

PULMONARY PROTECTION IN CARDIAC SURGERY

**Imperial College London, certifies that this is the approved
thesis submitted for the degree**

Doctor of Philosophy (Ph.D.)

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ABSTRACT

Background: Pulmonary dysfunction is a common complication of cardiac surgery. Pulmonary protection strategies have evolved over the years; however, their efficacy remains controversial.

Objective: To compare interventions aiming at reducing pulmonary dysfunction in patients undergoing coronary artery bypass grafting. The (BiPAP) trial [ISRCTN 85570732] investigated the efficacy of NIV with BiPAP in addition to usual care, versus usual care alone. The ventilate trial [ISRCTN 34428459] investigated LFV versus usual care during cardiopulmonary bypass.

Outcome measures: In the BiPAP trial, the primary outcome was time until fit for discharge. In the ventilate trial, the primary outcome was inflammation measured by NF- κ B p65 activation in lung biopsies pre- and post- CPB. In a sub-study, pulmonary arteries were isolated from lung biopsies, to characterise changes in structure and function related to the CPB.

Results: The median duration of the hospital stay until fit-for-discharge was 5 days for the BiPAP group (IQR, 4-6) and 6 days for the usual care group with a reduced incidence of adverse events. In the ventilate trial LFV (adjusted for pre-CPB level) compared to the standard care group had no effect on NF- κ B p65 activation in lung biopsies. In a sub-study, the pattern of anatomical and physiological changes in pulmonary artery obtained from lung biopsy pre- and post-CPB was described.

Conclusions: The BiPAP trial, improved recovery time. The ventilate trial did not significantly improve clinical or inflammatory markers. For the first time in human

we describe the structural and functional changes in pulmonary arteries from lung biopsy associated with CPB. A better understanding of available modalities of lung protection and/or combinations could result in the development of customised strategies for the different cohorts of patients with the potential to maximise patient and institutional benefits.

SOURCES OF FUNDING

I.1 The BiPAP Trial

The equipment for the BLPAP used in the study was donated by Philips Respironics (Chichester, UK). The study was partly supported by the National Institute for Health Research Bristol Cardiovascular Biomedical Research Unit. Neither Philips Healthcare nor the National Institute for Health Research had any role in the design, conduct, analysis, or reporting of the trial. The trial was sponsored by Imperial College London. The trial was designed, conducted, analyzed, and interpreted independently.

I.2 The Ventilate Trial

The trial was sponsored by Imperial College London. The trial was designed, conducted, analysed, and interpreted independently of the sponsor. The authors had full access to the data and are responsible for submitting the publication. The views expressed are those of the authors and not necessarily those of the NHS, the National Institute of Health Research, or the Department of Health. The sub-analysis of the same study examined the structure and function of isolated human pulmonary arteries. The study was supported by the British Heart Foundation (BHF; FS/13/16/30199, PG/13/9/29990) and the Oxford BHF Centre of Research Excellence; the Garfield Weston Trust and the UK National Institute for Health Research (NIHR) Bristol Cardiovascular Biomedical Research Unit. Kim Dora is a BHF-funded Senior Basic Science Research Fellow; Emad Al Jaaly is a NIHR-funded Academic Clinical Fellow.

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The trial designs, investigations, observations and conclusions presented in this thesis are the result of my own work unless I have stated otherwise, and it has not been submitted for any degree previously.

All major work was performed at the National Heart and Lung Institute, Imperial College London and the Imperial College Healthcare NHS Trust. However, some experiments were conducted in the Department of Pharmacology, University of Oxford, Oxford.

Emad Al Jaaly

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DEDICATION

This thesis is wholeheartedly dedicated to my beloved parents, who have been my source of inspiration and gave me strength, who continually provide me with moral, spiritual and emotional support. To my brother and sisters for their lifelong encouragement. To my wife for her help, care and immortal love and to my lovely children.

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Abbreviations

APF	Redox-sensitive fluorescent probe (3'-p-aminophenyl) fluorescein
ATP	Adenine triphosphate
BCR	B-cell receptor
BE	Base Excess
BiPAP	Bilevel Positive Airway Pressure
BM	Blood Glucose
BMI	Body mass index
BP	Blood Pressure
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Grafting
CI	Confidence Interval
cm	Centimetre (a unit of length in the metric system)
CPAP	Continuous Positive Airway Pressure
CPB	Cardiopulmonary Bypass
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
EPAP	Expiratory Positive Airway Pressure
FACS	Fluorescent Activated Cell Sorting
FEV₁	Forced expiratory volume in one second
FiO₂	Fractional concentration of inspired oxygen
HR	Heart Rate
HRP	Horseradish Peroxidase

