknowledged in the text.

This work has not been submitted in candidature for any other degree or professional qualification.

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October 2018

# ABSTRACT

Cardiopulmonary bypass (CPB) is associated with postoperative pulmonary dysfunction, predisposing patients to considerable morbidity and mortality. The association of CPB with pulmonary dysfunction is thought to be driven by the development of a systemic inflammatory response which is characterised by neutrophil dysregulation and inflammatory cytokine release. Emerging evidence suggests that the human lung may play a beneficial role in modulating circulating neutrophil function thus potentially preventing organ dysfunction which is driven by neutrophil dysregulation.

This thesis describes novel work using a one-lung ventilation model with direct pulmonary vein blood sampling in patients undergoing coronary artery bypass (CABG) surgery to explore the effects of ventilation on the innate immune system with focus on circulating neutrophil function and cytokine release. Adult patients awaiting 'on-pump' CABG surgery were recruited to two separate cohorts which differed in the intensity of one-lung ventilation to test our hypothesis that ventilation during CPB has beneficial immunomodulatory effects. Five blood samples were obtained from each patient during the operation: three central venous samples and one sample from both the left and right pulmonary veins.

CPB had no apparent effect on the phagocytic function or priming status of circulating neutrophils however there was an increase in pro-inflammatory and anti-inflammatory cytokine concentrations in the post-CPB blood samples. Lung ventilation during cardiopulmonary bypass did not impact upon pulmonary blood neutrophil phagocytosis or priming status, nor did it alter postoperative plasma cytokine levels. These results suggest that lung ventilation during cardiopulmonary bypass has no apparent immunomodulatory benefit and that other therapeutic strategies to prevent post-CPB organ dysfunction should be explored.

A second smaller laboratory study was undertaken alongside the clinical study which explored the effects of recombinant human interferon gamma (IFN $\gamma$ ) on the phagocytic function of neutrophils. Using an *in vitro* model, phagocytic function of neutrophils from healthy volunteers was impaired by pre-incubation with the  $\beta_2$ -agonist, salbutamol. This model was then used to explore the effect of IFN $\gamma$  on the ability to restore phagocytic function and the downstream cell signalling pathways involved in neutrophil phagocytosis. IFN $\gamma$  restored

phagocytosis of neutrophils pre-incubated with salbutamol and appeared to facilitate this by restoring RhoA activity, a key component of complement mediated phagocytosis. Moreover, Jak1, Jak2 and Stat1 all appeared to be important components of the cell signalling pathway involved in IFN $\gamma$ -mediated restoration of neutrophil phagocytosis. This work suggests that there is a potential therapeutic role for IFN $\gamma$  in patients with impaired neutrophil phagocytosis such as that induced by severe sepsis and provides a platform for further exploration of the downstream cell signalling pathways involved.

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# ABBREVIATIONS

ANOVA	Analysis of variance
AnV	Annexin V
AP	Ammonium persulphate
APC	Allophycocyanin or antigen presenting cell
ARDS	Acute Respiratory Distress Syndrome
ARP	Actin related proteins
ATP	Adenosine triphosphate
AU	Arbitrary units
BAL	Bronchoalveolar lavage
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
C5a	Complement factor 5a
cAMP	Cyclic adenosine monophosphate
CABG	Coronary artery bypass graft
CD	Cluster of differentiation
CDC42	Cell division control protein 42 homolog
COPD	Chronic obstructive pulmonary disease
CPAP	Continuous positive airway pressure
CPB	Cardiopulmonary bypass
CR3	Complement receptor 3
CXR	Chest x-ray
DAPI	4', 6-diamidino-2-phenylindole
ELISA	Enzyme-linked immunosorbent assay
Fc $\gamma$	Fc gamma
FEV <sub>1</sub>	Forced expiratory volume in 1 second
FiO <sub>2</sub>	Fraction of inspired oxygen
FITC	Fluorescein isothiocyanate
FOB	Fibre-optic bronchoscope
FRC	Functional residual capacity

FVC	Forced vital capacity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTPase	Guanosine triphosphate phosphohydrolase
HBSS	Hanks' balanced salt solution
HLA-DR	Human leukocyte antigen – antigen D related
HRP	Horseradish peroxidase
IBW	Ideal body weight
ICAM	Intercellular adhesion molecule
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
Ig	Immunoglobulin
IL	Interleukin
IQR	Interquartile range
ITU	Intensive treatment unit
IMDM	Iscove's modified Dulbecco's medium
IRF1	Interferon regulatory factor 1
Jak	Janus family of kinases
MFI	Median fluorescence intensity
MIG	Monokine induced by interferon gamma
MMP	Matrix metalloproteinase
MPAP	Mean pulmonary artery pressure
Na <sub>3</sub> VO <sub>4</sub>	Sodium orthovanadate
NaF	Sodium fluoride
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NK	Natural killer
OLV	One-lung ventilation
PAF	Platelet activating factor
PAMP	Pathogen-associated molecular patterns
PaO <sub>2</sub>	Partial pressure of oxygen

PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PEEP	Positive end expiratory pressure
PI	Propidium iodide
PMN	Polymorphonuclear cell
PMSF	Phenylmethanesulfonyl fluoride
PRRs	Pattern recognition receptors
PVRI	Pulmonary vascular resistance index
Rap1	Ras-related protein 1
RBC	Red blood cell
RhoA	Ras homolog gene family, member A
ROS	Reactive oxygen species
<i>S.aureus</i>	<i>Staphylococcus aureus</i>
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
Stat	Signal transducer and activator of transcription
TBST	Tris-buffered saline tween
TEMED	N, N, N', N'-tetramethylethylenediamine
TH	T-helper
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
T <sub>x</sub> B <sub>2</sub>	Thromboxane B <sub>2</sub>

# CHAPTER 1: INTRODUCTION

## 1.1 OVERVIEW

This thesis explores aspects of the immunological impact of surgical trauma. It describes two studies: a clinical study and a laboratory study. The clinical study, which forms the major part of this work, explores the immunomodulatory impact of lung ventilation during cardiopulmonary bypass. The smaller laboratory study explores the effects of recombinant human interferon gamma (IFN $\gamma$ ) on neutrophil phagocytosis.

This first chapter provides an overview of the literature which led to the hypotheses for both the clinical and laboratory studies. The chapter begins with an overview of surgery involving cardiopulmonary bypass (CPB) and the risk of post-operative pulmonary dysfunction. The role of the innate immune system (specifically neutrophils) in the pathogenesis of this pulmonary dysfunction is then discussed. An outline of how lung ventilation during CPB may regulate the immune system to attenuate pulmonary dysfunction is then followed by a description of the model of one-lung ventilation with direct pulmonary vein sampling used to test our clinical study hypotheses.

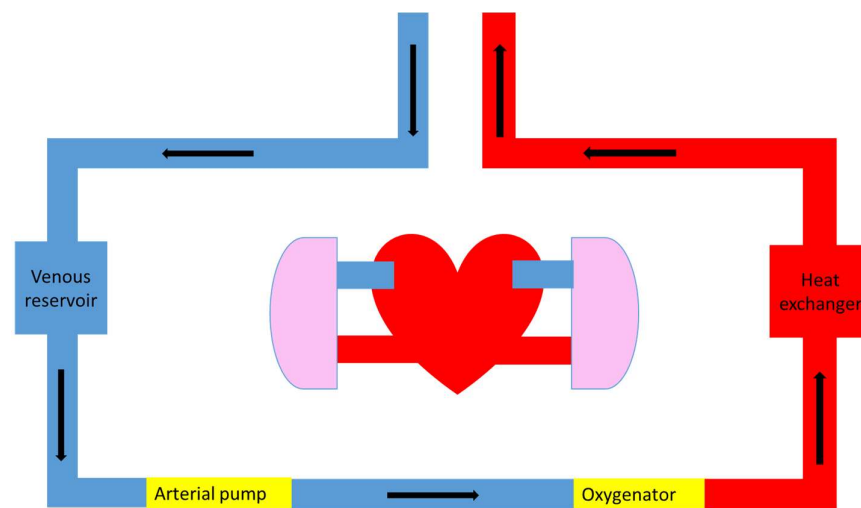
The effect of surgery with CPB on serum IFN $\gamma$  levels is then described leading to an overview of IFN $\gamma$  biology and IFN $\gamma$  cell signalling pathways. The impact of IFN $\gamma$  on leukocyte and neutrophil function is then described followed by an overview of the proposed in-vitro model to test our laboratory study hypotheses.

The chapter concludes with the aims and hypotheses for both the clinical study and the laboratory study.

## 1.2 CARDIOPULMONARY BYPASS AND PULMONARY DYSFUNCTION

Coronary artery bypass graft (CABG) procedures have been used to treat coronary artery disease for more than 60 years.(1, 2) This surgical procedure involves the use of healthy blood vessel conduits to divert blood around a narrowed coronary artery to improve blood flow to the myocardium.

Since the 1950s, cardioplegia and the use of a cardiopulmonary bypass (CPB) machine has been the only way of achieving a motionless and bloodless field during surgery.(3) This method involves the use of an extracorporeal circulation circuit to maintain circulation via mechanical pumps and oxygenation via an artificial oxygenator.(Figure 1)



*Figure 1: Schematic illustration of the circulatory and gas exchange system during cardiopulmonary bypass. On initiation of cardiopulmonary bypass, deoxygenated blood from the body is syphoned into a venous reservoir under gravity via a right atrial cannula. The blood is then pumped into an oxygenator and heat exchanger within the cardiopulmonary bypass circuit before being returned to the patient via an arterial cannula usually placed in the distal ascending aorta. An aortic cross clamp placed in the ascending aorta prevents flow of blood back into the heart. Under normal circumstances the lungs remain unventilated during CPB and blood flow via the pulmonary artery ceases. Cardiac activity is also stopped via a cardioplegic solution infused around the heart during CPB. As a result, the heart and lungs are essentially isolated from the circulatory system during CPB thus achieving a relatively bloodless and motionless field for the operating surgeon. The black arrows demonstrate the direction of blood flow.*

Over the years, clinical advances in the management of patients undergoing CABG procedures have vastly improved outcomes,(2, 4) and the median survival is now 17.6 years.(5) However, CABG procedures represent major surgery, and are therefore associated with numerous peri- and post-operative complications. Hence, the development of safer minimalist procedures such as percutaneous coronary intervention (PCI) has precipitated a decline in the number of CABG procedures carried out in the United Kingdom from just over 25,000 per year a decade ago to 17,000 in 2014.(Figure 2)(1)



**Figure 2: Number of coronary artery bypass operations and percutaneous coronary interventions per year, United Kingdom 1980 to 2013 (adapted from Cardiovascular Disease Statistics, British Heart Foundation 2015)(1)**

Despite the advent of PCI, CABG procedures remain the intervention of choice for patients with complex coronary artery lesions or left main stem disease.(1) Work to minimise the rate of complications associated with CABG procedures therefore remains highly relevant.

Pulmonary complications are common following CABG procedures and contribute substantially to postoperative morbidity and mortality.(6) While a degree of pulmonary dysfunction is to be expected following any major surgery (since the conditions of general anaesthesia precipitate atelectasis),(7) surgery involving CPB causes additional pulmonary injury and delayed functional recovery.(8) The vulnerability of the lungs to injury following CABG procedures is multifactorial and complex. Contributing factors include those that are



distinctly related to cardiac surgery such as the effects of the median sternotomy incision, breaching of the pleura to harvest the internal mammary artery and cessation of lung ventilation during the extracorporeal circuit time.(6) General surgical factors including the use of anaesthesia and mechanical ventilation further compound the risk of postoperative pulmonary complications.

Pulmonary dysfunction following cardiac surgery may manifest only as mild post-operative hypoxaemia with little clinical impact. However, a considerable proportion of patients develop more severe pulmonary complications including pleural effusions (27-95%), atelectasis (16.6-88%), pneumonia (4.2-20%) and pulmonary embolism (0.04-3.2%).(6) Moreover, around 2% of cases will develop severe pulmonary dysfunction manifesting as acute respiratory distress syndrome (ARDS) which is associated with a case fatality rate of 50%.(9)

Despite advances in operative techniques and post-operative care since the early days of cardiac surgery,(2) lung injury in this setting continues to present a substantial clinical problem.

## **1.3 OVERVIEW OF NEUTROPHIL BIOLOGY**

Neutrophils can be regarded as being key mediators in two substantial postoperative pulmonary complications following CABG surgery: in the prevention and resolution of pneumonia and in the pathogenesis of ARDS.

As the most abundant circulating leukocyte in humans, neutrophils play a fundamental role in the innate immune response. Central to their function is the ability to be recruited to sites of inflammation where they respond to invading bacterial and fungal species by phagocytosis and subsequent destruction of their target via a combination of cytotoxic mechanisms.(10) The importance of neutrophils in maintaining health is exemplified by patients with congenital or acquired neutropenia who are particularly prone to life-threatening bacterial and fungal infection.(11)

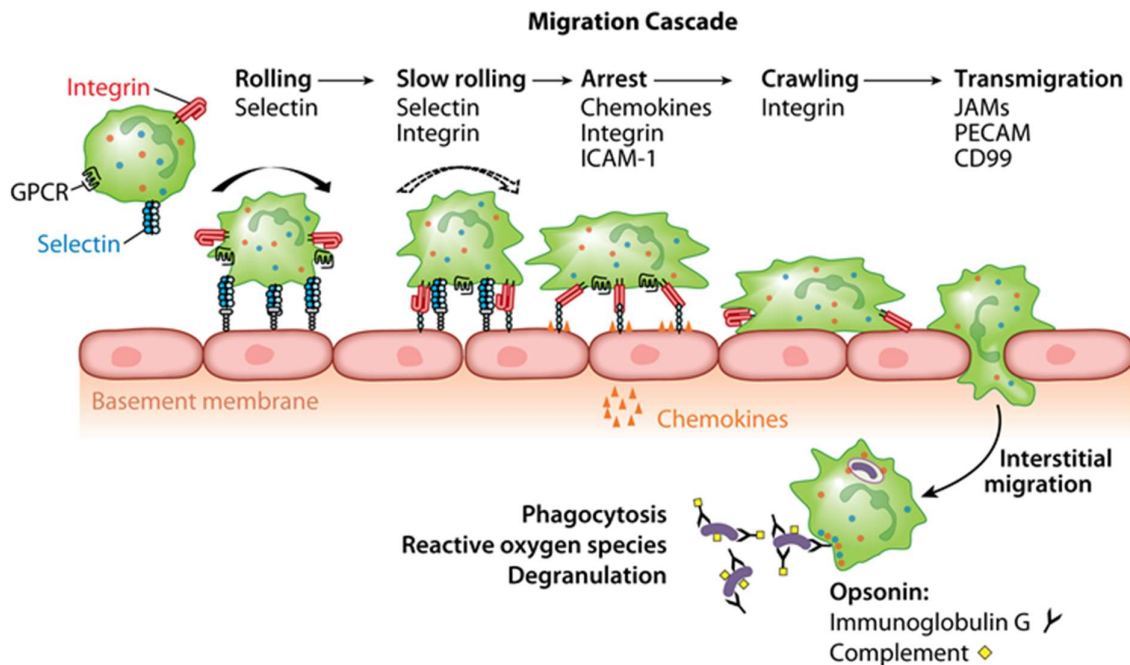
Neutrophils are formed from myeloid precursors within the bone marrow in response to cytokines, in particular G-CSF.(10) They are produced at a rate of one hundred billion per day which can increase further during bacterial infection. Neutrophils are relatively short-lived cells with a half-life of approximately 8-12 hours in the circulation. The removal of aged neutrophils from the circulation occurs mainly in the liver, spleen and bone marrow.(12)

### **1.3.1 Neutrophil recruitment**

Mature neutrophils are guided towards sites of inflammation by various chemoattractants such as complement component 5a (C5a), interleukin 8 (IL-8), leukotrienes and bacterial peptides. They travel in the vasculature and subsequently exit into target tissues via a sequential process known as the neutrophil recruitment cascade. This multistep process involves interaction between neutrophil surface receptors and ligands induced on activated endothelium in response to inflammatory stimuli (primarily intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2)).(10) This process consists of an initial low-affinity 'rolling' of neutrophils along the vascular endothelium which is mediated by the transient binding of neutrophil selectins, namely CD62P (P-selectin), CD62E (E-selectin) and CD62L (L-selectin) with fucosylated ligands expressed on the vascular endothelium.

Subsequent firm adhesion is mediated by the up-regulation of neutrophil surface  $\beta$ -integrins, particularly CD11b/CD18 (MAC-1) which bind to intercellular adhesion molecules (ICAM) on the vascular endothelium.(13) Firm arrest precedes intraluminal crawling and subsequent

transmigration across the endothelium predominantly via endothelial cell-cell junctions (paracellular transmigration) or through the endothelium (transcellular transmigration).(Figure 3)(10)



**Figure 3: Multistep adhesion cascade of neutrophil recruitment.** Initial attachment and rolling of neutrophils on the vascular endothelium is mediated by the interaction of selectins with their fucosylated ligands. Subsequent up-regulation of  $\beta$ -integrins, which primarily interact with ICAM-1 and ICAM-2 expressed on the vascular endothelium, promotes firm arrest of the neutrophil. Diapedesis then occurs predominantly via endothelial cell-cell junctions (paracellular transmigration) to reach the interstitial space where the neutrophil is guided by chemoattractants towards the target source. Abbreviations: ICAM, intercellular adhesion molecule; JAM, junctional adhesion molecule; PECAM, platelet endothelial cell adhesion molecule; GPCR, G-protein-coupled receptor. Adapted from Mayadas et al (10).

### 1.3.2 Neutrophil pathogen recognition and phagocytosis

Neutrophils hold the ability to recognise and subsequently phagocytose pathogens via several mechanisms. These mechanisms include Fc-receptor mediated phagocytosis and complement-receptor mediated phagocytosis.(14)

Fc-receptor phagocytosis involves the recognition of target particles coated in immunoglobulin (Ig) by the Fc receptor.(15) Fc receptors are classified according to which class of immunoglobulin they bind; Fc $\gamma$  receptors bind to IgG, Fc $\alpha$  receptors bind to IgA and Fc $\epsilon$  receptors bind to IgE. Fc $\gamma$  receptors are the predominant Fc receptors found on the surface of neutrophils. Following ligand binding, signalling occurs via numerous cellular components. These include tyrosine kinases, Rho GTPases (Ras homologous guanosine triphosphate phosphohydrolases) such as Rac and CDC42 (cell division control protein 42 homolog) and the actin nucleation complex Arp2/3 (actin related proteins 2 and 3) eventually culminating in actin polymerisation.(16) The cytoskeletal rearrangement resulting from actin polymerisation results in extension of the neutrophil plasma membrane in the form of pseudopods around the target particle to form a vacuole described as the phagosome.(15)

Complement mediated phagocytosis differs from Fc-receptor mediated phagocytosis. Complement receptors, namely CR3 (consisting of CD11b and CD18 subunits) on the neutrophil surface bind iC3b-opsonised pathogens resulting in activation of RhoA which acts independently of tyrosine kinase.(16) RhoA subsequently induces activity of Rho kinase thus activating myosin and the recruitment of Arp2/3 resulting in actin polymerisation and the formation of a phagosome.

Upon formation of the phagosome following either Fc-receptor mediated or complement mediated phagocytosis, numerous pre-formed granules fuse with the membrane to produce a phagolysosome and a cascade of events ensues. These include release of numerous hydrolytic enzymes and the *de novo* generation of reactive oxygen species (ROS) generated by formation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex which initiates pathogen killing.

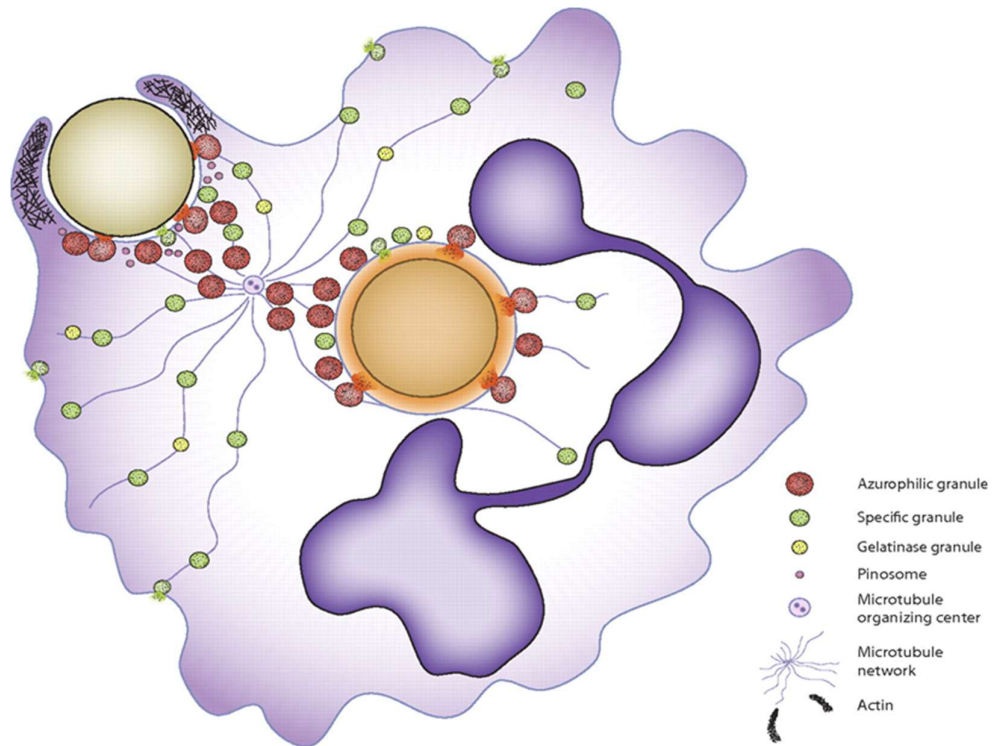
The formation of ROS has formed the basis of several experimental assays used as surrogate measurements of neutrophil phagocytosis. This includes the nitroblue (NBT) assay where neutrophils containing insoluble blue NBT formazan deposits are counted having been formed by reduction of the water-soluble yellow-coloured nitroblue tetrazolium (Y-NBT) by

superoxide produced upon phagolysosome formation.(17). A chemiluminescence assay based on the ability of reactive oxygen species to catalyse the oxidation of a substrate resulting in light emission has also been used to quantify phagocytosis.(18)

### **1.3.3 Neutrophil cytotoxic functions**

Neutrophils have a characteristically segmented nucleus, and cytoplasm which contains three types of granules categorised on their enzyme content and function. The primary (or azurophilic) granules are the most potent: following internalisation of a target, they fuse with the fully formed phagosome and release their antimicrobial contents including myeloperoxidase, antimicrobial peptides and serine proteases such as neutrophil elastase.(15) Secondary (or specific) granules facilitate the breakdown of the bacterial cell wall and prevent bacterial growth via substances such as lactoferrin. Tertiary (or gelatinase) granules predominantly contain receptors involved in directed migration of the neutrophil across the endothelium to the site of inflammation.(Figure 4) (15)

Neutrophils also contain highly mobile secretory vesicles. These vesicles play an important part in the initial cell adhesion to vascular endothelium by transporting molecules such as  $\beta_2$ -integrin to the neutrophil surface upon neutrophil activation.(12)



**Figure 4: Granule targeting and membrane traffic during phagocytosis.** Following internalisation of a target particle, primary (azurophilic) granules rapidly fuse with the forming and fully-formed phagosomes. The secondary (specific) granules are more diffusely localised and secreted. The contents of the tertiary (gelatinase) granules and secretory vesicles have mostly been released during the extravasation process when migrating to the site of inflammation. The microtubule network which emanates from the microtubule organising centre is likely to be involved in directing some of the granule traffic. Adapted from Nordenfelt et al (15).

### **1.3.4 Neutrophil priming**

Neutrophil priming refers to a process whereby the response of neutrophils to an activating stimulus is enhanced by prior exposure to small 'non-activating' concentrations of this or another stimulus.(19) Neutrophils can therefore be regarded as existing in one of three states: quiescent, primed or activated.(20) While un-primed circulating neutrophils remain biologically active, their microbicidal ability in response to inflammatory stimuli may be as small as 5% of the enhanced response of primed neutrophils.(13) The two-step process of achieving full neutrophil microbicidal capacity is understood to be the key mechanism by which the highly destructive antimicrobial properties of neutrophils are balanced with prevention of inappropriate or excessive activation, which may lead to tissue damage and resultant organ dysfunction. Dysregulation of this control mechanism is hypothesised as being the origin of many chronic inflammatory conditions including rheumatoid arthritis and chronic obstructive pulmonary disease (COPD).(20)

In the primed state neutrophils are essentially 'ready to go' and although priming is not associated with a functional response, it is a complex process during which numerous biochemical events occur.(20) These events include cytoskeletal actin remodelling resulting in decreased neutrophil deformability. This increased 'stiffness' has been shown to promote the retention of neutrophils within the microcirculation.(21)

Experimental models have demonstrated that priming promotes neutrophil integrin expression and induces CD62L shedding; these changes result in the increased adhesive properties of neutrophils during inflammation.(13)

A further consequence of priming is the enhancement of neutrophil respiratory burst, a process of release of reactive oxygen species, which facilitates effective intracellular pathogen killing.(22) The priming process is understood to assist with the formation of the NADPH oxidase system resulting in generation of reactive oxygen species thus explaining the enhanced response of primed neutrophils compared to un-primed neutrophils. Moreover, the adhesive state of primed neutrophils has been shown to augment respiratory burst activity suggesting that neutrophil adhesion plays an important role in maximising microbicidal response and potential for tissue damage.(23)

Importantly, neutrophil priming has been shown to be a reversible state. This is exemplified in experimental studies demonstrating reversible neutrophil priming using platelet activating

factor (24) and in more recent clinical studies investigating the pulmonary de-priming of neutrophils.(25) The ability for neutrophils to undergo de-priming and revert to their quiescent state reflects the homeostatic balance between effective pathogen killing and prevention of inadvertent host tissue damage. Exploring potential therapeutic interventions aimed at de-priming neutrophils in conditions where tissue damage results from dysregulation of this homeostatic balance is therefore of clinical relevance.



## **1.4 PNEUMONIA FOLLOWING CARDIOPULMONARY BYPASS**

Pneumonia is the most common infection following cardiac surgery (26) with an incidence ranging from 4% to 20%.(6) Improved surgical techniques and post-operative management have reduced the incidence of pneumonia following cardiac surgery. Nevertheless, the development of pneumonia following cardiac surgery continues to pose a substantial clinical problem and has been associated with increased mortality and prolonged intensive care and hospital stay.(27)

Neutrophils are central to first line immune defence and in the prevention and resolution of pneumonia, however very little is known about the effect of CPB on neutrophil phagocytic function. Literature reporting a reduced incidence of pneumonia following off-pump bypass surgery (CABG surgery performed on a beating heart and without the need for cardiopulmonary bypass) raises the question as to whether surgery with CPB adversely affects leukocyte function thus contributing to post-operative infection.(28) Exploring the effect of surgery with CPB on neutrophil phagocytic function may therefore identify a potential therapeutic target to reduce post-CPB-related pulmonary complications such as pneumonia.

### **1.4.1 The effect of cardiopulmonary bypass on neutrophil phagocytosis**

A small number of historic studies have explored the impact of CPB on phagocytic function and yielded conflicting results. Among the studies reporting a reduction in granulocyte phagocytic function following CPB is that by Hamano (29). This study measured the phagocytic function of blood granulocytes in 14 patients undergoing cardiac surgery with CPB at various time points ranging from pre-operatively to seven days post-operatively using the chemiluminescence method. A control group consisted of seven patients undergoing surgical abdominal aortic aneurysm repair. The study demonstrated that granulocyte phagocytic function of patients undergoing CPB was significantly impaired postoperatively until twelve hours following CPB but returned to baseline by 24 hours. No change in phagocytic capacity was detected in the control group throughout the study period.

Silva (30) reported similar conclusions of a transient defect in PMN phagocytic capacity in his study of nitroblue tetrazolium (NBT) reduction in 12 patients undergoing CPB. PMN leukocytes were obtained one day before, during and up to six days after cardiopulmonary bypass. A transient but significant decrease in NBT reduction was found during CPB but returned to baseline levels by day one post-CPB with no further change over the study period.

These early studies suggest that neutrophil phagocytic capacity is transiently reduced during CPB. However, determination of the cause of this dysfunction goes beyond the scope of the studies. It may be that medication or anaesthetic agents administered cause a transient dysfunction in phagocytic capacity, or perhaps mechanical damage from the bypass circuit causes neutrophil dysfunction which results in mobilisation of functional neutrophils from the bone marrow therefore explaining the normalisation of the results postoperatively.

A more recent study by Rothenburger assessed the impact of CABG surgery with CPB on leukocyte phagocytic capacity in a cohort of 79 patients and yielded contrasting results.(31) Flow cytometric analysis using fluorescein isothiocyanate (FITC)-conjugated *Escherichia coli* was used to determine phagocytic capacity at various time points from pre-operatively to seven days post-operatively. The authors demonstrated no significant difference between pre-operative baseline and post-operative phagocytic capacity on day one. However, a significant increase exceeding the baseline levels was found at day three and seven postoperatively.

It is difficult to directly compare the aforementioned studies because of the different methods used to measure phagocytic capacity and the wide range of cardiac operations included. The earlier studies used measures of oxidative burst (such as chemiluminescence and NBT reduction) as a proxy for phagocytic capacity. Neutrophil oxidative burst involves the *de novo* generation of reactive oxygen intermediates generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which initiates pathogen killing following phagocytosis.(16) It is therefore questionable as to whether measures of oxidative burst are a suitable proxy measure of phagocytosis as the results may be attributable to transient defects in oxidative burst function rather than to phagocytic mechanisms.

The effect of CPB, especially in the context of modern-day CABG procedures, is not well understood and more research needs to be undertaken to clarify the impact of CPB on neutrophil phagocytic capacity.

## **1.5 ACUTE RESPIRATORY DISTRESS SYNDROME FOLLOWING CARDIOPULMONARY BYPASS**

One of the most severe peri-operative complications of CABG procedures is the development of a systemic inflammatory response. In the majority of cases, this inflammation remains at a subclinical level; however, its most extreme form can lead to a systemic inflammatory response syndrome (SIRS) associated with major organ dysfunction and death.(32)

There are multiple aetiological factors associated with the development of a systemic inflammatory response following CABG procedures. The use of an extracorporeal circuit has been implicated as a substantial contributor to the inflammatory response following CABG procedures as a result of factors including contact of blood constituents with the artificial material of the bypass circuit, haemodilution, and cooling of blood during CPB.(7, 32, 33) Other potentiating factors for systemic inflammation following CPB include ischaemia-reperfusion injury and release of endotoxins from ischaemic tissues.(7) General anaesthesia and mechanical surgical trauma further compound this risk.

The terms 'post-pump syndrome' and 'systemic inflammatory response syndrome secondary to CPB' refer to a state of multi-organ dysfunction following bypass that cannot be directly attributed to an identifiable cause such as infection or ischaemia. This is understood to develop due to activation of the innate immune system as a result of the aforementioned interacting triggers. Numerous organ systems including the cardiac, renal and coagulation systems may sustain injury, however the most common clinical manifestation of the SIRS response following CPB is seen in the respiratory system.(34)

### **1.5.1 Pulmonary ischaemia during cardiopulmonary bypass**

Under normal physiological conditions, the human lung receives a blood supply from the pulmonary and bronchial arteries. The pulmonary arteries supply most of the blood to the lungs, while the bronchial arteries contribute only 3-5% of the total blood flow.(7) During the extracorporeal time on CPB, pulmonary artery blood flow ceases (see Figure 1, section 1.2) and the lungs become dependent on the small bronchial artery blood supply to meet metabolic demands.(9) Studies have demonstrated that despite adequate perfusion pressures, bronchial artery blood flow reduces even further during CPB (35) resulting in almost complete exclusion of the lungs from an oxygenated blood supply during this period.(7)

Experimental models in both animals and humans have demonstrated an association between CPB and the development of lung ischaemia. Using a swine model, Serraf demonstrated an accumulation of lactate and a significant decrease in pulmonary parenchymal ATP energy stores during CPB, providing evidence of ischaemia.(36) As further evidence of ischaemia during CPB, Gasparovic demonstrated that pulmonary lactate production was significantly elevated for the first 6 hours following CPB in 40 adults.(37)

Parallel to these findings, researchers have explored the idea that pulmonary perfusion during CPB may alleviate both lung ischaemia and post-operative lung injury. In a controlled study of children undergoing surgery for congenital heart disease with pulmonary hypertension, continuous lung perfusion with oxygenated blood during total CPB was shown to preserve the PaO<sub>2</sub>/FiO<sub>2</sub> ratio and reduce the duration of postoperative mechanical ventilation compared to controls.(38) Plasma levels of circulating adhesion molecules were also significantly reduced, namely ICAM-1 and soluble granule membrane protein 140. This suggests that pulmonary perfusion during CPB may reduce neutrophil-endothelial interaction mediated by adhesion molecules in the pulmonary vasculature.

In an adult cohort, perfusion of the pulmonary artery with hypothermic oxygenated blood for 10 minutes prior to the initiation of total CPB resulted in a well preserved alveolar-arterial oxygen gradient and oxygenation index compared to controls.(39) It is important to note however, that the treatment group also received mechanical ventilation during the period of pulmonary perfusion; it is therefore difficult to separate the effects of ventilation from those of pulmonary artery perfusion.

Wei and colleagues examined the effect of intermittent lung perfusion with a hypothermic protective solution. The study showed that this relieved lung injury and improved pulmonary gas exchange parameters in children undergoing CPB for surgical correction of tetralogy of Fallot.(40) The researchers also described reduced levels of inflammatory mediators in the serum and bronchoalveolar lavage (BAL) fluid of the experimental group. A particularly interesting finding from this study was that expression of ICAM-1 adhesion molecules on pulmonary vascular endothelium was significantly reduced in the lung perfusion group. This ties in with the concept that lung ischaemia up-regulates vascular endothelial adhesion molecules, which in turn promotes neutrophil-endothelial cell adhesion and resultant pulmonary injury.(41)

### **1.5.2 Neutrophil priming and recruitment to the lungs during CPB**

The priming of neutrophils during CPB is thought to play an important role in the development of peri-operative inflammatory organ dysfunction and specifically in the promotion of lung injury.(8)

Priming induces numerous changes within the neutrophil, one of which is the alteration in expression of cell surface adhesion molecules. There is typically a down-regulation of L-selectin and a reciprocal up-regulation of the  $\beta_2$  integrin complex, specifically CD11b/CD18 (MAC-1). These changes promote firm ligation of neutrophils to vascular endothelium, their subsequent recruitment into tissues and, upon stimulation, release of proteolytic enzymes and reactive oxygen species leading to tissue damage.(42)

Previous studies have reported changes in cell surface adhesion molecules following CPB. Ilton demonstrated a significant increase in neutrophil CD11b expression in central venous blood during bypass compared to preoperative levels in patients undergoing CABG with CPB.(42) He concluded that this up-regulation in CD11b is likely to contribute to pulmonary neutrophil sequestration following bypass and is therefore a potential therapeutic target. Studies by Ng(43) and Pavelkova(44) echo these findings by demonstrating a significant up-regulation of neutrophil CD11b in the early postoperative period compared to preoperative levels in patients undergoing CPB. Whether neutrophil L-selectin is down-regulated during CPB remains unclear as this has been found in some studies(44, 45) but not others.(42, 46)

The release of pro-inflammatory cytokines (such as tumour necrosis factor (TNF)-alpha, IL1, IL2, IL6 and IL8) and complement activation products during CPB contributes to neutrophil-

mediated tissue damage.(44) These mediators promote recruitment of neutrophils into tissues as well as mobilising them from the bone marrow and marginating pools.(44) This enhanced population of circulating neutrophils may be trapped in the pulmonary capillaries and a cascade of vascular endothelial cell swelling, extravasation of proteins, release of proteolytic enzymes and congestion of the alveoli with inflammatory debris ensues.(7)

The anatomy of the pulmonary vasculature further potentiates neutrophil-mediated lung damage. The mean diameter of the smallest pulmonary capillaries is 5.5µm which is smaller than the average diameter of systemic capillaries and smaller than the mean diameter of neutrophils (7.2µm).(11) As a result of this difference in diameter, neutrophils must change shape in order to transit the pulmonary capillary network. However, when neutrophils are primed they undergo cytoskeletal actin remodelling causing them to become less pliable thus hindering transit through the pulmonary vasculature.(20) This change promotes the retention of neutrophils within the capillary bed and resultant tissue damage.

Summers et al demonstrated the concept of pulmonary retention of primed neutrophils through the application of gamma scintigraphy.(25) Using autologous radiolabelled neutrophils, they investigated the pulmonary transit time of primed and unprimed neutrophils in healthy human subjects. The study illustrated that there was minimal delay in the pulmonary retention of unprimed neutrophils transiting the healthy human pulmonary vasculature but significant and prolonged pulmonary retention of primed neutrophils. The lungs are particularly susceptible to neutrophil damage via this mechanism following resumption of blood flow after CPB.

### **1.5.3 Disturbance in respiratory mechanics**

Disturbances in lung mechanics are an unavoidable consequence of surgery with CPB and lead to further pulmonary insult. The cessation of mechanical ventilation during CPB causes loss of structural integrity of the lung resulting in alveolar collapse, retention of secretions and atelectasis.(6) Breaching of the pleura during harvesting of the internal mammary artery causes even greater lung collapse and further compounds these effects. In addition, factors such as the median sternotomy incision leading to post-operative pain, poor expectoration and reduced pulmonary compliance contribute further to postoperative pulmonary dysfunction.(9)

## **1.6 STRATEGIES TO ATTENUATE PULMONARY DYSFUNCTION FOLLOWING CABG PROCEDURES**

Given the substantial implication of the neutrophilic inflammatory response in the development of post-CABG organ dysfunction, numerous ways to attenuate the inflammatory cascade have been extensively researched with varying degrees of success. These include modifications to the extracorporeal circuit, such as coating it with heparin, using different types of pump and using a leukocyte depletion filter on the extracorporeal circuit.(9, 47) Multiple pharmacological therapies have also been investigated including use of corticosteroids, aprotinin, monoclonal anti-cytokine antibodies and pentoxifylline.(9, 47)

Conducting surgery on a beating heart without the need for CPB (off-pump surgery) has also been explored over the past two decades as an alternative means to avoid the inflammatory response observed following CABG procedures. Studies have demonstrated significantly lower levels of serum inflammatory mediators and neutrophil elastase levels, improved cardiac function and reduced blood loss with off-pump CABG compared to on-pump procedures.(48) However, no significant effect of off-pump surgery on post-operative pulmonary function has been observed. Cox evaluated the alveolar-arterial oxygen gradient in patients randomised to either on-pump or off-pump CABG and demonstrated no difference at any time point in the two groups from induction of anaesthesia to six hours post-extubation.(49) Two further randomised studies have demonstrated no difference in postoperative arterial blood gases, PaO<sub>2</sub>/FiO<sub>2</sub> ratios or pulmonary function tests between off-pump and on-pump CABG groups.(50, 51)

This evidence suggests that in part, respiratory impairment may occur independently of CPB and CPB may not be responsible for as much of the pulmonary injury as was initially thought. To this end, other potential lung preservation strategies aside from eliminating CPB have been explored. Maintaining lung ventilation during CPB has been a particular focus of research in aiming to ameliorate pulmonary dysfunction in this setting and this is discussed in greater detail in section 1.7.

## 1.7 THE ROLE OF VENTILATION DURING CPB

Discontinuation of mechanical ventilation during cardiopulmonary bypass procedures is common practice and usually results in the lungs collapsing to their functional residual capacity.(52) If the pleura is disrupted, for example on internal mammary artery harvesting, the degree of lung collapse is even greater.

There are clear benefits from ceasing mechanical ventilation during CPB. These include reduced movement and blood in the operative field resulting in an increased opportunity for surgical precision. Despite this, there is a significant body of literature suggesting that hypoventilation may contribute to the pulmonary dysfunction associated with cardiopulmonary bypass. The origins of this pulmonary dysfunction in relation to hypoventilation are thought to be multifactorial and associated with the development of atelectasis, hydrostatic pulmonary oedema, reduced lung compliance and lung ischaemia.(8) Hence, many researchers have explored the hypothesis that ventilation during CPB reduces postoperative lung injury using numerous ventilation strategies including continuous positive airway pressure (CPAP) and continuous low volume ventilation. (53)

Studies evaluating the application of CPAP during CPB as a potential treatment to minimise pulmonary dysfunction include that by Loeckinger,(54) which randomised 14 patients listed for elective CABG procedures to two groups: one group received CPAP at 10cm H<sub>2</sub>O during CPB and in the other group the lungs were left open to the atmosphere. The team demonstrated that the CPAP group had significantly less ventilation/perfusion mismatch, lower pulmonary shunt values and better gas exchange indices measured four hours after completion of CPB.

Conversely, Figueiredo and team (55) concluded that CPAP at 10cm H<sub>2</sub>O does not improve post-operative gas exchange in patients undergoing CPB. This study randomised 30 patients undergoing CABG procedures to either CPAP at 10cm H<sub>2</sub>O or no CPAP during CPB. In both groups, arterial blood samples for gas analysis and pulmonary gas exchange index calculations were collected at four different time points: pre-bypass, and 30 minutes, 12 hours and 24 hours post-CPB. While the CPAP group had an improved PaO<sub>2</sub> at 30 minutes post CPB, no significant sustained effect on pulmonary gas exchange was observed.



The conclusions of the Figueiredo study are echoed by Altmay and colleagues (56) who evaluated the effects of CPAP at 10cm H<sub>2</sub>O versus no CPAP in a larger cohort of 120 patients undergoing CABG procedures. Although this study demonstrated a lower shunt fraction and lower alveolar-arterial oxygen partial pressure difference during surgery in the CPAP group, these differences were not sustained post-operatively. Similarly, Berry and colleagues (57) evaluated the use of applying CPAP at 5cm H<sub>2</sub>O with 21% or 100% oxygen during CPB. While alveolar-arterial oxygen partial pressure difference was statistically lower in the CPAP groups at 30 minutes post CPB this effect was not sustained to four and eight hours after surgery.

Based on the available evidence, any benefit from CPAP in this context is short-lived with a questionable impact on the clinical outcome of treated patients. Therapeutic strategies other than CPAP have been explored with similar results.