hrs	hours
I:E	Inspiration to expiration ratio
IL	Interleukin
IS	Incentive Spirometry
κB-RE	NF-κB response elements
K	Potassium
kPa	Kilopascal
Kco	Carbon Monoxide Transfer Coefficient
LFV	Low Frequency Ventilation
min	minutes
NEMO	NF-κB essential modulator
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NIV	Non-Invasive Ventilation
NO	Nitric Oxide
NYHA	New York Heart Association
PAO₂-PaO₂	Alveolar-Arterial Oxygenation Gradients
PA-a O₂	Alveolar-Arterial oxygen partial pressure difference
PBS	Phosphate Buffered Saline
pCO₂	Partial pressure of carbon dioxide; refers to the amount of carbon dioxide in the blood
PEEP	Positive End-Expiratory Pressure
pH	A measure of the acidity or alkalinity of a solution
pO₂	Partial pressure of oxygen; refers to blood oxygen concentration
ROS	Reactive Oxygen Species

RR	Respiration Rate
RT	Reverse Transcription
RV	Residual Volume
SAO₂	Oxygen Saturation in the blood (measured from the finger)
SD	Standard Deviation
SIO₂	Oxygen Saturation in the blood (measured from the blood line)
TBS	Tris-Buffered Saline
TCR	T-cell receptor
TLC	Total Lung Capacity
TNFα	Tumour Necrosis Factor alpha
TV	Tidal Volume
VA	Alveolar Volume
VC	Vital Capacity
V/Q	Ventilation/Perfusion ratio
WBC	White Blood Cell Count

CHAPTER I. THESIS DEVELOPMENT AND DESCRIPTION

After my graduation from the medical school, I became attracted to cardiac surgery and decided to pursue a career in this field. In 2008 I obtained a junior trainee position in cardiothoracic surgery at Imperial College NHS Trust, Hammersmith Hospital, hoping to progress into a specialist registrar position. During ward rounds I quickly noticed the serious pulmonary complications that some patients experience after cardiac surgery. I wanted to investigate this problem but realised that my understanding of research was rather limited, and my academic skills were inadequate. I worked to learn about research in a broad sense, acquiring expertise in the art of literature search and paper-writing. I studied lung physiology and pathology during and after cardiac surgery under the effect of CPB. Consequently, I started to think about possible strategies to ameliorate pulmonary dysfunction following cardiac surgery.

Late in 2008, I met two consultant respiratory physicians (Dr Rob Shiner and Dr Philip Ind), with whom I discussed the possibility of using elective NIV with bi-level positive airway pressure ventilation for patients undergoing CABG to reduce post-operative lung dysfunction. We designed a trial based on the hypothesis that NIV with bilevel positive pressure ventilation (BiPAP) after CABG would shorten the duration of post-operative stay by improving lung physiology, gas exchange and reducing basal atelectasis to a clinically important extent.

Early in 2010, nearing the end of the recruitment phase of this first trial Professor G.D. Angelini, joined Imperial College NHS Trust. This gave me the opportunity to work as part of a larger research team including statisticians, epidemiologists and

basic scientists. I was coached in statistics by Professor Barny Reeves and Dr Francesca Fiorentino.

My interest in academia increased; and when Professor Angelini, showed me an experimental study he had conducted using LFV during CPB in a pig model, I proposed to evaluate this intervention in a clinical setting with patients undergoing CABG. We hypothesised that using LFV during CPB would reduce inflammatory activation, lung dysfunction and ischemia/reperfusion injury compared with conventional non-ventilation of the lungs during CPB. This was the basis for my second randomised control trial involving 36 patients (The ventilate trial). We established a collaboration with Professor I. Adcock to learn and perform the laboratory experiments on lung tissue and blood samples. The ventilate trial was novel and original in obtaining lung biopsies something that to my knowledge had not be done before in human during heart surgery. Subsequently I established a link with Professor Kim Dora in Oxford an expert in pulmonary vessels functional and structural anatomy.

Details of my contribution to the BiPAP Trial

I was involved fully in the design of the study and in obtaining ethical approval and developed a consent form and information sheet for patients. I designed an Excel spread sheet for data collection, contacted the company that sponsored the trial (Philips Respironics) and negotiated for the use of the number of BiPAP machines required for the trial. I was soon challenged by another issue; the nursing staff at the cardiac intensive care unit of the Hammersmith hospital had never used BiPAP and didn't know how to operate the machines. Hence, I organised 12 teaching sessions for all the nurses with a senior instructor from Philips Respironics. I had to learn how to operate the machines and trouble shoot technical problems. I coordinated all phases of the trial between nurses who see patients in pre-assessment clinics, patient's admission team, anaesthesiologists, cardiac intensive care unit nurses and the statistician who created the randomization tables. I collected all the data and performed the bedside spirometry for all patients following surgery on days 1, 2 and 3.

Details of my contribution to the Ventilate Trial

I oversaw the ethics approval process. I worked in Professor Adcock's laboratories to learn and perform the experiments on lung tissue and blood samples. Learning, performing and validating basic science experiments was all new for me. Conducting the trial was my daily work, from identifying suitable patients that fulfilled the criteria, then obtaining their consent, coordinating the randomisation along with Dr Francesca Fiorentino, then informing the consultant anaesthesiologist in theatre about the allocation and scrubbing in each operation to perform the lung biopsies.

Laboratory assays

The blood assays work is based on Fluorescence-activated cell sorting (FACS) which is a specialized category of flow cytometry. It provides a technique for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell kind. I did establish a standard step-by-step draft method to perform all the FACS work (oxidative stress in Leukocytes & detection of activated phospho-p38 MAPK and NF- κ B p65 sub-units). I took all the blood samples and conducted the blood FACS experiments for the first 12 patients at the Imperial College Laboratory at the Hammersmith site. With help from Dr Andrew Durham I performed the rest of the samples' analysis in the Imperial college laboratory at the South Kensington site. Finally, we both shared the work on the heme assay.

Lung tissues assays

I performed all lung biopsies during surgery and preserved the lung samples at the Imperial college laboratories at the Hammersmith site. After completing the lung biopsies, I gained from Miss Elen Jazrawi's, expertise on tissue work, learning the steps and assays needed for the extraction of protein samples (Bradford Protein Assay, Polyacrylamide gel electrophoresis, Western Transfer Semi-dry Blotting, Immunodetection and Band Quantitation). I performed personally the protein extraction from the first 20 biopsies, and Miss Elen Jazrawi, helped me with the rest of the work. We worked together on the NF- κ B Transcription Factor activation assay, the semi-quantitative measurement of p38 MAPK- α pT180/Y182 and total p38 MAPK using the phospho-tracer assay and caspase 3 activity assay. For the RNA extraction, ELISA work, I am grateful for the time I spent with Dr Andrew Durham, who helped me understand the methods. Due to the volume of lab work, I needed his help performing the RNA extraction, and ELISA work.

Finally, I worked on validating the work and writing the results and detailed methods in my thesis.

Details of my contribution to isolated human pulmonary artery structure and function work

During the time I spend in the set-up of the ventilate trial, I became aware of the novel work of Professor K. Dora and her research team in the Oxford Pharmacology department, on the study of the structure and function of pulmonary arteries in animal models (pigs).

We discussed the possibility of doing this in clinical samples and I became fully involved in the study design and contributed to the ethics approval and tissue transport paperwork. I was not involved with the laboratory work, but I coordinated the progress of the work and contributed significantly to the writing of the published paper.

Final comment: this has been an incredible learning experience. I have gained new skills by collaborating with basic scientists, clinicians, statisticians and epidemiologists, without whom this work, and thesis would not have been possible.

My Ph.D. thesis consists of the following six chapters: -

Chapter I: - Thesis development and description

Chapter II: - Introduction

Chapter III: - The BiPAP trial (the effect of adding postoperative non-invasive ventilation to usual care to prevent pulmonary complications in patients undergoing CABG: a randomised controlled trial)

Chapter IV: - The ventilate trial (a low frequency ventilation during cardiopulmonary bypass for pulmonary protection: a randomised controlled trial)

Chapter V: - Contributions to Dora et al's study on isolated human pulmonary artery structure and function pre- and post-cardiopulmonary bypass surgery

Chapter VI: - Thesis Discussion, Conclusion and Future Direction

Reference list**Appendix: -**

Research presentations

Research publications

Copies of research publications

CHAPTER II. INTRODUCTION

II.1 Background

Pulmonary dysfunction is a common complication of cardiac surgery. The mechanisms involved in the development of pulmonary dysfunction are multifactorial and can be related to the activation of inflammatory and oxidative stress pathways (Figure II.1). Clinical manifestation varies between mild atelectasis to severe respiratory failure. Managing pulmonary dysfunction post cardiac surgery is a multistep process that starts before surgery and continues during both the operative and postoperative phases [1]. Different pulmonary protection strategies have evolved over the years. However, the wide acceptance and clinical application of such techniques is hindered by the poor level of evidence or the sample size of the studies. A better understanding of available modalities and/or combinations could result in the development of customised strategies for the different cohorts of patients with the potential to maximise patient and institutional benefits [1, 2].