For example, low tidal volume ventilation has been evaluated in a small number of studies. Such studies include that by Gagnon et al (58) which randomised patients to receive either volume controlled ventilation (3ml/kg) without PEEP or no ventilation during CPB in patients undergoing CABG. This team measured numerous parameters including pulmonary vascular resistance index (PVRI), PaO<sub>2</sub>/FiO<sub>2</sub>, mean pulmonary artery pressure (MPAP), post-operative length of stay and post-operative pulmonary complications. Notably, there were no statistically significant post-operative haemodynamic or clinical differences between the two groups. Similarly, John and colleagues (59) published an interim report on their prospective randomised trial evaluating the role of continued ventilation at 5ml/kg of body weight during CPB. Numerous indicators of lung injury including pulmonary gas exchange indices, static and dynamic compliance, and extravascular lung water were measured alongside clinical endpoints of intubation time, inpatient stay duration and incidence of chest complications. In the interim cohort of 23 patients, post-CPB extravascular lung water and time to extubation were significantly smaller in the group that was ventilated during CPB; however, no differences were observed in the other outcome measures. This study has strength as efforts were made to standardise many variables including having the same operating surgeon and anaesthetist. It is important to note, however, that the final study results of this randomised trial have yet to be published.

Based on the available evidence, which predominantly evaluates indices of perioperative pulmonary function, it would appear that the use of CPAP or continuing ventilation during the period of CPB has, at best, short-lived benefits and a questionable clinical impact.

## **1.8 VENTILATION DURING CARDIOPULMONARY BYPASS AND IMMUNOMODULATION**

The evidence for maintaining ventilation during CPB is sparse when outcomes of perioperative lung function are evaluated. However, there has been recent interest in the potential benefits of continued ventilation on neutrophil function and inflammatory cytokine release.

### **1.8.1 Ventilation during CPB and inflammatory mediators**

In a prospective study, Ng investigated the effects of maintaining lung ventilation during CPB on inflammatory and proteolytic responses.<sup>(60)</sup> This study randomised 50 patients undergoing CABG with CPB to continuous ventilation and non-ventilation groups with the primary endpoint of measuring pro-inflammatory mediators (IL8, matrix metalloproteinase (MMP)-9 and thromboxane B<sub>2</sub> (TxB<sub>2</sub>)) and anti-inflammatory mediators (IL10 and tissue inhibitor of metalloproteinase (TIMP)-1) in the plasma and BAL fluid at various time points before and after aortic de-clamping. The team demonstrated that continued ventilation throughout CPB resulted in significantly lower IL8 levels four hours after de-clamping of the aorta. Significantly higher levels of IL10 and TIMP-1 were observed at six hours after de-clamping of the aorta in the ventilation group. These findings suggest that continuous ventilation dampens the pro-inflammatory response in the early post-operative period. Importantly, the authors did not demonstrate any difference between the groups in clinical outcome measures, including ventilator time or length of intensive care stay. However, the study was underpowered for these clinical outcomes.

In a similar study, Durukan examined the effects of continued low frequency, low tidal volume ventilation on the inflammatory response post-CPB.<sup>(3)</sup> The study randomised 59 patients awaiting CABG procedures to either continuous ventilation during CPB or no ventilation. Serum interleukins (IL6, IL8 and IL10), serum lactate and alveolar-arterial oxygen measurements were obtained at various time points from before surgery up to six hours post-surgery. Overall, no significant differences were noted in the outcome measures between each group, suggesting low-frequency, low-tidal volume ventilation during CPB does not attenuate the inflammatory response.

Notably, these studies differ in ventilation strategies used and in their outcome measures. Further studies need to be done to draw any firm conclusions on the role of ventilation on attenuation of inflammatory mediators in this setting.

### **1.8.2 Ventilation during CPB and neutrophil priming**

One study by Ng and colleagues investigated the effect of ventilation during CPB on the activation of peripheral blood and alveolar neutrophils by measurement of CD11b cell surface adhesion markers.<sup>(43)</sup> This study randomised 46 patients undergoing CABG with CPB to continuous ventilation during CPB (5ml/kg, FiO<sub>2</sub> 0.5 and 5 ventilations per minute) or non-ventilation. BAL and radial artery blood samples were obtained at induction of anaesthesia and four hours following de-clamping of the aorta. The study found a significant increase in blood neutrophil CD11b levels following CPB compared with baseline values; however, no difference was observed between the ventilated and non-ventilated groups. Further studies using a range of different ventilator settings and techniques need to be conducted to validate these conclusions. However, these emerging findings suggest that other factors such as contact of neutrophils with the extracorporeal circuit or lack of pulmonary artery perfusion may be more important in causing neutrophil activation than the arrest of ventilation during CPB.

### **1.8.3 Ventilation during bypass and the effect on neutrophil phagocytosis**

There is currently no published research on the effect of ventilation during CPB on neutrophil phagocytosis. Our laboratory has recently conducted unpublished research in a small number of patients undergoing CABG with one lung ventilation to directly evaluate the effect of ventilation on the function of neutrophils returning from the lungs via the pulmonary veins following bypass. This preliminary study, which involved five patients, demonstrated a marked reduction in peripheral blood neutrophil phagocytic capacity following CPB. However, neutrophil phagocytic capacity appeared to be significantly higher in the pulmonary vein sample obtained from the ventilated lung compared to the sample from the non-ventilated lung. Cell surface markers of neutrophil activation and serum levels of inflammatory and anti-inflammatory cytokines in blood obtained from each lung were evaluated, but no significant difference was detected.

While no definite conclusions can be drawn from this preliminary study, owing to the very small sample size, this opens up the exciting prospect that lung ventilation during CPB may

have an important role to play in restoration of neutrophil phagocytosis. The direct effects of impaired neutrophil phagocytosis following CPB on post-operative lung injury, infection rates and long-term clinical outcome are poorly understood. However, further exploration of these preliminary findings may facilitate a better understanding in this area.

## 1.9 REGULATION OF NEUTROPHIL FUNCTION BY THE HEALTHY HUMAN LUNG

The theory that the human lung plays a role in regulation of neutrophil function is a relatively new concept that has emerged over the past few years. Novel work by Summers and colleagues suggests that the healthy human lung may selectively retain primed neutrophils, facilitate their de-priming and subsequently release them back into the systemic circulation in a quiescent state, thus protecting the systemic circulation from their potential toxic effects.(25)

In a recent study, neutrophils isolated from the peripheral blood of healthy subjects were either primed with granulocyte-macrophage colony-stimulating factor (GM-CSF; n=8), primed with platelet activating factor (PAF; n=5) or remained un-primed (n=8), before being radiolabelled and re-injected. Pulmonary neutrophil transit time was then measured using gamma scintigraphy.

The study demonstrated that un-primed neutrophils had a pulmonary transit time of 14.2 seconds and less than 5% first pass retention in the lung. In contrast, over 97% of neutrophils primed *ex vivo* with GM-CSF were retained on first pass through the lung with no evidence of washout over two minutes. Remarkably, these neutrophils appeared to be slowly released from the pulmonary vasculature over time such that 48% of the maximal gamma-camera signal detected from the lungs was still present at 40 minutes. Neutrophils primed *ex vivo* with PAF were initially retained but subsequently released at a faster rate such that only 14% remained in the lungs at 40 minutes. This suggests that neutrophil priming can markedly prolong pulmonary transit time in a manner that is specific to the priming agent. These findings led the team to hypothesise that the lung may have a regulatory role in terms of de-priming neutrophils and should this mechanism fail, release of primed neutrophils into the circulation may occur and lead to organ damage.

A limitation of this work is that the neutrophils used were primed *ex vivo*. It is possible that these behave differently to neutrophils primed *in vivo*; however, there are few data available to support this. In fact, a previous study by Haslett in a rabbit model found that neutrophils primed *in vivo* or *ex vivo* demonstrate comparable sequestration in the pulmonary vascular bed.(61) It could also be argued that while neutrophils primed *ex vivo* appeared to take

significantly longer to traverse the pulmonary vasculature, the lung may not have had any effect on facilitating their de-priming before release into the systemic circulation, as this was not directly measured. To address this limitation, the study was extended to a separate group of subjects with ARDS (n=8), patients with sepsis but not ARDS (n=6) and perioperative controls (n=5). Whole blood samples were simultaneously taken from the internal jugular vein (blood entering the lungs) and the radial artery (blood having left the lungs) in each of the patients. Markers of neutrophil priming including CD11b and CD62L cell surface expression and neutrophil shape change were measured in the arterial and venous samples and expressed as the trans-pulmonary gradient (ratio of the arterial value over the venous value).

There was no difference between the three groups with regard to trans-pulmonary CD11b cell surface expression or neutrophil shape change. However, subjects with ARDS had a significant decrease in trans-pulmonary gradient of CD62L cell surface expression compared with patients with sepsis but not ARDS and preoperative controls. This suggests that patients with ARDS lose the ability to retain and de-prime neutrophils. One interpretation of this finding is that the pulmonary de-priming of neutrophils may fail when pulmonary function is impaired, resulting in more primed neutrophils entering the systemic circulation.

The pulmonary retention of primed neutrophils during on-pump CABG procedures is well described, but the impact of ventilation during CPB on the de-priming of pulmonary neutrophils has not been studied to date. Further study in this area may shed light on the role of the lung in regulating neutrophil priming status.

## **1.10 ONE-LUNG VENTILATION EXPERIMENTAL MODEL DURING CARDIOPULMONARY BYPASS**

During CABG procedures, a bloodless and motionless surgical field is achieved using an extracorporeal circulation circuit. The heart and lungs are arrested during CPB and delivery of oxygenated blood to the patient is maintained by a mechanical pump and artificial oxygenator within the extracorporeal circuit.(3)

Typically, bilateral mechanical ventilation ceases at the point at which the patient has been fully established on CPB. Pulmonary artery blood flow also ceases at this point. On discontinuation of CPB (following completion of all distal vessel anastomoses) bilateral mechanical ventilation is re-commenced and there is resumption of blood flow to both lungs via the pulmonary arteries.

Studying the cardiopulmonary bypass model provides an unparalleled opportunity to determine the effects of ventilation during CPB on blood neutrophil function and inflammatory mediators. The beauty of using this model is that following the resumption of blood flow via the pulmonary artery after CPB, the blood exiting each lung can be studied separately in the individual pulmonary veins. Furthermore, one lung can be selectively ventilated during CPB while the other is selectively deflated. This provides a unique opportunity to compare neutrophil function and inflammatory mediators in blood coming from a lung that has been ventilated during CPB and a lung that has remained deflated during CPB. To this end, the patient acts as their own control.

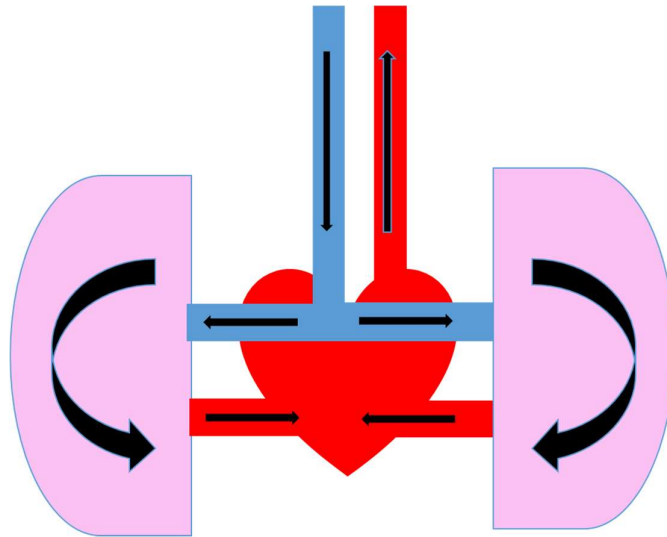
The following series of schematic illustrations highlights the changes in the circulatory and gas exchange systems during CPB. The experimental model of one-lung ventilation during CPB and subsequent sampling of blood exiting each lung following resumption of normal blood flow is also illustrated (Figure 5-Figure 8). Figure 6 in this sequence is a duplicate of Figure 1 which featured earlier in this chapter for ease of reference.

One-lung ventilation during CABG operations offers experimental advantage over and above other types of on-pump cardiac surgery (e.g. valve replacements) as the resumption of full pulmonary and systemic blood flow following CPB in CABG patients is usually standardised and relatively quick. Heart valve operations often require a period of partial bypass before

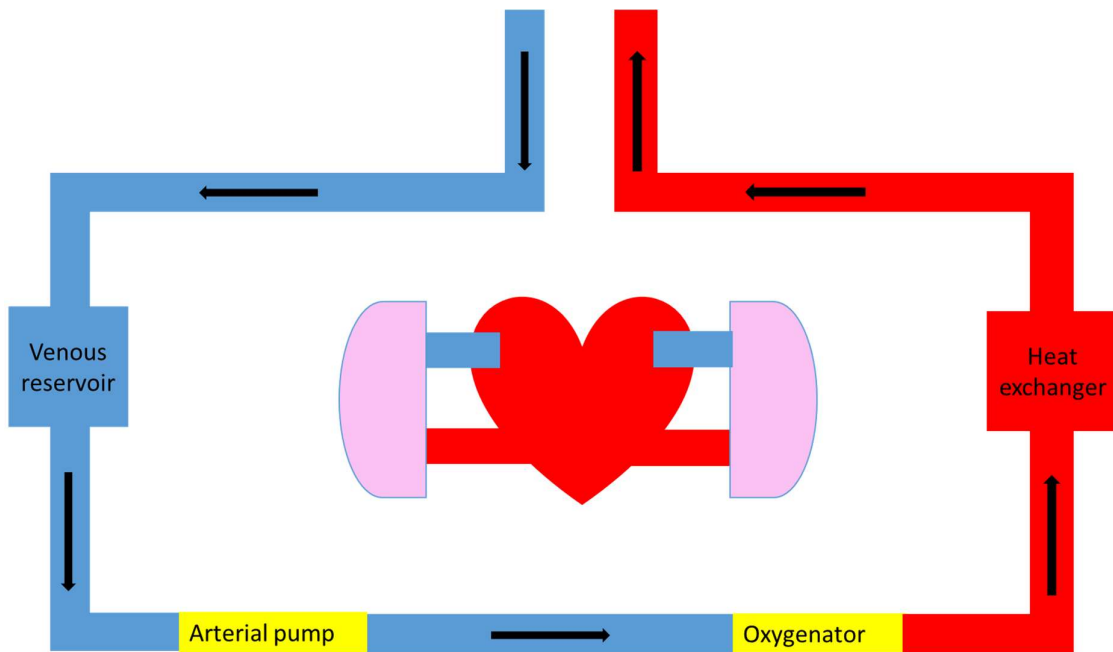


CPB is fully stopped and this would make results of the effects of one-lung ventilation (OLV) difficult to interpret.

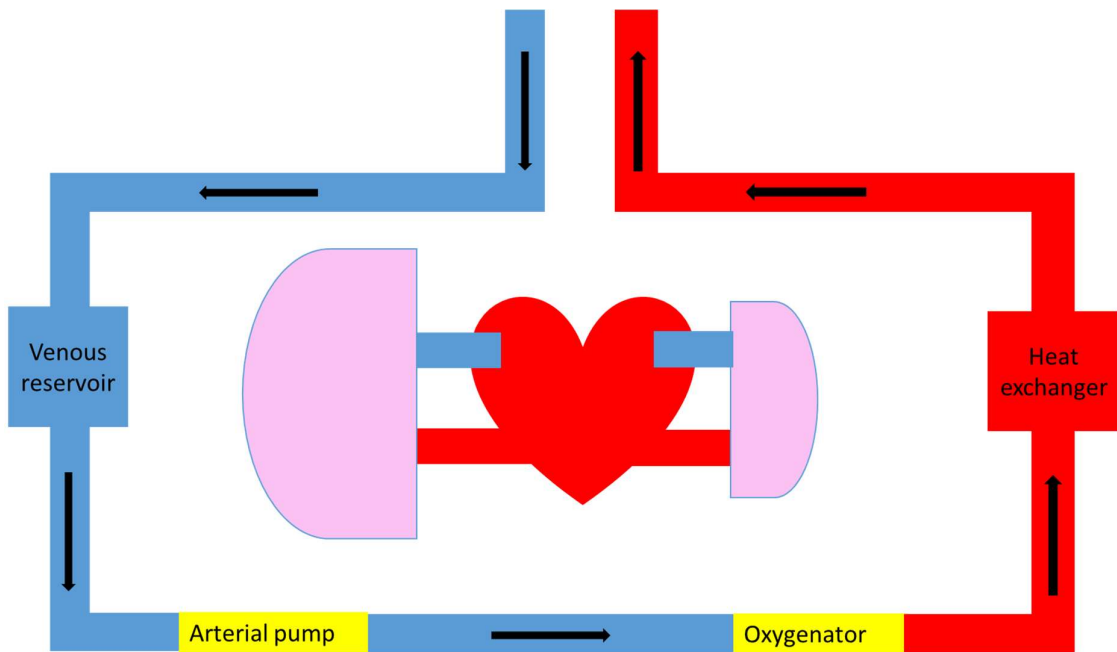
Notably, there is no published literature on the use of one-lung ventilation to assess the effects of ventilation on neutrophil function or inflammatory mediators in blood directly sampled from individual pulmonary veins, thus highlighting the novelty of this experimental model.



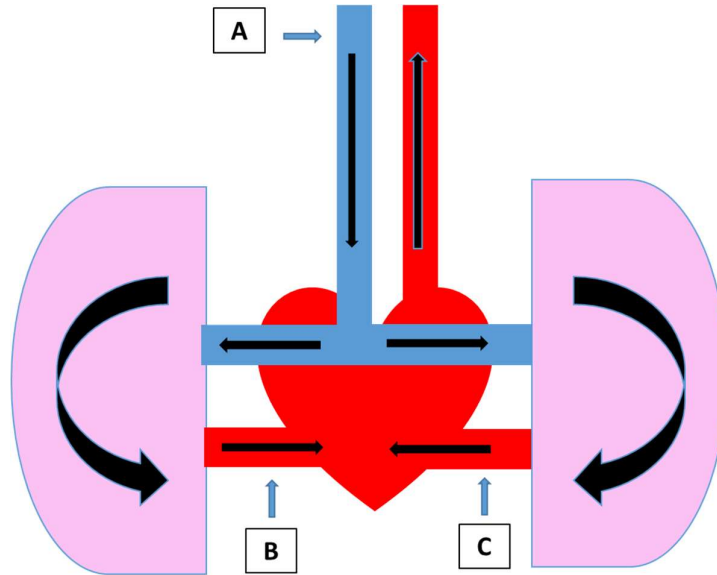
*Figure 5: Schematic illustration of the normal human cardiopulmonary circulation. Deoxygenated blood (blue) from the body enters the right atrium via the venae cavae. The right ventricle then pumps this deoxygenated blood to the left and right lungs via the left and right pulmonary arteries respectively. Following a process of gas exchange within the lungs (pink), oxygenated blood (red) is delivered to the left atrium from each lung via the left and right pulmonary veins. The left ventricle then pumps the oxygenated blood to the body. The black arrows illustrate the direction of blood flow.*



*Figure 6: Schematic illustration of the circulatory and gas exchange system during cardiopulmonary bypass. On initiation of cardiopulmonary bypass, deoxygenated blood from the body is syphoned into a venous reservoir under gravity via a right atrial cannula. The blood is then pumped into an oxygenator and heat exchanger within the cardiopulmonary bypass circuit before being returned to the patient via an arterial cannula usually placed in the distal ascending aorta. An aortic cross clamp placed in the ascending aorta prevents flow of blood back into the heart. Under normal circumstances the lungs remain unventilated during CPB and blood flow via the pulmonary artery ceases. Cardiac activity is also stopped via a cardioplegic solution infused around the heart during CPB. As a result, the heart and lungs are essentially isolated from the circulatory system during CPB thus achieving a relatively bloodless and motionless field for the operating surgeon. The black arrows demonstrate the direction of blood flow.*

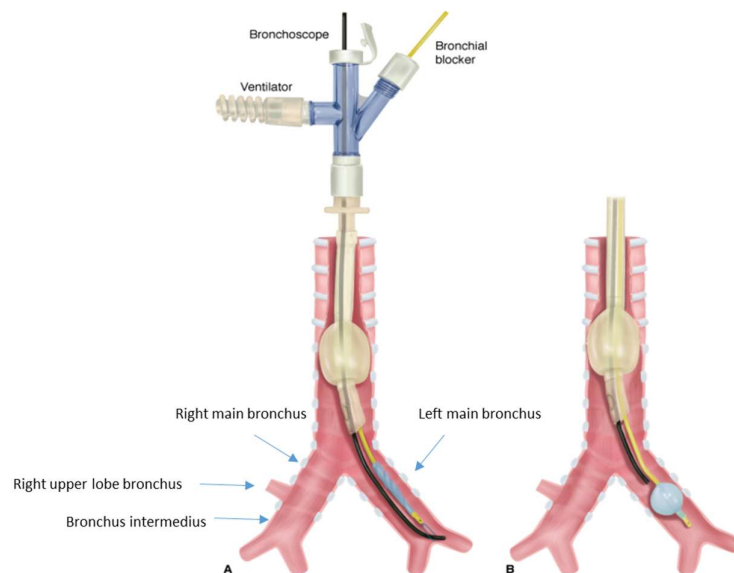


*Figure 7: Schematic illustration of one-lung ventilation during cardiopulmonary bypass. Under normal circumstances, bilateral ventilation ceases following the initiation of CPB. By placing an endobronchial blocker within the left main bronchus the left and right lungs can be isolated thus allowing ventilation to be initiated to the right lung whilst maintaining full deflation of the left lung during the period of CPB. Irrespective of the one-lung ventilation system, there continues to be no blood flow to either lung via the pulmonary arteries during CPB. Following CPB, the endobronchial blocker within the left main bronchus can be deflated and bilateral ventilation resumed to both lungs as usual for the remainder of the operation. The black arrows demonstrate the direction of blood flow during the period of CPB.*



**Figure 8: Schematic illustration of blood sampling sites following resumption of normal cardiopulmonary blood flow on completion of CPB.** Following completion of all distal vessel anastomoses, bilateral mechanical ventilation and cardiac activity is resumed. The patient is subsequently weaned off CPB resulting in the restoration of the normal human cardiopulmonary circulation. A blood sample from the central line which is typically placed in the internal jugular vein (a branch of the superior vena cava) is representative of the blood entering the right side of the heart and thus the common afferent blood entering each lung via the pulmonary arteries [A]. Efferent blood exiting each lung in the right [B] and left [C] pulmonary veins can be sampled separately. In summary, each lung has a common afferent blood supply via the pulmonary arteries but separate efferent blood exiting in the pulmonary veins. The effect of maintaining lung ventilation during CPB compared to maintaining lung deflation can be evaluated by comparing the blood entering and exiting each lung.

One-lung ventilation can be achieved during on-pump CABG procedures by placing an endobronchial blocker within a main bronchus via the endotracheal tube. Placement of the endobronchial blocker within the left main bronchus (to keep the left lung deflated during CPB) is preferable to a right sided endobronchial blocker for two main reasons. Firstly, the left main bronchus is longer (~5cm) compared to the right main bronchus (~2-3cm) which facilitates easier placement of the endobronchial balloon and reduces the possibility of endobronchial balloon misplacement during the operation (Figure 9). Secondly, the left pleura is opened in most CABG cases to harvest the left internal mammary artery as a conduit vessel. Opening the left pleura ensures greater collapse (below functional residual capacity (FRC)) of the left lung as opposed to solely stopping ventilation to that lung. This ensures maximal difference between the ventilated and deflated lung.



**Figure 9: Illustration of the placement of an endobronchial blocker within the left main bronchus under direct visualisation using a fibre-optic bronchoscope (FOB).** [A] Placement of an endobronchial blocker in the left main bronchus via a single lumen endotracheal tube using a FOB [B]. Following placement, the FOB is withdrawn, and the balloon inflated under direct visualisation. The diagram illustrates the longer left main bronchus compared to the right main bronchus which terminates when it bifurcates into the right upper lobe bronchus and bronchus intermedius. Adapted from Tobin, *Principles and Practice of Mechanical Ventilation*.(62)

### 1.10.1 One-lung ventilation strategy

Mechanical ventilation is known to have potential adverse effects on pulmonary function. Historically, anaesthetists ventilated patients in the peri-operative period using large tidal

volumes (often as high as 15mls/kg of ideal body weight). This is considerably higher than the normal ambulant tidal volume of 6mls/kg of ideal body weight.(63) The rationale behind using large tidal volumes was that it was thought to reduce intra-operative atelectasis nevertheless, a large body of subsequent research has demonstrated that ventilating with larger tidal volumes has potential deleterious pulmonary effects including the development of acute lung injury and ARDS.(63) To prevent such complications, lung-protective ventilation is now the standard of care widely adopted by anaesthetists. This involves the use of two-lung ventilation tidal volumes between 4-8mls/kg and plateau ventilatory pressures of less than 30cmH<sub>2</sub>O.(64, 65). The optimal tidal volume for lung protective ventilation using a one-lung ventilation model is still under debate however, current evidence strongly supports the use of low tidal volume strategies. (66) (67)

## **1.11 CARDIOPULMONARY BYPASS AND INTERFERON GAMMA**

Interferon gamma (IFN $\gamma$ ) exhibits multiple biological effects and plays a central role in coordinating critical components of the innate and acquired immune response.(68)

Previous studies have demonstrated a reduction in serum levels of IFN $\gamma$  following surgery with cardiopulmonary bypass leading to the hypothesis that this may contribute to the postoperative immune dysfunction and infection that commonly complicates surgery with CPB.(69)

Connecting the finding of reduced postoperative neutrophil phagocytosis in our 2012 preliminary study with literature describing reduced levels of IFN $\gamma$  following surgery with CPB, raises the question as to whether the two may be linked.

The remainder of this chapter outlines the background to the smaller IFN $\gamma$  laboratory study which aims to assess the effects of recombinant human IFN $\gamma$  on restoring neutrophil phagocytosis using an *in vitro* model.

### **1.11.1 Interferon gamma biology**

Interferons are a family of cytokines that are released by numerous host cells upon infection. They have multiple effects on a diverse range of immune cells and play an important role in both adaptive and innate immune responses.(70)

In mammals, interferons are classified into two groups according to their receptor specificity and sequence homology.(70, 71) Type 1 interferons consist of IFN- $\alpha$ , IFN- $\beta$ , IFN- $\omega$  and IFN- $\tau$ . They are structurally similar and bind to a common interferon-alpha receptor (IFNAR) where they primarily exhibit anti-viral effects. IFN $\gamma$  is the only type 2 interferon. It is structurally different from type 1 interferons and binds to the IFN $\gamma$  receptor. As a pleiotropic cytokine, it exerts a diverse array of antiviral, antiproliferative and immunomodulatory effects on normal and tumour cells.(68) Natural killer (NK) cells and antigen presenting cells (APC), which are components of the innate immune response, are the main source of IFN- $\gamma$  production in early host defence.(72) T-helper cell type 1 (TH1) lymphocytes become the main source of IFN $\gamma$  production in the adaptive immune response.(71) Interleukin 12 (IL12) and interleukin 18 (IL18) control IFN $\gamma$  production and serve to increase levels of IFN $\gamma$  during the immune response.(71)



### **1.11.2 Interferon gamma receptor and signalling pathway**

The IFN $\gamma$  receptor consists of two ligand binding IFNGR1 chains and two signal- transducing IFNGR2 chains (Figure 10). The receptor requires the presence of associated signalling machinery to facilitate signal transduction as it lacks intrinsic phosphatase/ kinase activity.(71) The Jak1 and Jak2 members of the Janus family of kinases are associated with the IFN $\gamma$  receptor and are activated upon ligand binding.(68)

Following IFN $\gamma$  binding, Jak2 autophosphorylates, leading to transphosphorylation of Jak1. Subsequently, a Stat1 (signal transducer and activator of transcription 1) pair is recruited to the receptor which becomes phosphorylated leading to dissociation of two Stat1 homodimers from the receptor. The Stat1 homodimers then enter the nucleus to initiate or suppress a diverse array of IFN $\gamma$  related genes. Such genes include those resulting in upregulation of cellular proteins for example ICAM-1 and monokine induced by IFN $\gamma$  (MIG) which serves as a chemoattractant for T cells.(71) Other IFN $\gamma$  related genes are transcription factors eg interferon regulatory factor-1 (IRF1) which drive the next wave of transcription.

While IFN $\gamma$  is understood to primarily signal via the Jak-Stat pathway to facilitate signal transduction, other non-stat pathways are described but are less well defined.(68) These include the Rap1 signalling pathway described by Alsayed and colleagues in which IFN $\gamma$  activates the c-Cbl protooncogene product (CBL) resulting in downstream activation of Rap 1 (a small G-protein that possesses tumour suppressor activity).(68)

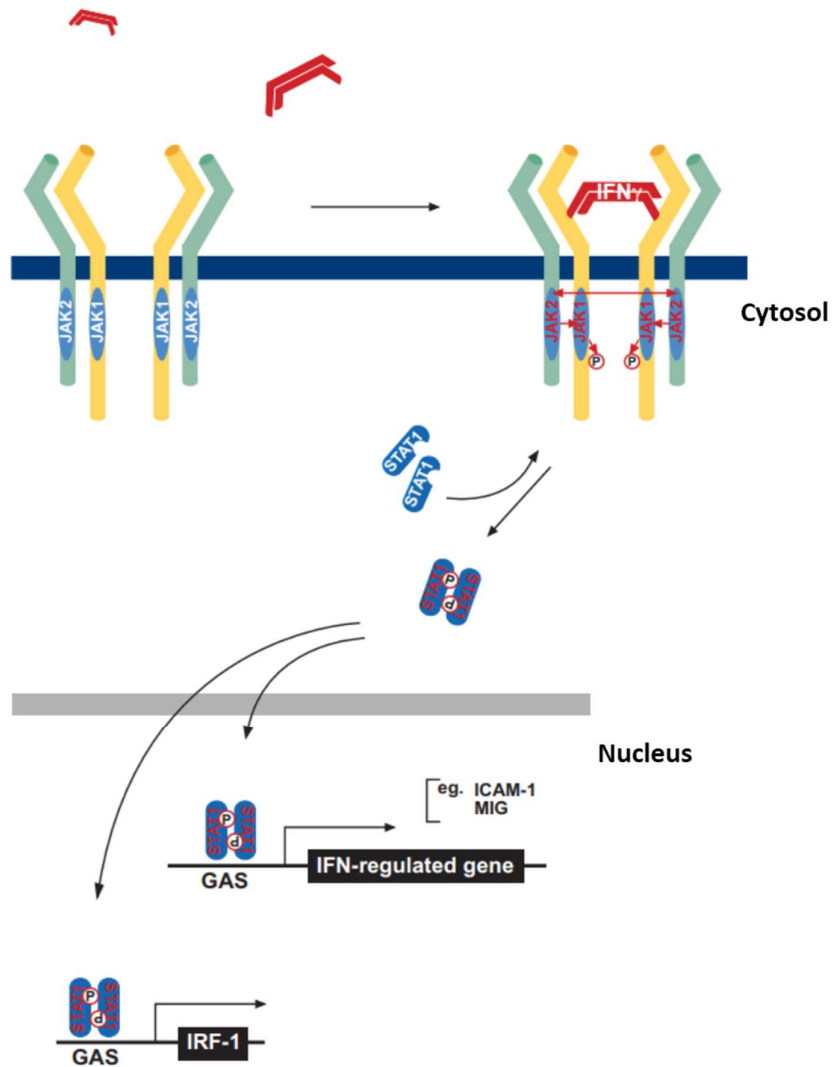


Figure 10: **Schematic representation of IFN $\gamma$  signal transduction via the Jak-Stat pathway.** Binding of IFN $\gamma$  to the IFN $\gamma$  receptor (IFNGR1, yellow and IFNGR2, green) results in autophosphorylation of Jak2 leading to transphosphorylation of Jak1. The activated Jak1 results in recruitment of a Stat1 pair, which is phosphorylated and induces dissociation of a Stat1 homodimer from the receptor. The Stat1 homodimer then travels to the nucleus to initiate or suppress IFN $\gamma$  related genes. Abbreviations: IFN $\gamma$ , Interferon-gamma; JAK, Janus family of kinases; Stat1, signal transducer and activator of transcription 1; GAS, IFN $\gamma$  activation sites. Adapted from Schroder et al.(71)

### **1.11.3 Interferon gamma and immune function**

The majority of IFN $\gamma$  research has focused on its effect on cells of the adaptive immune response, particularly activated macrophages where it has been shown to exert multiple biological effects. These include upregulation of macrophage antigen presenting capacity and induction of antimicrobial mechanisms.(71, 73) IFN $\gamma$  has also been shown to enhance the activity of cells such as NK cells and B-lymphocyte cells (where it promotes immunoglobulin secretion and class switching).(71) Moreover, the availability of recombinant human IFN $\gamma$  has facilitated the exploration of its clinical utility on cell types such as monocytes. This is exemplified by Docke and colleagues who demonstrate that administration of subcutaneous IFN $\gamma$  to selected patients with sepsis and reduced monocytic HLA-DR (Human Leukocyte Antigen – antigen D Related) expression can result in restoration of monocytic activity. This suggests that treatment with IFN $\gamma$  may have immunomodulatory potential and may enhance the clearance of sepsis in carefully selected patients.(74)

The effect of IFN $\gamma$  on cells of the innate immune system such as neutrophils has been studied to a lesser degree, nevertheless IFN $\gamma$  has been shown to enhance neutrophil oxidative burst in both human and animal models.(75-77) This research has in part led to the use of recombinant human IFN $\gamma$  in clinical practice: for example in the treatment of patients with chronic granulomatous disease (a primary immunodeficiency resulting from mutations in the genes encoding for components of the NADPH oxidase system which is critical for neutrophil pathogen killing).(78) IFN $\gamma$  has also been shown to regulate the expression of many cell surface markers involved in neutrophil adherence and extravasation (79) as well as inhibiting neutrophil apoptosis (80) thus illustrating the dynamic potential IFN $\gamma$  possesses to modify innate immune cell function.

## **1.12 INTERFERON GAMMA AND THE EFFECTS ON NEUTROPHIL PHAGOCYTOSIS**

Despite the immunomodulatory effects of IFN $\gamma$  on neutrophil function, there is a relatively small body of research assessing the direct effect of IFN $\gamma$  on neutrophil phagocytosis.

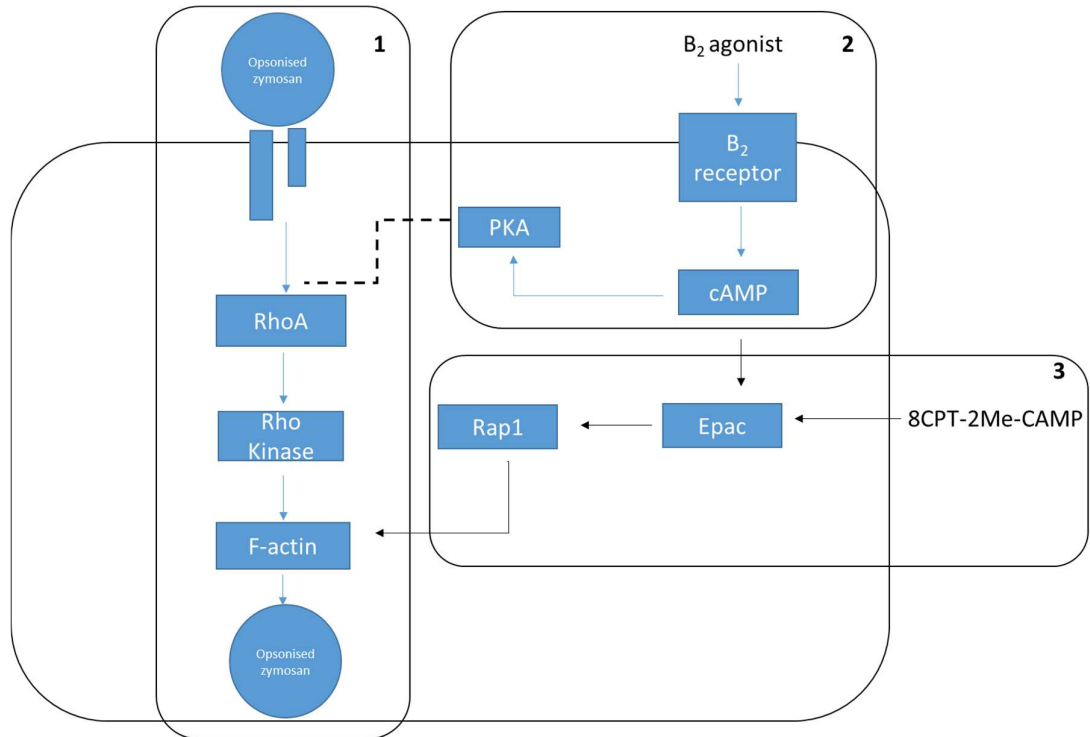
Using a murine model, Marchi and colleagues demonstrated that recombinant IFN $\gamma$  enhanced phagocytic capacity of neutrophils in response to zymosan and opsonised zymosan stimuli. Moreover, they observed an increase in Fc $\gamma$  receptor, complement receptor and dectin-1 receptors and an increase in neutrophil reactive oxygen species (ROS) production following treatment with IFN $\gamma$ .(77) *In vitro* treatment of neutrophils with IFN $\gamma$  for 20-30 minutes has been shown by Shalaby and colleagues to increase neutrophil phagocytosis of latex beads (81) and similarly, has been shown to enhance neutrophil phagocytosis of *Plasmodium falciparum* merozoites.(82)

While these studies have yielded promising results of the potential augmentative capacity of IFN $\gamma$  on neutrophil phagocytosis, more research is needed to consolidate these findings and to investigate the potential restorative capacity of IFN $\gamma$  on neutrophils with impaired phagocytosis. Moreover, investigation into the cell signalling mechanisms behind the apparent effects of IFN $\gamma$  on neutrophil phagocytosis is needed to facilitate a greater understanding of the potential pharmacological role for recombinant human IFN $\gamma$  in clinical situations where neutrophil phagocytosis is impaired.

## **1.13 IN-VITRO MODEL TO ASSESS THE EFFECTS OF INTERFERON GAMMA ON NEUTROPHIL PHAGOCYTOSIS**

Impaired neutrophil phagocytosis has been demonstrated in critically ill patients with severe systemic inflammation and has been shown to predict subsequent nosocomial infection within the intensive care setting.(83, 84) In order to investigate potential therapies to restore neutrophil phagocytosis without depending on patient blood samples which are often difficult to obtain, *in vitro* models facilitating experimental impairment and subsequent restoration of neutrophil phagocytosis are of great utility. Scott and colleagues describe an experimental model using the  $\beta_2$ -agonist salbutamol to impair phagocytic capacity of adherent neutrophils for opsonised zymosan particles.(85) Using this model, they demonstrated that impairment of neutrophil phagocytosis by salbutamol was associated with significantly reduced RhoA activity (a small GTPase protein inherently involved in coordinating the terminal steps of cytoskeletal organisation and effective phagocytosis)(16) and that this impairment was mediated through cyclic adenosine monophosphate (cAMP). Furthermore, by applying activators to redirect cAMP in this experimental model, they sought to characterise novel pathways by which impaired neutrophil function can be reversed (Figure 11).

Similarly, this *in vitro* model for reducing complement mediated neutrophil phagocytosis provides a platform to explore the effect of IFN $\gamma$  on neutrophil phagocytic capacity and facilitates the characterisation of downstream regulatory cell signalling pathways. Theoretically, by inhibiting key molecules known to be involved in IFN $\gamma$  cell signalling, (eg Jak1, Jak2 and Stat1) inroads can be made with regards to mapping out the potential pathways by which IFN $\gamma$  impacts upon neutrophil phagocytosis.



**Figure 11: A schematic representation of normal phagocytosis by neutrophils (1), inhibition of phagocytosis by salbutamol (2) and activation of Epac directing signalling through Rap1 to restore phagocytosis even when RhoA is simultaneously blocked (3).** Section 1 illustrates normal complement mediated phagocytosis. After engagement of opsonised zymosan with the complement receptor,(86) signalling through RhoA results in alterations in the filamentous actin cytoskeleton necessary for complement mediated phagocytosis. Section 2 illustrates the proposed mechanism by which salbutamol, a  $\beta_2$ -agonist can experimentally impair neutrophil phagocytosis. On activation of the  $\beta_2$  receptor, cAMP acts via PKA to inhibit RhoA activation thus preventing cytoskeletal organisation and effective phagocytosis. Section 3 illustrates a proposed alternative intracellular pathway for phagocytosis when RhoA is blocked. By activating Epac with an analogue of cAMP (8CPT-2ME-CAMP), RhoA is bypassed and signalling via Rap1 results in actin cytoskeletal rearrangement and subsequent phagocytosis. Abbreviations: F-actin, filamentous actin; PKA, protein kinase A; Rap1, Ras-related protein 1; cAMP, cyclic adenosine monophosphate; Epac, exchange protein directly activated by cAMP. Adapted from Scott et al.(85)

## **1.14 AIM AND HYPOTHESES – CLINICAL STUDY**

The clinical study aims to explore whether there is an immunological benefit of lung ventilation during CPB in patients undergoing CABG procedures by using a novel one-lung ventilation model with direct pulmonary vein blood sampling.

The hypotheses are:

- The phagocytic capacity of neutrophils in pulmonary vein blood is significantly improved when returning from a lung which has been ventilated during CPB compared to a lung which has been selectively deflated during CPB.
- The phagocytic capacity of neutrophils in central venous blood is significantly reduced following CPB.
- Neutrophils returning from a lung which has been ventilated during CPB are less primed than neutrophils returning from a selectively deflated lung.
- Pulmonary vein blood returning from a lung which has been ventilated during CPB contains lower levels of pro-inflammatory cytokines and higher levels of anti-inflammatory cytokines than pulmonary vein blood returning from a non-ventilated lung.

## **1.15 AIM AND HYPOTHESES – INTERFERON GAMMA LABORATORY STUDY**

Alongside the clinical study, a smaller lab-based study will be described, aiming to evaluate the effect of recombinant human IFN $\gamma$  on neutrophil phagocytosis and to explore the downstream cell signalling pathways involved.

The hypotheses are:

- Recombinant human IFN $\gamma$  restores the phagocytic capacity of neutrophils with impaired phagocytic function induced by the  $\beta_2$ -agonist, salbutamol.
- IFN $\gamma$  enhances RhoA activity to restore phagocytosis in neutrophils pre-treated with the  $\beta_2$ -agonist, salbutamol.
- The effect of IFN $\gamma$  on neutrophil phagocytosis is mediated via the Jak-Stat pathway.



# CHAPTER 2: MATERIALS AND METHODS

## 2.1 OVERVIEW

The chapter begins with a description of the materials and methods used for the laboratory study which explores the effects of recombinant human IFN $\gamma$  on phagocytic function of human neutrophils. This is followed by a description of the materials and methods used for the clinical study, which explores the immunological role of lung ventilation during CPB. The chapter concludes with a description of the methods used for statistical analysis of both the laboratory and clinical studies.

## 2.2 INTERFERON GAMMA LABORATORY STUDY

### 2.2.1 Ethical approval and regulation

Ethical approval for laboratory work on healthy volunteer blood samples was granted by County Durham & Tees Valley REC (reference number 12/NE/0121). All laboratory work was conducted in accordance with Newcastle University's biological and chemical safety policies.

### 2.2.2 Screening and recruitment of healthy volunteers

Healthy volunteers were invited to donate 30-160mls of blood by advertisement through Newcastle University. Interested volunteers were issued with a participant information sheet and invited to attend the Clinical Research Facility within the Newcastle upon Tyne Hospitals NHS Foundation Trust on a given morning to donate blood if they met the inclusion criteria. Written consent was obtained prior to venepuncture by a designated researcher. The exclusion criteria for healthy volunteers are outlined in Table 1.

Table 1: Exclusion criteria for healthy volunteers

Exclusion criteria
Under 18 years of age
Taking regular, prescribed medication (oral contraceptive pill was permissible in female patients)
Diagnosis of anaemia in the past year
Donation of more than 1 litre of blood in the previous year
Donation of blood (eg to the Blood Transfusion Service or to research studies) within the previous 90 days

Blood samples were collected with a 21-gauge needle and syringe and placed in a Falcon tube containing the anticoagulant sodium citrate (end concentration 0.4%) before being transported to the Simpson laboratory within Newcastle University.

### 2.2.3 Laboratory materials and protocols

#### Materials, reagents and equipment

1. Dextran solution was from Pharmacosmos (Holbæk, Denmark).
2. Percoll™ Plus (17-5445-01) was from GE Healthcare Life Sciences (Illinois, USA).

3. Dulbecco's phosphate buffered saline (PBS) without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (BW17512F12), Hanks' balanced salt solution (HBSS) without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (BW04-3156) and Iscove's modified Dulbecco's medium (IMDM) (BW12-722F) were from BioWhittaker (Maryland, USA).
4. Sodium chloride solution, (S6546), calcium chloride solution (21115), citrate concentrated solution (S5770), sodium fluoride (S7920), trypan blue (302643), Giemsa stain (G4507), zymosan A from *Saccharomyces cerevisiae* (Z4270), salbutamol (58260), acetone (W332615), protease cocktail inhibitor (P8340), N, N, N', N'-tetramethylethylenediamine (TEMED) (T9281), phenylmethanesulfonyl fluoride (PMSF) (P7626), ammonium persulphate (AP) (A3678), sodium dodecyl sulphate (SDS) (L3771), unconjugated rabbit anti-actin polyclonal antibody (A2668), bromophenol blue (B0126),  $\beta$ -mercaptoethanol (M6250), nitrocellulose western blotting membranes (10600002) and 15ml clear glass vials (27162), 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (D9542) and propidium iodide (PI) (P4170) were from Sigma-Aldrich (Gillingham, UK).
5. Recombinant human IFN $\gamma$  protein (285-IF-100/CF) was from R+D systems (Minneapolis, USA).
6. Sodium orthovanadate (508605) was from Calbiochem (San Diego, USA).
7. 30% acrylamide/bis solution, 37.5:1 (161-0158) was from Bio-Rad laboratories (California, USA).
8. Colour protein standard (P77125) was from New England Biolabs (Massachusetts, USA).
9. Unconjugated rabbit anti-human-RhoA monoclonal antibody (IgG) (2117P) was from Cell Signaling Technology (Massachusetts, USA).
10. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit polyclonal antibody (IgG) (43C-CB1106-FIT) was from Stratech (Newmarket, UK).
11. Pierce™ bicinchoninic acid (BCA) protein assay kit (23225), Pierce™ western blotting substrate (10590624), ethanol (10041814), Falcon™ 15 ml and 50ml conical tubes (352097 and 352098), Falcon™ 5ml round-bottom polystyrene flow cytometry tubes

(10186360), Sorvall™ Primo™ benchtop centrifuge (75005184), the Hausser Scientific™ hemocytometer (A014835), CL-Xposure™ film (34089) and pHrodo® Green *Staphylococcus aureus* (*s.aureus*) Bioparticles® Conjugate (p3537) were from Thermo Fisher Scientific (Massachusetts, USA).

12. G-LISA® RhoA activation assay Biochem Kit™ (BK124) was from Cytoskeleton (Colorado, USA).
13. Skimmed milk powder was from Marvel (Premier International Foods Ltd., Spalding, UK).
14. Ruxolitinib (SM87-2) was from Cambridge Bioscience (Cambridge, UK).
15. GGTI-298 (Rap1 GTPase inhibitor, 2430) was from Tocris Bioscience (Bristol, UK).
16. Fludarabine (14128) was from Cayman Chemical (Michigan, USA).
17. Polystyrene tissue culture plates (6-well and 24-well) (83.3920 and 83.3922) and polypropylene Eppendorfs (2ml) (72.695) were from Sarstedt (Numbrecht, Germany).
18. JB Nova unstirred waterbath was from Grant Instruments Limited, (Cambridgeshire, UK).
19. Shandon cytospin 3 was from Thermo Shandon Limited (Cheshire, UK).
20. Leitz Laborlux II biological microscope was from SpectroGraphic Limited (Leeds, UK).
21. BMG Fluostar Optima plate reader was from BMG Labtech (Offenburg, Germany).
22. Mini-Protean II multiscree apparatus was from Bio-Rad (California, USA).
23. BD PharmLyse™ (555899), CellWASH (349524), BD FACSCanto™ II and BD FACS™ lyse wash assistant were from BD Biosciences (New Jersey, USA).
24. Annexin V (AnV) binding buffer (422201), Allophycocyanin (APC)-conjugated annexin V (640919), fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD11b Antibody (IgG1) (301329), FITC-conjugated mouse IgG1, κ Isotype control (981802), APC-conjugated mouse anti-human CD62L antibody (IgG1) (304810), APC-

conjugated mouse IgG1,  $\kappa$  Isotype control (981806) were from BioLegend (California, USA).

25. V-plex pro-inflammatory panel 1 (human) kit (K15049D-1) was from Meso Scale Diagnostics (Maryland, USA).

### **Isolation of blood neutrophils**

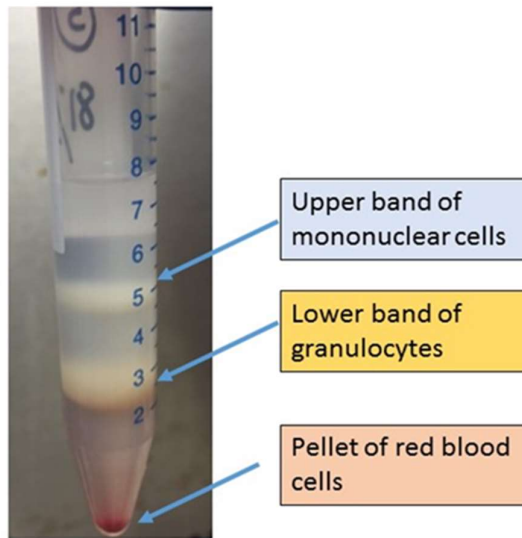
Neutrophils were isolated from citrated whole blood using a Percoll density gradient technique.<sup>(87)</sup> After centrifugation of whole blood at 300g for 20 minutes at 37°C and brake speed zero, the upper layer of platelet-rich plasma was transferred to a glass vial and 220 $\mu$ ls of 1M calcium chloride was added per 10mls of plasma. The vial was gently inverted and incubated in a waterbath at 37°C to facilitate the production of autologous serum.

Dextran (6%) was added to the remaining cell pellet at a volume of 2.5mls per 10mls of cell pellet and 0.9% sodium chloride warmed to 37°C was added to make up to the initial blood volume. The sample was inverted gently and left at room temperature for 30 minutes.

After 30 minutes of red blood cell (RBC) sedimentation, the resultant leukocyte-rich upper layer was transferred to a new Falcon tube, washed with warmed 0.9% sodium chloride and centrifuged at 200g for 5 minutes (at 37 °C and brake speed zero).

The supernatant was then discarded, and the remaining cell pellet suspended in 2.5mls of 55% Percoll and carefully layered onto a 2.5mls 70% Percoll layer which had previously been layered onto a 2.5mls 81% Percoll layer. The components of the cell pellet were then fractionated by centrifugation of the Percoll gradient (700g, brake speed zero for 20 minutes at 37°C).

The mononuclear cell layer (which forms at the 55%/70% Percoll interface following centrifugation) was discarded and the granulocyte layer (which forms at the 70%/81% Percoll interface) was harvested into a new Falcon tube and washed in Hanks' Balanced Salt Solution (HBSS) without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  at 200g for 5 minutes (Figure 12). The supernatant was discarded and the neutrophil-enriched pellet re-suspended in an appropriate volume of HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in preparation for cell counting.

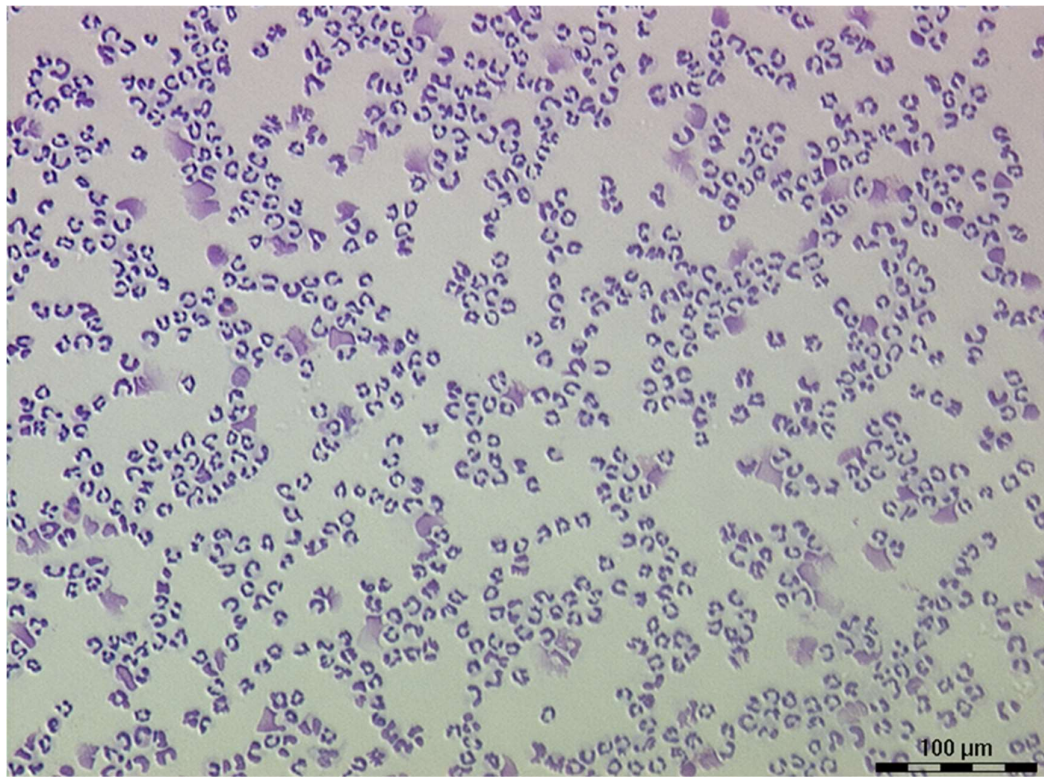


*Figure 12: Example of neutrophil isolation from whole blood using the Percoll gradient technique. The picture demonstrates the formation of an upper band of mononuclear cells between the 55%/70% Percoll layers and a lower band of granulocytes between the 70%/ 81% Percoll layers. A small pellet of remaining red blood cells can also be identified. (Picture obtained on 31.03.16 from a processed sample of healthy volunteer blood)*

### **Neutrophil cell count and purity**

Following isolation, 5µls of 0.4% trypan blue solution was added to 100µls of neutrophils suspended in HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> and counted on a haemocytometer to determine the total number of live cells (which do not take up trypan blue stain) obtained for each experimental sample.

Cytospin slides were prepared by adding 150µls of neutrophil suspension to a cytospin chamber before centrifugation at 300rpm for 3 minutes. The slides were then air-dried, fixed in acetone for 10 minutes, air-dried again and stained with Giemsa. Neutrophil cell purity was determined using light microscopy of cytospin slides and any sample with a purity of less than 95% was excluded from the analysis (Figure 13).



*Figure 13: Photomicrograph of a neutrophil cytospin demonstrating a >95% neutrophil cell purity. Giemsa staining highlights the blue multilobular nuclei of the individual neutrophils. (Picture obtained on 15.12.16 using healthy volunteer neutrophils).*

### **Neutrophil phagocytosis assay using light microscopy**

This neutrophil phagocytosis assay was based on a technique previously described. (63) Five million isolated neutrophils suspended in HBSS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were centrifuged at 200g for 5 minutes. The supernatant was discarded and the cell pellet re-suspended at 1 million/ml with warmed Iscove's modified Dulbecco's medium (IMDM) containing 1% (v/v) autologous serum.

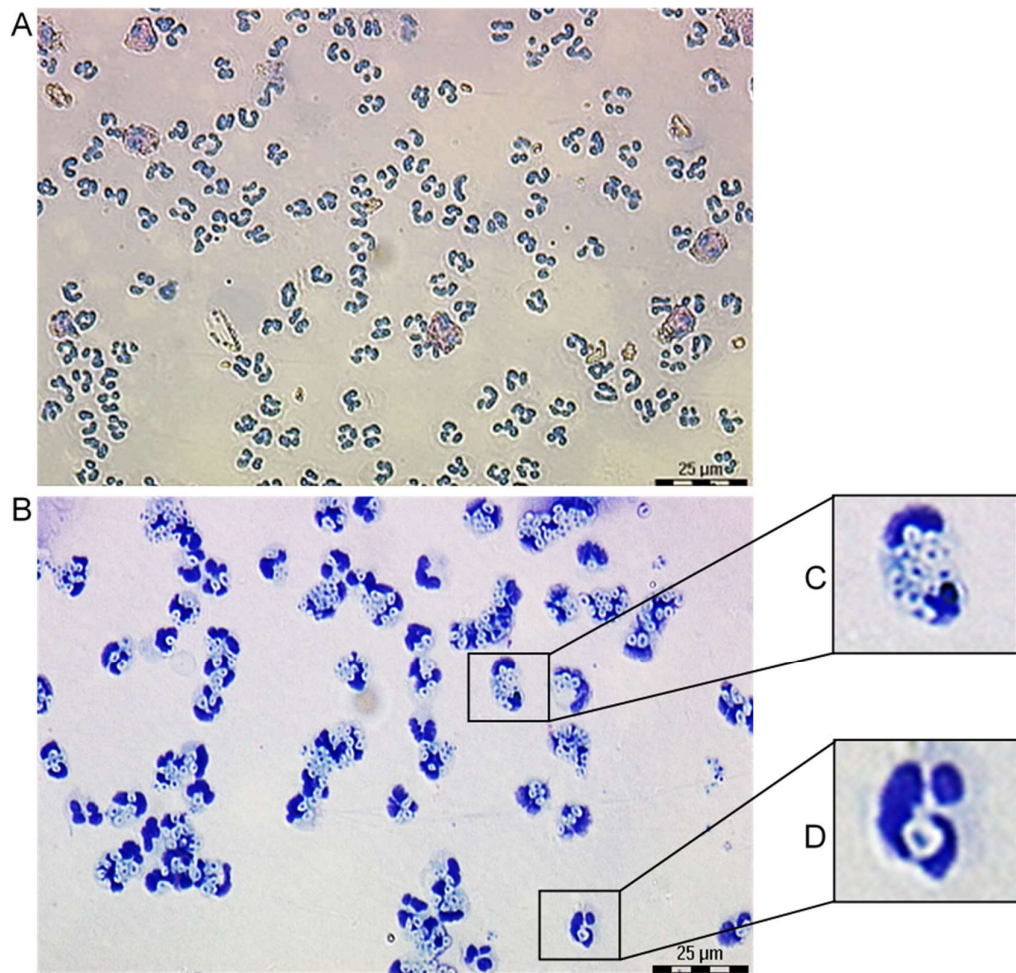
The suspension was gently inverted before aliquots of 500 $\mu\text{l}$ s were placed into 4 wells of a 24-well tissue culture plate (i.e. 1 control well and 3 experimental wells). The plate was incubated for 30 minutes in a 5%  $\text{CO}_2$  incubator at 37°C. During this time, zymosan particles were serum-opsonised by diluting to a concentration of 0.2mg/ml in IMDM and 50% (v/v) autologous serum and pre-incubated in a 37°C water bath for 30 minutes.

After incubation, 50µls of serum-opsonised zymosan suspension was added to the three experimental wells to achieve a neutrophil:zymosan particle ratio of 1:10 prior to incubation for a further 30 minutes.

The wells were washed with phosphate buffered saline (PBS) and left to air-dry for 10 minutes before being fixed with 100% ethanol. The neutrophils were then stained with Giemsa and washed with de-ionised water.

Neutrophil phagocytic capacity was quantified using light microscopy to count the number of neutrophils containing 2 or more ingested zymosan particles. Duplicate counts were performed on 4 fields with a minimum of 100 neutrophils counted per field and the mean of the four counts was calculated (Figure 14).





*Figure 14: Photomicrographs illustrating the light microscopic quantification of neutrophil phagocytosis using serum-opsonised zymosan particles. [A] Adherent neutrophils incubated without zymosan particles and stained with Giemsa. [B] Adherent neutrophils incubated with zymosan particles and stained with Giemsa. [C] Example of a neutrophil that has phagocytosed multiple zymosan particles. [D] Example of a neutrophil that has phagocytosed one zymosan particle. (Picture obtained on 15.12.16 using healthy volunteer neutrophils).*

## **Assessing the effects of salbutamol and recombinant human IFN $\gamma$ on neutrophil phagocytosis**

The short-acting  $\beta_2$  agonist salbutamol was used to impair neutrophil phagocytosis from healthy volunteer subjects as previously described.(85)

A dose response analysis was performed by pre-incubating neutrophils with salbutamol at concentrations ranging from 10nM to 10 $\mu$ M for 30 minutes at 37°C before the addition of serum-opsonised zymosan for a further 30 minutes. Phagocytosis was then quantified using the light microscopy assay as described above. The concentration of salbutamol which resulted in the largest impairment of neutrophil phagocytosis was used for all further experiments.

Recombinant human IFN $\gamma$  was applied for 30 mins at concentrations ranging from 0.1ng/ml to 100ng/ml to neutrophils that had been pre-treated with salbutamol before the addition of serum opsonised zymosan for a further 30 minutes. Phagocytosis was determined using the light microscopy assay as previously described and the concentration that resulted in the largest restoration of neutrophil phagocytosis was used for all further experiments.

### **Inhibitor work to map out cell signalling pathways**

To explore downstream cell signalling mechanisms involved in phagocytosis, the effect of inhibiting key signalling molecules known to be involved in IFN $\gamma$  signal transduction was assessed.

Isolated adherent neutrophils were incubated for 30 minutes in a 5% CO $_2$  incubator at 37°C with salbutamol (end concentration 10 $\mu$ M) and either ruxolitinib, a Jak 1 and 2 inhibitor (end concentration 1 $\mu$ M), fludarabine, a Stat1 specific activation inhibitor (end concentration 50 $\mu$ M) or GGTI-298, a Rap1 GTPase inhibitor (end concentration 10 $\mu$ M). Following this, 1ng/ml (50nM) of recombinant human interferon gamma was added for 30 minutes at 37°C prior to the addition of serum-opsonised zymosan for a further 30 minutes. Phagocytosis was quantified using the light microscopy assay as described above.

### **Preparation of neutrophil lysates**

Neutrophil lysates were prepared prior to measurement of RhoA activity (using an enzyme-linked immunosorbent assay (ELISA)) and total RhoA (using western blot) to determine

whether IFN $\gamma$  mediated restoration of neutrophil phagocytosis signals via a RhoA dependent pathway.

1ml aliquots of isolated neutrophils (suspended at 5 million/ml in warmed IMDM and 1% (v/v) autologous serum) were placed into a tissue culture plate for each experimental condition as follows: control; salbutamol alone; IFN $\gamma$  alone; and salbutamol and IFN $\gamma$ . Salbutamol was added to the appropriate wells to achieve a final concentration of 10 $\mu$ M. The plate was then incubated for 30 minutes at 37°C in a 5% CO $_2$  incubator.

Following incubation, IFN $\gamma$  was added to the appropriate wells to achieve a final concentration of 50nM and incubated for a further 30 minutes. 50 $\mu$ ls of serum-opsonised zymosan suspension was then added to the appropriate wells to achieve a neutrophil:zymosan ratio of 1:10. A corresponding well for each condition without the addition of zymosan particles served as negative controls. The plate was incubated for 5 minutes at 37°C in a 5% CO $_2$  incubator and then placed on ice.

Medium from each well was transferred into Eppendorf tubes and kept on ice prior to centrifugation at 300g for 5 minutes at 4°C before the supernatant was discarded. 1ml of wash solution (PBS, 1mM phenylmethanesulfonyl fluoride (PMSF) and 1mM sodium orthovanadate (Na $_3$ VO $_4$ )) was added to each well and the remaining adherent neutrophils scraped off the plate before being added to the respective Eppendorf tube and centrifuged again at 300g for 5 minutes at 4°C.

The supernatant was discarded and 100 $\mu$ ls of lysis solution containing lysis buffer, 5mM Na $_3$ VO $_4$ , 50mM sodium fluoride (NaF) and 1% (v/v) protease inhibitor cocktail was added to each Eppendorf tube before being kept on ice for 20 minutes. Samples were then centrifuged at 10,000g for 5 minutes at 37°C to generate an insoluble pellet and a soluble supernatant (neutrophil lysate) and stored at -80°C for future analysis.

### **Measurement and equalisation of protein concentrations in neutrophil lysates**

Protein concentration in neutrophil cell lysates was determined using a bicinchoninic acid (BCA) assay for the colorimetric detection and quantitation of total protein as per the manufacturer's instructions. This method is based upon the reduction of Cu $^{2+}$  to Cu $^{1+}$  by protein in alkaline conditions. The chelation of BCA with Cu $^{1+}$  results in a purple-coloured water-soluble product which, when exposed to a wavelength of 562nm, exhibits a linear

correlation with protein concentrations (detection range 20-2000µg/ml). Known concentrations of a common protein (bovine serum albumin (BSA)) were used to formulate a standard curve from which the sample protein concentration was determined.

The protein concentration in each sample was equalised to the concentration of the most dilute sample with lysis buffer using the following equation (where A is the higher concentration lysate (mg/ml) and B is the concentration of the most dilute lysate (mg/ml)):

$$(A - B) / (B) \times (\text{volume of A in } \mu\text{l}) = \text{___}\mu\text{l}$$

### **Measurement of RhoA activity in neutrophil lysates (ELISA)**

RhoA activity in neutrophil lysates was quantified using an ELISA (G-LISA® RhoA activation Assay Biochem Kit™) according to the manufacturer's instructions. This assay is based on the principle that active, GTP-bound Rho binds to the plate which is coated with Rho GTP-binding protein, whereas inactive GDP-bound Rho is removed during washing. A RhoA-specific primary antibody is then used to detect the bound, active RhoA followed by a secondary HRP-conjugated antibody for the colorimetric quantification of RhoA activity. Absorbance in each of the neutrophil cell lysate samples was read at 490nm using a BMG Fluostar Optima plate reader.

### **Measurement of total β-actin and total RhoA in neutrophil lysates (western blotting)**

Total β-actin (loading control) and total RhoA in neutrophil lysates were assessed using western blot analysis. Separating and stacking gels were constructed at 0.75mm thick using the BioRad Mini-Protean II apparatus according to previously described methods.(88) The separating gel was cast at 12% acrylamide (37.5:1 acrylamide:bis-acrylamide) in a solution of 1.5M Tris pH 8.8, 0.1% (w/v) ammonium persulphate (AP), 0.1% (w/v) sodium dodecyl sulphate (SDS) and 0.04% (v/v) N, N, N', N'-tetramethylethylenediamine (TEMED). The stacking gel was cast at 5% acrylamide in a solution of 0.5M Tris pH 6.8, 0.1% (w/v) AP, 0.1% (w/v) SDS and 0.1% (v/v) TEMED.

During gel polymerisation, the protein samples were solubilised in 1 x SDS-PAGE loading buffer (10% (v/v) glycerol, 1% (v/v) SDS, 100mM β-mercaptoethanol, 0.05% (w/v) bromophenol blue

and 62.6mM Tris pH6.8) and boiled at 100°C for 5 minutes. The gels were then loaded with 3µls of molecular weight markers and 20µls of each experimental sample and run at 30mA for 1 hour in running buffer solution containing 0.1% (w/v) SDS, 190mM glycine and 25mM Tris.

Gels were placed on a nitrocellulose membrane and run at 100V for 1 hour in cold transfer buffer solution containing 190mM glycine and 25mM Tris. The membrane was washed with 1x tris-buffered saline tween (TBST) and blocked in a 5% (w/v) skimmed milk powder solution in TBST for 1 hour. Primary antibody probing was achieved using rabbit anti-RhoA (1/1000 in 5% TBST milk) and rabbit anti-β actin (1/500 in 5% TBST milk) and incubated overnight at 4°C.

Membranes were washed in 1x TBST prior to the addition of a goat anti-rabbit HRP-conjugated secondary antibody for 1 hour (1/20,000 in 5% TBST milk). The membrane was then incubated with Pierce™ western substrate for 5 minutes for the chemiluminescent detection of bound antibody prior to being placed in a metal cassette box. A CL-Xposure™ film was then exposed to the membrane by placing it in the metal cassette box for 1 minute before being developed and air dried.

## **2.3 CLINICAL STUDY**

### **2.3.1 Ethical approval and regulation**

The clinical study was approved by the North East – York Research Ethics committee on 17th November 2015 (reference: 15/NE/0319). The Newcastle upon Tyne Hospitals NHS Foundation Trust granted NHS management permission for the research to take place within the trust on 21st January 2016 (R&D reference: 7549). The study was also listed on the ISRCTN registry on 5th November 2015 (ISRCTN 70523327).

Laboratory work on healthy volunteer blood was undertaken under prior ethical approval by County Durham & Tees Valley REC (reference: 12/NE/0121).

### **2.3.2 Screening and recruitment**

#### **Sample size**

A pragmatic sample size of 35 patients was chosen for the clinical study. The literature suggests that a sample size of 30 is acceptable for pilot studies of this nature.(89, 90) Experience from the initial preliminary study indicated that it was feasible to recruit approximately one patient every two weeks and hence 35 patients was considered an achievable sample in the time available.

We anticipated that the clinical study would require methodological and practical optimisation, therefore the first five recruited patients would form the optimisation cohort and the remaining patients would form the experimental cohort. However, considering the early results from the experimental cohort, this cohort was split into a ‘first’ and ‘second’ experimental cohort with subtly different ventilation protocols. The justification for this change is discussed in in the results section.

#### **Screening of elective patients**

Patients attending the daily cardiothoracic pre-assessment clinic at the Freeman Hospital, Newcastle were screened for eligibility and eligible patients were offered a full explanation of the study and a patient information sheet.

Patients were then contacted by telephone at least 24 hours after explanation of the study to determine whether they wished to consent to enter the study. If verbal consent was given,

arrangements were made for written consent to be obtained: this was normally done when the patient was admitted on the day prior to their operation.

Theatre lists on the Trust's e-record system were regularly checked to confirm the date of planned admissions.

### **Screening of non-elective patients**

In-patients awaiting a non-elective/semi-urgent CABG at the Freeman Hospital, Newcastle were screened for eligibility via liaison with the cardiothoracic coordinator (who is responsible for arranging in-patient CABG procedures). The 'In-house urgent CABG waiting list' on the Trust's intranet system was accessed regularly for screening purposes. Eligible inpatients were approached and offered a full explanation of the study and a patient information sheet. Non-elective patients were given at least four hours between the initial explanation and giving written consent.

### **Inclusion and exclusion criteria**

Inclusion and exclusion criteria are listed in Table 2 and all patients who had verbally agreed to participate in the study were checked against these criteria on the day prior to their operation and before written consent was obtained. Patients undergoing repeat/redo CABG surgery were excluded due to different cardiac vessel anatomy compared to first time CABG patients. Patients on immunosuppressant medication were excluded due to the potential confounding effect of altered leukocyte function, however patients taking less than 7.5mg of glucocorticoids were included as this dose was considered to be less than the daily endogenous production of glucocorticoids.<sup>(91)</sup> Only patients scheduled for a morning operating list were included due to the time required for collection of the blood samples and subsequent laboratory processing.

Table 2: Inclusion and exclusion criteria

Inclusion Criteria	Exclusion Criteria
Adult patients awaiting elective or non-elective on-pump coronary artery bypass graft procedures	Patients under 18 years of age
	Emergency surgery precluding ability to obtain informed, written consent
	Patients unable to provide informed written consent
	Patients undergoing repeat or 'redo' CABG surgery
	Patients taking regular immunosuppressant medications or medications likely to significantly alter leukocyte function (eg glucocorticoids >7.5mg, disease-modifying anti-rheumatic drugs (DMARDS), or biological agents).
	Operation by surgeon not participating in the study
	Operation scheduled for an afternoon list



### **2.3.3 Clinical, surgical and anaesthetic protocols**

#### **Clinical data collection**

Following patient consent, medical notes were reviewed and clinical data recorded including weight, height, smoking status, medical co-morbidities, medications and spirometric lung function values (forced expiratory volume in 1 second (FEV<sub>1</sub>), forced vital capacity (FVC) and FEV<sub>1</sub>/FVC ratio). Ideal body weight was calculated using the Devine formula.(92)

A logistic EuroSCORE (93) (a formula used to predict the risk of death after a cardiac operation) was calculated for each patient and served as an indicator of the patient's operative risk.

#### **Pre-surgical protocol**

The operating surgeon was contacted to check that they had no medical objection to the patient entering the study. An anaesthetist who was familiar with the study protocol and trained in the insertion of a left bronchus-blocking balloon was contacted to ensure they were available to perform the study procedures. The cardiothoracic theatre managers were contacted to ensure an intubation bronchoscope and bronchus-blocking balloon were available and that all members of theatre staff were informed of the study patient prior to the operation.

#### **Study interventions**

Patients participating in the study had the following interventions (which differed from standard practice) during their CABG procedure. All other elements of the CABG procedure were entirely as standard.

1. A 9Fr Cohen endobronchial blocking balloon (Cook Medical, Bloomington, Indiana, USA) was guided into the left main bronchus using a fibre-optic bronchoscope following intubation by the anaesthetist. At the point where ventilation to both lungs is normally stopped and the patient is commenced on CPB, the bronchus-blocking balloon was inflated, ensuring that there was no ventilation to the left lung. Ventilation was then resumed to the right lung during the period the patient was on CPB. After CPB had finished, the left bronchus-blocking balloon was deflated and ventilation was resumed to both lungs, as per standard practice.
2. The left pleural cavity was normally breached during the CABG procedures for the surgeon to access the left internal mammary artery to use as a graft vessel. This

ensured maximal deflation of the left lung prior to inflation of the left bronchus-blocking balloon. In study patients where the left pleura had not been breached by accessing the left internal mammary artery, the surgeon made a small incision in the left pleura to ensure maximal deflation of the left lung prior to inflation of the bronchus-blocking balloon.

3. Blood samples were obtained at various points during the CABG procedure. The timing and sites of these blood samples are outlined in the following sections.

### **Anaesthetic procedures**

Routine anaesthetic procedures were followed for each patient. Following insertion of peripheral venous and radial artery cannulae the patients were pre-oxygenated with  $\text{FiO}_2$  1.0. Induction of anaesthesia was at the discretion of the consultant anaesthetist but was typically achieved by administering intravenous midazolam, fentanyl, and etomidate or propofol. Skeletal muscle relaxation was typically achieved using atracurium. Prophylactic intravenous flucloxacillin was given at the time of induction of anaesthesia to all study participants. Patients were then intubated and ventilated as per the standardised lung protective ventilation protocol as detailed below. Anaesthesia was maintained throughout the operation by isoflurane. No patient received additional corticosteroids before, during or after the operation.

### **Surgical and CPB procedures**

Systemic tranexamic acid was administered prior to skin incision and subsequent median sternotomy. The left internal mammary artery was dissected down with simultaneous saphenous vein harvesting from the leg. After administration of intravenous unfractionated heparin, the aorta was cannulated with an armoured, angled cannula. An armoured two-stage venous cannula was then placed in the right atrium through the appendage. Cardiopulmonary bypass was then commenced with systemic cooling (via the CPB machine) to  $34^\circ\text{C}$ . The extracorporeal circuit consisted of a roller pump (Stockert S5 model supplied by LivaNova, London, UK) and membrane oxygenator (CAPIOX<sup>®</sup>RX model supplied by Terumo Cardiovascular Group, Ann Arbor, Michigan, USA).

A mean arterial pressure target of 50-80mmHg was set for each patient. Pump-flow was adjusted on an individual basis to achieve adequate perfusion as guided by regular measurements of mixed venous oxygen saturations. After aortic cross-clamping (to prevent

back-flow of blood into the heart), cold blood cardioplegia was administered to the aortic root via a separate cannula and myocardial protection was augmented with topical cold saline and ice slush. Following completion of all distal vessel anastomoses the aortic cross-clamp was removed and with the heart in sinus rhythm and normothermia achieved, CPB was gradually discontinued over approximately 5 minutes. Protamine sulphate was administered to reverse the effects of heparin followed by decannulation of the heart. Atrial and right ventricular pacing wires were placed prophylactically prior to mediastinal and left pleural drain placement and chest closure.

### **Ventilation and blood sampling protocols**

The step-by-step ventilation and blood sampling protocols are outlined below for each of the optimisation and experimental cohorts. Following venepuncture, all blood samples were immediately added to a Falcon tube, which contained the anticoagulant sodium citrate (end concentration 0.4%), inverted gently 5 times and left in a cool bag until transport to the lab after the final sample had been obtained.

#### ***Optimisation cohort:***

The initial ventilation and blood sampling protocol for the optimisation cohort was as follows:

1. Patient anaesthetised, intubated and bilateral ventilation commenced with standardised settings as follows:
  - a. Pressure controlled ventilation
  - b. FiO<sub>2</sub> to maintain SaO<sub>2</sub> between 96-99%
  - c. Tidal volume: 6-8mls/kg of ideal body weight
  - d. Airway pressure: <25 cmH<sub>2</sub>O
  - e. Positive end expiratory pressure (PEEP): 5-8 cmH<sub>2</sub>O
  - f. 10-12 ventilations per minute
2. Central venous line placed in internal jugular vein. Blood sample 1 obtained (25mls of venous blood from the central line) by the anaesthetist. The first 3mls of blood was discarded to account for residual flush in the line and this was the case for all subsequent central vein samples.

3. Bronchus-blocking balloon placed in the left main bronchus following intubation and the position of the balloon confirmed by fibre-optic bronchoscopy by the anaesthetist. However, the balloon was not inflated at this point. Bilateral ventilation was continued as per the standardised ventilation settings at this point.
4. Surgery commenced.
5. The left pleural cavity was confirmed as opened by the surgeon (a small incision was made in the left pleura by the surgeon if not opened when accessing the left internal mammary artery: this was to achieve maximal collapse of the left lung when bilateral ventilation was stopped).
6. Patient placed on bypass and at the time when ventilation is normally discontinued:
  - a. Both lungs deflated
  - b. Surgeon confirms when left lung is fully deflated
  - c. Left bronchus blocking balloon inflated
  - d. Ventilation to the right lung commenced with standardised settings as follows:
    - i. Pressure-controlled ventilation
    - ii.  $\text{FiO}_2$ : 0.21
    - iii. Tidal volume: 2mls/kg of ideal body weight
    - iv. 7 ventilations per minute
    - v. PEEP: 5cm  $\text{H}_2\text{O}$ .
7. CABG procedure commenced on CPB.
8. After 15 minutes of aortic cross-clamp time the second 25mls blood sample was obtained from the central venous line by the anaesthetist
9. CABG completed.
10. Both lungs re-ventilated as per the following standardised settings after deflation of left bronchus blocking balloon:
  - a. Pressure-controlled ventilation

- b.  $\text{FiO}_2$  at discretion of anaesthetist to maintain  $\text{SaO}_2$  between 96-99%
  - c. Tidal volume: 6-8mls/kg of ideal body weight
  - d. Airway pressure: <25 cmH<sub>2</sub>O
  - e. PEEP: 5-8 cmH<sub>2</sub>O
  - f. 10-12 ventilations per minute.
11. Patient weaned off bypass (approximately 5 minutes after bilateral ventilation recommenced).
12. After patient weaned off bypass, but before intravenous protamine given, the following blood samples were obtained:
- a. 25mls blood sample (sample 3) from central line taken by the anaesthetist
  - b. 25mls blood sample (sample 4) from a right pulmonary vein, taken by the surgeon with a 21-gauge needle and 30mls syringe
  - c. 25mls blood sample (sample 5) from a left pulmonary vein taken by the surgeon with a 21-gauge needle and 30mls syringe
- NB: The surgeon was advised on whether to sample the left or right pulmonary vein first: alternate sides for each study participant.*
13. Intravenous protamine given and normal chest closure.
14. At the end of the procedure, all labelled samples (1-5) were immediately transported to the Simpson Lab in Newcastle University for further analysis.

A summary of the blood sampling protocol for the first experimental cohort is outlined in Table 3.

Table 3: Blood sampling protocol for optimisation cohort

Sample	Description	Volume	Person to retrieve sample
1	<p><b>Venous blood:</b></p> <p>Taken from the patient's central line immediately following central line insertion (ie after induction of anaesthesia)</p>	25mls	Anaesthetist
2	<p><b>Venous blood:</b></p> <p>Taken from patient's central line 15 minutes after aortic cross-clamp applied (ie during CPB).</p>	25mls	Anaesthetist
3	<p><b>Venous blood:</b></p> <p>Taken from patient's central line</p> <p><b>AFTER</b> grafts have been completed, taken off CPB and normal ventilation has resumed</p> <p>but</p> <p><b>BEFORE</b> protamine sulphate has been given.</p>	25mls	Anaesthetist
4	<p><b>Venous blood:</b></p> <p>Taken from <b>RIGHT</b> pulmonary vein (ie sampling blood that has been through the previously ventilated lung) at same time as sample 3 ie</p> <p><b>AFTER</b> grafts have been completed, taken off CPB and normal ventilation has resumed</p> <p>but</p> <p><b>BEFORE</b> protamine sulphate has been given.</p>	25mls	Surgeon
5	<p><b>Venous blood:</b></p> <p>Taken from <b>LEFT</b> pulmonary vein (ie sampling blood that has been through the deflated lung) at same time as sample 3 ie</p> <p><b>AFTER</b> grafts have been completed, taken off CPB, normal ventilation has resumed but</p> <p><b>BEFORE</b> protamine sulphate has been given.</p>	25mls	Surgeon

***First experimental cohort:***

The ventilation and blood sampling protocol was adjusted for the first experimental cohort as detailed below. These changes were made due to unforeseen challenges with some of the ventilation settings and blood sampling times which came to light during the optimisation cohort. The justification for the protocol adjustments is discussed further in the results section.

1. Patient anaesthetised, intubated and bilateral ventilation commenced with standardised settings as follows:
  - a. Volume-controlled ventilation
  - b. FiO<sub>2</sub> to maintain SaO<sub>2</sub> between 96-99%
  - c. Tidal volume: 6-8mls/kg of ideal body weight
  - d. Airway pressure: <25cmH<sub>2</sub>O
  - e. PEEP: 5-8cmH<sub>2</sub>O
  - f. 10-12 ventilations per minute.
2. Central venous line placed in internal jugular vein. Blood sample 1 obtained (25mls of venous blood from the central line) by the anaesthetist. The first 3mls of blood was discarded to account for residual flush in the line and this was the case for all subsequent central vein samples.
3. Bronchus-blocking balloon placed in the left main bronchus following intubation and the position of the balloon confirmed by fibre-optic bronchoscopy by the anaesthetist. However, the balloon was not inflated at this point. Bilateral ventilation continued as per the standardised ventilation settings at this point.
4. Surgery commenced.
5. Just before intravenous heparin given, blood sample 2 obtained (25mls of venous blood from central line) by the anaesthetist.
6. The left pleural cavity was confirmed as opened by the surgeon (a small incision was made in the left pleura by the surgeon if not opened when accessing the left internal

mammary artery: this was to achieve maximal collapse of the left lung when bilateral ventilation was stopped).

7. Patient placed on bypass and at the time when ventilation is normally discontinued:
  - a. Both lungs deflated
  - b. Surgeon confirms when left lung is fully deflated
  - c. Left bronchus-blocking balloon inflated
  - d. Ventilation to the right lung commenced with standardised settings as follows:
    - i. Volume-controlled ventilation
    - ii.  $FiO_2$ : 0.21
    - iii. Tidal volume: 2mls/kg of ideal body weight
    - iv. 7 ventilations per minute
    - v. PEEP: 5cmH<sub>2</sub>O.
8. CABG procedure commenced on CPB.
9. CABG completed.
10. Both lungs re-ventilated as per the following standardised settings after deflation of the left bronchus-blocking balloon:
  - a. Volume-controlled ventilation
  - b.  $FiO_2$  at discretion of anaesthetist to maintain SaO<sub>2</sub> 96-99%
  - c. Tidal volume: 6-8mls/kg of ideal body weight
  - d. Airway pressure: <25cmH<sub>2</sub>O
  - e. PEEP: 5-8cmH<sub>2</sub>O
  - f. 10-12 ventilations per minute.
11. Patient weaned off bypass (approximately 5 minutes after bilateral ventilation recommenced).
12. Intravenous protamine given after patient weaned off bypass.



13. Two minutes after protamine given, 3 blood samples taken before chest closure as follows:

- a. 25mls blood sample (sample 3) from central line taken by the anaesthetist
- b. 25mls blood sample (sample 4) from a right pulmonary vein taken by the surgeon with 21-gauge needle and 30mls syringe
- c. 25mls blood sample (sample 5) from a left pulmonary vein taken by the surgeon with 21-gauge needle and 30mls syringe.

*NB: The surgeon was advised on whether to sample the left or right pulmonary vein first: alternate sides for each study participant.*

14. Normal chest closure

15. At the end of the procedure all labelled samples (1-5) were immediately transported to the Simpson Lab in Newcastle University for further analysis.

A summary of the blood sampling protocol for the first experimental cohort is outlined in Table 4.

Table 4: Blood sampling protocol for first experimental cohort

Sample	Description	Volume	Person to retrieve sample
1	<p><b>Venous blood:</b></p> <p>Taken from the patient's central line immediately following central line insertion (ie after induction of anaesthesia)</p>	25mls	Anaesthetist
2	<p><b>Venous blood:</b></p> <p>Taken from the patient's central line just before heparin given (ie just prior to the initiation of CPB)</p>	25mls	Anaesthetist
3	<p><b>Venous blood:</b></p> <p>Taken from patient's central line:</p> <p><b>AFTER</b> grafts have been completed, normal ventilation resumed, taken off CPB and protamine given but</p> <p><b>BEFORE</b> chest closure</p>	25mls	Anaesthetist
4	<p><b>Venous blood:</b></p> <p>Taken from <b>RIGHT</b> pulmonary vein (ie sampling blood that has been through the previously ventilated lung) at same time as sample 3 ie:</p> <p><b>AFTER</b> grafts have been completed, normal ventilation resumed, taken off CPB and protamine given but</p> <p><b>BEFORE</b> chest closure</p>	25mls	Surgeon
5	<p><b>Venous blood:</b></p> <p>Taken from <b>LEFT</b> pulmonary vein (ie sampling blood that has been through the deflated lung) at same time as sample 3 ie:</p> <p><b>AFTER</b> grafts have been completed, normal ventilation resumed, taken off CPB and protamine given but</p> <p><b>BEFORE</b> chest closure</p>	25mls	Surgeon

### ***Second experimental cohort:***

Based on the preliminary results from the nine patients recruited to the first experimental cohort, a revised ventilation protocol was adopted for the remaining recruited patients: this formed the second experimental cohort. This revised ventilation protocol differed from the first experimental cohort protocol as follows;

1. The  $\text{FiO}_2$  for the right lung ventilation during CPB was increased to 0.5
2. The tidal volume for the right lung ventilation during CPB was increased to 4mls/kg ideal body weight.

All other aspects of the ventilation and blood sampling protocol remained the same as for the first experimental protocol as outlined in Table 4.

### **2.3.4 Laboratory procedures**

Following collection of the final blood sample, all samples were immediately transported to the Simpson laboratory within the Institute of Cellular Medicine, Newcastle University. All samples were pseudo-anonymised with a study identification number and patient details corresponding to this number were stored in a locked office in compliance with ethical requirements.

#### **Simultaneous isolation of blood neutrophils**

Neutrophils were simultaneously isolated from each of the five 25mls blood samples using a Percoll density gradient technique as previously described in section 2.2.3.

Prior to the formation of autologous serum, 4mls of the upper platelet-rich plasma layer (derived from the initial centrifugation of whole blood) was added to a Falcon tube and immediately placed on ice for subsequent preparation and storage of platelet-free plasma for each sample.

To ensure that neutrophils for each of the five samples were exposed to Percoll for the shortest time possible, the gradients for samples 1-3 were made first and then placed in a centrifuge after which the gradients for samples 4 and 5 were made and placed in another centrifuge. This method enabled sequential neutrophil harvesting and ensured comparable exposure times of neutrophils to the Percoll reagent.

Neutrophil counting and purity were determined as previously described (section 2.2.3) and any sample with a purity of less than 95% was excluded from the analysis.

### **Simultaneous neutrophil phagocytosis assay by light microscopy**

Five simultaneous phagocytosis assays were performed using the method outlined in section 2.2.3. Zymosan was opsonised with serum from the respective blood sample. The tissue culture plate was gently agitated by rotating the bottom of the plate by hand at 10-minute intervals during the incubation time.

Neutrophil phagocytic capacity was then quantified using light microscopy to count the number of neutrophils containing two or more ingested zymosan particles. Duplicate counts were performed on four fields with a minimum of 100 neutrophils counted per field for each of the samples 1-5.

### **Apoptosis assay on isolated neutrophils by flow cytometry**

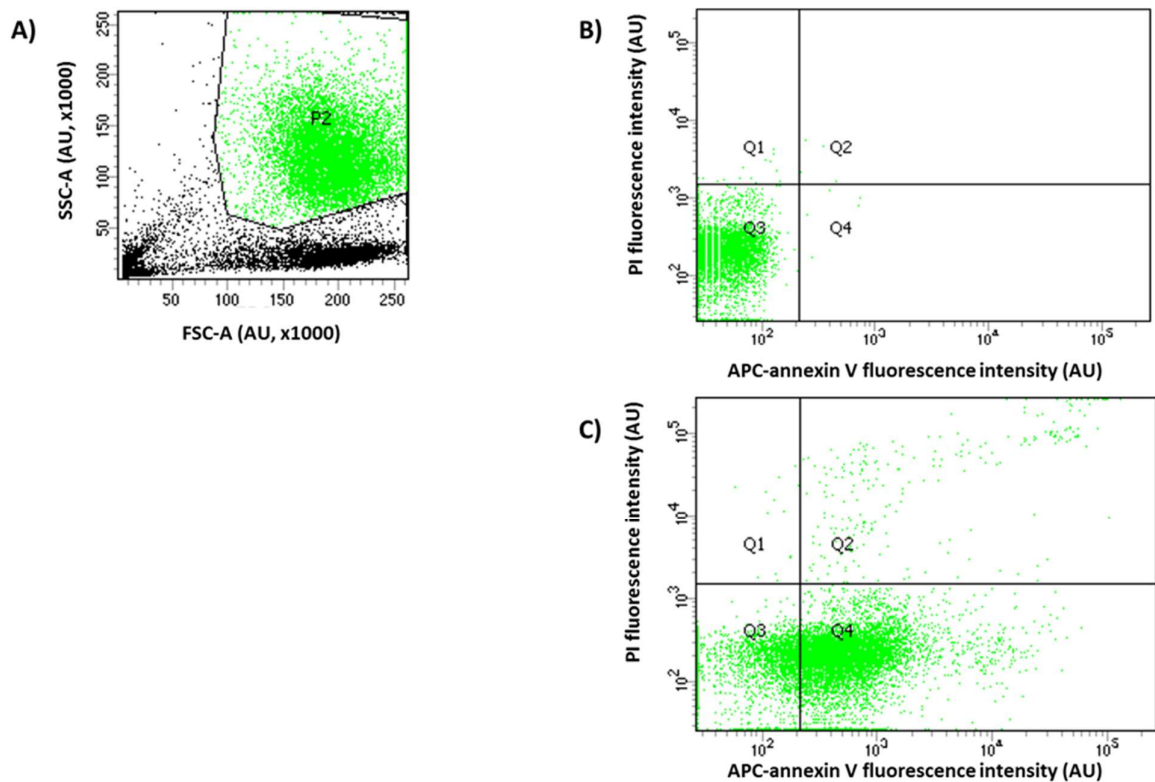
An apoptosis assay was performed on isolated neutrophils for each of the five samples.

Neutrophils suspended in IMDM and 1% (v/v) autologous serum at a concentration of 1 million/ml were added to two Eppendorf tubes for each experimental sample (Eppendorf 1 and Eppendorf 2). The samples were centrifuged at 200g for 5 minutes at 37°C and washed with PBS. Neutrophils from Eppendorf 1 were re-suspended in 300µl of Annexin V binding buffer alone, which served as an unstained control. Neutrophils in Eppendorf 2 were re-suspended in 100µl of Annexin V binding buffer containing 5µl of APC Annexin V. All samples were then transferred to flow cytometry tubes and incubated at room temperature in the dark for 10 minutes. Following this, a further 200µl of Annexin V binding buffer containing 1µl of propidium iodide (PI, end concentration 0.5µg/ml) was added to Eppendorf 2. The samples were then kept in the dark at 4°C until analysis.

Flow cytometric analysis was performed using a BD FACSCanto™ II with fluorescence excitation 488nm / emission 585nm for PI and fluorescence excitation 635nm / emission 660nm for APC Annexin V. The neutrophil population was gated using characteristic forward and side scatter properties and the acquisition set to record 10,000 events. Further gating strategies identified the percentage of neutrophils in this population that were live (Annexin V negative, PI negative), early apoptotic (Annexin V positive, PI negative), late apoptotic

(Annexin V positive, PI positive) and dead (Annexin V negative, PI positive) as illustrated in Figure 15.

For the second experimental cohort, neutrophil apoptosis was assessed again at 20 hours following isolation to measure the rate of apoptosis across the samples. Neutrophils suspended at 1million/ml in IMDM and 1% (v/v) autologous serum were incubated for 20 hours following isolation at 37°C in a 5% CO<sub>2</sub> incubator. Apoptosis was then assessed using the methods and gating strategies as described above.



**Figure 15: Gating strategy for flow cytometric evaluation of apoptosis on isolated neutrophils using APC-annexin V and propidium iodide.** [A] The neutrophil population was identified using characteristic forward and side scatter properties (P2). [B] Further gating on the neutrophil population served as a negative control and identified gates for live neutrophils (Q3), early apoptotic neutrophils (Q4), late apoptotic neutrophils (Q2) and dead neutrophils (Q1). [C] A positive control sample of isolated neutrophils stained with APC-annexin V and PI demonstrating most of the neutrophils in early apoptosis. Abbreviations: AU (arbitrary units), FSC-A (forward scatter area), SSC-A (side scatter area), PI (propidium iodide), APC (Allophycocyanin).

## **Phagocytosis assay by flow cytometry on whole blood**

This neutrophil phagocytosis assay was based on a technique previously described.(94, 95) Each of the five samples of blood was processed as follows. 150µls of citrated whole blood was equally divided between 3 Eppendorf tubes and placed on ice for 10 minutes.

10µls of 1mg/ml pHrodo™ *S. aureus* bioparticle conjugate was added to the first Eppendorf tube which was immediately placed back on ice for 1 hour to serve as an inhibitor control. 10µls of 1mg/ml pHrodo™ *S. aureus* bioparticle conjugate was added to the second Eppendorf tube which was immediately placed in a 37°C shaking waterbath for 1 hour. The third Eppendorf tube was incubated at 37°C in a shaking waterbath but did not contain pHrodo™ *S. aureus* bioparticle conjugate and served as a negative control.

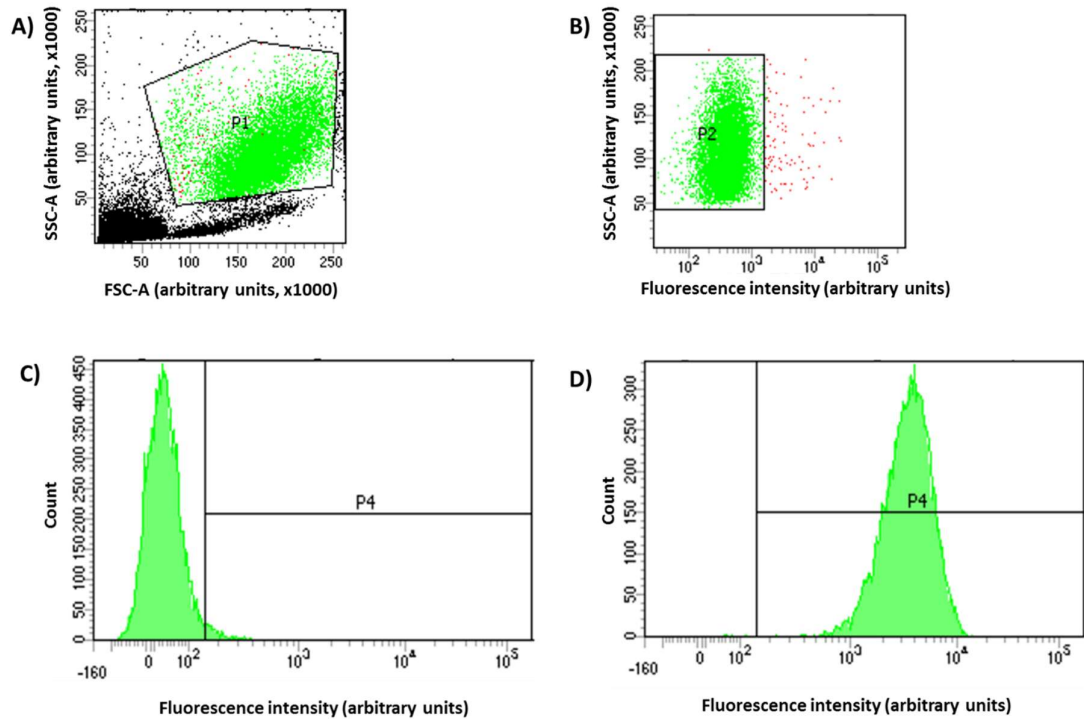
After 1 hour of incubation, all tubes were placed on ice for 10 minutes to stop further neutrophil phagocytosis. Red blood cells were then lysed with 1.5mls of BD pharmlyse™ solution per tube and kept in the dark at room temperature for 10 minutes.

The contents were then transferred from Eppendorf tubes to flow cytometry tubes before being placed in a BD FACS™ Lyse Wash Assistant where they were automatically washed once and re-suspended in 300µls of PBS. The tubes were kept on ice until analysis and all incubation steps were performed protected from light.

Immediately before each tube was analysed, 3µls of DAPI (4', 6-diamidino-2-phenylindole) dye solution was added (end concentration 1µM) to stain DNA content thus enabling necrotic neutrophils to be excluded from the analysis.

Flow cytometric analysis was performed using a BD FACSCanto™ II with fluorescence excitation 488nm / emission 530nm. The neutrophil population was gated using characteristic forward and side scatter properties and then gated again on the DAPI negative population to identify viable neutrophils for analysis. Further gating strategies identified the percentage of viable neutrophils that were pHrodo™ bioparticle 'bright' (representing the percentage of phagocytosing neutrophils) as well as the median fluorescence intensity (MFI) for each sample (representing the efficacy of phagocytosis) (Figure 16).

Percentage positivity values for the 4°C inhibitory control samples were subtracted from the 37°C samples to correct for the effect of surface-bound pHrodo™ *S. aureus* bioparticles thus obtaining net values for the percentage of phagocytosis for each of the experimental blood samples.



**Figure 16: Gating strategy for flow cytometric evaluation of neutrophil phagocytosis on whole blood using pHrodo™ *S.aureus* bioparticles.** [A] The neutrophil population (P1) was initially identified using characteristic forward and side scatter properties. [B] Further gating using DAPI identified the live neutrophil population (P2). [C] Histogram demonstrating live neutrophil pHrodo™ fluorescence intensity for the 4°C inhibitory control sample (a gate for pHrodo™ positive neutrophils (P4) was identified using a negative control sample which did not contain pHrodo™). [D] Histogram demonstrating the increase in live neutrophil pHrodo™ fluorescence intensity for the 37°C sample.

### **CD11b and CD62L cell surface adherence markers on whole blood**

CD11b and CD62L cell surface expression were analysed using flow cytometry. For each blood sample, 50µls of citrated whole blood was added to 2 Eppendorf tubes. To one Eppendorf tube, 10µls of FITC-conjugated mouse anti-human CD11b and 5µls of APC-conjugated mouse anti-human CD62L were added. To the other Eppendorf tube, an equal concentration of each isotype control (FITC-IgG1 and APC-IgG1) was added. All samples were incubated in the dark at 4°C for 1 hour.

Red blood cells were then lysed with 1.5mls of BD pharmlyse™ solution and kept in the dark at room temperature for a further 10 minutes. Samples were then transferred to flow cytometry tubes before being placed in a BD FACS™ Lyse Wash Assistant where they were automatically washed once and re-suspended in 300µls of PBS. Following washing, the samples were kept on ice until analysis.

Immediately before analysis, 3µls of DAPI dye solution (end concentration 1µM) was added to stain DNA content thus enabling dead neutrophils to be excluded from the analysis. Analysis was performed using a BD FACSCanto™ II at a fluorescence excitation 488nm / emission 530nm for FITC and fluorescence excitation 635nm / emission 660nm for APC using the gating strategy as described above.

### **Plasma cytokine analysis**

Plasma samples were maintained on ice immediately following harvesting and stored at -80°C for future analysis. Cytokine analysis was performed on platelet-free plasma, which was diluted in PBS to a ratio of 1:2. The V-plex proinflammatory panel 1 (human) kit (Meso Scale Diagnostics, Rockville, Maryland, USA) was used according to the manufacturer's instructions for the quantitative determination of 10 cytokines important in the regulation of inflammation and immune function as detailed in Table 5.



Table 5: V-plex proinflammatory panel 1 cytokines and their detection ranges

<b>Cytokine</b>	<b>Detection range (pg/ml)</b>
IFN $\gamma$	0.20–938
IL1 $\beta$	0.04–375
IL2	0.09–938
IL4	0.02–158
IL6	0.06–488
IL8	0.04–375
IL10	0.03–233
IL12p70	0.11–315
IL13	0.24–353
TNF $\alpha$	0.04–248

### **2.3.5 Collection of post-operative data**

Post-operative clinical data were collected from patients' medical notes following their discharge from the intensive treatment unit (ITU). This included time to extubation, length of ITU stay, total blood loss from the mediastinal and left pleural drains at 12 hours post operation and the report from the first post-operative CXR (which was issued by a radiologist). Information on the medications administered intra-operatively was collected from the notes retrospectively.

### **2.3.6 Comparison control cohort**

Clinical and operative data from patients who underwent first-time CABG surgery within the period of the study but who had not been recruited to the study were collected from the Trust's Cardiothoracic Data Registry. This group served as a comparison control cohort when evaluating the safety and clinical impact of the study interventions.

### **2.3.7 Healthy volunteer comparative work**

Flow cytometric evaluation of CD11b and CD62L cell surface markers was performed on neutrophils from a small group of healthy volunteers using the same methods as previously described. This enabled comparison of neutrophil priming status from healthy volunteers with the baseline (sample 1) patient samples.

## 2.4 STATISTICAL ANALYSIS

Data from the IFN $\gamma$  laboratory study was analysed using a repeated measures one-way ANOVA with either Tukey's or Dunnett's multiple comparisons test in keeping with previous publications in this field.(85)

For the clinical study, non-parametric statistical tests were applied to all data as normality could not be assumed in the small patient population. The Friedman test with Dunn's post-hoc multiple comparisons was used for comparing values between the baseline blood sample (sample 1) and samples 2-5. The Wilcoxon matched pairs signed-rank test was used to evaluate differences between the right (sample 4) and left (sample 5) pulmonary blood samples. The Mann-Whitney U test was used to evaluate differences in clinical and perioperative parameters between the patient group and the comparative cohort group. Patients with incomplete datasets were excluded from the statistical analysis (i.e. patients for whom at least one of the five experimental blood samples was not obtained or where laboratory data for one or more of the five experimental blood samples was not available).

A 2-tailed  $p$  value of less than 0.05 was considered statistically significant for both the laboratory and clinical studies. All data are represented as mean values  $\pm$ SEM (standard error of the mean) for the laboratory study and median values  $\pm$ IQR (interquartile range) for the clinical study unless otherwise documented.

All data were analysed using version 7.01 of the GraphPad Prism statistical software program for Windows (GraphPad software, La Jolla, California, USA).

# CHAPTER 3: RESULTS OF INTERFERON GAMMA LABORATORY STUDY

## 3.1 OVERVIEW

This chapter reports results of the IFN $\gamma$  laboratory study which describes the effects of recombinant human IFN $\gamma$  on the phagocytic function of human neutrophils.

The main aims of this study were:

1. To establish whether recombinant human IFN $\gamma$  restores the phagocytic function of human neutrophils.
2. To explore the effect of recombinant human IFN $\gamma$  on cell signalling pathways involved in neutrophil phagocytosis.
3. To explore the potential role of IFN $\gamma$  as a therapeutic strategy for restoring neutrophil phagocytic function in patients following CABG surgery and further afield.

The chapter begins by presenting results of previous dose-response experiments undertaken to determine the concentration of salbutamol required to maximally impair phagocytic function of neutrophils from healthy volunteers. The effect of IFN $\gamma$  on neutrophils with impaired phagocytic function is then presented followed by the effect of IFN $\gamma$  on neutrophil RhoA activity. The effect on phagocytosis of inhibiting key cell signalling molecules known to be involved in IFN $\gamma$  signal transduction is then explored. The chapter ends with a summary of the conclusions drawn from the IFN $\gamma$  laboratory study.

### 3.2 B<sub>2</sub>-AGONIST INDUCTION OF PHAGOCYTOTIC IMPAIRMENT IN ISOLATED NEUTROPHILS

Previous research by our group has demonstrated that pre-incubating neutrophils with the  $\beta_2$ -agonist salbutamol, results in a dose dependent impairment of phagocytosis of serum-opsonised zymosan particles.(85) Based on this earlier dose response work by Scott and colleagues, all experiments in this section of the work use salbutamol at an end concentration of 10 $\mu$ M in aiming to maximally impair neutrophil phagocytosis *in vitro* (Figure 17).

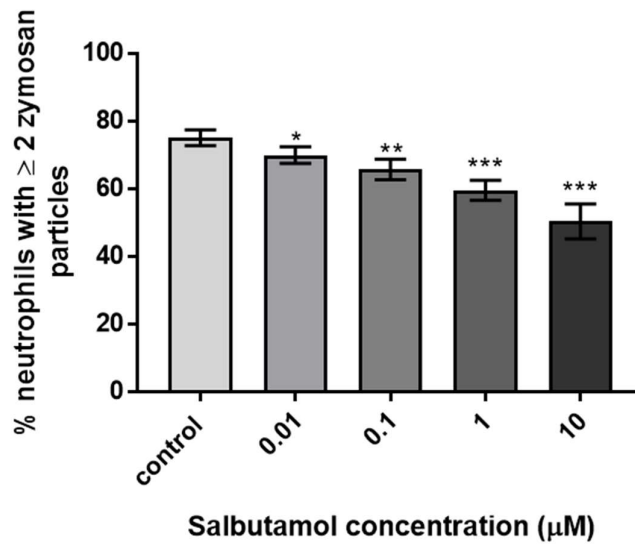


Figure 17: **Salbutamol, a short acting  $\beta_2$ -agonist, impairs neutrophil phagocytosis in a dose-dependent manner** (original data from Scott et al. (85) presented with permission). Isolated adherent human neutrophils were pre-incubated with salbutamol for 30 minutes at concentrations ranging from 0.01 $\mu$ M to 10 $\mu$ M in a 5% CO<sub>2</sub> incubator at 37°C. Serum-opsonised zymosan suspension (50 $\mu$ ls) was then added to achieve a neutrophil:zymosan particle ratio of 1:10 before incubation for a further 30 minutes. Phagocytosis was quantified using the light microscopy technique as previously described. Data are presented as mean (columns) and SEM (bars); n=9; statistical analysis was by repeated measures one-way ANOVA with Dunnett's multiple comparisons test relative to the control column (no salbutamol). \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001

### 3.3 THE EFFECTS OF INTERFERON GAMMA ON NEUTROPHILS WITH IMPAIRED PHAGOCYTOTIC FUNCTION

Preliminary dose response experiments carried out on healthy volunteer neutrophils by my lab colleague Jon Scott (Figure 18) demonstrated that maximum restoration of phagocytosis could be achieved with an end concentration of 1ng/ml (50nM) of IFN $\gamma$ .

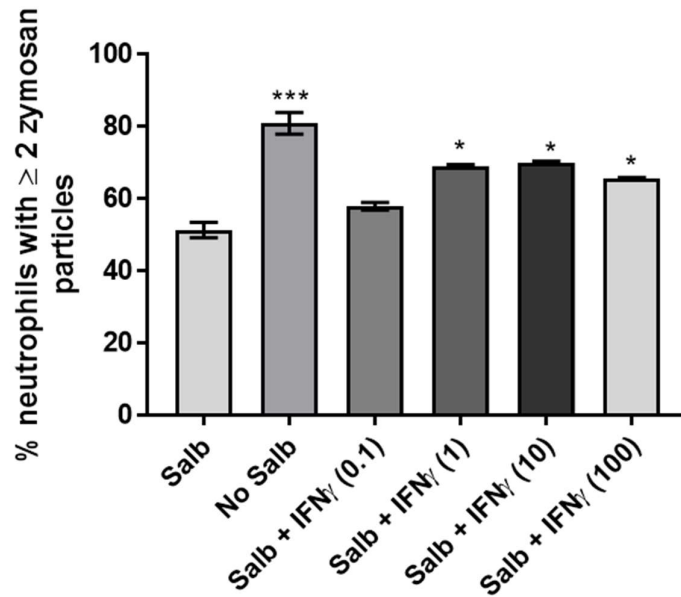


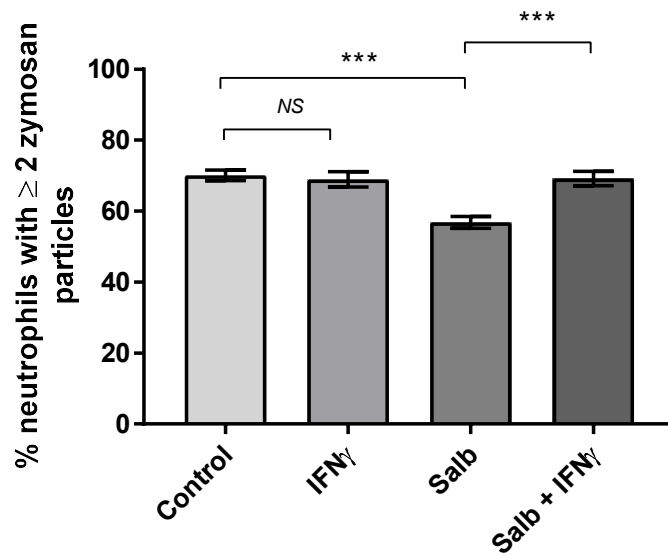
Figure 18: Dose response analysis for the effect of interferon gamma on neutrophil phagocytosis (original data from experiments by Mr Jon Scott, presented with permission). Isolated adherent human neutrophils were pre-incubated with salbutamol (10 $\mu$ M) for 30 minutes in a 5% CO $_2$  incubator at 37°C. Neutrophils were then incubated with recombinant human interferon gamma at concentrations ranging from 0.1ng/ml to 100ng/ml for 30 minutes prior to the addition of serum-opsonised zymosan for a further 30 minutes. Phagocytosis was quantified using the light microscopy technique as previously described. Data are presented as mean (columns) and SEM (bars); n=4; statistical analysis was by repeated measures one-way ANOVA with Dunnett's multiple comparisons test relative to the salbutamol alone column. \*P<0.05, \*\*\*P<0.001. Abbreviations: Salb (salbutamol), IFN $\gamma$  (interferon gamma).

In this section of the work a concentration of 1ng/ml (50nM) was therefore used to further explore the effect of IFN $\gamma$  on neutrophils with impaired phagocytic function using the light microscopic assay previously described in Chapter 2, section 2.2.3.

Neutrophils isolated from the blood of ten healthy volunteers (7 female; mean age 39.3 years; age range 30-60 years) were used for this section of the work. A full data set was obtained for

all subjects as all experimental wells were suitable for phagocytic quantification by light microscopy. Median neutrophil purity was  $\geq 95\%$  for all subjects. A random sample of the cell culture plates was re-counted by my laboratory colleague Mr Jon Scott, who was blind to the experimental wells and his counts were comparable to the original counts.

Mean phagocytic capacity for the control sample was 70.1% (SEM $\pm$ 1.5) (Figure 19). This result is consistent with previously published literature for levels of healthy volunteer phagocytosis using this assay.(84, 85) The addition of IFN $\gamma$  had no apparent augmentative effect on the phagocytic function of neutrophils from healthy volunteers compared to the control sample. Salbutamol significantly impaired neutrophil phagocytosis to a mean phagocytic capacity of 56.9% (SEM $\pm$ 1.7) (Figure 19). It is notable that despite the significant phagocytic impairment induced by pre-incubation with salbutamol in these healthy subjects, the phagocytic capacity remained slightly higher than expected when compared to previous literature using the same concentration of salbutamol to impair neutrophil phagocytosis.(85) As illustrated in Figure 19, IFN $\gamma$  significantly restored the phagocytic capacity of neutrophils that had been pre-incubated with salbutamol to a level comparable with the control sample. This result suggests that recombinant human IFN $\gamma$  fully restores neutrophil phagocytic impairment induced by salbutamol in this *in vitro* model.



**Figure 19: Recombinant human interferon gamma restores phagocytosis of neutrophils pre-incubated with salbutamol.** Neutrophils were incubated with interferon gamma (50nM; IFN $\gamma$ ), Salbutamol (10 $\mu$ M; Salb) or a combination of both. For the combination well containing both salbutamol and interferon gamma, isolated neutrophils were first incubated with salbutamol for 30 mins prior to the addition of interferon gamma for a further 30 minutes. Serum-opsonised zymosan particles were then added to each sample for a further 30 minutes prior to quantification of phagocytosis using light microscopy as previously described. Data are presented as mean (columns) and SEM (bars); n=10; statistical analysis was by repeated measures one-way ANOVA with Tukey's multiple comparisons test. \*\*\*P < 0.001, NS=non-significant.



### **3.4 THE EFFECTS OF INTERFERON GAMMA ON RHOA ACTIVITY**

To explore the downstream mechanisms behind the above results, a separate group of healthy volunteer subjects was studied to assess RhoA activity in neutrophil cell lysates.

As described in Chapter 1 section 1.13, the rationale behind exploring RhoA (a small GTPase) relates to its inherent involvement in coordinating the terminal steps of actin polymerisation and subsequent complement-mediated phagocytosis. Previous work suggests that salbutamol may act by inhibiting RhoA activity which in turn impairs neutrophil phagocytosis.(85) Hence, characterising the effect of IFN $\gamma$  on RhoA activity may provide insight into the possible mechanisms by which it appeared to restore neutrophil phagocytosis.

Neutrophils isolated from the blood of 3 healthy volunteer subjects (2 female; mean age 32.3 years; range 24-42 years) were used for this section of the work. Due to the small number of patients in this section, the results are reported descriptively.

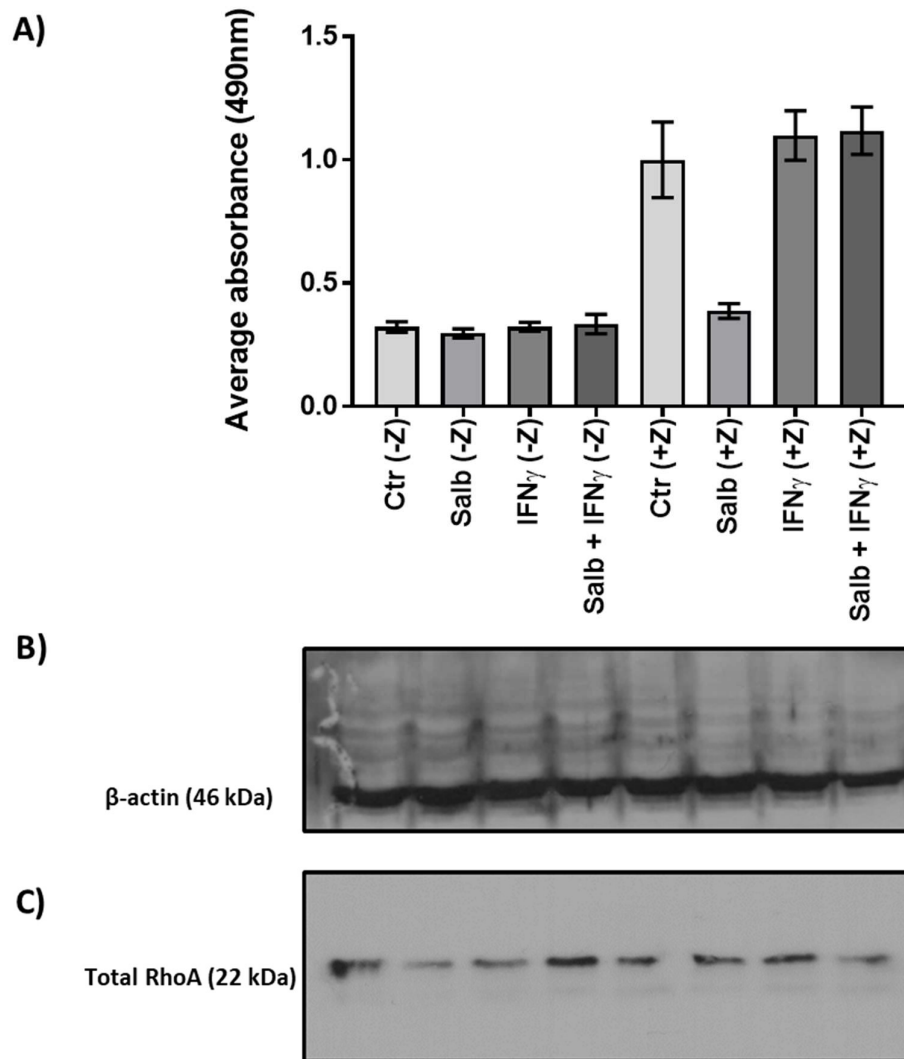
RhoA activity (as measured using an ELISA method described in Chapter 2, section 2.2.3) was relatively low for all negative control samples which did not contain zymosan (Figure 20, panel A). The presence of zymosan alone increased RhoA activity above all negative control samples thus supporting the concept that complement-mediated zymosan uptake is characterised by RhoA activation (Figure 20, panel A).

There was a notable reduction in RhoA activity of the sample pre-incubated with salbutamol before the addition of zymosan compared to the control well containing zymosan alone (positive control). However, it is curious that despite salbutamol impairing neutrophil phagocytic function to the order of 60% in the light microscopy experiments, there appeared to be negligible activation of RhoA in the salbutamol and zymosan well at a level comparable to the negative control samples.

RhoA activity appeared to be restored to the level of the positive control sample following the addition of IFN $\gamma$  to neutrophils pre-incubated with salbutamol. (Figure 20, panel A). These findings suggest that IFN $\gamma$  restores phagocytic function in neutrophils pre-incubated with salbutamol via a RhoA-dependent pathway and adds further weight to the apparent restorative effects seen in the previous experiments.

To validate these results, western blots were undertaken to ensure that there were no discrepancies in total RhoA levels across all samples which would have confounded the results

obtained for the levels of activated RhoA. Representative western blots for both  $\beta$ -actin (loading control) and total RhoA expression are shown in Figure 20, panels B and C respectively and demonstrate relatively uniform total Rho A expression across all samples.



**Figure 20: Interferon gamma appears to restore RhoA activity in neutrophils pre-treated with salbutamol.** Isolated adherent neutrophils were incubated with Salbutamol (10 $\mu$ M; salb), interferon gamma (50nM; IFN $\gamma$ ), or a combination of both. For the combination well containing both salbutamol and interferon gamma, isolated neutrophils were first incubated with salbutamol for 30 mins prior to the addition of interferon gamma for a further 30 minutes. For each condition, either no Zymosan particles (negative control) or Zymosan particles were added for 5 minutes prior to preparation of cell lysates as previously described (see section 2.2.3). RhoA activity was assessed by ELISA.  $\beta$ -actin and total RhoA were assessed by Western blotting; n=3; [A] Data are presented as mean (columns) and SEM (bars); n=3; [B] Representative western blot for total  $\beta$ -actin (loading control) and associated western blot for total RhoA [C] (for each western blot the 8 lanes are in the same order as presented in A). Abbreviations: Ctr (control), Z (Zymosan particles), salb (salbutamol), IFN $\gamma$  (interferon gamma).

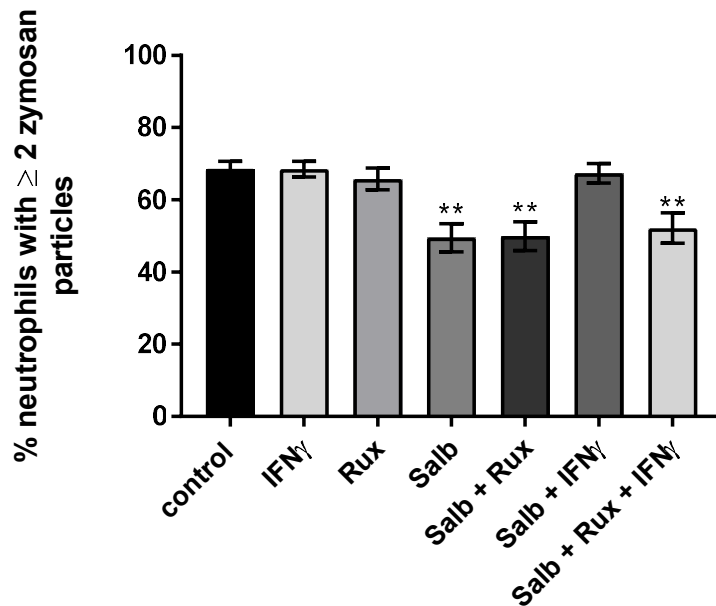
### **3.5 THE EFFECTS OF INHIBITING KEY CELL SIGNALLING MOLECULES INVOLVED IN INTERFERON GAMMA SIGNAL TRANSDUCTION**

As described in Chapter 1, section 1.11.2, Jak 1 and 2 have been described as key molecules involved in facilitating signal transduction to the nucleus following the binding of IFN $\gamma$  to the IFN $\gamma$  receptor. To characterise the apparent restorative effect IFN $\gamma$  has on neutrophil phagocytosis, the effects of inhibiting these key cell signalling molecules were explored to make further inroads into mapping out potential mechanisms behind this finding.

A separate group of 7 healthy volunteers was used for this section of the work (3 female; mean age 35.7 years; range 21-51 years). Ruxolitinib was used to selectively inhibit Jak 1 and 2 (96) and was used at an end concentration of 1 $\mu$ M following previous dose response experiments carried out by my lab colleague Mr Jon Scott.

As illustrated in Figure 21, pre-incubating neutrophils with IFN $\gamma$  or ruxolitinib alone had no apparent effect on neutrophil phagocytosis relative to the control sample. As previously observed, pre-incubating neutrophils with salbutamol significantly impaired neutrophil phagocytic function relative to the control sample. Neutrophils pre-incubated with salbutamol and ruxolitinib had impaired phagocytosis comparable to that seen with salbutamol alone.

The addition of IFN $\gamma$  to neutrophils pre-incubated with salbutamol resulted in restoration of neutrophil phagocytic capacity comparable to that of the control sample. Notably, the restorative effects of IFN $\gamma$  were not observed in neutrophils pre-incubated with salbutamol and ruxolitinib suggesting that Jak 1 and 2 molecules are necessary for IFN $\gamma$  to exert its apparent restorative effects on neutrophil phagocytosis (Figure 21).



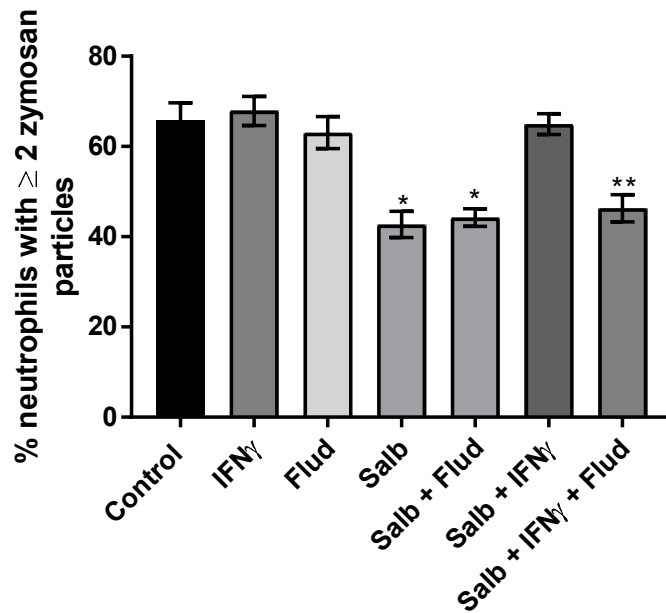
**Figure 21: Ruxolitinib, a selective inhibitor of Janus kinase 1 and 2, inhibits restoration of neutrophil phagocytosis by interferon gamma.** Neutrophils were incubated with interferon gamma, ruxolitinib, salbutamol or a combination of these agents. For the well containing salbutamol, ruxolitinib and interferon gamma, neutrophils were pre-incubated for 30 minutes with both salbutamol and ruxolitinib before the addition of interferon gamma for a further 30 minutes. For all other wells, the agents were added in the same order and applied for the same duration and at the same time as in the well containing all three agents. Serum-opsonised zymosan was then added to each well for a further 30 minutes prior to quantification of phagocytosis by light microscopy. Data are presented as mean (columns) and SEM (bars); n=7; statistical analysis was by repeated measures one-way ANOVA with Dunnett's multiple comparisons test relative to the control column; \*\*P<0.01. Abbreviations/end concentrations: IFN $\gamma$  (interferon gamma/50nM), Rux (ruxolitinib/1 $\mu$ M), Salb (salbutamol/10 $\mu$ M).

To further characterise the apparent restorative effect IFN $\gamma$  has on neutrophil phagocytosis, the effects of inhibiting Stat1, another key molecule known to be involved in IFN $\gamma$  cell-signalling was explored.

A separate group of four healthy volunteers was used for this section of the work. (2 female; mean age 30.3 years; age range 21-50 years). Fludarabine, a Stat1 specific activation inhibitor was used at an end concentration of 50 $\mu$ M following initial dose response experiments.

As demonstrated in Figure 22, pre-incubating neutrophils with IFN $\gamma$  or fludarabine alone had no apparent effect on neutrophil phagocytosis relative to the control sample. Pre-incubating neutrophils with salbutamol significantly impaired phagocytic function relative to the control sample. Neutrophils pre-incubated with salbutamol and fludarabine had impaired phagocytic function comparable to the sample pre-incubated with salbutamol alone. As seen in previous experiments, the addition of IFN $\gamma$  to neutrophils pre-incubated with salbutamol resulted in a

restored phagocytic function comparable to that of the control sample. Nevertheless, the apparent restorative effects of IFN $\gamma$  were not observed in the samples pre-incubated with salbutamol and fludarabine. This result suggests that Stat1 may also play an important role in IFN $\gamma$  signalling to improve complement mediated phagocytosis.

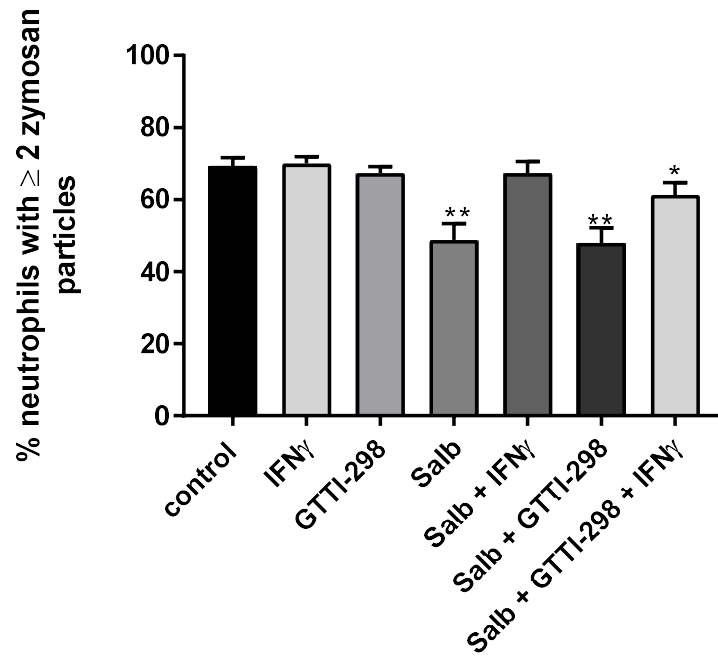


**Figure 22: Fludarabine, a Stat1 specific activation inhibitor, inhibits the restoration of neutrophil phagocytosis by interferon gamma.** Neutrophils were incubated with interferon gamma, fludarabine, salbutamol or a combination of these agents. For the well containing salbutamol, interferon gamma and fludarabine, neutrophils were pre-incubated for 30 minutes with both salbutamol and fludarabine before the addition of interferon gamma for a further 30 minutes. For all other wells, the individual agents were added in the same order and applied for the same duration and at the same time as for the well containing all three agents. Serum-opsonised zymosan was then added to each well for a further 30 minutes prior to quantification of phagocytosis by light microscopy. Data are presented as mean (columns) and SEM (bars); n=4; statistical analysis was by repeated measures one-way ANOVA with Dunnett's multiple comparisons test relative to the control column; \*P<0.05, \*\*P<0.01. Abbreviations/end concentrations: Salb (salbutamol/10 $\mu$ M), IFN $\gamma$  (interferon gamma/50nM), Flud (fludarabine/50 $\mu$ M)

As illustrated in Chapter 1, section 1.13, Figure 11, previous work by our group has demonstrated that activating Rap1, a small GTPase, may restore phagocytic function in neutrophils pre-incubated with salbutamol by bypassing signalling via RhoA to facilitate actin cytoskeletal rearrangement.(85)

In a group of 6 healthy volunteers (3 female; mean age 35.7 years; age range 21-51 years), the effects of inhibiting Rap1 were explored using the Rap1 inhibitor GGTI-298, at an end concentration of 10 $\mu$ M (following initial dose response experiments carried out by my colleague Mr Jon Scott) with the aim of further characterising IFN $\gamma$  cell-signalling pathways.

As shown in Figure 23, preincubation of neutrophils with IFN $\gamma$  or the Rap1 inhibitor GGTI-298 alone had no apparent effect on neutrophil phagocytosis relative to the control and as previously observed, IFN $\gamma$  fully restored phagocytic function in neutrophils pre-incubated with salbutamol. The restorative effect of IFN $\gamma$  on neutrophil phagocytosis was partly inhibited by the Rap1 inhibitor GGTI-298, suggesting that IFN $\gamma$  has potential to enhance neutrophil phagocytosis in the absence of Rap1 activation, albeit not back to the levels observed in the control sample.



**Figure 23: GTTI-298, a Rap-1 GTPase inhibitor, inhibits full restoration of neutrophil phagocytosis by interferon gamma.** Neutrophils were incubated with interferon gamma, GTTI-298, salbutamol or a combination of these agents. For the well containing all three agents, neutrophils were incubated for 30 minutes with both salbutamol and GTTI-298 before the addition of interferon gamma for a further 30 minutes. For all other wells, the individual agents were added in the same order, at the same time and for the same duration as for the well containing all three agents. Serum-opsonised zymosan was then added to each well for a further 30 minutes prior to quantification of phagocytosis by light microscopy. Data are presented as mean (columns) and SEM (bars); n=6; statistical analysis was by repeated measures one-way ANOVA with Dunnett's multiple comparisons test relative to the control column; \*P<0.05, \*\*P<0.01. Abbreviations/end concentrations: Salb (salbutamol/10 $\mu$ M), IFN $\gamma$  (interferon gamma/50nM), GGT-298 (Rap1 inhibitor/10 $\mu$ M)



### 3.6 SUMMARY OF THE INTERFERON GAMMA LABORATORY STUDY

In conclusion, results from the IFN $\gamma$  laboratory study reinforce the concept that incubating neutrophils from healthy volunteers with salbutamol (a short acting  $\beta_2$  agonist) results in reproducible *in vitro* impairment of neutrophil phagocytic function.

The addition of IFN $\gamma$  at an end concentration of 1ng/ml (50nM) appeared to fully restore salbutamol induced impairment of neutrophil phagocytosis. However, IFN $\gamma$  did not augment the phagocytic capacity of neutrophils from healthy volunteers relative to the control sample.

RhoA activation in neutrophils pre-incubated with salbutamol was substantially impaired to a level comparable to the negative control samples suggesting that salbutamol inhibits complement mediated phagocytosis by inhibiting RhoA activity. Moreover, in neutrophils pre-incubated with salbutamol the addition of IFN $\gamma$  appeared to restore RhoA activity to a level comparable to the positive control.

Finally, Jak1, Jak 2 and Stat1 all seem to be important molecules involved in the restorative effects of IFN $\gamma$  on neutrophil phagocytosis. Inhibition of Rap1 partly inhibited the restoration of phagocytosis by interferon gamma suggesting that this molecule may also be involved in the cell-signalling pathway.

These findings suggest that there is a potential therapeutic role for IFN $\gamma$  in patients with impaired neutrophil phagocytosis such as that observed in our 2012 preliminary clinical bypass study as previously described in Chapter 1.

The findings from this chapter are discussed further in Chapter 7.

# CHAPTER 4: CLINICAL STUDY OPTIMISATION

## 4.1 OVERVIEW

This chapter reports the process of optimisation of the clinical study.

The main aims were:

1. To ensure a workable and reproducible laboratory protocol for the clinical study.
2. To recruit 5 patients listed for 'on-pump' CABG surgery to an 'optimisation cohort' which would serve to iron out any technical challenges with the clinical study protocol and address any potential confounding factors.

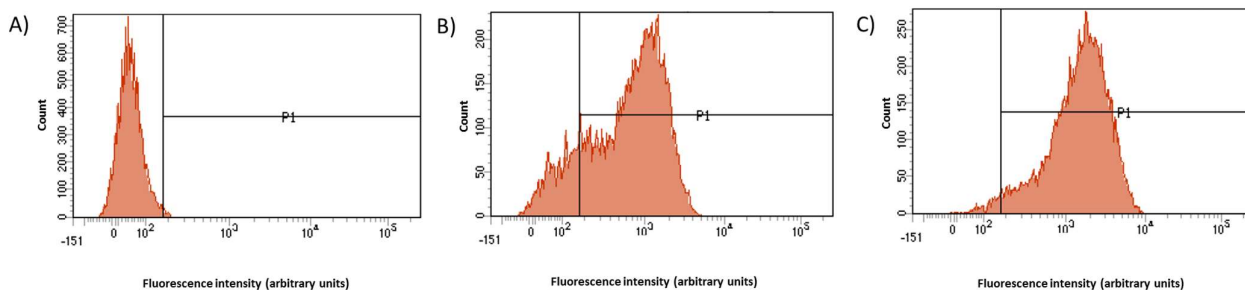
The chapter begins with the results of image flow cytometry experiments employed to refine the quantification of neutrophil phagocytosis using pHrodo bioparticles. An overview of patient screening and recruitment to the optimisation cohort is then detailed, followed by a description of measures taken to address technical challenges identified with the protocol.

As study optimisation focused on refining the methods rather than investigating the experimental questions, this chapter provides a descriptive analysis of the results and the protocol amendments necessary prior to commencing recruitment to the first experimental cohort.

## 4.2 LABORATORY PROTOCOL OPTIMISATION

Refinement of the laboratory experiments for the clinical study was initially carried out using healthy volunteer blood samples before commencing patient recruitment to the optimisation cohort. The laboratory refinement involved optimisation of the individual experiments as well as repeated practice at performing five simultaneous neutrophil isolations from individual patients to ensure a workable and reproducible protocol.

While the planned methods for most experiments resulted in satisfactory outcomes, the flow cytometric evaluation of neutrophil phagocytosis using whole blood required substantial optimisation. This was owing to repeatedly obtaining high fluorescence intensity readings for pHrodo in the 4°C inhibitory control sample (Figure 24). This result was unexpected and resulted in the inability to quantify the true phagocytic capacity of neutrophils in the 37°C sample.



*Figure 24: Histograms demonstrating the gating strategy for flow cytometric evaluation of neutrophil phagocytosis on whole blood using pHrodo S.aureus bioparticles. [A] Negative control, [B] 4°C inhibitory control sample demonstrating an unexpectedly high fluorescence intensity for pHrodo similar to that of the 37°C pHrodo sample [C] 37°C pHrodo sample*

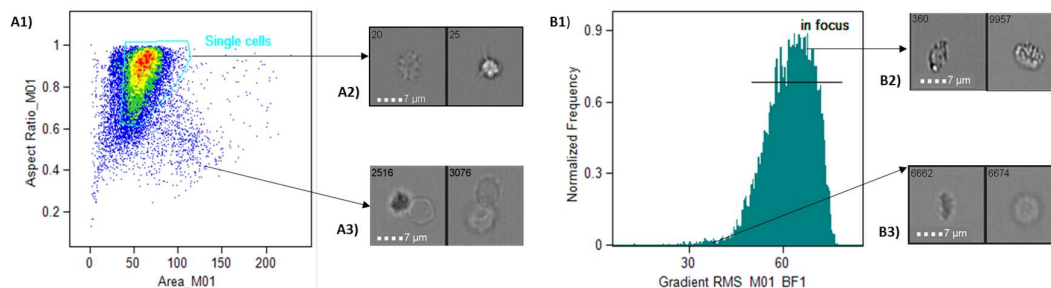
The principle of using pHrodo bioparticles to quantify phagocytosis is based on their pH sensitivity. pHrodo bioparticles fluoresce brightly in acidic environments such as inside neutrophil phagosomes, however they are non-fluorescent in pH neutral environments such as the extracellular environment. Quantification of neutrophil phagocytosis can therefore be achieved by measuring the fluorescence intensity of pHrodo bioparticles when incubated in optimum conditions (i.e. 37°C) relative to a sample maintained on ice to inhibit phagocytosis. The unexpectedly high fluorescence intensity of pHrodo in the 4°C inhibitory control sample could have been due to unexpected neutrophil phagocytosis or unexpected fluorescence in the extracellular environment. Despite numerous adjustments to the protocol this problem

persisted, therefore image flow cytometry was employed to obtain an objective assessment of pHrodo particle location. Image flow cytometry combines the conventional features of flow cytometry with high resolution microscopy therefore enabling morphological and spatial information at single cell resolution.(97) The results of this image flow cytometry work are now described.

#### 4.2.1 Image flow cytometry to optimise phagocytosis assay using pHrodo bioparticles

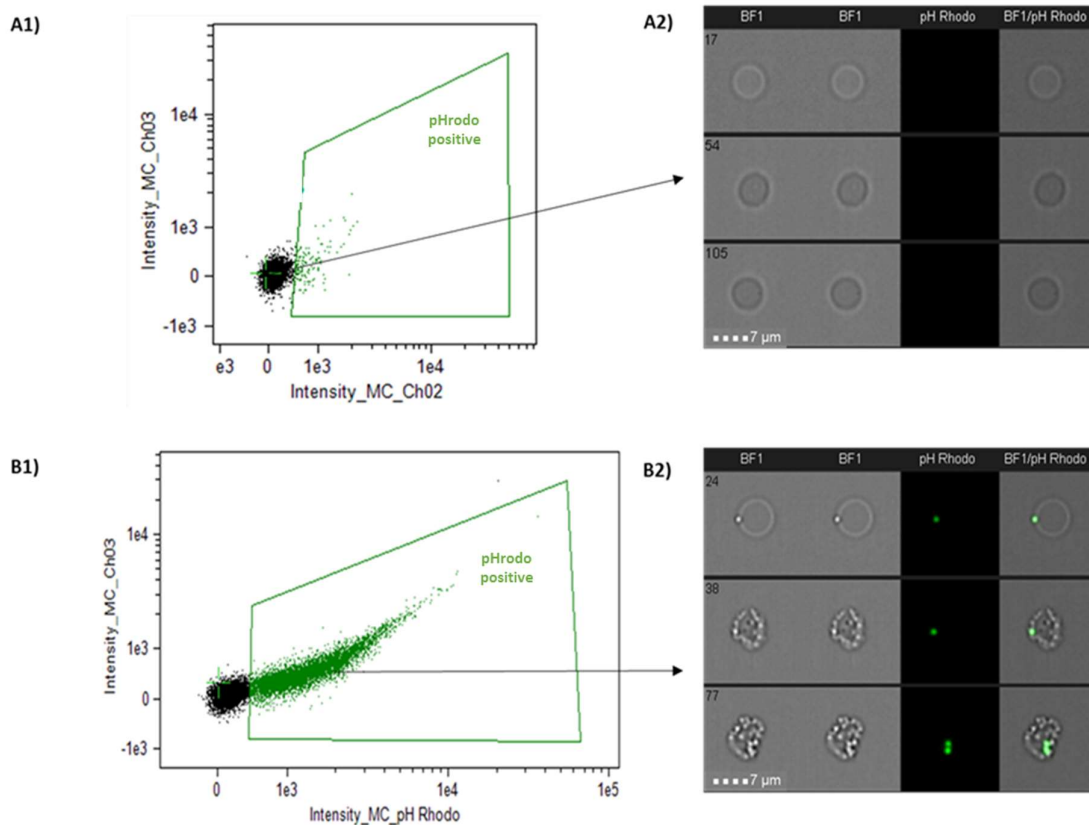
A phagocytosis assay was performed on whole blood as per the previously described methods (see section 2.2.3). The samples were kept on ice prior to analysis using an imaging flow cytometer (Imagestream<sup>®</sup>X Mark II Imaging Flow Cytometer from Amnis, Seattle, USA). Results were analysed using the IDEAS<sup>®</sup> software package (Amnis, Seattle, USA).

Single cells were initially identified based on individual cell area and aspect ratio (the proportional relationship between the width and height of the cellular image). Further gating identified focused cells based on the gradient RMS (the root mean square of the rate of change of the image intensity profile). Using manual interrogation of the bright-field image, those with a gradient RMS of greater than 50 were accepted as being in focus (Figure 25).



**Figure 25: Gating strategy for single, focused cells using image flow cytometry.** [A1] Single blood cells were gated based on area and aspect ratio. [A2] Representative images of gated single cells (mid-area, high aspect ratio). [A3] Representative images of doublets (high area, low aspect ratio) which were excluded from the analysis. [B1] Further gating identified focused single cells based on a gradient RMS of greater than 50. [B2] Representative images of focused single cells. [B3] Representative images of unfocused single cells which were excluded from the analysis.

Cells positive for pHrodo bioparticles were then identified by further gating strategies using the total integrated fluorescence of the image (Intensity Mask (MC)) as described in Figure 26.



**Figure 26: Identification of pHrodo positive cells.** [A1] Gating strategy for pHrodo positive cells in the 37°C negative control sample (no pHrodo bioparticles) using the intensity mask. [A2] Representative images of pHrodo negative cells in the 37°C negative control sample. [B1] Identification of pHrodo positive cells in the 37°C pHrodo positive sample [B2] Representative images of pHrodo positive cells in the 37°C positive sample.

Determination of the location of pHrodo bioparticles in pHrodo positive cells was then achieved by refining the default bright-field mask to mark the interior of the cell. From that, the 'internalisation feature' in the Amnis IDEAS® software was applied to each cell to determine whether the bioparticle was bound to the surface of the cell (outside the mask area) or internalised (inside the mask area) (Figure 27).

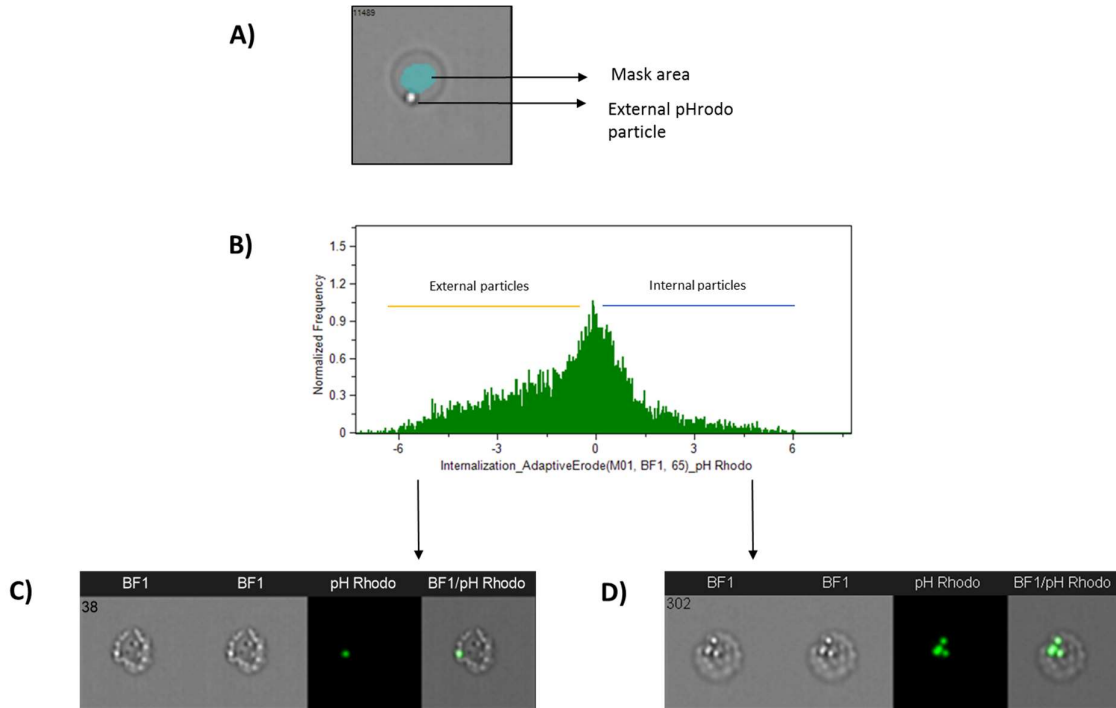
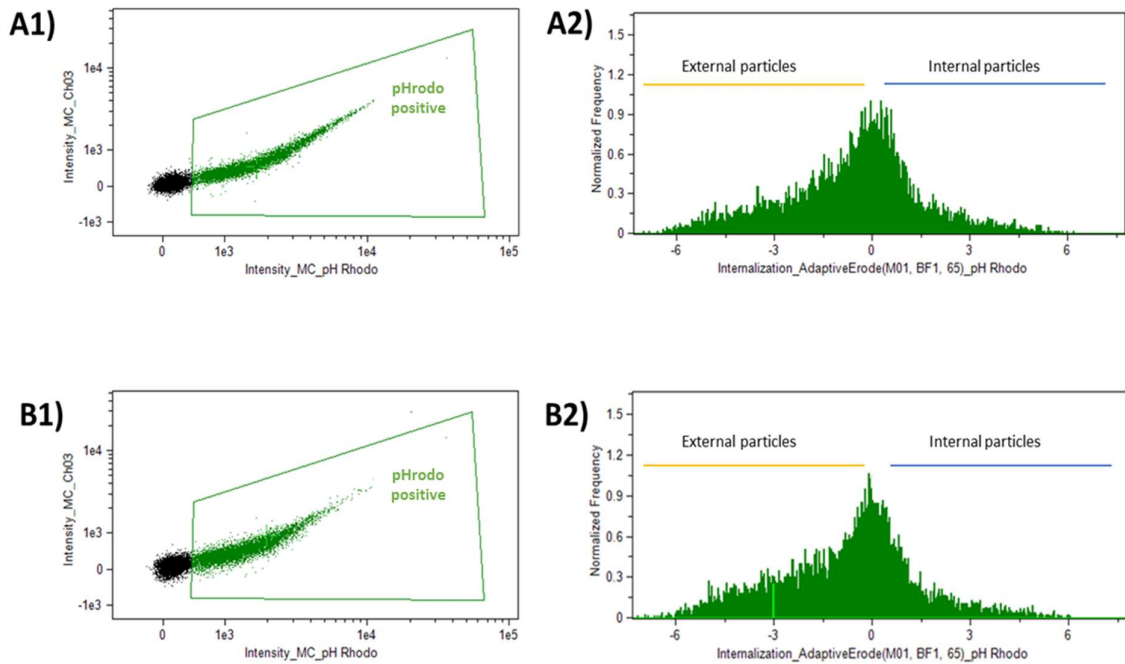


Figure 27: **Determining the location of pHrodo bioparticles.** [A] A mask area was created to demark the interior of the cell based on the bright-field image and applied to all pHrodo positive cells to determine whether the bioparticle was bound to the surface of the cell (outside the mask area) or internalised (inside the mask area). [B] Graphical representation of internalised bioparticles (Adaptive erode > 0) and external bioparticles (Adaptive erode < 0). [C] Representative images of a cell with an adaptive erode of < 0 demonstrating the pHrodo particle on the surface of the cell. [D] Representative images of a cell with an adaptive erode of > 0 demonstrating multiple internalised pHrodo bioparticles.

Comparison of the location of pHrodo bioparticles in the 4°C inhibitory control sample and the 37°C sample demonstrated a similar proportion of both external and internal particles (Figure 28). These results suggested that the high proportion of pHrodo positivity in both the 4°C and 37°C sample was due to pHrodo bioparticle fluorescence in the extracellular environment rather than due to phagocytosis thus explaining the previous unexpected flow cytometry results. Moreover, the lower than expected proportion of internal particles in the 37°C sample relative to the 4°C sample may have been reflective of the experimental conditions resulting in suboptimal phagocytosis in this sample.



**Figure 28: Determining the location of pHrodo particles at 4°C and 37°C.** [A1] Identification of pHrodo positive cells in the 4°C inhibitory control sample. [A2] Graphical representation of the proportion of internal and external particles in the 4°C inhibitory control sample. [B1] Identification of pHrodo positive cells in the 37°C positive sample. [B2] Graphical representation of the proportion of internal and external particles in the 37°C positive sample.

To address this issue, the pH of each of the components of this assay was tested: a neutral pH was obtained across all assay components. Despite this, a commercially available wash buffer (CellWASH, BD Biosciences, New Jersey, USA, cat: 349654) was trialed instead of our own in-house wash buffer (PBS, 0.2% BSA and 1 $\mu$ M sodium azide), keeping all other methods the same. This change resulted in a significant improvement in the flow cytometry results with satisfactory inhibition of pHrodo fluorescence in the 4°C inhibitory sample relative to the 37°C sample, thus enabling the true neutrophil phagocytic capacity to be assessed. The optimised flow cytometry results yielded following this adjustment are illustrated in Figure 29.

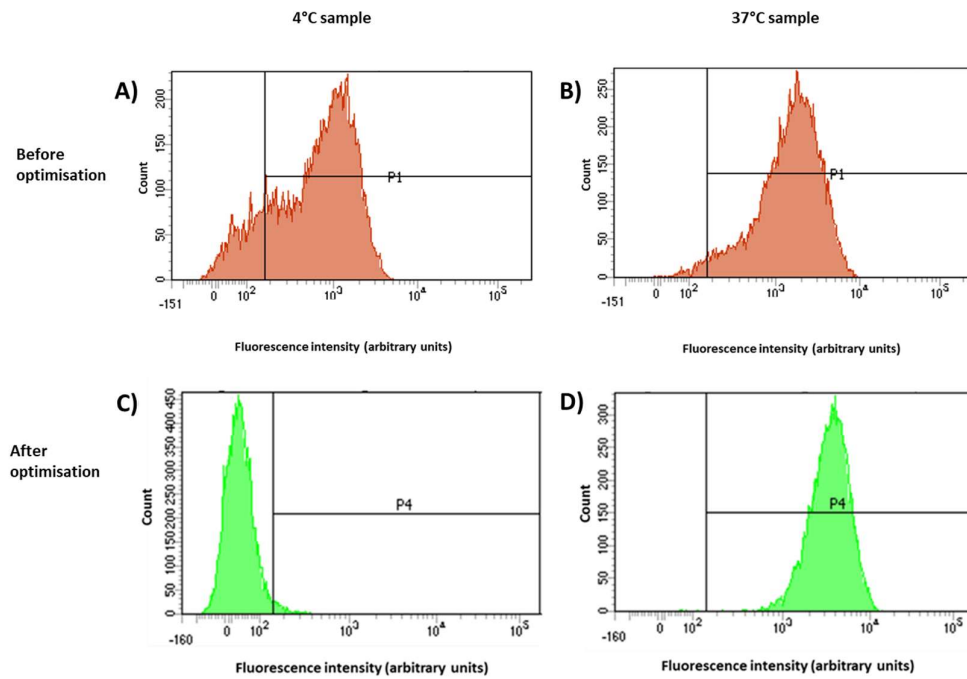


Figure 29: **Histograms of the optimised flow cytometric evaluation of neutrophil phagocytosis on whole blood using pHrodo *S. aureus* bioparticles.** [A] Demonstrates the unexpected high degree of pHrodo fluorescence in the 4°C inhibitory control sample before optimisation. [B] 37°C sample before optimisation. [C] 4°C inhibitory control sample after optimisation demonstrating negligible pHrodo fluorescence. [D] 37°C sample after optimisation.

In summary, using image flow cytometry to obtain an objective assessment of pHrodo bioparticle location resulted in optimisation of the flow cytometric assay of neutrophil phagocytosis on whole blood prior to commencing recruitment to the clinical study.



### 4.3 CLINICAL PROTOCOL OPTIMISATION

After refinement of the laboratory protocol, a period of clinical protocol optimisation followed.

#### 4.3.1 Screening and recruitment

Patient screening and recruitment to the optimisation cohort are illustrated in Figure 30. Four of the five recruited patients received the intervention of right lung ventilation. One patient did not receive the one-lung ventilation intervention as the surgical procedure was converted intraoperatively to an 'off-pump' procedure. Four of the five patients were recruited as 'elective' procedures having been screened and identified in the cardiothoracic pre-assessment clinic. One patient was recruited as an inpatient following a recent myocardial infarction and was the only 'urgent' case within this cohort.

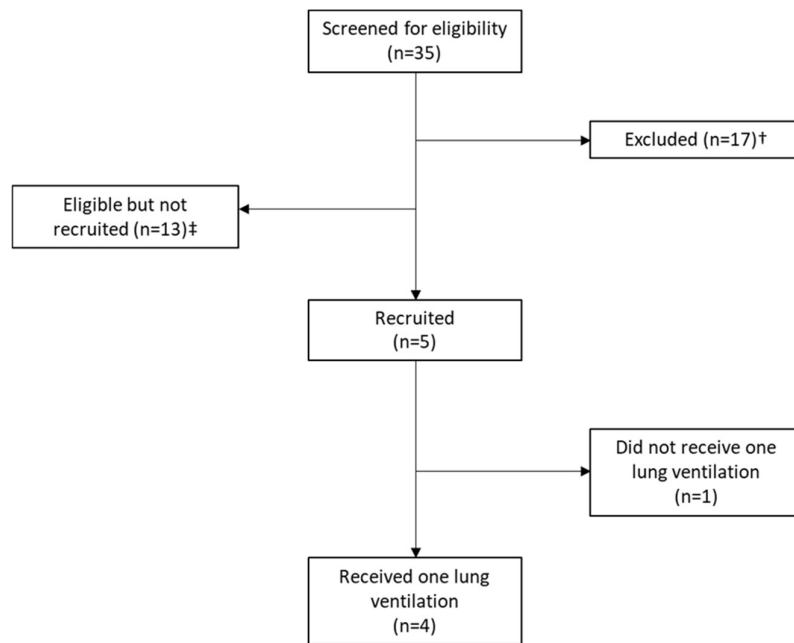


Figure 30: **Flow diagram demonstrating patient screening and recruitment to the optimisation cohort.** †Reasons for exclusion: surgeon not participating in study (n=5), listed too late for consent (n=4), surgery converted to 'off-pump' procedure (n=2), immunosuppressant drugs (n=2), simultaneous valve replacement (n=2), surgery cancelled for medical reasons (n=2). ‡Reasons eligible patients not recruited: declined (n=7), clinical commitments of research fellow (n=4), surgery cancelled/postponed (n=1), scheduled at same time as another study patient (n=1).

### **4.3.2 Baseline clinical characteristics**

Baseline clinical characteristics of patients recruited to the optimisation cohort are illustrated in Table 6. The baseline characteristics were deemed to be representative of the target patient population and no obvious confounding baseline characteristics were identified within this cohort.

Table 6: Baseline clinical characteristics of patients recruited to the optimisation cohort

<b>Baseline characteristic</b>	
Gender (number of patients)	
Male	5
Female	0
Age (years)	74.0 (65.0-82.0)
Height (centimetres)	173.0 (168.9-179.0)
Weight (kg)	86.9 (80.5-91.0)
Ideal body weight (kg)†	68.7 (64.6-74.1)
Smoking status (number of patients)	
Current smoker	0
Ex-smoker	3
Never smoker	2
Lung function	
FEV <sub>1</sub> (litres)	2.9 (2.1-3.1)
FVC (litres)	4.1 (3.5-4.7)
FEV <sub>1</sub> predicted (%)	95.0 (74.5-107.5)
FEV <sub>1</sub> /FVC (%)	68.0 (52.0-76.5)
Logistic EUROscore <sup>§</sup>	2.0 (0.5-5.0)
Urgency of procedure (number of patients)	
Elective	4
Urgent	1
Co-morbidities (number of patients)	
Cardiovascular‡	
Hypertension	4
Atrial Fibrillation	2
Previous TIA	1
Respiratory	
COPD	1
Other	
Diabetes (type 2)	1
Benign prostatic hypertrophy	2
Low grade prostate cancer	1
Hypothyroidism	1
Osteoarthritis	1

Data are presented as median (interquartile range) unless otherwise documented in the table, n=5

† Ideal body weight as calculated by the Devine formula (92)

‡ Cardiovascular co-morbidities other than ischaemic heart disease

§ Logistic Euroscore: a formula used to predict the risk of death after a cardiac operation (93)

Abbreviations: TIA: transient ischaemic attack, COPD: chronic obstructive pulmonary disease, FEV<sub>1</sub>: forced expiratory volume in 1 second, FVC: forced vital capacity

### **4.3.3 Operative optimisation**

#### **Ventilator settings for one-lung ventilation**

Following recruitment of the first patient, an unforeseen problem arose with the original ventilator settings for the one-lung ventilation period. It became apparent that using pressure-controlled ventilation to deliver one-lung ventilation to the right lung during CPB was not possible. This was because the ventilator could not be programmed to effectively deliver an ultra-low tidal volume of 2mls/kg of ideal body weight using the pressure-controlled mode for this patient as this volume was too small for the ventilator to function correctly in the pressure-controlled mode. This was evidenced by repeated ventilator alarms during the one lung ventilation period. Following consultation with the lead anaesthetist we decided to change from pressure-controlled ventilation to volume-controlled ventilation for the remaining patients. This strategy worked effectively, and a good tidal volume for the one lung ventilation period was attainable for the remaining recruited patients in this cohort.

#### **Timing of blood samples**

For the four patients who underwent one-lung ventilation within this cohort, all five experimental blood samples were retrieved. Nevertheless, an unforeseen challenge arose when plasma obtained from the post-CPB blood samples (samples 3, 4 and 5) would not form serum following the addition of calcium chloride (see Chapter 2, section 2.3.3, Table 3 for initial blood sampling protocol). This was the case for the first two recruited patients and occurred because the samples contained heparin, which is routinely administered intraoperatively to prevent blood clot formation during the extracorporeal circuit time. To solve this problem the post-CPB blood samples were retrieved after protamine was administered intraoperatively so that the effects of heparin were reversed prior to sample collection (see Chapter 2, section 2.3.3, Table 4 for the revised blood sampling protocol). While this resulted in a slight delay in retrieving the samples following CPB, it ensured that the samples were as comparable as possible before commencing the laboratory work and facilitated the formation of autologous serum (which was essential for the laboratory experiments) after the addition of calcium chloride for the post-CPB samples.

## Duration of intervention

Analysis of operative data within the optimisation cohort confirmed that the median duration of the right-lung ventilation intervention during CPB was 70 minutes and that the median duration between cessation of CPB and retrieval of the post-CPB blood samples was six minutes as shown in Table 7. The research team agreed that the duration of the right lung ventilation intervention was suitable to test the hypothesis and that the post-CPB blood samples were retrieved within a satisfactory period following CPB.

Table 7: Operative data from patients in the optimisation cohort

Operative variable	Optimisation cohort
Duration of bilateral lung ventilation before CPB (mins)	81.0 (70.8-92.0)
CPB time (mins)	76.5 (62.5-86.0)
Aortic cross-clamp time (mins)	40.0 (33.8-47.0)
Number of grafts	3.0 (3.0-3.8)
Duration of right lung ventilation during CPB (mins)	70.0 (60.5-82.5)
Duration between bilateral ventilation restarting and obtaining postoperative blood samples (mins) †	5.5 (5.0-11.3)
Duration between end of CPB and obtaining postoperative blood samples (mins) †	6.0 (3.0-12.0) *

*Data are presented as median (interquartile range), n=4 (\* n=3 as one patient not completely off bypass before sample obtained)*  
*† Postoperative samples refer to samples 3, 4 and 5*

## Acceptability of protocol to anaesthetic and surgical staff

The optimisation cohort enabled us to run through the protocol and practicalities of the research with theatre staff, anaesthetists and surgeons. The bronchus blocking balloon was successfully positioned in all cases, reassuring us that left lung deflation using this method was suitable for the purposes of the study.

Several surgeons had opted not to participate in the study as the protocol involved lifting the heart after the patient had come off CPB to obtain the left pulmonary vein sample. This is because the process of lifting the heart after CPB can be associated with a transient drop in mean arterial pressure while the sample is being retrieved. Within the optimisation cohort, one surgeon had agreed to participate in the study, however during the operation became reluctant to lift the heart to retrieve the left pulmonary vein sample after the patient had come off bypass. Therefore, the sample was retrieved while the patient was still on bypass. As obtaining the left pulmonary vein sample after the patient was completely off bypass was key to addressing our research question, the optimisation cohort highlighted that we needed to clarify with the participating surgeons that the research involved transiently lifting the heart following CPB to avoid the disappointment of not obtaining the left pulmonary vein sample at the correct time.

#### **4.4 SUMMARY OF CLINICAL STUDY OPTIMISATION**

In conclusion, clinical study optimisation was invaluable in refining the study protocols prior to commencing recruitment to the first experimental cohort.

Image flow cytometry was used to visualise pHrodo particle location, which resulted in resolution of an initial problem with the flow cytometric analysis of neutrophil phagocytosis using pHrodo bioparticles.

The optimisation cohort enabled the ventilation strategy and timing of blood samples to be refined. Further, it reassured us that other aspects of the protocol were as workable and reproducible as possible prior to commencing recruitment to the first experimental cohort to test our hypotheses. The final protocols used for the first and second experimental cohorts are detailed in Chapter 2.

The results of the first experimental cohort are now described in Chapter 5.

# CHAPTER 5: RESULTS OF FIRST EXPERIMENTAL COHORT

## 5.1 OVERVIEW

This chapter reports the results of the first experimental cohort of the clinical study, which aimed to assess the immunological impact of lung ventilation during CPB using a one-lung ventilation model. Throughout this chapter, right lung ventilation during CPB was stipulated as follows: volume-controlled ventilation,  $FiO_2$  0.21, tidal volume 2mls/kg ideal body weight, 7 ventilations per minute and PEEP of 5 cmH<sub>2</sub>O.

The main aims of the first experimental cohort were:

1. To evaluate the impact of lung ventilation during CPB on blood neutrophil phagocytic capacity and priming status.
2. To evaluate the impact of lung ventilation during CPB on plasma pro-inflammatory and anti-inflammatory cytokine profile.
3. To ensure the safety of the experimental model.

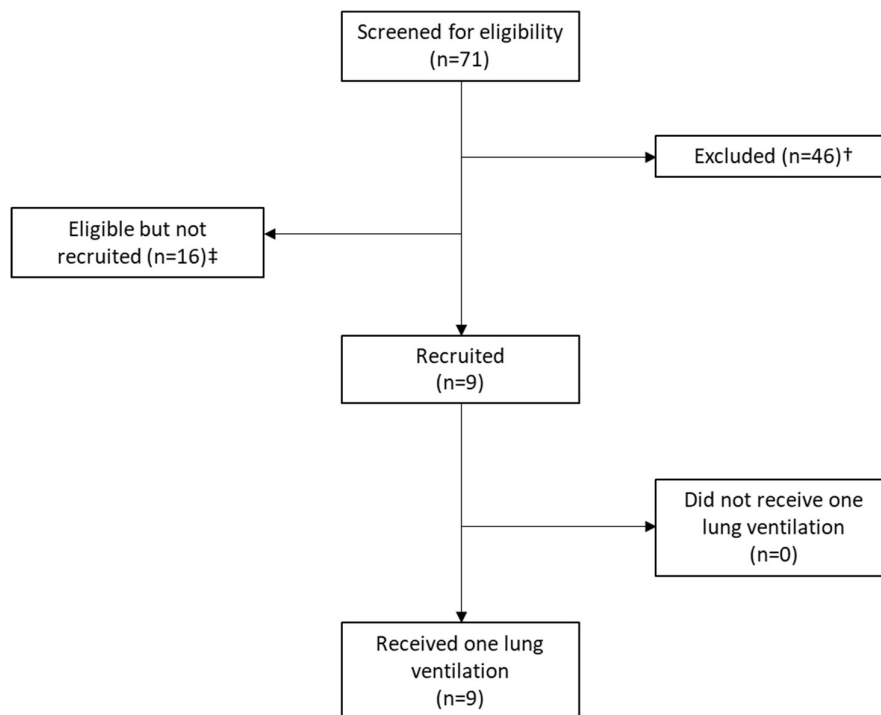
The chapter begins with an overview of patient screening and recruitment followed by a description of baseline clinical and operative characteristics. Data on neutrophil yield and viability are presented, followed by the results of blood neutrophil phagocytosis, neutrophil priming status and plasma cytokine measurements across all five experimental blood samples. Data on clinical outcome measures including pulmonary vein blood gas analysis and postoperative chest radiograph findings are then presented. The chapter ends with a summary of the conclusions drawn from the first experimental cohort.



## 5.2 SUBJECTS

### 5.2.1 Screening and recruitment

Nine patients were recruited to the first experimental cohort. Patient screening and recruitment are illustrated in Figure 31. There were no withdrawals from this cohort and all nine patients received the intervention of right lung ventilation during CPB as per the standardised protocol. One patient developed a cardiac arrhythmia resulting in hypotension after discontinuation of CPB rendering it unsafe to lift the heart to obtain the left pulmonary vein blood sample. All five blood samples were obtained in the remaining eight patients.



*Figure 31: Flow diagram demonstrating patient screening and recruitment to the first experimental cohort. †Reasons for exclusion: surgeon not participating in study (n=29), operation on afternoon list (n=10), immunosuppressant drugs/immunosuppressed (n=4), listed too late for consent (n=2), possible simultaneous valve replacement (n=1). ‡ Reasons eligible patients not recruited: operation cancelled/postponed after written consent (n=4), operation cancelled/postponed before written consent (n=4), scheduled at same time as another potential study patient (n=4), clinical commitments of research fellow (n=3), declined (n=1).*

### **5.2.2 Baseline clinical characteristics**

Baseline clinical characteristics of patients in the first experimental cohort are illustrated in Table 8. Notably, median lung function values demonstrated normal spirometry hence objective air flow obstruction was not an overriding clinical finding within this cohort. The risk of incomplete deflation of the left lung (due to air trapping from obstructive airways disease) before inflation of the bronchus blocking balloon was therefore minimised. Most patients had multiple co-morbidities other than ischaemic heart disease necessitating a CABG operation.

Table 8: Baseline clinical characteristics of patients recruited to the first experimental cohort.

<b>Baseline characteristic</b>	
Gender (number of patients)	
Male	7
Female	2
Age (years)	62.0 (57.5-78.0)
Height (centimetres)	171.4 (162.0-178.0)
Weight (kg)	77.0 (70.5-89.8)
Ideal body weight (kg)†	67.2 (56.0-73.2)
Smoking status (number of patients)	
Current smoker	0
Ex-smoker	4
Never smoker	5
Lung function	
FEV <sub>1</sub> (litres)	2.8 (2.3-3.9)
FVC (litres)	4.2 (3.2-5.0)
FEV <sub>1</sub> predicted (%)	97.0 (93.0-119.5)
FEV <sub>1</sub> /FVC (%)	79.0 (68.0-80.5)
Logistic EUROscore §	1.2 (1.0-4.3)
Urgency of procedure (number of patients)	
Elective	8
Urgent	1
Co-morbidities (number of patients)	
Cardiovascular‡	
Previous MI	6
Previous PCI	3
Hypertension	7
Heart failure with EF<40%	1
Respiratory	
Asthma	3
Other	
Diabetes (type 2)	3
GORD	3
Osteoarthritis	3
Liver cirrhosis	1
PMR	1

Data are presented as median (interquartile range) unless otherwise documented in the table, n=9

† Ideal body weight as calculated by the Devine formula (92)

‡ Cardiovascular co-morbidities other than ischaemic heart disease

§ Logistic Euroscore: a formula used to predict the risk of death after a cardiac operation (93)

Abbreviations: MI: myocardial infarction, PCI: percutaneous coronary intervention, EF: ejection fraction, GORD: gastro-oesophageal reflux disease, PMR: polymyalgia rheumatica

### **5.2.3 Operative data**

A bronchus-blocking balloon was successfully positioned in the left main bronchus of all study patients and there were no reported complications from this intervention. In all patients, the left pleura was successfully breached, and maximal left lung deflation was achieved prior to inflation of the left bronchus-blocking balloon. Right lung ventilation was commenced immediately after inflation of the left bronchus blocking balloon and continued until weaning of CPB in all patients as per the study protocol for the first experimental cohort.

There were no significant intraoperative complications within the first experimental cohort. One patient developed a cardiac arrhythmia with resultant hypotension after discontinuation of CPB which responded to cardiac pacing. Another patient developed mild intraoperative chest wall bleeding which was controlled with cauterisation prior to obtaining the pulmonary vein samples.

The operative data of patients within this cohort are detailed in Table 9. The median duration of right lung ventilation during CPB was 61 minutes (IQR 49-67.5). The median duration between bilateral ventilation recommencing (as the patient was weaned off CPB) and obtaining the post-operative blood samples was 13 minutes (IQR 7-16). The duration of this bilateral ventilation period was largely dictated by the stability of the patient during the CPB weaning process. The median duration between the restoration of normal cardiopulmonary circulation after the end of CPB and obtaining the postoperative blood samples was 7 minutes (IQR 6-13.5) which again was dictated by patient stability and the speed with which intravenous protamine was administered.

Table 9: Operative data from patients in the first experimental cohort

<b>Operative variable</b>	<b>First experimental cohort</b>
Duration of bilateral lung ventilation before CPB (mins)	98.0 (83.5-105.5)
CPB time (mins)	65.0 (59.0-74.0)
Aortic cross-clamp time (mins)	37.0 (29.5-41.0)
Number of grafts	3.0 (2.0-3.5)
Duration of right lung ventilation during CPB (mins)	61.0 (49.0-67.5)
Duration between bilateral ventilation restarting and obtaining postoperative blood samples (mins) †	13.0 (7.0-16.0)
Duration between end of CPB and obtaining postoperative blood samples (mins) †	7.0 (6.0-13.5)

*Data are presented as median (interquartile range), n=9*  
† Postoperative samples refer to samples 3, 4 and 5 as previously described

### 5.3 NEUTROPHIL YIELD AND VIABILITY

The desired volume of blood (25mls) was obtained for each sample. In all patients, there were no discernible differences in the dilution of the five blood samples as evidenced by comparable haematocrits after the initial whole blood centrifugation.

There was a trend towards a greater median number of neutrophils isolated from the post bypass samples (samples 3-5) compared to the pre-bypass samples (samples 1-2) but this did not reach statistical significance. (Figure 32, panel A).

Typically, the PMN cell layer (which was formed using the Percoll gradient technique) was well defined in the pre-bypass samples and separation from the upper PBMC layer was usually straightforward. In the post-CPB samples, the PMN cell layer was often less well defined and there was occasional difficulty in separating it from the PBMC layer. This is reflected in the wider range of numbers of neutrophils isolated in the post-CPB samples compared to the pre-CPB samples. For one patient, there were too few neutrophils isolated from sample 5 for any further laboratory analyses. The median neutrophil purity was greater than 95% in all five samples (Figure 32, panel B). For ease of reference, samples 1 and 2 were the pre-CPB samples (sample 1 obtained from the central venous line immediately following induction of anaesthesia and sample 2 obtained from the central venous line immediately prior to the administration of intravenous heparin and before the initiation of cardiopulmonary bypass). Samples 3, 4 and 5 were the post-CPB samples (sample 3 was obtained from the central venous line following the cessation of cardiopulmonary bypass and after the administration of protamine, samples 4 and 5 were obtained directly from the right and left pulmonary veins respectively at the same time as sample 3). Regardless of whether the left or right pulmonary vein sample was obtained first by the surgeon (alternate side first for each study patient) all results have been corrected so that sample 4 in the results figures equates to the right (ventilated lung) pulmonary vein sample and sample 5 equates to the left (deflated lung) pulmonary vein sample.

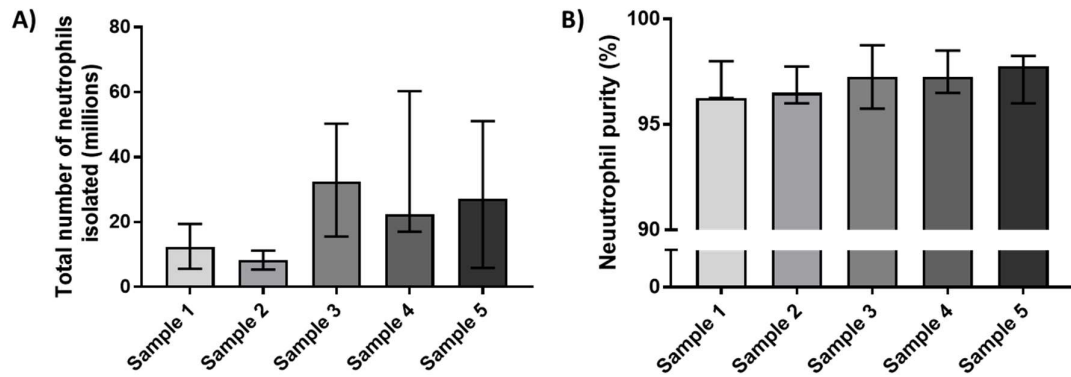


Figure 32: **Neutrophil yield [A] and purity [B] across all five blood samples.** Data are presented as median (columns) and interquartile range (bars);  $n=8$  [A] and  $n=7$  [B]; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample).

The viability of isolated neutrophils was assessed using APC-conjugated annexin V and propidium iodide as previously described. The percentage of viable neutrophils ( $AnV^-/PI^-$ ) early apoptotic neutrophils ( $AnV^+/PI^-$ ), late apoptotic neutrophils ( $AnV^+/PI^+$ ) and dead neutrophils ( $AnV^-/PI^+$ ) was determined for each experimental sample.

There was a trend towards a greater percentage of live neutrophils in the post-CPB samples compared to the pre-CPB samples reaching statistical significance in sample 5 (Figure 33, panel A). Similarly, there were fewer early and late apoptotic neutrophils in the post-CPB samples compared to the pre-CPB samples reaching significance in sample 5 and samples 4 and 5 respectively (Figure 33, panel B and C). Most neutrophils were either live/viable or early apoptotic in all five samples. Only a small percentage of neutrophils were in late apoptosis and a negligible percentage were dead/non-viable. Notably, there was no difference in the viability of neutrophils extracted from the left and right pulmonary vein blood samples.

The median duration between obtaining sample 1 and 2 was 49 minutes (IQR 34.5-55.0) and the median duration between obtaining sample 2 and the post-CPB samples (3, 4, and 5) was 97 minutes (IQR 89.0-124.5). A complete dataset for viability analysis was available for six of the nine recruited patients (for one patient the left pulmonary vein sample was not retrieved as previously described and for two patients, too few cells were harvested in one of the five experimental samples which precluded a full viability dataset in these patients).

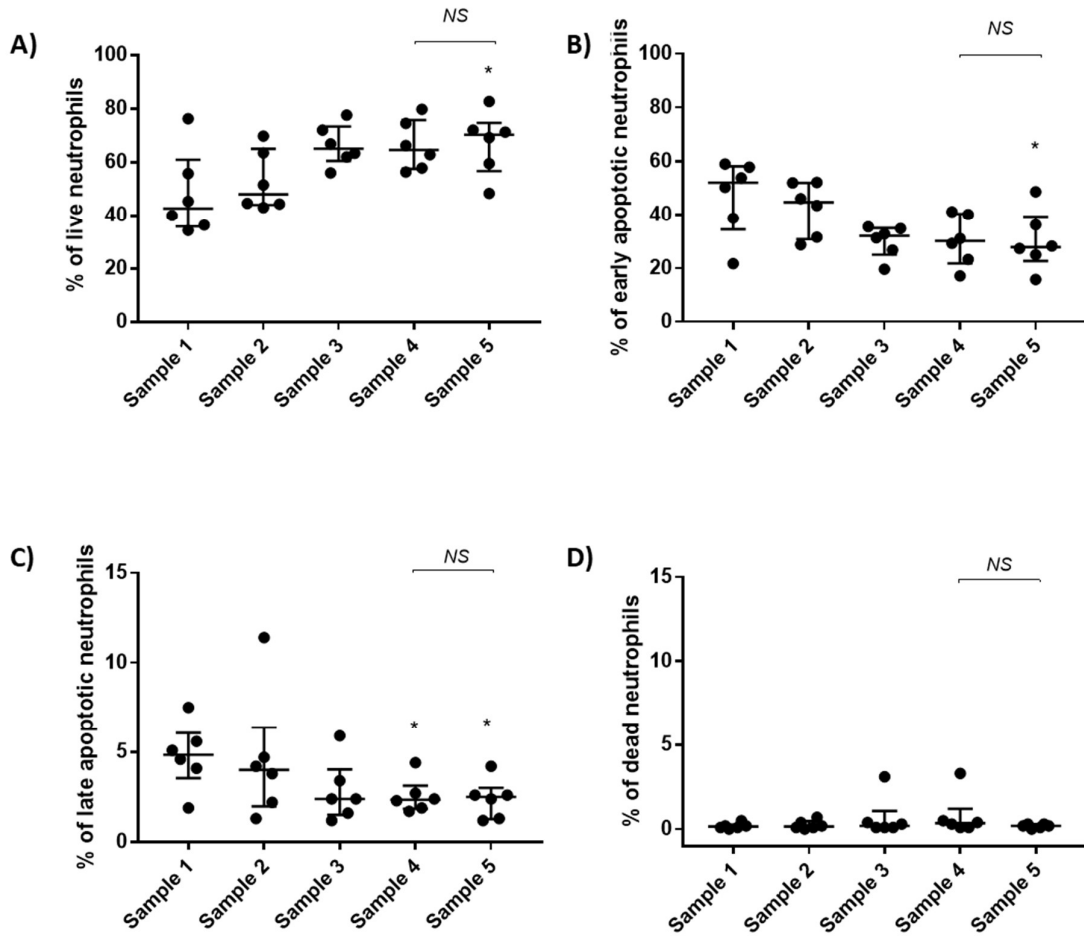


Figure 33: **Neutrophil viability analysis illustrating the percentage of live [A], early apoptotic [B], late apoptotic [C] and dead [D] neutrophils across all five blood samples.** Data are presented on scatter plots with median (middle bar) and interquartile range (whiskers);  $n=6$ ; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5).  $*p<0.05$ , NS=non-significant.



## **5.4 THE EFFECTS OF CPB AND LUNG VENTILATION ON NEUTROPHIL PHAGOCYTOSIS**

Neutrophil phagocytosis was assessed using two independent methods: a light microscopic assay performed on isolated adherent neutrophils and a whole blood flow cytometric assay as previously described (see Chapter 2).

A full data set across all five samples was obtained in six of the nine patients for the light microscopic analysis (for one patient, sample 5 was not obtained due to inability to lift the heart as previously described, for another there was an inadequate yield of neutrophils obtained in sample 5, and for another there was poor adherence of neutrophils to the tissue culture plate).

In contrast to our hypothesis, the capacity of adherent neutrophils to phagocytose opsonised zymosan particles was maintained throughout the CABG procedure with median phagocytic capacity being well within the range observed in healthy volunteers (60-80%) across all five experimental samples (Figure 34). The finding of normal phagocytic capacity in the post-CPB venous blood (sample 3), negated the possibility of assessing the effects of lung ventilation during CPB on restoring impaired neutrophil function. Nevertheless, there was no statistical difference in the phagocytic capacity of neutrophils extracted from the right and left pulmonary veins compared to the pre- and post-CPB central vein samples.

There was more variability in results obtained from the left pulmonary vein (sample 5) with two patients having a phagocytic capacity of less than 30%. It is notable that for these patients, the medium within the tissue culture well for sample 5 became considerably congealed following the incubation steps. It was apparent on microscopy that the zymosan particles had not penetrated through the medium in these samples resulting in the removal of most of the particles during the washing step. It is unclear as to why the medium congealed in these samples, but this may explain these outlying results.

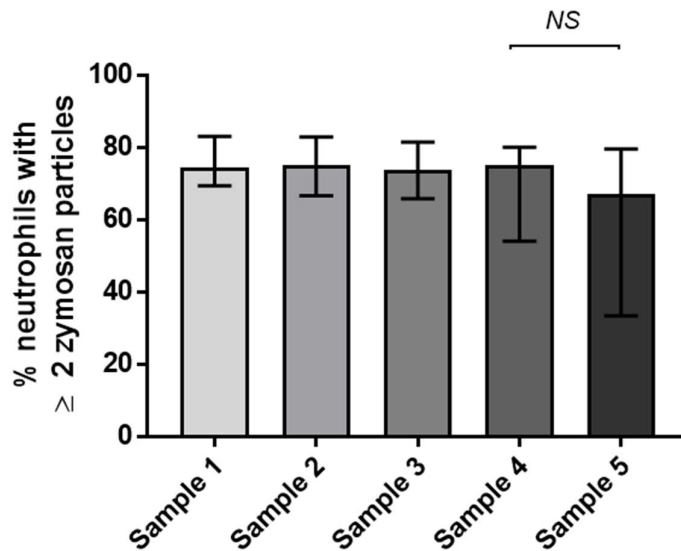


Figure 34: **Light microscopic analysis of neutrophil phagocytic capacity across all five blood samples.** Data are presented as median (columns) and interquartile range (bars); n=6; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5).

All five samples were obtained in eight of the nine patients for the flow cytometric analysis of neutrophil phagocytosis using whole blood. For the phagocytic ability assessment, two of the patients were excluded due to falsely high readings in the 4°C control samples. The analysis of the remaining six patients demonstrated a preserved phagocytic ability across all five blood samples. This was evidenced by the net percentage of neutrophils which were positive for pHrodo *S.aureus* bioparticles between the samples incubated at 4°C (control) and the samples incubated at 37°C (Figure 35, panel A).

The phagocytic capacity analysis included the eight patients for which all five blood samples were obtained. Neutrophil phagocytic capacity was maintained throughout the CABG procedure as evidenced by the median fluorescence intensity (MFI) of pHrodo *S.aureus* bioparticles across all five samples (Figure 35, panel B). There was a small but significant increase in the phagocytic capacity for the post-CPB central venous sample (sample 3) compared to the pre-CPB baseline sample (sample 1). There was no difference in neutrophil phagocytic capacity between the right and left pulmonary vein samples. These results support those obtained in the light microscopic analysis of phagocytosis on isolated adherent neutrophils.

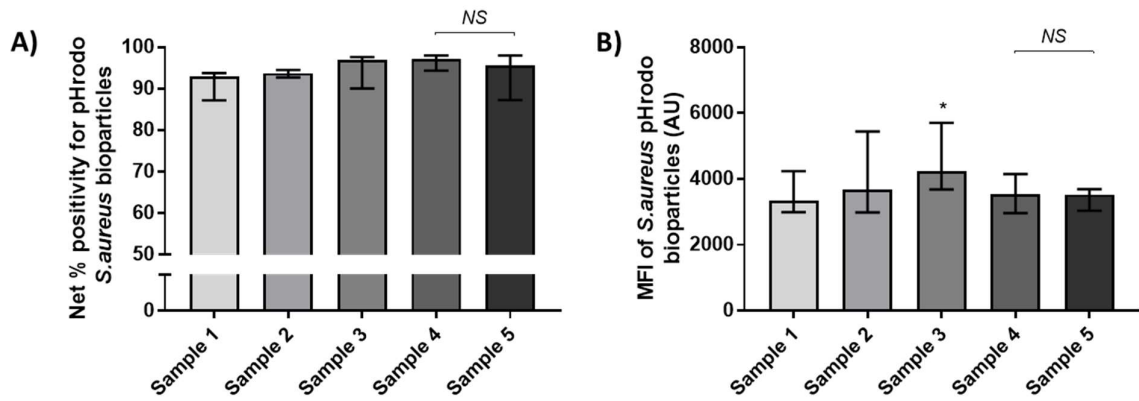


Figure 35: Flow cytometric evaluation of neutrophil phagocytic ability [A] and phagocytic capacity [B] across all five blood samples. Data are presented as median (columns) and interquartile range (bars);  $n=6$  [A] and  $n=8$  [B]. Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5). \* $p<0.05$ , NS=non-significant.

To ascertain whether patient neutrophil phagocytic capacity at baseline reflected the levels observed in healthy individuals, flow cytometric evaluation of phagocytic capacity was performed in a separate group of six healthy volunteers (median age 37.5 years, IQR 32.3-44.3) using the methods previously described. Notably, there was no significant difference in the phagocytic capacity of neutrophils in the pre-CPB baseline sample when compared to this group of healthy volunteers, suggesting that phagocytic capacity was normal in the pre-CPB samples and was well maintained throughout the CABG operation.

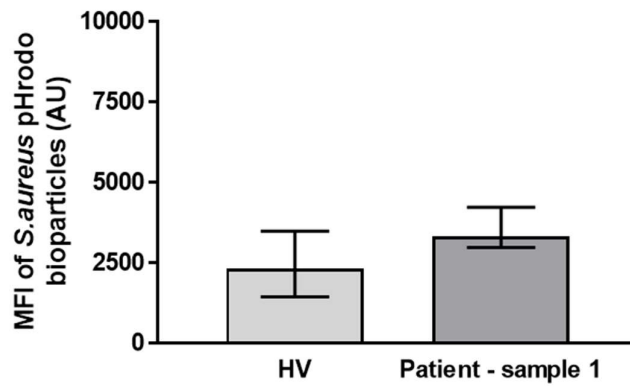


Figure 36: Comparison of neutrophil phagocytic capacity between healthy volunteers (HV) and the pre-bypass (baseline) blood sample for the first experimental cohort patients. Data are represented as median (columns) and interquartile range (bars);  $n=6$  (healthy volunteers) and  $n=8$  (patients). Statistical analysis was by the Mann-Whitney U test;  $p=0.18$ .

## 5.5 THE EFFECTS OF CPB AND LUNG VENTILATION ON NEUTROPHIL PRIMING STATUS

Neutrophil priming status was assessed using the intensity of expression of CD11b and CD62L cell surface adhesion markers. A full data set for all five blood samples was obtained in eight of the nine patients.

The expression of CD11b did not significantly change throughout the duration of the CABG operation, with the intensity of expression remaining consistent across all five experimental blood samples. Moreover, there was no significant difference in the expression of CD11b in neutrophils returning in the pulmonary venous blood from the right (ventilated) lung following CPB and those returning in the pulmonary venous blood from the left (deflated) lung following CPB (Figure 37, panel A).

As shown in Figure 37, panel B, the expression of CD62L was significantly higher in samples 3 and 4 compared to sample 1, suggesting that post-CPB neutrophils were less primed than the pre-CPB neutrophils. There was no significant difference in CD62L expression between the right (ventilated) and left (deflated) lungs following CPB.

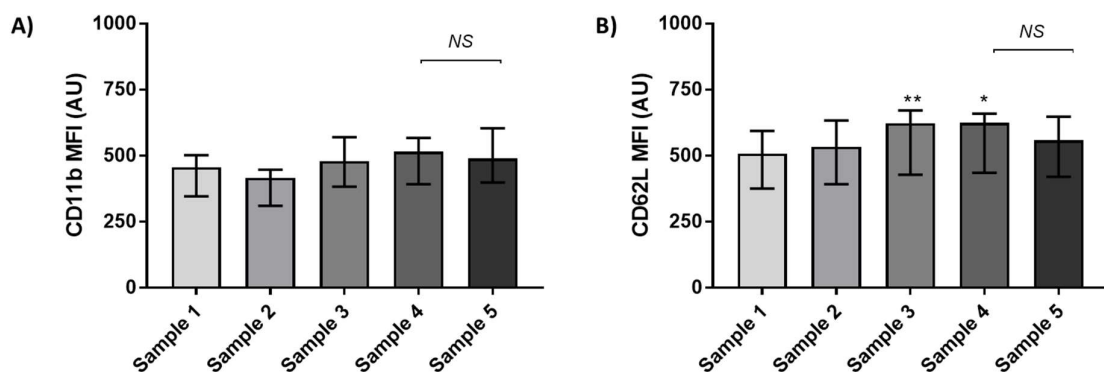


Figure 37: Flow cytometric evaluation of neutrophil priming status by expression of CD11b [A] and CD62L [B] cell surface markers across all five blood samples. Data are represented as median (columns) and interquartile range (bars);  $n = 8$ ; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5). \* $p < 0.05$ , \*\* $p < 0.01$ , NS = non-significant.

In summary, there was no evidence of an increase in the priming status of neutrophils between the pre-CPB and post-CPB central venous blood samples. Notably, the intervention of lung ventilation during CPB (as per the settings stipulated in the first experimental cohort protocol) had no discernible effect on neutrophil priming status as measured by the relative expression of CD11b and CD62L cell surface adhesion markers.

To ascertain whether patient neutrophil priming status at baseline reflected the levels observed in healthy individuals, analysis of CD11b and CD62L cell surface expression was performed in a separate group of six healthy volunteers (median age 37.5 years, IQR 32.3-44.3) using the same methods. As illustrated in Figure 38, there was no significant difference in neutrophil CD11b or CD62L expression profile between the pre-CPB baseline patient samples and the healthy volunteer samples.

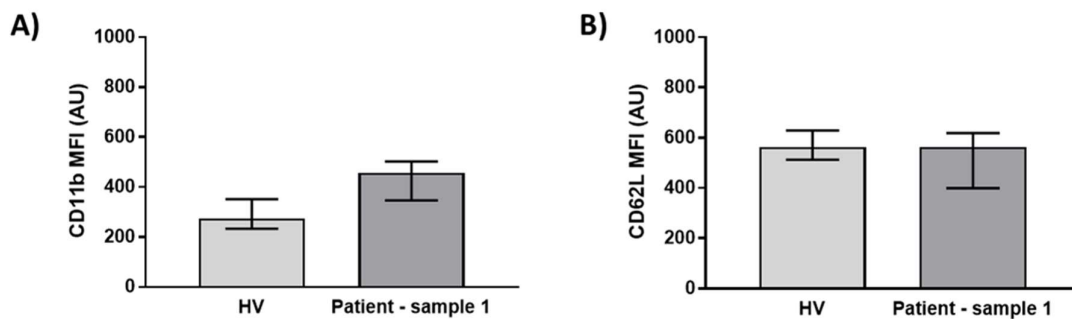


Figure 38: Comparison of CD11b [A] and CD62L [B] cell surface expression between healthy volunteers (HV) and the pre-bypass (baseline) blood sample for the first experimental cohort patients. Data are represented as median (columns) and interquartile range (bars);  $n=6$  (healthy volunteers) and  $n=8$  (patients). Statistical analysis was by the Mann-Whitney U test;  $p=0.11$  [A] and  $p=0.67$  [B].

## 5.6 THE EFFECTS OF CPB AND LUNG VENTILATION ON PLASMA CYTOKINE CONCENTRATIONS

The concentration of pro-inflammatory and anti-inflammatory cytokines in plasma prepared from each of the five experimental blood samples was assessed. All five plasma samples were obtained for eight of the nine patients within this cohort (one patient was excluded from the analysis as only four samples were obtained due to the inability to retrieve the left pulmonary vein sample as previously described).

There were significantly higher plasma concentrations of IL8, IL6 and IL10 in the post-CPB blood samples (samples 3, 4, and 5) compared to the pre-CPB baseline blood sample (sample 1). However, there were no significant differences in the concentration of these cytokines between blood exiting the right lung, which was ventilated during CPB (sample 4) and blood exiting the left lung, which remained deflated during CPB (sample 5) (Figure 39, panels A-C).

There was a small but significant reduction in the plasma concentration of IFN $\gamma$  in the post-CPB blood samples compared to the pre-CPB baseline blood sample. However, there was no significant difference in the plasma concentration of IFN $\gamma$  between the ventilated (sample 4) and deflated (sample 5) lung (Figure 39, Panel D).

There were no significant differences in the plasma concentrations of TNF $\alpha$ , IL1 $\beta$ , IL12p70, IL2, IL4 and IL13 compared to the respective pre-CPB baseline sample, nor was there any significant difference in the concentration of these cytokines between the ventilated and deflated lung (Figure 40, panels A-F). All five values for patients with at least one undetectable cytokine level were excluded from the final analysis for that specific cytokine.

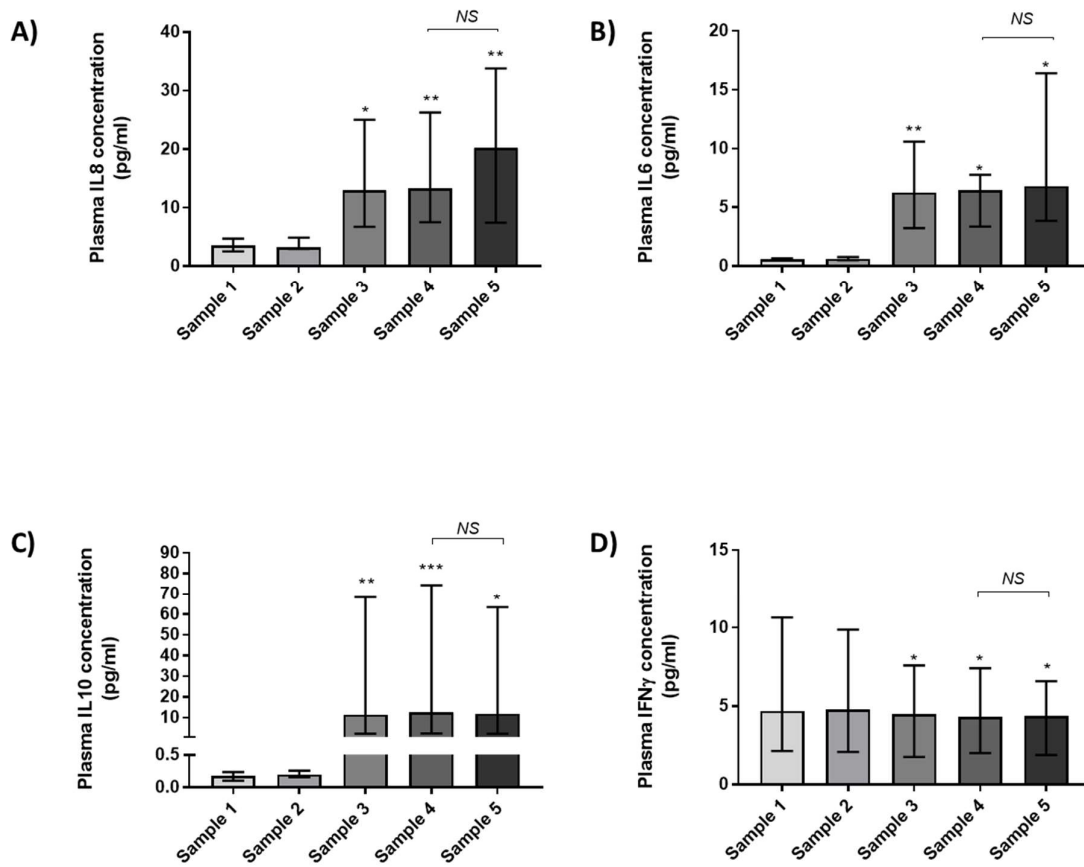


Figure 39: **Plasma concentrations of IL8 [A], IL6 [B], IL10 [C] and IFN $\gamma$  [D] across all five blood samples.** Data are presented as median (columns) and interquartile range (bars); n=8; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5). \*p<0.05, \*\*p<0.005 and \*\*\*p<0.001.



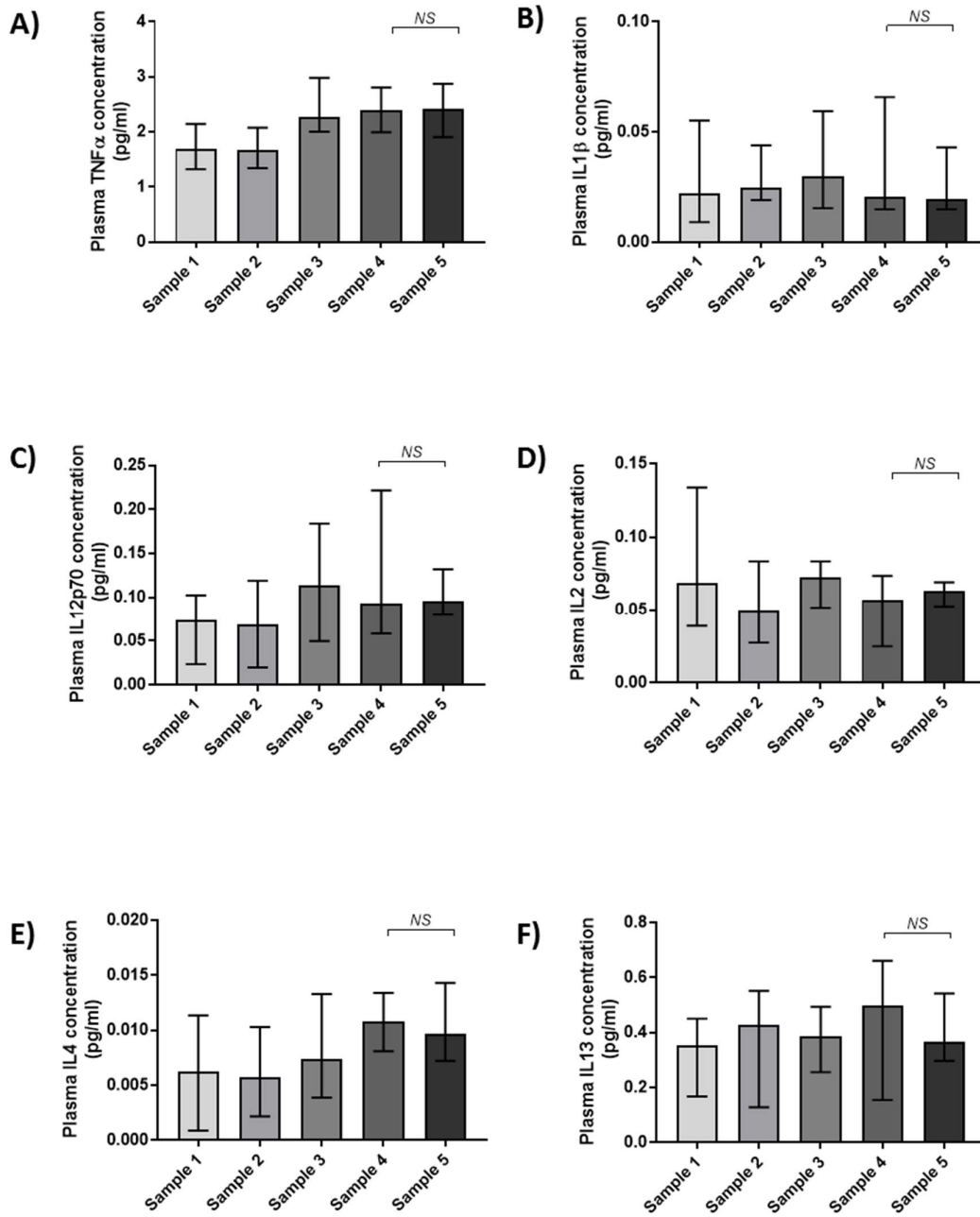


Figure 40: Plasma concentrations of TNF $\alpha$  [A], IL1 $\beta$  [B], IL12p70 [C], IL2 [D], IL4 [E] and IL13 [F] across all five blood samples. Data are presented as median (columns) and interquartile range (bars); n=8 (TNF $\alpha$ ), n=7 (IL1 $\beta$ , IL12 and IL2), n=6 (IL4) and n=4 (IL13); Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5).

## **5.7 THE EFFECTS OF INTRAOPERATIVE LUNG VENTILATION ON PULMONARY VEIN BLOOD GAS ANALYSIS**

Blood gas analysis was performed immediately after obtaining the left and right pulmonary vein blood samples. The objective of this analysis was to assess whether there was a measurable difference in post-CPB alveolar gas exchange between the left lung, which remained deflated during CPB, and the right lung, which was ventilated during CPB.

Paired left and right pulmonary vein samples for blood gas analysis were obtained in eight of the nine patients. There were no significant differences in pulmonary vein blood pH, pO<sub>2</sub>, lactate, or HCO<sub>3</sub> between the left and right lungs (Table 10). It is notable that the pO<sub>2</sub> in both the left and right pulmonary vein blood was above normal limits, perhaps reflecting the high levels of FiO<sub>2</sub> administered in the immediate post-CPB period (median 0.55, IQR 0.50-0.64).

A statistically significant increase in pCO<sub>2</sub> was observed in blood from the right pulmonary vein compared to the left (median 5.2 (IQR 4.9-5.3) versus 4.6 (IQR 3.5-5.1) (p=0.008) (Table 10). Nonetheless, the pCO<sub>2</sub> values for both lungs remained within normal limits and therefore this finding is of questionable clinical significance. One possible explanation for this finding is that, despite ultra-low tidal volume ventilation, there may have been a degree of air-trapping within the right lung, resulting in a small increase in pulmonary vein blood pCO<sub>2</sub> following resumption of pulmonary blood flow after CPB.

Table 10: Comparison of blood gas parameters for the left (deflated) and right (ventilated) lung following resumption of pulmonary blood flow after CPB

<b>Parameter</b>	<b>Left pulmonary vein</b>	<b>Right pulmonary vein</b>	<b>p-value</b>
FiO <sub>2</sub>	0.55 (0.50-0.64)	0.55 (0.50-0.64)	-
pH	7.4 (7.4-7.4)	7.4 (7.4-7.5)	0.25
pO <sub>2</sub> (kPa)	30.2 (26.7-36.5)	31.5 (19.35-35.9)	0.84
pCO <sub>2</sub> (kPa)	4.6 (3.5-5.1)	5.2 (4.9-5.3)	0.008
Lactate (mmol/L)	1.6 (1.4-2.0)	1.3 (1.2-1.8)	0.16
HCO <sub>3</sub> (mmol/L)	23.0 (21.6-24.4)	23 (21.7-24.4)	0.77

*Data are presented as median (interquartile range); n=8.  
Statistical analysis was by the Wilcoxon matched-pairs signed rank test.*

## **5.8 IMPACT OF ONE-LUNG VENTILATION ON CLINICAL OUTCOME MEASURES**

To evaluate the safety and clinical impact of our study interventions, operative and perioperative clinical outcome data were compared to a control cohort. This control cohort consisted of patients who underwent first-time CABG surgery within the period of the study but who had not been recruited to the study. These data were sourced from the Trust's Cardiothoracic Data Registry by a data manager who was independent of the study. The data manager was provided with a list of names of those patients recruited to the study to avoid their inclusion when forming the comparative control cohort.

Eight patients formed the control cohort. The control cohort and first experimental cohort were comparable with regards to age, height, logistic EUROscore and number of grafts performed as detailed in Table 11. However, the body weight of the control cohort was significantly higher than the experimental cohort (92.5kg, (IQR 83-104.7) vs 77kg (IQR 70.5-89.8)).

Table 11: Comparison of the baseline clinical characteristics for the first experimental cohort and control cohort

Baseline characteristic	First experimental cohort	Control cohort	p-value
Gender – no. of pts (%)			
Male	7 (77.8)	7 (87.5)	-
Female	2 (22.2)	1 (12.5)	
Age (years)	62.0 (57.5-78.0)	63.5 (60.8-80.3)	0.56
Height (cm)	162.0 (161-176)	165.8 (160-174)	0.56
Weight (kg)	77.0 (70.5-89.8)	92.5 (83.0-104.7)	0.03
Smoking status – no. of pts (%)			
Never smoker	5 (55.6)	2 (25.0)	
Ex-smoker	4 (44.4)	5 (62.5)	-
Smoker	0	1 (12.5)	
Logistic EUROscore	1.23 (1.03-4.31)	2.24 (1.76-4.20)	0.50
Number of grafts	3.0 (2.0-3.5)	3.0 (2.3-3.8)	0.68
<i>Data are presented as median (interquartile range) unless otherwise documented in the table, n=9 (patient cohort) and n=8 (comparative cohort). Statistical analysis was by the Mann-Whitney U-test.</i>			

There were no statistically significant differences in the operative outcome measures of total cardiopulmonary bypass time and total aortic cross-clamp time between the two cohorts as shown in Figure 41, panels A and B. In keeping with these findings, the subjective opinion from operating surgeons contributing to the first experimental cohort was that the right lung ventilation protocol did not adversely impact on the progress of the operation.

Total blood loss from the mediastinal and left pleural drain was similar in both groups and served as a surrogate for assessing the safety of extracting blood from the pulmonary veins following CPB (Figure 41, panel C). There were no statistically significant differences in the time to extubation (the time from the last recorded observations in theatre to the recorded extubation time) between the control and experimental cohort (Figure 41, panel D).

The duration of ITU stay was comparable in both groups with most patients spending one night on ITU before discharge back to the ward the following day (Figure 41, panel E). One patient in the experimental cohort was maintained on ITU for a total of 4 nights because of poor oxygenation requiring treatment with high flow oxygen, atrial fibrillation necessitating treatment with amiodarone, and fluid overload which required intravenous diuretics. It is notable that this was the same patient who developed a cardiac arrhythmia resulting in hypotension after discontinuation of CPB rendering it unsafe to lift the heart to obtain the left pulmonary vein blood sample. This patient was also the only non-elective case within this cohort, as this patient required semi-urgent surgery following a non-ST elevation myocardial infarction (NSTEMI) 12 days prior to the operation. The length of stay for this patient seems likely to be because of their poor clinical condition rather than because of the study interventions.

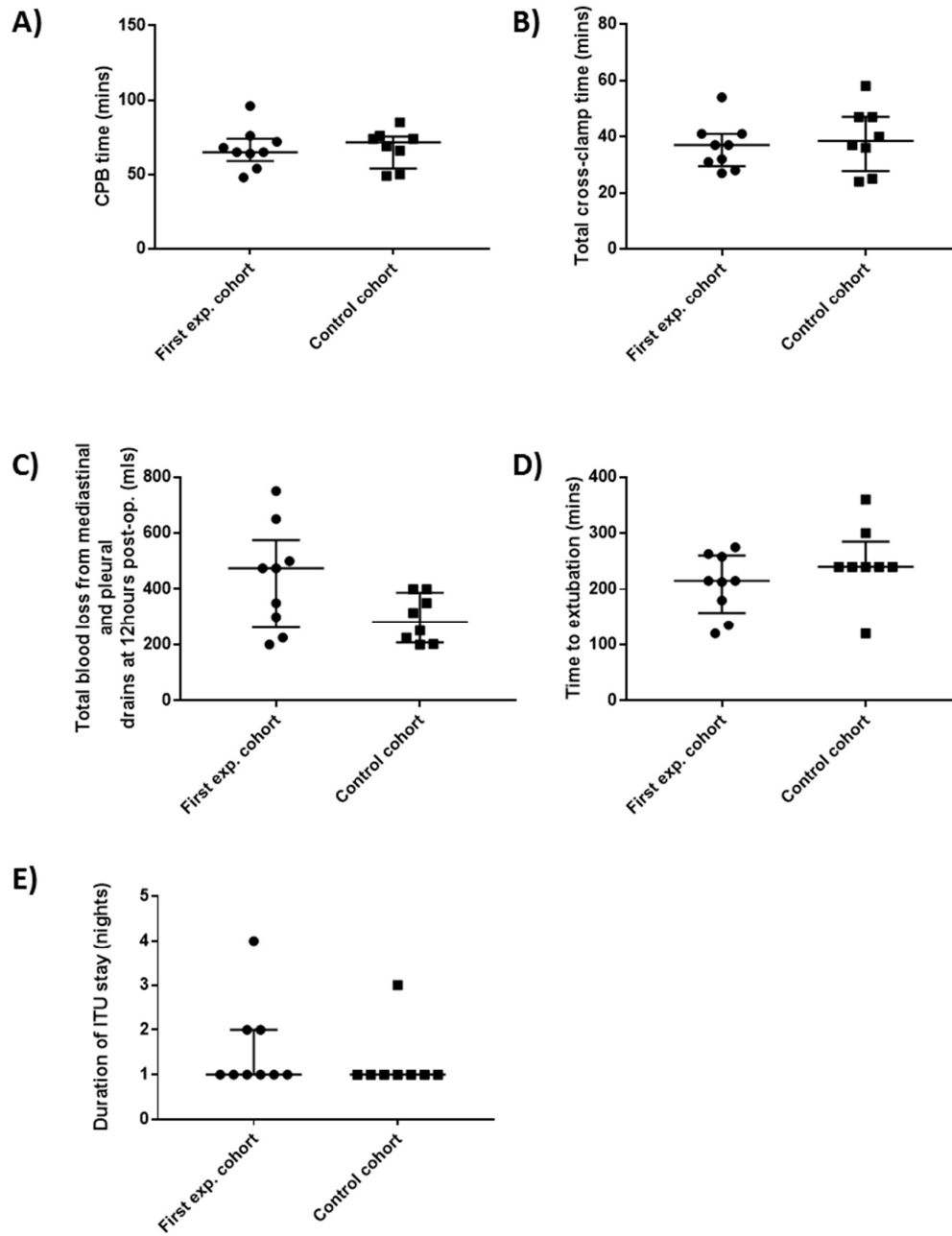


Figure 41: Comparison of clinical outcome measures between the first experimental cohort and the control cohort. Data are presented as scatter plots with median (middle bar) and interquartile range (whiskers); n=9 (first experimental cohort) and n=8 (control cohort). Statistical analysis was by the Mann-Whitney U test. [A]  $p=0.55$ , [B]  $p=0.73$ , [C]  $p=0.12$ , [D]  $p=0.24$ , [E]  $p=0.51$ .

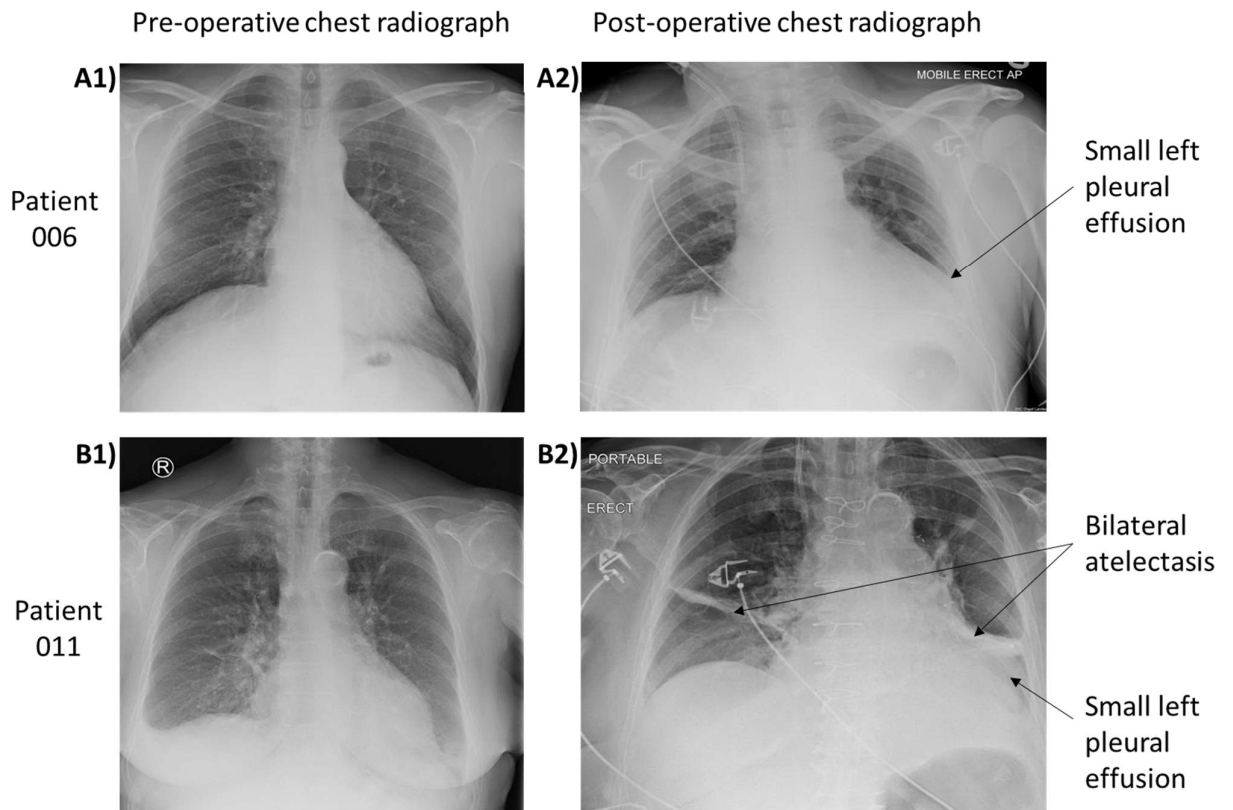
Radiology reports of the first post-operative mobile chest radiograph were reviewed to evaluate the impact of right lung ventilation during CPB on perioperative lung parenchymal and pleural changes. Most patients within the patient and control cohorts had at least one reported pleural or parenchymal change in their post-operative chest radiograph compared to their pre-operative screening chest radiograph. Only one patient in each group had no reported post-operative radiological abnormality (Table 12).

The most common postoperative radiology findings were atelectasis, small to moderate pleural effusions or a combination of both (Figure 42). The pattern of post-operative radiology findings was similar in both the patient and control cohort however a larger sample size is required to consolidate these findings. Notably, there were no unexpected chest radiograph findings within the patient cohort.

*Table 12: Classification of post-operative mobile CXR report for the patient and comparison cohort*

<b>Postoperative mobile CXR report</b>	<b>Patient cohort (N=9)</b>	<b>Control cohort (N=8)</b>
No parenchymal or pleural abnormality	1	1
Left atelectasis alone	2	2
Right atelectasis alone	0	0
Bilateral atelectasis alone	0	0
Left pleural effusion alone	1	1
Left atelectasis and left effusion	1	0
Left atelectasis and bilateral pleural effusion	1	1
Bilateral atelectasis and left effusion	1	0
Bilateral atelectasis and bilateral effusion	2	0
Bilateral effusions alone	0	1
Pulmonary oedema	0	1
Pulmonary oedema, left atelectasis and bilateral effusions	0	1





**Figure 42: Comparison of pre-operative and post-operative chest radiographs for patient 006 [A1, A2] and patient 011 [B1, B2].** [A1] Pre-operative PA chest radiograph for study patient 006 demonstrating clear lung fields. [A2] Post-operative AP mobile chest radiograph for study patient 006 demonstrating the development of a small post-operative left pleural effusion. [B1] Pre-operative PA chest radiograph for study patient 011 demonstrating a small right pleural effusion and borderline cardiomegaly. [B2] Post-operative AP mobile chest radiograph for study patient 011 demonstrating the development of post-operative bilateral midzone atelectasis and a small left pleural effusion. The heart size appears larger on the post-operative films and is most likely due to these films being mobile AP films compared to the pre-operative departmental PA films. Abbreviations: AP (Antero-posterior), PA (Postero-anterior).

## **5.9 SUMMARY OF THE FIRST EXPERIMENTAL COHORT**

Results from the first experimental cohort suggest that neutrophil phagocytic function is well preserved during on-pump CABG procedures. The maintenance of neutrophil phagocytic capacity in the post-CPB central vein sample precluded the ability to assess the potential restorative effect of lung ventilation during CPB on neutrophils returning in pulmonary vein blood. Nevertheless, our results suggest that irrespective of whether a lung remains deflated or is ventilated during CPB, neutrophil phagocytic capacity is not adversely affected.

In contrast to our hypothesis, neutrophil priming status did not appear to increase during on-pump CABG procedures as evidenced by expression of CD11b and CD62L cell surface adhesion markers nor did lung ventilation impact upon pulmonary vein blood neutrophil priming status.

We observed a significant increase in pro-inflammatory and anti-inflammatory cytokines in plasma prepared from post-CPB blood samples. However, lung ventilation during CPB had no discernible effect on levels of pro-inflammatory or anti-inflammatory cytokines measured directly from pulmonary vein blood within this cohort.

There were no clinically significant differences in post-CPB pulmonary vein blood gas parameters between a ventilated and deflated lung using our novel one-lung ventilation model and although the number of patients within this cohort was small, clinical safety measures were comparable to a control cohort.

## 5.10 PROTOCOL ADJUSTMENTS

An important consideration which became apparent within this cohort was whether a tidal volume of 2mls/kg IBW was enough to truly test our hypothesis. As previously described in chapter 1, section 1.10.1, lung protective ventilation strategies are now standard practice in aiming to prevent potential complications of mechanical ventilation such as ARDS. The recommended tidal volume for protective two-lung ventilation is 4-8mls/kg IBW. While lung protective strategies for one-lung ventilation are yet to be defined, low tidal volume strategies are recommended, hence our decision on a tidal volume of 2mls/kg IBW for our one-lung ventilation model. Notably, a tidal volume of 2mls/kg IBW was also used in the 2012 preliminary study which formed the basis for this work.

Nevertheless, in this cohort, our ventilatory strategy appeared to only partially inflate the right lung on direct visualisation during the operation, raising the question as to whether an adequate volume of ventilation was reaching the alveoli. Our low tidal volume strategy of 2mls/kg may have invalidated the capacity to test our hypothesis and so we subsequently refined the ventilation strategy further in aiming to achieve better alveolar ventilation.

The refined ventilation protocol incorporated two changes and was adopted for the remaining recruited patients, thus forming the second experimental cohort. Firstly, the tidal volume for the right lung ventilation during CPB was increased to 4mls/kg IBW and secondly, the FiO<sub>2</sub> for the right lung ventilation during CPB was increased from 0.21 to 0.5. Justification for these parameters were based on research by Ng and colleagues who demonstrated a reduction in plasma pro-inflammatory cytokines with continuous bilateral lung ventilation during CPB using a similar ventilatory protocol of 5mls/kg, FiO<sub>2</sub> 0.5 and 5 ventilations per minute.(60)

Finally, for the second experimental cohort, one small addition was made to the laboratory protocol. This involved the analysis of neutrophil apoptosis at 20 hours following the baseline apoptosis assay to analyse the rate of apoptosis. This was to explore whether the higher percentage of viable neutrophils observed in the post-CPB samples compared to the pre-CPB samples in the first cohort was simply related to the timing of the blood samples or whether post-CPB neutrophils display a pro-survival phenotype which may contribute to the post-operative systemic inflammatory response.

The results of the second experimental cohort are now detailed in Chapter 6.

# CHAPTER 6: RESULTS OF SECOND EXPERIMENTAL COHORT

## 6.1 OVERVIEW

This chapter reports the results of the second experimental cohort of the clinical study which aimed to assess the immunological impact of lung ventilation during CPB based on our refined one-lung ventilation protocol. The rationale for the refinements to the ventilation protocol were based on the findings from the first experimental cohort and is detailed in Chapter 5 section 5.10. Throughout this chapter, right lung ventilation during CPB was stipulated as follows: volume-controlled ventilation,  $FiO_2$  0.5 (increased from 0.21 in the first experimental cohort), tidal volume 4mls/kg ideal body weight (increased from 2mls/kg in the first experimental cohort), 7 ventilations per minute and PEEP 5cmH<sub>2</sub>O.

The aims of the second experimental cohort were:

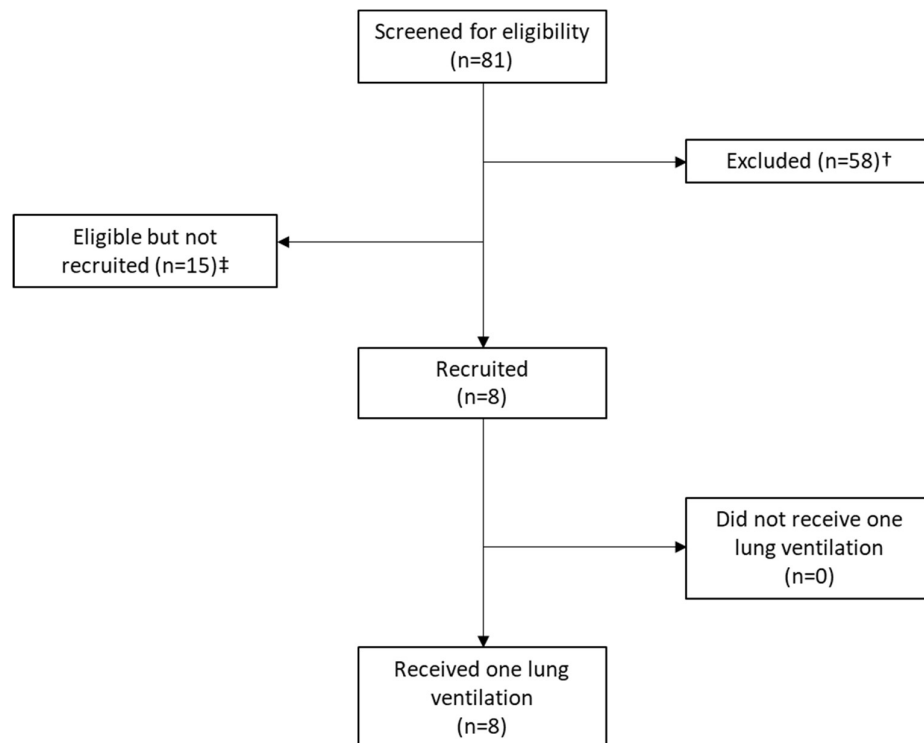
1. To evaluate the impact of lung ventilation during CPB on blood neutrophil phagocytic capacity and priming status using our refined ventilation protocol.
2. To evaluate the impact of lung ventilation during CPB on plasma pro-inflammatory and anti-inflammatory cytokine profile using our refined ventilation protocol.
3. To ensure the safety of the experimental model using our refined ventilation protocol.

The chapter begins with an overview of patient recruitment and baseline clinical and operative characteristics. Data on neutrophil yield, viability, phagocytosis, priming status and plasma cytokine concentrations are then detailed followed by clinical outcome data. The chapter ends with a summary of the conclusions drawn from the second experimental cohort.

## 6.2 SUBJECTS

### 6.2.1 Screening and recruitment

Eight patients were recruited to the second experimental cohort. Screening and recruitment are illustrated in Figure 43. All eight patients received the intervention of one-lung ventilation throughout the period of CPB as per the refined ventilation protocol. All five experimental blood samples were obtained in six of the eight patients (for two of the patients, the left pulmonary vein sample (sample 5) was not obtained).



*Figure 43: Flow diagram demonstrating patient screening and recruitment to the second experimental cohort. †Reasons for exclusion: surgeon not participating in study (n=42), operation on afternoon list (n=11), listed too late for consent (n=5). ‡Reasons eligible patients not recruited: operation cancelled/postponed before written consent (n=5), scheduled at same time as another study patient (n=2), clinical commitments of research fellow (n=8).*

### **6.2.2 Baseline clinical characteristics**

The baseline clinical characteristics of patients in the second experimental cohort are outlined in Table 13. The values obtained in this cohort were comparable to those in the first experimental cohort.

Table 13: Baseline clinical characteristics of patients recruited to the second experimental cohort.

<b>Baseline characteristic</b>	
Gender (number of patients)	
Male	8
Female	0
Age (years)	65.5 (60.3-74.5)
Height (centimetres)	173.0 (166.3-175.8)
Weight (kg)	84.2 (76.5-101.4)
Ideal body weight (kg)†	68.7 (63.7-71.2)
Smoking status (number of patients)	
Current smoker	0
Ex-smoker	5
Never smoker	3
Lung function	
FEV <sub>1</sub> (litres)	2.9 (2.5-3.0)
FVC (litres)	4.1 (3.1-4.3)
FEV <sub>1</sub> predicted (%)	93.5 (80.5-101.5)
FEV <sub>1</sub> /FVC (%)	73.5 (69.0-82.5)
Logistic EUROscore§	2.4 (1.6-4.5)
Urgency of procedure (number of patients)	
Elective	7
Urgent	1
Co-morbidities (number of patients)	
Cardiovascular‡	
Previous MI	5
Previous PCI	3
Hypertension	6
Atrial Fibrillation	1
Respiratory	
Asthma	1
COPD	1
Pleural plaques	1
Other	
Diabetes mellitus (type 2)	3
GORD	1
Osteoarthritis	2
CKD	1
TIA	1
Folliculitis	1

Data are presented as median (interquartile range) unless otherwise documented (n=8)

† Ideal body weight as calculated by the Devine formula (92)

‡ Cardiovascular co-morbidities other than ischaemic heart disease

§ Logistic Euroscore: a formula used to predict the risk of death after a cardiac operation (93)

Abbreviations: MI: myocardial infarction, PCI: percutaneous coronary intervention, COPD: chronic obstructive pulmonary disease, GORD: gastro-oesophageal reflux disease, CKD: chronic kidney disease, TIA: transient ischaemic attack

### **6.2.3 Operative data**

A bronchus-blocking balloon was positioned in the left main bronchus and the left pleura was successfully breached in all eight patients within this cohort. After CPB was initiated, the bronchus-blocking balloon was immediately inflated and right lung ventilation as per the stipulated protocol was commenced. Right lung ventilation continued until weaning of CPB in all patients as per the study protocol.

Within this cohort the left pulmonary vein sample (sample 5) was not obtained for two of the eight patients. One patient had extensive mediastinal and cardiac fat resulting in the operating surgeon being unable to locate the left pulmonary vein. For another, a low blood pressure following the cessation of CPB rendered it unsafe to lift the heart to obtain the left pulmonary vein sample.

The operative details of patients within this cohort are shown in Table 14. The median duration of the intervention of right lung ventilation during CPB was 56.5 minutes (IQR 45.3-74.5) which was comparable to the duration of intervention for the first experimental cohort. As previously described in Chapter 5, the duration between bilateral ventilation recommencing after CPB and obtaining the post-operative blood samples was dictated by the stability of the patient and the timing of administration of intravenous protamine.



Table 14: Operative data from patients in the second experimental cohort

<b>Operative variable</b>	<b>Second experimental cohort</b>
Duration of bilateral lung ventilation before CPB (mins)	116.5 (78.8-131.3)
CPB time (mins)	61.0 (52.0-81.3)
Aortic cross-clamp time (mins)	36.0 (32.3-41.8)
Number of grafts	3.0 (2.0-3.0)
Duration of right lung ventilation during CPB (mins)	56.5 (45.3-74.5)
Duration between bilateral ventilation restarting and obtaining postoperative blood samples (mins) †	12.5 (9.3-16.3)
Duration between end of CPB and obtaining postoperative blood samples (mins) †	9.0 (5.0-12.8)

*Data are presented as median (interquartile range), n=8*  
† Postoperative samples refer to samples 3, 4 and 5 as previously described

### 6.3 NEUTROPHIL YIELD AND VIABILITY

For all but one blood sample taken within this cohort the desired volume of blood was obtained (25mls). For one patient, only 10mls of blood was obtained from the left pulmonary vein despite a substantial attempt by the surgeon to extract blood from this vessel. Despite the relatively low volume of blood obtained for this one sample, there was a sufficient volume of blood and adequate number of neutrophils extracted to undertake all planned experiments.

There was a statistically significant greater median number of neutrophils isolated from the post-CPB samples (samples 3-5) compared to the first pre-CPB sample (sample 1) as illustrated in Figure 44, panel A. This trend was also observed in the first experimental cohort but did not reach statistical significance (see Figure 32, panel A). Median neutrophil purity was above 95% across all five experimental blood samples however it is notable that the purity for several individual samples within this cohort was between 90% to 95% (Figure 44, panel B). A

pragmatic decision to include these samples was made so as not to reduce the small sample size even further.

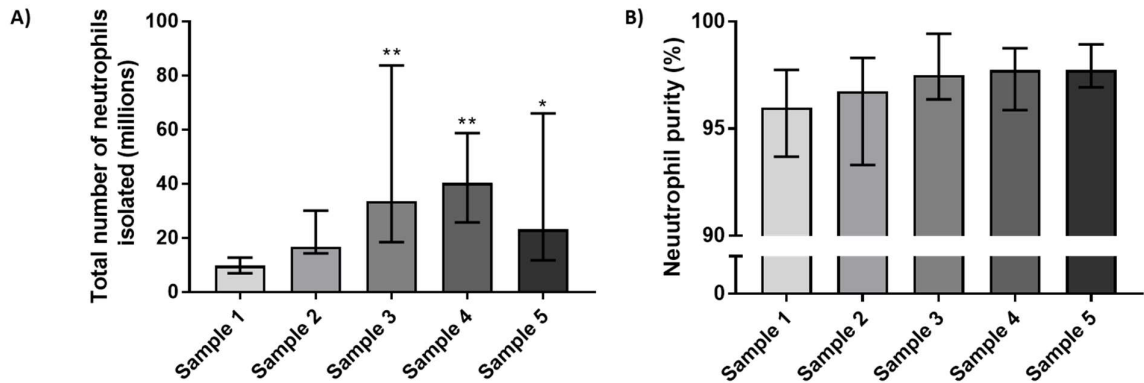


Figure 44: **Neutrophil yield [A] and purity [B] across all five blood samples.** Data are presented as median (columns) and interquartile range (bars); n=6 [A] and n=6 [B]; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). \*p<0.05, \*\*p<0.005.

Neutrophil viability analysis for the percentage of live, early apoptotic, late apoptotic and dead neutrophils for patients in this cohort was performed at two separate time points. The baseline apoptosis experiment was at 0 hours (ie the time at which the first apoptosis experiment was performed). A further apoptosis assay was performed at 20 hours following the initial apoptosis experiment to investigate whether the higher percentage of viable neutrophils observed in the post-CPB samples compared to the pre-CPB samples in the first cohort was related to the timing of the blood samples or whether post-CPB neutrophils display a pro-survival phenotype

For the apoptosis assay performed at 0 hours, there was a trend towards a greater percentage of live neutrophils in the post-CPB samples reaching statistical significance in sample 4 (Figure 45, panel A1). Moreover, there were fewer early and late apoptotic cells in the post-CPB samples reaching significance in sample 4 and samples 3 and 4 respectively (Figure 45, panels B1 and C1). The percentage of dead neutrophils was relatively small across all five blood samples (Figure 45, panel D1). There was no statistically significant difference in the viability

of neutrophils extracted from the left and right pulmonary vein blood samples. A complete data set for neutrophil viability analysis at 0 hours was available for six of the eight recruited patients within this cohort. These results followed a similar trend to the results obtained for the first experimental cohort.

At 20 hours, the number of live neutrophils had reduced across all five samples with the majority having moved to the early apoptotic stage. The trend of more live cells and fewer early and late apoptotic cells in the in the post-CPB samples compared to the pre-CPB samples persisted at 20 hours (Figure 45, panels A2, B2 and C2). The percentage of dead neutrophils remained essentially unchanged between the 0-hour and 20-hour apoptosis analysis (Figure 45, panels D1 and D2). A complete data set for neutrophil viability analysis at 20 hours was available for five of the eight recruited patients within this cohort. As demonstrated in Figure 46, the shift in neutrophil viability between 0-hours and 20-hours was also evident on light microscopic analysis.

While there was a trend toward the post-CPB samples having more live neutrophils and fewer early and late apoptotic neutrophils, the difference in median percentage of neutrophils at the 0-hour and 20-hour apoptosis analysis for each of the five samples remained constant (Figure 47). These results suggest that the apoptotic rate of neutrophils was the same across all five samples and that the difference in percentage of live, early and late apoptotic neutrophils observed between the pre- and post-CPB samples was most likely related to the timing of the samples rather than the post-CPB neutrophils displaying a pro-survival phenotype.

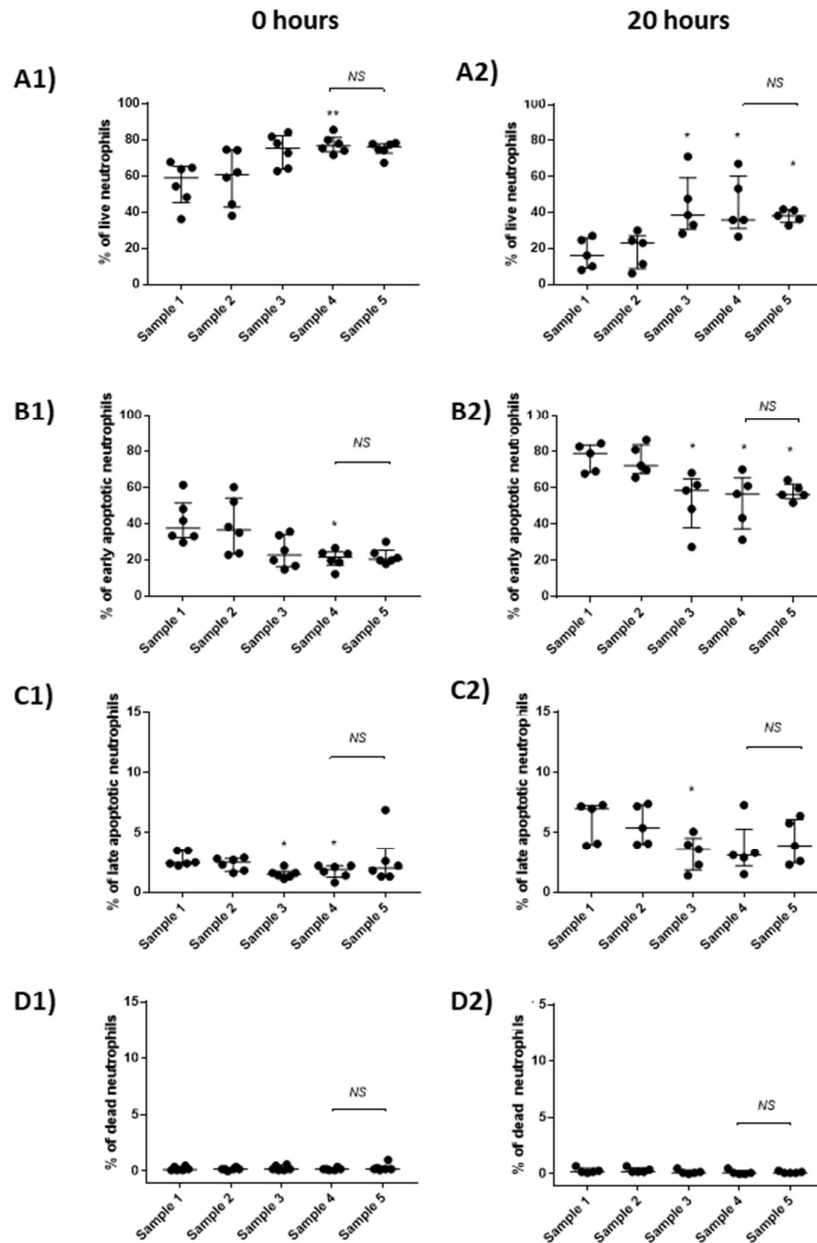


Figure 45: **Neutrophil viability analysis illustrating the percentage of live [A], early apoptotic [B], late apoptotic [C] and dead [D] neutrophils across all five blood samples at 0 hours and 20 hours.** Data are presented on scatter plots with median (middle bar) and interquartile range (whiskers); n=6 (0 hrs) and n=5 (20hrs); Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of sample 2-5 to sample 1 (baseline sample) The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5). \*p<0.05, \*\*p<0.01, NS=non-significant.

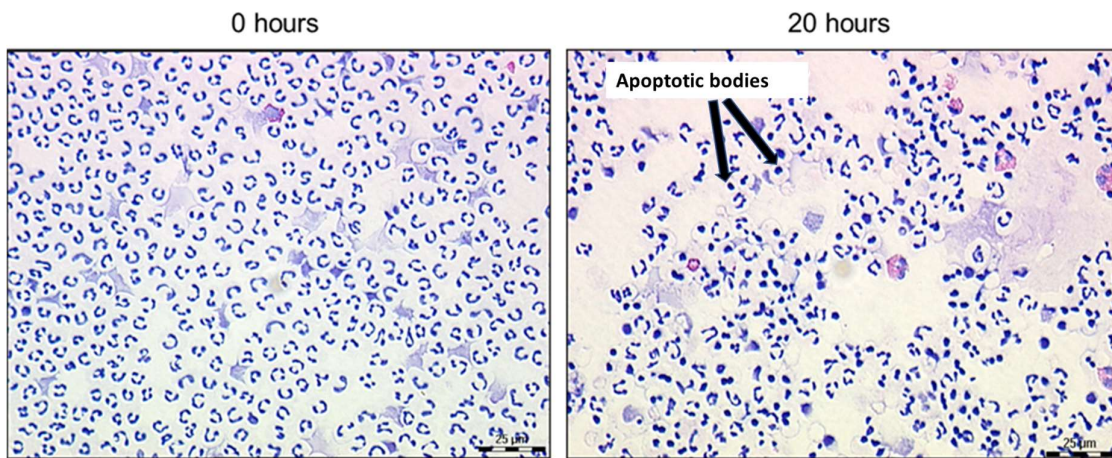


Figure 46: **Representative photomicrographs of neutrophil cytopsins at 0 hours and 20 hours following isolation from whole blood.** At 0 hours the multilobulated nuclei of isolated neutrophils interlinked by thin chromatin bridges are clearly evident. At 20 hours, a substantial proportion of neutrophils have become apoptotic as evidenced by condensation of nuclear chromatin resulting in fragmentation of the nucleus and the formation of apoptotic bodies (black arrows).

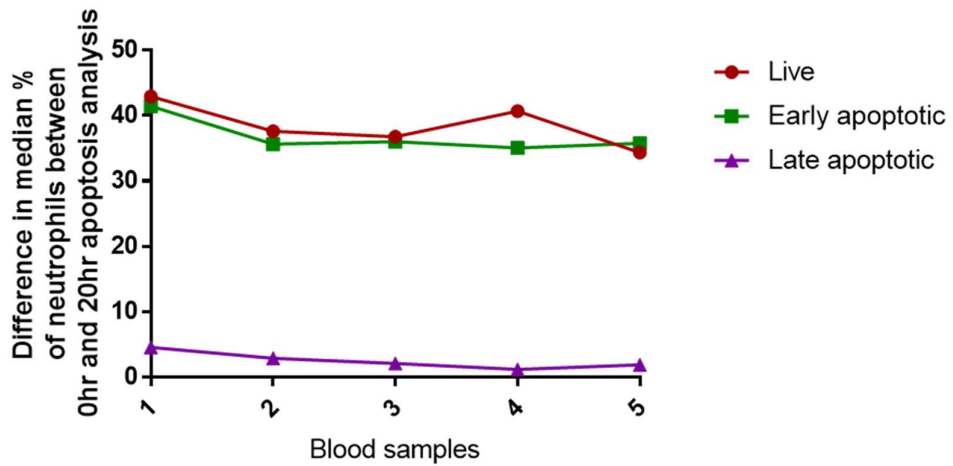


Figure 47: **Difference in median percentage of live, early and late apoptotic neutrophils between 0-hour and 20-hour apoptosis analysis for each of the five blood samples.**

## **6.4 THE EFFECTS OF CPB AND LUNG VENTILATION ON NEUTROPHIL PHAGOCYTOSIS**

As previously described, neutrophil phagocytosis was assessed using both a light microscopic assay performed on isolated adherent neutrophils and a whole blood flow cytometric assay.

A full data set for all five experimental blood samples was obtained for six of the eight recruited patients in this cohort (for one patient, the left pulmonary vein sample was not obtained, for another, the left pulmonary vein sample was not obtained, and isolated neutrophils failed to adhere to the tissue culture wells for samples 1 and 2).

In keeping with the findings of the first experimental cohort, and in contrast to our hypothesis, the capacity of isolated adherent neutrophils to phagocytose zymosan particles was well preserved across all five experimental blood samples (Figure 48). All samples had a median phagocytic capacity comparable to that of isolated neutrophils from healthy volunteers. Moreover, there was no statistically significant difference in the phagocytic capacity of isolated neutrophils extracted from the left and right pulmonary venous blood.

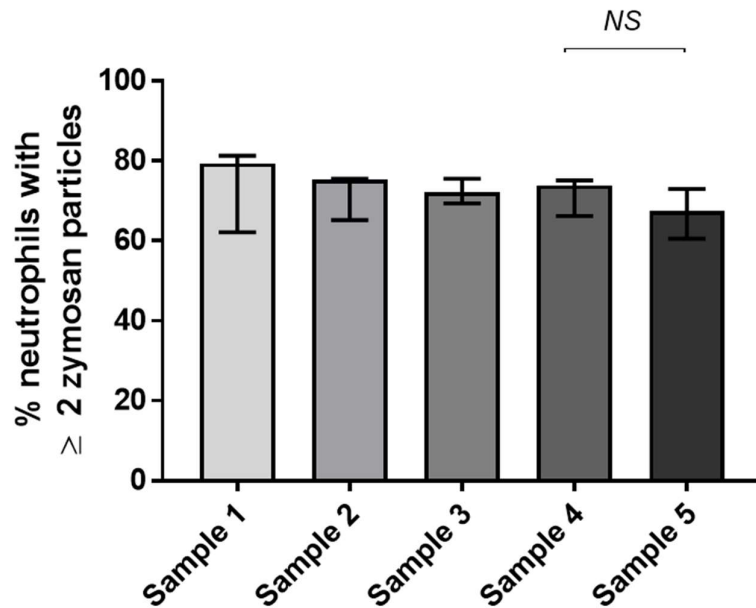


Figure 48: **Light microscopic analysis of neutrophil phagocytic capacity across all five blood samples.** Data are presented as median (columns) and interquartile range (bars); n=6; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5).

For the flow cytometric analysis of neutrophil phagocytosis using whole blood, a full data set for all five experimental blood samples was obtained in six of the eight patients due to the inability to obtain the left pulmonary vein blood samples for two patients as previously described.

There was a preserved phagocytic ability across all five experimental blood samples as evidenced by the net percentage of neutrophils which were positive for pHrodo *S.aureus* bioparticles between the 4°C (control) and the samples incubated at 37°C. A small but significant increase in phagocytic ability was obtained between blood samples 1 and 5 (Figure 49, panel A). Neutrophil phagocytic capacity was also well maintained throughout the CABG procedure as demonstrated by comparable median fluorescence intensity (MFI) of pHrodo *S.aureus* bioparticles across all five experimental blood samples (Figure 49, panel B).

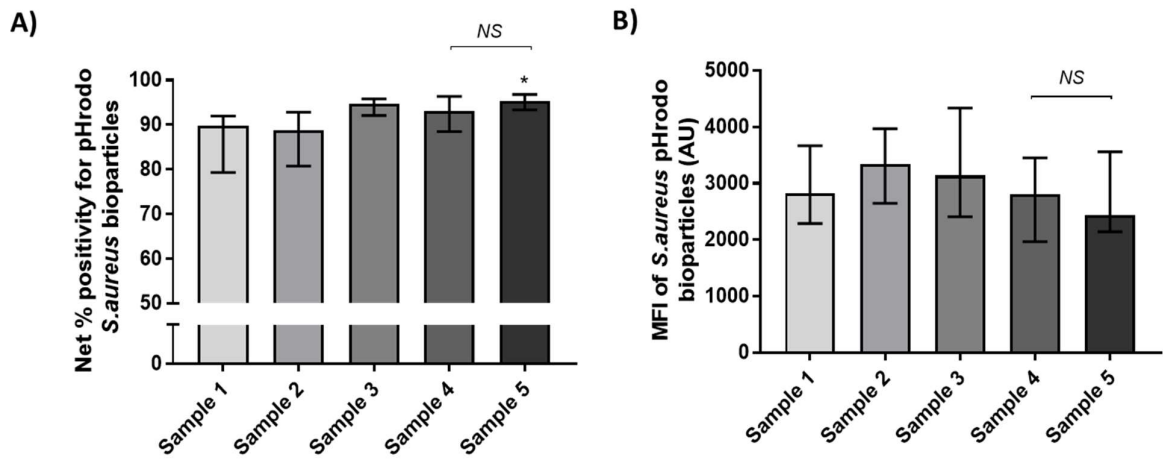


Figure 49: Flow cytometric evaluation of neutrophil phagocytic ability [A] and phagocytic capacity [B] across all five blood samples. Data are presented as median (columns) and interquartile range (bars);  $n=6$  [A] and  $n=6$  [B]. Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5).  $*p<0.05$ , NS=non-significant.



## 6.5 THE EFFECTS OF CPB AND LUNG VENTILATION ON NEUTROPHIL PRIMING STATUS

A full data set of all five blood samples was obtained in six of the eight patients within this cohort for evaluation of neutrophil priming status. The relative intensity of expression of CD11b cell surface adhesion marker did not significantly change across all five experimental blood samples nor was there any difference in expression of CD11b between the right (ventilated) and left (deflated) lung following CPB (Figure 50, panel A). These results are counter to our hypothesis yet mirror the findings obtained in the first experimental cohort. As shown in Figure 50, panel B, the expression of CD62L was significantly higher in the post-bypass samples compared to the pre-bypass samples suggesting that post-bypass neutrophils were less primed than pre-bypass neutrophils. Again, this result contrasts with our hypothesis that post-bypass neutrophils would be more primed and have relatively lower expression of CD62L cell surface adhesion marker expression. Notably, our intervention of lung ventilation had no impact on neutrophil cell surface expression of CD62L. A similar pattern of results for CD62L expression was also obtained in the first experimental cohort.

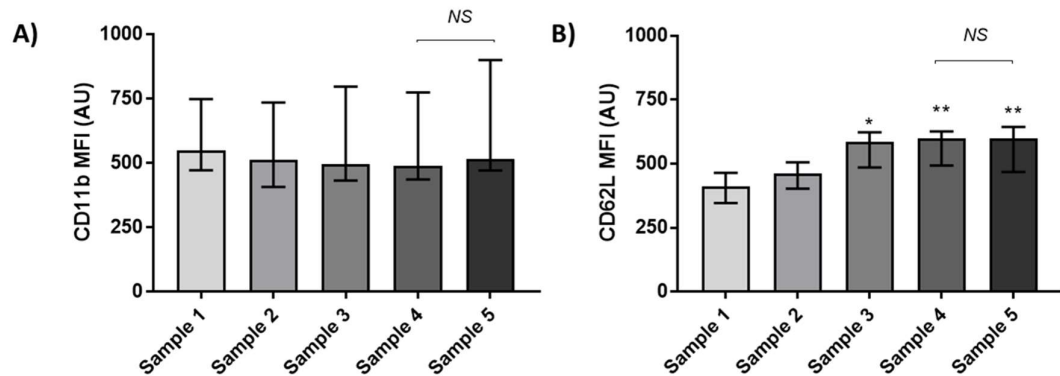


Figure 50: Flow cytometric evaluation of neutrophil priming status by expression of CD11b [A] and CD62L [B] cell surface markers across all five blood samples. Data are represented as median (columns) and interquartile range (bars);  $n = 6$ ; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5). \* $p < 0.05$ , \*\* $p < 0.01$ , NS = non-significant.

## 6.6 THE EFFECTS OF CPB AND LUNG VENTILATION ON PLASMA CYTOKINE CONCENTRATIONS

The concentration of pro-inflammatory and anti-inflammatory cytokines was assessed in plasma prepared from each of the five experimental blood samples. Within this cohort, all five plasma samples were obtained for only six of the eight patients due to an inability to obtain the left pulmonary vein sample in two patients as previously described. At the time of writing, only four of the six patients with complete datasets within this cohort were analysed for cytokine concentrations due to a significant delay in obtaining the equipment necessary to run the analysis on the two final patients recruited to this cohort. As a result, this section reports the results of four out of the eight patients recruited to this cohort.

There was a trend towards higher levels of IL8, IL6 and IL10 in the post-bypass blood samples (samples 3, 4 and 5) relative to the pre-bypass baseline sample (sample 1). As illustrated in Figure 51 (panels A, B and C), statistical significance was reached in a number of these measurements despite the small number of patient samples analysed. These results are in keeping with those obtained from the first experimental cohort. Despite the more intense ventilation protocol in this cohort compared to the first experimental cohort, there continued to be no significant difference in the levels of IL8, IL6 and IL10 between blood exiting the right lung which was ventilated during CPB (sample 4) and blood exiting in the left lung which was deflated throughout CPB (sample 5).

There was no significant difference in the plasma levels of IFN $\gamma$  across all five samples within this cohort (Figure 51, panel D). This result contrasts with the small but significant reduction detected in the post-bypass samples from the first experimental cohort. Moreover, there were no significant differences in the plasma concentrations across all five experimental blood samples for TNF $\alpha$ , IL1 $\beta$ , IL12p70 or IL2 (Figure 52). Multiple unrecordable levels were obtained for the IL4 and IL13 cytokine analysis within this cohort resulting in little meaningful data to analyse.

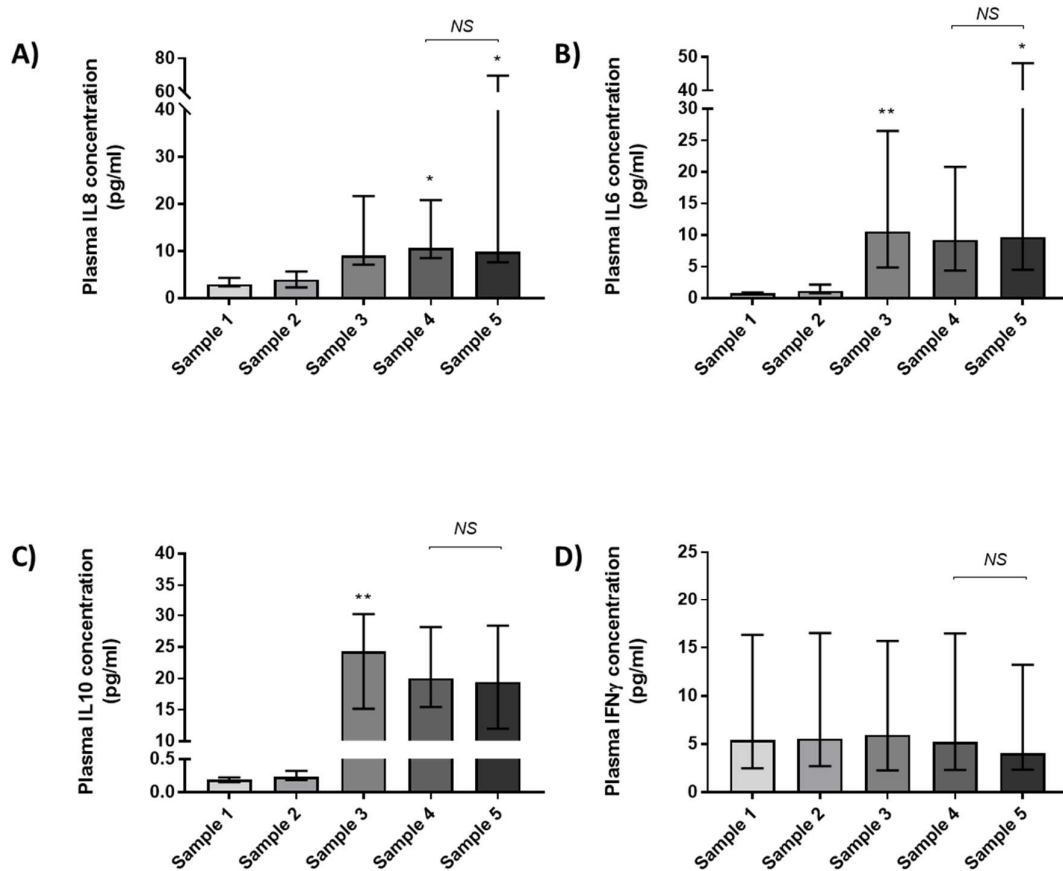


Figure 51: **Plasma concentrations of IL8 [A], IL6 [B], IL10 [C] and IFN $\gamma$  [D] across all five blood samples.** Data are presented as median (columns) and interquartile range (bars): n=4; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5). \*p<0.05, \*\*p<0.005 and NS=non-significant.

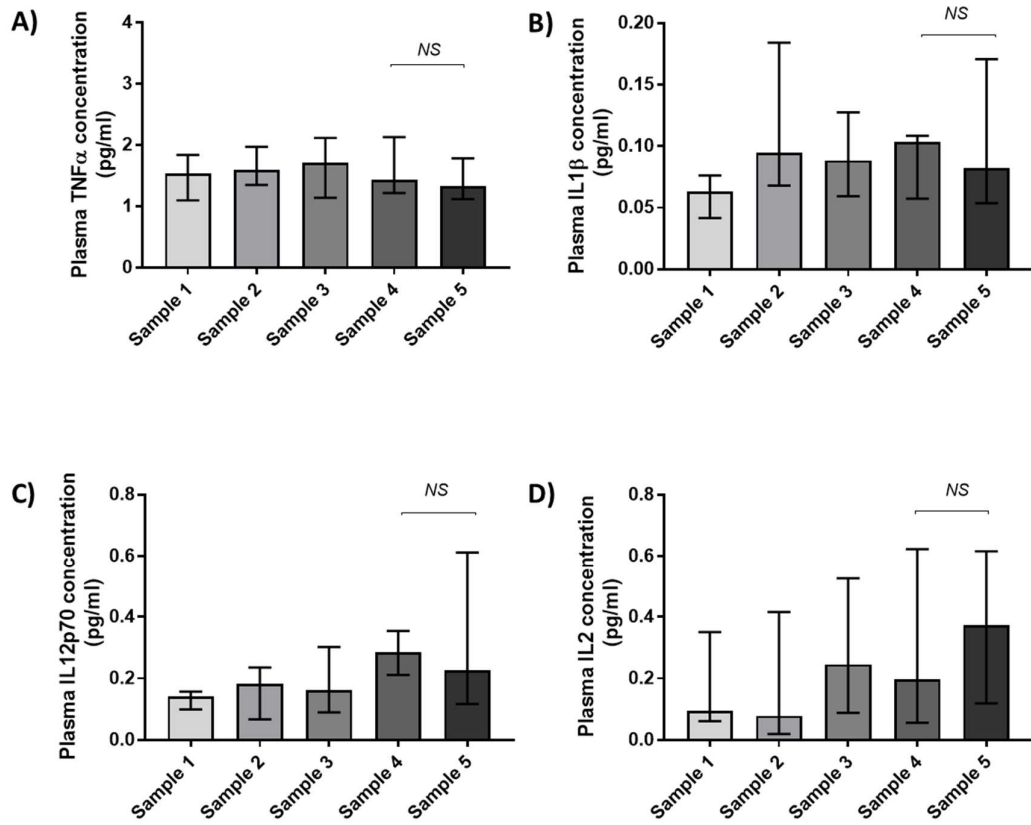


Figure 52: Plasma concentrations of TNF $\alpha$  [A], IL1 $\beta$  [B], IL12p70 [C] and IL2 [D] across all five blood samples. Data are presented as median (columns) and interquartile range (bars); n=4; Friedman test with Dunn's post-hoc multiple comparisons was used for comparisons of samples 2-5 to sample 1 (baseline sample). The Wilcoxon matched pairs signed rank test was used for comparisons between the ventilated lung (sample 4) and the deflated lung (sample 5).

## 6.7 THE EFFECTS OF INTRAOPERATIVE LUNG VENTILATION ON PULMONARY VEIN BLOOD GAS ANALYSIS

Paired left and right pulmonary vein blood gas analysis was available for five of the eight patients within this cohort. For two patients, the left pulmonary vein sample was not obtained as previously described and for one patient there was not enough blood obtained from the left pulmonary vein to enable a blood gas analysis to be performed.

There were no statistically significant differences between pulmonary vein blood pH, pO<sub>2</sub>, pCO<sub>2</sub>, lactate or HCO<sub>3</sub> between the left and right lungs following resumption of pulmonary blood flow after cessation of CPB (Table 15). The pulmonary vein blood gas samples were obtained in the immediate post-CPB period and the FiO<sub>2</sub> levels administered at this time were at the discretion of the anaesthetist.

Table 15: Comparison of blood gas parameters for the left (deflated) and right (ventilated) lung following resumption of pulmonary blood flow after CPB

Parameter	Left pulmonary vein	Right pulmonary vein	p-value
FiO <sub>2</sub>	0.5 (0.5-0.7)	0.5 (0.5-0.7)	-
pH	7.4 (7.3-7.4)	7.4 (7.3-7.4)	0.50
pO <sub>2</sub> (kPa)	23.5 (11.0-30.3)	27.0 (15.1-44.4)	0.13
pCO <sub>2</sub> (kPa)	5.0 (4.4-6.6)	5.3 (5.1-6.3)	0.75
Lactate (mmol/L)	1.5 (1.1-2.0)	1.6 (1.1-2.2)	0.25
HCO <sub>3</sub> (mmol/L)	22.4 (21.0-23.5)	22.5 (21.6-24.0)	0.63

*Data are presented as median (interquartile range); n=5  
Statistical analysis was by the Wilcoxon matched-pairs signed rank test.*

## 6.8 IMPACT OF ONE-LUNG VENTILATION ON CLINICAL OUTCOME MEASURES

The safety and clinical impact of the study interventions for the second experimental cohort were compared to a control cohort. This control cohort consisted of eight patients who were identified from the Trust's Cardiothoracic Data Registry by a data manager who was independent of the study. It consisted of age and gender-matched controls who underwent first time CABG surgery within the period of the second experimental cohort. As detailed in Table 16, there were no significant differences in baseline clinical characteristics between the patient and control groups.

Table 16: Comparison of the baseline clinical characteristics for the second experimental cohort and the control cohort

Baseline characteristic	Second experimental cohort	Control cohort	p-value
Gender – no. of pts (%)			
Male	8 (100.0)	8 (100.0)	-
Female	0 (0.0)	0 (0.0)	
Age (years)	65.5 (60.3-74.5)	69.5 (63.3-78.5)	0.40
Height (cm)	173.0 (166.3-175.8)	171.0 (170.0-174.0) *	0.63
Weight (kg)	84.2 (76.5-101.4)	79.0 (65.9-92.4)	0.34
Smoking status – no. of pts (%)			
Never smoker	3	3	
Ex-smoker	5	5	-
Smoker	0	0	
Logistic EUROscore	2.4 (1.6-4.5)	2.2 (1.2-3.6)	0.88
Number of grafts	3.0 (2.0-3.0)	3.0 (2.3-3.0)	0.88

*Data are presented as median (interquartile range), n=8 (patient cohort) and n=8 (comparative cohort).  
Statistical analysis was by the Mann-Whitney U-test.  
\*n=7 (no available height value for 1 patient)*

There were no statistically significant differences in operative outcome measures for total cardiopulmonary bypass time, total aortic cross-clamp time, total blood loss from mediastinal

and pleural drains at 12 hours, time to extubation or duration of ITU stay (Figure 53, panels A-D). These results are in keeping with the results of the first experimental cohort. It is notable that the subjective opinion from operating surgeons contributing to the second experimental cohort was that the more intense one-lung ventilation strategy did not negatively impact on the progress of the operation however the movement and ventilation of the right lung was more evident compared to the first experimental cohort.

While the duration of ITU stay was comparable in both groups, there was a greater spread of data for patients in the second experimental cohort with one patient resident on ITU for 7 post-operative days (Figure 53, panel E). This was due to the patient developing pneumonia and acidaemia which required continuous positive airway pressure support via high flow nasal cannulae following extubation. Notably, this patient had a significantly prolonged time to extubation (686 minutes) compared to other patients within the second experimental and control cohorts (Figure 53, panel E).

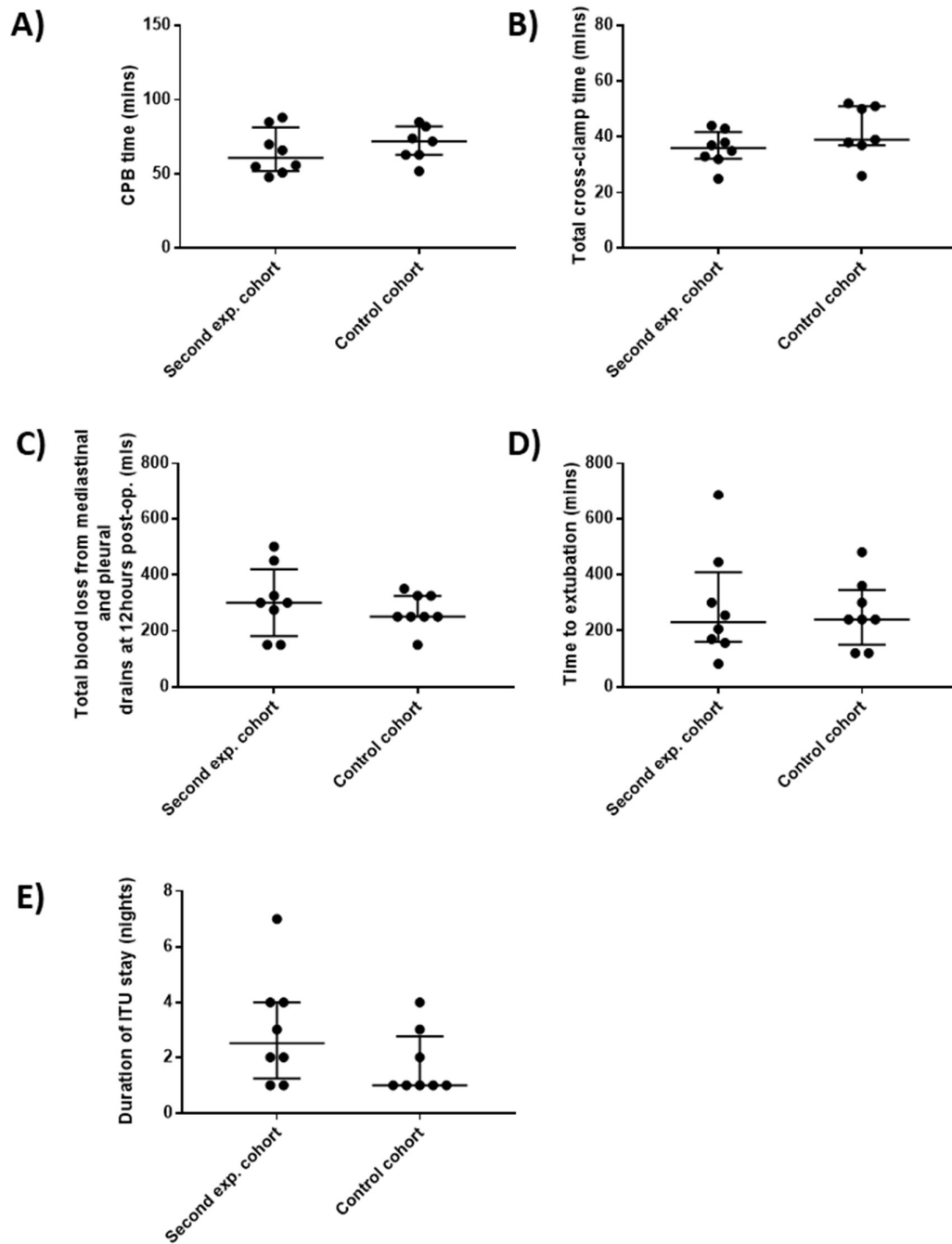


Figure 53: Comparison of clinical outcome measures between the second experimental cohort and the control cohort. Data are presented on scatter plots with median (middle bar) and interquartile range (whiskers);  $n=8$  (second experimental cohort),  $n=7$  (control cohort for panels A and B due to missing data for CPB time and total cross-clamp time in one control cohort patient) and  $n=8$  (control cohort for panels C, D and E). Statistical analysis was by the Mann-Whitney U test. [A]  $p=0.48$ , [B]  $p=0.14$ , [C]  $p=0.55$ , [D]  $p=0.98$ , [E]  $p=0.18$ .



Despite the small numbers of patients, there were no discernible differences in post-operative mobile CXR reports between the patient group and the control group for the second experimental cohort. The most common chest radiograph findings were left atelectasis, left pleural effusion or a combination of both which parallels the findings of the first experimental cohort (Table 17).

*Table 17: Classification of post-operative mobile CXR report for the patient and comparison cohorts*

<b>Postoperative mobile CXR report</b>	<b>Patient cohort (N=8)</b>	<b>Control cohort (N=8)</b>
No parenchymal or pleural abnormality	0	0
Left atelectasis alone	2	0
Right atelectasis alone	0	0
Bilateral atelectasis alone	0	0
Left pleural effusion alone	2	5
Left atelectasis and left effusion	2	1
Left atelectasis and bilateral pleural effusion	0	1
Bilateral atelectasis and left effusion	0	0
Bilateral atelectasis and bilateral effusion	1	1
Bilateral effusions alone	1	0
Pulmonary oedema	0	0
Pulmonary oedema, left atelectasis and bilateral effusions	0	0

## 6.9 SUMMARY OF THE SECOND EXPERIMENTAL COHORT

Conclusions from the second experimental cohort echo those of the first experimental cohort. The phagocytic function of blood neutrophils remained well preserved during on-pump CABG procedures at a level comparable to healthy volunteers. Moreover, neutrophils in pulmonary vein blood had preserved phagocytic function irrespective of whether the lung was ventilated or remained deflated during CPB.

Neutrophil priming status did not increase throughout the course of the operation as evidenced by CD11b and CD62L cell surface adhesion markers. Higher levels of neutrophil CD62L in the post-bypass samples suggest that these neutrophils were in fact less primed than pre-bypass neutrophils which contrasts with our hypothesis.

As demonstrated in the first experimental cohort, there was a trend towards increased levels of pro-inflammatory (IL8, and IL6) and anti-inflammatory (IL10) cytokines in the post bypass samples compared to the pre-bypass samples. However, despite the more intense one-lung ventilation protocol during CPB which was adopted in this cohort, there was no difference in the levels of these cytokines measured directly from the pulmonary vein blood of the ventilated or deflated lung thus refuting our hypothesis.

Post-bypass pulmonary vein blood gas parameters remained unchanged between the ventilated and deflated lung despite the reinforced one-lung ventilation protocol in this cohort.

Finally, the safety of our clinical model is reinforced by comparable clinical outcome measures between the patient group and a separate control group with regard to postoperative clinical data and chest radiograph findings.

A combined discussion on all four results chapters is now detailed in chapter 7.

# CHAPTER 7: DISCUSSION AND FUTURE WORK

## 7.1 OVERVIEW

This final chapter focuses on a discussion and analysis of the results obtained for both the IFN $\gamma$  laboratory study and the clinical study.

The results of the IFN $\gamma$  laboratory study are discussed first followed by an analysis of the results from the clinical study. The chapter concludes with recommendations for future work based on the findings of the clinical study.

## 7.2 INTERFERON GAMMA LABORATORY STUDY

In keeping with the findings of Scott and colleagues,(85) we demonstrate that neutrophil phagocytic impairment is induced by pre-incubating neutrophils from healthy volunteers with the  $\beta_2$ -agonist salbutamol. Our results strengthen the utility of this *in vitro* model to facilitate the exploration of potential therapeutic agents such as IFN $\gamma$ , on neutrophil phagocytosis.

As previously described in chapters 5 and 6, surgery with CPB did not result in neutrophil phagocytic impairment in our clinical study. This was an unexpected finding and differed from the initial results of the 2012 preliminary study. Therapeutics to improve neutrophil phagocytosis in the setting of surgery with CPB are not indicated based on our results. Nevertheless, phagocytic impairment in critically ill patients has been shown to predict subsequent nosocomial infection in the ITU setting (84) suggesting that our *in vitro* work on the effect of IFN $\gamma$  on neutrophil phagocytosis may be of benefit in this population. Moreover, a recent randomised controlled trial examining the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in a subset of critically ill patients provides proof of principle for a pharmacological effect on neutrophil phagocytic function in this population.(98)

In our study, recombinant human IFN $\gamma$  appeared to restore complement mediated neutrophil phagocytosis back to baseline levels. While most research into the effect of IFN $\gamma$  on neutrophil phagocytosis has focussed on its augmentative effect on phagocytosis,(77, 81, 82) our study contributes further by demonstrating a restorative effect on neutrophils with impaired phagocytosis. However, it is curious that despite previous research demonstrating an augmentative potential of IFN $\gamma$  on neutrophils with *normal* phagocytic function, IFN $\gamma$  did not augment neutrophil phagocytosis over and above normal baseline levels in our study. Possible explanations for these findings may relate to the limitations of our microscopic analysis of neutrophil phagocytosis using zymosan particles as it is inherently limited by the resolution of the microscope. Another possible explanation may be that the neutrophils were phagocytosing at maximum capacity for zymosan particles which may have negated our ability to assess the augmentative potential of IFN $\gamma$  using this method. Further experimentation using other measurements of neutrophil phagocytosis such as flow cytometric analysis may serve to support our light microscopy findings. It is important to acknowledge that while neutrophil phagocytic function was assessed using a light microscopic assay of zymosan uptake, neutrophil phagocytic capacity (the number of zymosan particles phagocytosed by each

neutrophil) was not quantified. It is therefore possible that IFN $\gamma$  may have impacted on phagocytic capacity in neutrophils with normal and impaired phagocytosis and more work will need to be conducted to evaluate this.

Our cell signalling work to explore the effect of salbutamol on RhoA activity supports the findings from previous research by Scott and colleagues by demonstrating that pre-incubating neutrophils with salbutamol results in inhibition of RhoA activation.(85) As previously illustrated in Chapter 1, Figure 10, RhoA plays a salient part in the terminal steps of cytoskeletal organisation to facilitate phagocytosis and salbutamol appears to impair phagocytosis by inhibiting RhoA activation. Scott and colleagues went on to demonstrate that when RhoA is blocked, signalling can occur via alternative intracellular pathways such as that involving Rap1 (which acts independently of RhoA) leading to cytoskeletal rearrangement and subsequent complement-mediated phagocytosis. Interestingly, IFN $\gamma$  appeared to restore RhoA activity in our study, suggesting that IFN $\gamma$  may signal via intracellular pathways to re-activate RhoA in the presence of salbutamol resulting in restored phagocytosis.

Curiously, the level of RhoA activation that was observed in the experimental well containing both salbutamol and zymosan was comparable to the control samples which contained no zymosan suggesting that there was no activation of RhoA. However, the corresponding light microscopy experiment demonstrated that in the well containing both salbutamol and zymosan, phagocytosis was of the order of 60% and so a degree of RhoA activation would be expected, albeit reduced compared to baseline. It is important to acknowledge that the small sample size in this experiment limits the conclusions that we can draw, however these results suggest that perhaps mechanisms other than complement-mediated phagocytosis may be occurring during zymosan uptake which may be independent of RhoA-mediated pathways. This could explain the phagocytosis percentage of around 60% yet no evidence of RhoA activation in the sample with salbutamol and zymosan. Although neutrophil uptake of zymosan particles is thought to occur largely via complement-mediated phagocytosis, there remains the possibility that some particles are, for example, being phagocytosed via Fc receptors which is independent of RhoA.

The addition of ruxolitinib (a selective inhibitor of Jak1 and 2) inhibited the restorative effects of IFN $\gamma$ , thus demonstrating that Jak1 and 2 play a key role in IFN $\gamma$  cell signalling to restore neutrophil phagocytosis. Notably, Janus kinase inhibitors such as ruxolitinib are now used in

clinical practice as treatments to block the pro-inflammatory effects of cytokines including IFN $\gamma$  which drive conditions such as rheumatoid arthritis and myeloproliferative disorders.(99)

Work by Frank and colleagues demonstrates that fludarabine (a Stat1-specific activation inhibitor) results in loss of Stat1 activation by cytokines in peripheral blood mononuclear cells.(100) We observed similar results in our *in vitro* model by demonstrating that the restorative effect of IFN $\gamma$  was not observed in neutrophils treated with fludarabine. Our work suggests that IFN $\gamma$  restores neutrophil phagocytosis via a Jak/Stat-dependent cell signalling pathway.

A limitation of this work resides in our use of an *in vitro* model which may not necessarily reflect *in vivo* conditions. For example, neutrophil impairment may occur via different mechanisms *in vivo* in critically ill patients, and therefore patients may not respond to therapies which have been shown *in vitro* to restore phagocytic function in neutrophils pre-incubated with salbutamol.

The exploratory nature of these experiments and the very small sample sizes results in the need for further research to add weight to these conclusions and to explore the downstream cell signalling mechanisms involved. Nevertheless, these preliminary findings are in keeping with previous work highlighting the beneficial immunomodulatory potential of IFN $\gamma$  on cells of the immune system such as macrophages, monocytes and NK cells (71, 73, 74) and support further exploration of this promising therapeutic approach for carefully selected critically ill patients with neutrophil phagocytic impairment.

## **7.3 CLINICAL STUDY**

### **7.3.1 Neutrophil phagocytic capacity**

This research suggests that neutrophil phagocytic capacity in adults undergoing on-pump CABG procedures is well preserved throughout the operative period. Moreover, maintaining lung ventilation during the period of CPB confers no disadvantage nor advantage to blood neutrophil phagocytic capacity in this setting. These findings imply that despite numerous potential mechanical, pharmacological and physiological insults associated with on-pump CABG surgery, blood neutrophils retain their innate ability to phagocytose target particles. Hence, interventions aimed at improving neutrophil phagocytic function to prevent infection following CABG procedures are not supported by our findings.

Our findings are consistent with earlier research demonstrating preserved phagocytic capacity following CPB (31, 101) but are inconsistent with the conclusions of both our 2012 preliminary study and previous research describing transiently decreased phagocytosis following CPB. (29, 30)

There is little doubt that CABG surgery involving CPB creates an environment that could potentially inhibit neutrophil phagocytic function. For example, previous work by Carter and colleagues has demonstrated that when neutrophils are exposed to non-physiological levels of shear stress such as those imposed by blood pumps and oxygenators, phagocytic index is reduced with increasing levels of shear stress. (102) Moreover, literature exploring the effects of anaesthetic and intraoperative pharmacological agents on neutrophil phagocytic capacity provides evidence of their potential deleterious effects on phagocytic function. (103-105)

It is therefore interesting that, despite numerous potential insults, neutrophil phagocytic capacity remained unaffected throughout on-pump CABG procedures in our study. This finding is perhaps testament to how neutrophils are highly adaptive to adverse conditions thus enabling them to function effectively as primary cells in immune defence. There is no doubt that this clinical model is complex and untangling the mechanisms by which neutrophils retain their ability to undertake complement mediated phagocytosis following surgery with CPB presents a significant challenge.

It is important to acknowledge that the main phagocytosis outcome measure in our study was that of complement-mediated phagocytosis using both zymosan particles and *S. aureus*

bioparticles. As previously described in chapter 1, historical research has used several different assays to measure phagocytosis, particularly by way of surrogate markers of phagocytosis such as NBT reduction and chemiluminescence.(17, 18) It is possible that different conclusions on the impact of CPB on neutrophil phagocytosis may be in part related to the different laboratory assays used and the fact that most of the research into the effect of CPB on neutrophil phagocytosis is now largely historical. Modernisation of the bypass technique and extracorporeal circuits may also be partly responsible for our findings.

It is notable that the phagocytosis results in our current study markedly differed from the results of the 2012 preliminary study.(106) The preliminary study, consisting of five patients, concluded that neutrophil phagocytosis was significantly reduced following on-pump CABG procedures. This dysfunction appeared to be partially restored in neutrophils exiting the right lung which was ventilated during CPB (Figure 54). Despite recruiting a cohort of comparable patients within the same centre, using similar parameters for the right lung ventilation and adopting a comparable blood sampling protocol, our current study has the contrasting conclusion that there is no intraoperative difference in phagocytosis across all experimental blood samples.

The significance of the phagocytic dysfunction illustrated in the preliminary study in the post bypass central vein and left pulmonary vein samples is notable as the mean percentage of neutrophils phagocytosing  $\geq 2$  zymosan particles was in the order of 35%. When comparing this value to other literature which uses the same assay, this would appear to be a profound reduction in phagocytic capacity similar to the degree of dysfunction observed in critically ill patients with severe systemic inflammation within an ITU setting.(83) As neutrophil phagocytic dysfunction has been shown to strongly predict subsequent nosocomial infection in critically ill ITU patients,(84) if the preliminary results were reflective of true clinical values, much higher levels of morbidity related to infections in patients undergoing routine on-pump CABG procedures may be expected. This raises the question as to whether the preliminary results were artefactual rather than truly reflective of phagocytic dysfunction or whether post-CPB phagocytic dysfunction is transient with function being regained shortly after surgery.



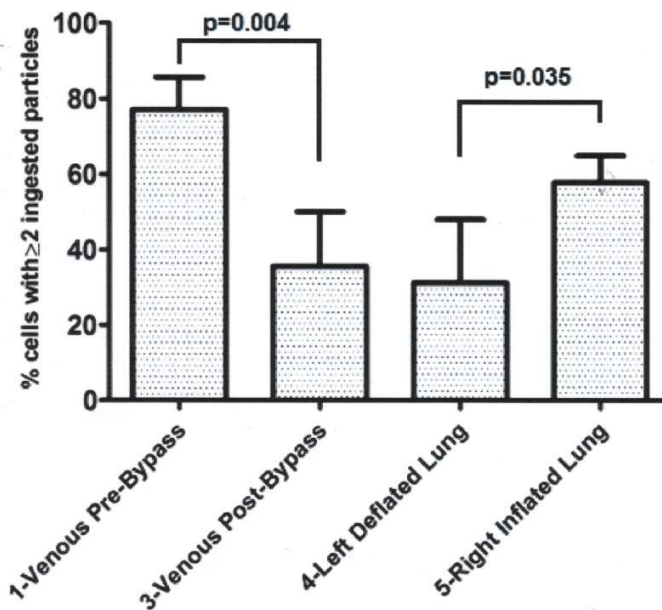


Figure 54: **Data from preliminary study** (cited with permission, Jones et al): Mean percentage of adherent neutrophils that phagocytosed  $\geq 2$  zymosan particles for each experimental blood sample. Data represented as mean  $\pm$  SD. (n=5) (106)

Neutrophil viability was not evaluated in the preliminary study. Hence, a plausible explanation for the results may be that the neutrophils were apoptotic or not viable therefore impairing phagocytic function. Nevertheless, viability analysis was conducted in the current study and did not detect any significant differences across all experimental samples.

Another explanation as to why our results differed from the preliminary study relates to the experimental conditions of the zymosan phagocytosis assay. It was noted in our first experimental cohort that for two patients the medium within the tissue culture wells for the left pulmonary vein sample (deflated lung) became more congealed during the incubation steps of the phagocytosis assay compared to the other experimental wells. On light microscopic examination of these samples, a substantial proportion of zymosan particles appeared to form a layer within this thicker medium rather than penetrate through to reach the adhered neutrophils at the bottom of the tissue culture well. The phagocytic capacity of neutrophils from the left (deflated) lung for these two patients was comparatively lower than for the other experimental samples suggesting that zymosan particles may have been unable to penetrate through this thicker medium during the incubation period subsequently resulting in the lower neutrophil phagocytic capacity observed. Based on these observations, it is possible that the findings of impaired neutrophil phagocytosis for the post-bypass samples in

the preliminary study (notably the post-bypass central venous sample and the left (deflated) pulmonary vein sample as illustrated in Figure 54) reflected failure of zymosan particles to penetrate through thicker medium to reach the adherent neutrophils, rather than reflecting true neutrophil impairment. It is difficult to ascertain why this process occurred in some samples but not others in our current study and it is not known whether it occurred at all in the preliminary study samples. Notably, the medium in which isolated neutrophils were incubated contained 1% autologous serum (which was prepared by adding calcium chloride to plasma from the respective experimental samples). This serum may have contained residual fibrin leading to thicker, more congealed medium in some samples but not others which may ultimately have resulted in the differing results between our current study and the preliminary study.

The significant difference in neutrophil phagocytic capacity between the left and right pulmonary vein blood in the preliminary study is worthy of consideration. The right lung ventilation strategy adopted in the preliminary study (tidal volume ventilation at 2mls/kg of ideal body weight (IBW), 7 ventilations per minute, FiO<sub>2</sub> of 21% and a PEEP of 5cmH<sub>2</sub>O) was based on lung-protective ventilation recommendations adjusted for a one-lung ventilation model as previously described in chapter 1. As the ideal body weight in this population is typically 60-70kg, at 2mls/kg this resulted in a tidal volume of approximately 120-140mls. Notably, the ventilatory strategy adopted in the preliminary study was exactly comparable to that adopted in the first experimental cohort of the current study. Nevertheless, in our current study this ventilatory strategy appeared to only partially inflate the right lung on direct visualisation during the operation, raising the question as to whether an adequate volume of ventilation was reaching the alveoli. This observation subsequently resulted in the decision to intensify the ventilation strategy in our second experimental cohort in aiming to achieve better alveolar ventilation. It is curious that the ventilation strategy adopted in the preliminary study resulted in the striking restoration of pulmonary blood neutrophil phagocytic function that was observed. An alternative explanation for the preliminary results may be that maintaining an intact pleura or administering PEEP to the right lung during CPB resulted in the neutrophil restoration rather than the direct effects of alveolar oxygenation during CPB. Unfortunately, the impact of lung ventilation on restoring impaired neutrophil phagocytosis could not be explored in our current study as phagocytic capacity was maintained throughout the procedure.

Literature illustrating the potential role of the healthy human lung in host defence suggests that primed circulating neutrophils are selectively retained before the lung facilitates their de-priming and subsequent release back into the circulation.(25) Interestingly, the pulmonary de-priming mechanism has been shown to take a substantial period of time; for example, Summers and colleagues demonstrate that almost half of neutrophils primed *ex vivo* with GM-CSF remain in the healthy human lung at 40 minutes.(25) Although not directly comparable, this period of neutrophil retention appears to be much longer than the time between resumption of pulmonary blood flow post-CPB and subsequent pulmonary vein blood sampling in the preliminary study. Based on the current literature in this field, it is unlikely that the significant restoration in phagocytic capacity observed in blood exiting the lung that was ventilated during CPB could be detected after a very short period of post-CPB pulmonary blood flow.

Our current study contributes further to understanding the effects of surgery with CPB on neutrophil phagocytosis as it is reflective of modern-day CABG procedures. In addition, we describe maintained phagocytic function throughout CPB using two different measures of neutrophil phagocytosis (a microscopic assay of zymosan particle uptake on isolated neutrophils and a whole blood cytometric method using *S. aureus* bioparticles) which strengthens our findings. Nevertheless, our study was not without limitation with regard to measuring neutrophil phagocytosis. First, while measuring phagocytosis reflects neutrophil antimicrobial function, the microbicidal function (for example bacterial killing ability) was not evaluated. Therefore, there remains the possibility that neutrophil phagocytic function was maintained intraoperatively but microbicidal function may have been impaired. Second, our measurement outcome was that of complement-mediated neutrophil phagocytosis which occurs via a substantially different mechanism compared to that of Fc receptor-mediated phagocytosis which was not evaluated in this study. It is therefore possible that different results may have been obtained had Fc receptor phagocytosis been assessed. Third, despite attempts to obtain a reliable baseline blood sample in the study patients, there remains the possibility that the first blood sample (which was obtained from the central line after the patient had been anaesthetised) was not reflective of baseline function. Obtaining a blood sample prior to anaesthetic induction (for example on the day before surgery) would have provided a more robust baseline sample however the technical complexities of the study negated this approach. Fourth, it is possible that subtle differences in phagocytic function

between the five experimental samples were overridden by the processing of the samples and therefore not entirely reflective of *in vivo* function. Finally, it is important to note that the results of both the current study and the 2012 preliminary study are inherently limited by small sample sizes and more work will need to be conducted to draw any firm conclusions on the impact of CABG surgery on neutrophil phagocytosis.

### **7.3.2 Neutrophil counts, viability and apoptosis**

A trend towards a greater number of neutrophils isolated from the post-CPB blood samples compared to the pre-CPB samples was observed in the first experimental cohort and reached statistical significance in the second experimental cohort. This increase in post-CPB circulating neutrophil counts is in keeping with previous literature and reflects the impact of a stress-response on circulating neutrophil numbers.(107) This effect is thought to be largely related to mobilisation of marginated neutrophils and the release of neutrophils from the bone marrow.(108)

Interestingly, there were similar numbers of neutrophils entering the lung (via the central venous blood) and exiting in the pulmonary veins of both the inflated and deflated lungs following CPB. This finding may be explained in part by the fact that we did not detect any differences in neutrophil priming status immediately following CPB, resulting in no detectable neutrophil sequestration or adherence within the pulmonary vasculature in terms of neutrophil counts. Limitations nevertheless exist with analysis of neutrophil counts to determine pulmonary retention of neutrophils, not least that it is a relatively crude measure which is largely determined by the quality of the neutrophil preparation from whole blood.

Delayed apoptosis has previously been described in primed or activated neutrophils, which prolongs the functional lifespan of the neutrophil.(13) In the setting of surgery with CPB, this delayed apoptosis could potentiate post-operative inflammation and subsequent organ damage. In the first and second experimental cohort, we observed a trend towards fewer apoptotic cells in the post-CPB samples suggesting that CPB may have a pro-survival effect on neutrophils. To investigate this further we analysed the rate of apoptosis over a 20-hour period in the second experimental cohort and concluded that the rate of apoptosis was similar in all five experimental samples. This suggested that the difference in the number of apoptotic cells between the pre-CPB and post-CPB samples was most likely secondary to the timing of the samples rather than related to any pro-survival effects of CPB. Our apoptosis results were inherently limited by the delay between retrieval and undertaking the laboratory analysis.

### 7.3.3 Neutrophil priming

Neutrophil priming as measured by the expression of cell surface CD11b remained unchanged throughout the CABG operation in both our first and second experimental cohorts. Curiously, CD62L cell surface markers appeared to increase in the post bypass samples indicating that these neutrophils were less primed than the pre-bypass samples.

These findings contrast with our hypothesis yet represent an important and interesting result. Direct neutrophil priming (because of factors such as exposure of blood to the extracorporeal circuit, pulmonary ischaemia and hypothermia) is speculated to be a key component of the post-operative inflammatory response in patients undergoing CABG surgery, yet we consistently did not observe this in our study population. Our results are in keeping with previous research demonstrating no significant upregulation in neutrophil CD11b (109) or down regulation in CD62L (42, 46) cell surface adhesion markers following surgery with CPB yet contrast with other studies concluding that CD11b is upregulated and CD62L is down-regulated.(42-45)

Our results suggest that the post-operative inflammatory response may involve a more complex interplay between components of the immune system and that increased activity of circulating neutrophils cannot solely be held responsible. This concept is perhaps best exemplified in studies examining the effects of off-pump CABG surgery (ie CABG surgery without the use of an extracorporeal circuit) where off-pump surgery failed to show benefit over on-pump surgery in preventing ARDS in the setting of cardiac surgery.(110, 111) This suggests that other factors rather than direct neutrophil priming by the conditions of the bypass circuit may be at play in the post-operative inflammatory response.

As previously alluded to, the finding of increased CD62L in the post-bypass samples is curious as we would not expect surgery with CPB to de-prime neutrophils. The most obvious explanation for this result is that the pre-bypass samples were obtained before the post bypass samples (for example this time gap was on average 97 minutes in the first experimental cohort). Therefore, down-regulation of surface CD62L may have occurred *in vitro* in the pre-CPB samples before laboratory analysis and may not be entirely representative of the *in vitro* characteristics at the time of extraction.

Neutrophil priming status did not increase during the CABG operation therefore negating our ability to assess whether lung ventilation during CPB has the potential to de-prime neutrophils and further work will need to be conducted to evaluate this hypothesis.

Limitations of our neutrophil priming analysis need consideration. First, the process of preparing whole blood samples for the flow cytometric evaluation of cell surface markers may have impacted directly on neutrophil cell surface markers resulting in the inability to detect subtle differences between the samples. Second, as the baseline sample was taken following anaesthetic induction, it is possible that this sample did not reflect true baseline function and perhaps obtaining a baseline sample the day before surgery for immediate analysis would have produced a more reliable baseline. Unfortunately, this method was not workable in our current model, both due to the high last-minute cancellation rate of procedures which would have resulted in the processing of blood samples of patients who would not proceed to surgery and the practical preparatory work required on the day before surgery. Although not directly comparable, there were no significant differences in CD11b and CD62L markers between the study patient baseline sample (sample 1) and a group of healthy volunteers suggesting that the baseline sample taken following anaesthetic induction was reliable.

Thirdly, the analysis of cell surface adhesion markers represents only one measure of neutrophil priming status and exploration of other priming measures such as reactive oxygen species production would have strengthened our conclusions.

Finally, significant advances in the care of patients undergoing CABG surgery have been made over the past 20 years therefore it is possible that newer circuits and more efficient CPB times are partly responsible for our findings.

### 7.3.4 Cytokine levels

In keeping with previous research, we observed a substantial increase in plasma concentrations of the pro-inflammatory cytokines IL8 and IL6 and the anti-inflammatory cytokine IL10 in the post-CPB blood samples.(112) This finding supports the well-established concept that cytokines play a pivotal role in modulating the inflammatory response in the early post-operative period thus serving as potential therapeutic targets in aiming to stem post-operative inflammation and end organ damage.

In our study it is interesting that lung ventilation during CPB had no impact on pulmonary vein blood cytokine concentrations. This was the case for both the first and second experimental cohorts which used different ventilation strategies as previously described. To the best of our knowledge, there has been no previous research examining the effects of lung ventilation on pulmonary vein blood cytokine concentrations using a one-lung ventilation model with direct pulmonary vein sampling and therefore no direct comparison with previous studies can be made. Nevertheless, our results contrast with the work of Ng and colleagues who demonstrated that continued bilateral ventilation during CPB resulted in a significantly lower systemic concentration of plasma IL8 and higher concentration of plasma IL10 compared to a control group receiving no ventilation during CPB.(60) This difference may be in part related to this team adopting a bilateral lung ventilation strategy based on *actual* body weight rather than *ideal* body weight which could have potentially resulted in the use of much higher tidal volumes. Moreover, this team noted that the beneficial effects of lung ventilation on the systemic cytokine profile were not sustained and therefore the results are of questionable clinical impact.

It could be argued that our ventilation strategy was not sufficient to observe any beneficial effects of ventilation on pulmonary vein blood cytokine levels. However, when no difference in cytokine concentrations was observed between the ventilated and unventilated lung using our initial ventilation strategy, we intensified our ventilation strategy for the second experimental cohort and yet the results were similar. Based on the principles of lung-protective ventilation as previously described in Chapter 1, section 1.10.1, any further increase in one-lung ventilation tidal volume above and beyond that which was employed in our second cohort would not have been without potential deleterious clinical effects.(113)



We did not detect any difference in the cytokine profile between either the right or left pulmonary vein blood samples (ie the blood exiting each lung) and the systemic central venous blood sample (ie the common blood entering each lung) following bypass. This contrasts with historic research describing higher IL8 and IL6 concentrations in pulmonary venous blood compared to right atrial blood (blood entering the lungs) following aortic de-clamping in patients undergoing CABG operations.(114) Our contrasting results may be explained by the pulmonary venous samples in our work being obtained much earlier than the aforementioned study and suggests that perhaps a longer period of reperfusion is necessary to detect inflammatory cytokine flux from the re-perfused lung following CABG. If this hypothesis were proven, then perhaps the full benefit of lung ventilation during CPB on cytokine release was not observed in on our study due to obtaining early pulmonary venous blood samples following reperfusion.

It is important to recognise that our small study population was a limitation to the cytokine analysis. While we detected a small but significant reduction in plasma IFN $\gamma$  concentrations in the first experimental cohort, this result was not reproduced in the second experimental cohort. Moreover, several cytokines such as TNF $\alpha$  which have previously been shown to increase during the post-operative inflammatory response remained unchanged across all five experimental samples and work will needs to be conducted to evaluate these findings further.

### 7.3.5 Clinical findings

Our study results suggest that there is no advantage nor disadvantage from one-lung ventilation during CPB on clinical outcome measures. These findings are in keeping with previous literature where the beneficial effects of bilateral mechanical ventilation during CPB on major clinical outcome measures has yet to be shown.(115)

Pulmonary vein blood gas measurements obtained directly from both the ventilated and deflated lung served to provide an immediate assessment of pulmonary gas exchange following resumption of pulmonary blood flow after CPB. Despite right lung ventilation during CPB, no difference in post-operative pulmonary gas exchange between the right and left lung was detected in either the first or second experimental cohorts. No previous research has evaluated post-operative pulmonary gas exchange using a model of direct pulmonary vein sampling with the left lung serving as each patient's own control. The most comparable studies have assessed post-operative  $\text{PaO}_2/\text{FiO}_2$  ratio as a surrogate for pulmonary gas exchange following bilateral ventilation during CPB. While Beer and colleagues demonstrated an improvement in  $\text{PaO}_2/\text{FiO}_2$  ratio when comparing ventilated and non-ventilated groups during CPB (116) other researchers did not show any statistically significant differences.(55, 58). The heterogeneity of different ventilation strategies adopted in these studies makes comparison difficult and more research exploring different ventilatory modalities and intensities is needed to determine any potential benefits that intraoperative ventilation may have on post-operative pulmonary gas exchange.

The hypothesis that ventilation during CPB may result in shorter extubation times and length of ITU stay was not supported by our results as these outcome measures were similar in both patient and control cohorts. Our results contrast with those of John (59) and Davoudi (117) who demonstrated shorter extubation times in patients ventilated during CPB but we remain mindful when assessing clinical outcomes of our study that only one lung was ventilated and so direct comparisons with studies exploring the impact of bilateral ventilation cannot be made.

The small sample size of our study population in addition to a wide range of different chest radiograph outcomes makes drawing any conclusion of the impact of lung ventilation on radiographic pulmonary complications rather challenging. However, we did not detect any differences between our patient groups and respective control cohorts with regards to CXR

findings which is in keeping with the findings of Gagnon and colleagues.(58) Left sided radiographic changes (particularly atelectasis) appeared to be more common than right sided changes in our study however this was true for both the control and patient cohorts. The higher prevalence of left basal atelectasis on chest radiographs following CPB has previously been described and has been attributed to factors such as the position of the heart and left diaphragmatic palsy from topical cooling strategies. (6)

Our comparable clinical outcome measures of total cardiopulmonary bypass time and total mediastinal and pleural drain blood loss for both the patient and control cohort supports the safety of a novel one-lung ventilation model with direct pulmonary vein sampling in this setting. This opens the potential to further evaluate the role of intraoperative ventilation during CPB in future studies.

### **7.3.6 Future work**

There is little doubt that more work needs to be conducted to draw any firm conclusions on the immunomodulatory potential of lung ventilation during surgery with CPB. Although our model was unique and had strength in that it enabled the patient to serve as their own control, it was a challenging experimental model to undertake which is an important consideration for any future work. The reasons for this include the difficulty of ensuring that all five blood samples were obtained (including the sample from the left pulmonary vein which was often challenging to obtain for anatomical reasons), as well as undertaking the complex surgical and ventilatory protocols. A suggested model to consider in any future work would involve a control cohort design where one cohort of patients undergoing on-pump CABG surgery received bilateral ventilation during CPB while a control group received no ventilation during CPB. In each group, blood entering the lung via the central venous blood could then be sampled following CPB which would represent blood entering the lungs. This could then be compared with blood from the radial arterial line (which is routinely inserted), to represent blood exiting the lungs. This would allow a transpulmonary assessment of blood constituents in both the ventilated and unventilated cohorts to assess the impact of ventilation during CPB without the requirement to obtain direct pulmonary vein samples.

It is noteworthy that previous research has demonstrated higher levels of anti-inflammatory cytokine concentrations in bronchoalveolar lavage (BAL) fluid in a group of patients ventilated during CPB compared to a control cohort receiving no ventilation during CPB.(60) This raises the question as to whether assessment of BAL fluid cytokine concentrations would be beneficial in assessing the direct effects of lung ventilation during CPB. Due to the technical complexities of our study we were unable to facilitate an assessment of the impact of ventilation during CPB on BAL cytokine concentrations. However, this is an interesting area for future research as high levels of inflammatory cytokines within BAL fluid following surgery with CPB may be responsible for the pooling of neutrophils in the alveolar space thus predisposing to pulmonary dysfunction and ARDS. A one-lung ventilation model with subsequent BAL of the ventilated and unventilated lung would serve as a model to investigate this further. In addition, there has been recent interest in future lung protection therapies such as inhaled hydrogen sulphide which has been shown in an animal model to protect against ventilator-associated lung injury.(118) Our novel one-lung ventilation model during CPB would provide a potential clinical platform to test this further by facilitating application

of hydrogen sulphide to one lung during CPB with subsequent assessment of BAL fluid cytokine concentrations while the unventilated lung serves as the patient's own control.

## **PUBLICATIONS ARISING FROM THIS WORK**

Funston, W., Ruchaud-Sparagano, M.H., Scott, J., O'Leary, D., Dark, J.H., Simpson, A.J. *The immunological impact of lung ventilation during cardiopulmonary bypass*. American Journal of Respiratory and Critical Care Medicine 2018;197: A2989

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