II.1.1 PULMONARY PHYSIOLOGY

Normal respiratory requirements of the human body can be easily met by tidal ventilation, which is approximately 4–8 ml/kg [3]. Variations in the levels of oxygen and carbon dioxide are regulated by voluntary control of respiratory muscles through the central nervous system (CNS) that sustain active control over breathing rate. Involuntary breathing control is regulated by the respiratory centre in the medulla oblongata and pons [4]. In normal circumstances, carbon dioxide levels stimulate the respiratory centre that influences the rate and depth of

breathing [5]. High partial pressures of carbon dioxide and changes in hydrogen ion (pH) in arterial blood gases stimulate the respiratory centre to breath faster and exchange excess carbon dioxide. Equally, low levels of carbon dioxide trigger shallower breathing until homeostasis is achieved [6, 7].

The lung possesses a unique structure and function. It contains air and blood, and fluid media that differ substantially in their specific densities. Exchange of gases is facilitated by 300 million alveoli surrounded by a blood capillary network [8]. The rate of oxygen transport depends upon the effectiveness of a four-stage processes which involves diffusion of oxygen into the alveoli; transfer of oxygen across the alveolar capillary membrane; transport of oxygen via haemoglobin (systemic capillaries) and movement of oxygen from haemoglobin to cells in the body. The effectiveness of these processes is dependent on normal airway passage, respiratory rate, adequate circulating volume ($N=5L/\text{minute}$), adequate alveolar ventilation ($N=4L/\text{minute}$) and alveolar cell surfactant that acts as a lubricant to prevent alveolar collapse and atelectasis [5, 8]. The lung counters the challenges of maintaining structural integrity under forces such as surface tension, intramural BP, and the weight of the blood that it contains [9]. Inside the alveoli, the terminal respiratory units, the blood-gas barrier consists of a thin (squamous) epithelial cell layer facing the air space, an intervening intercellular matrix, and an endothelial cell (EC) layer that faces the vascular space. The laminated, tripartite construction of the blood-gas barrier is extremely thin promoting gas exchange by passive diffusion [10-12].

II.1.2 PULMONARY PATHOPHYSIOLOGY IN CARDIAC SURGERY

The blood–gas barrier is strikingly strong, despite its thinness, allowing it to tolerate prevailing stresses and maintain structural integrity under physiological conditions [10-12]. This equilibrium is dramatically altered by the iatrogenic, prolonged lung collapse/atelectasis induced during CPB [13]. These conditions during CPB may affect passive diffusion of gasses at the blood–gas barrier [9-15]. Any disruption to the respiratory passage and circulating volume can lead to the ventilation-perfusion mismatch, increased PA-a O₂, increased extravascular lung water and impaired lung compliance. CPB may also induce poor alveolar blood supply with a high potential for marked alveolar ischemia triggering hypoxic pulmonary vasoconstriction (HPV) that plays a role in distributing the local blood flow to increase the efficiency of gas exchange [16-18].

Pulmonary dysfunction after cardiac surgery impacts both patient outcomes and health economics. Many patients will have altered pulmonary mechanics after surgery that may result in a wide range of clinical presentations; from mild atelectasis to life threatening acute lung injury or adult respiratory distress syndrome [19-22].

The degree of atelectasis/lung collapse and intrapulmonary shunt is associated with gas exchange impairment [14, 16]. This is the most common cause of acute respiratory failure after open heart surgery [23-25]. Acute respiratory failure is a sudden incapacity of the respiratory system to maintain adequate oxygenation with or without an impairment of carbon dioxide elimination [8]. Schell proposed two categories of acute respiratory failure [25]:-

Type 1 (called diffusion defect) occurs when lungs are not sufficiently exchanging gases, causing alveolar hypoventilation and ventilation leading to perfusion mismatch ($\text{PaO}_2 < 8 \text{ kPa}$).

Type 2 (called ventilatory pump failure) exhibits the failure of carbon dioxide removal with low level of oxygen ($\text{PaO}_2 < 8 \text{ kPa}$, $\text{PaCO}_2 > 6 \text{ kPa}$) called hypercapnic/hypoxemic acute respiratory failure [26].

Cardiac surgery patients may suffer post-operatively, from type 1 or Type 2 acute respiratory failure with dyspnoea, anxiety and hallmark symptoms that may or may not be present and at different altered levels of consciousness [25].

Midline sternotomy incisions abolish the integrity of the chest wall and affect respiratory mechanics leading to impaired respiration. Postoperative pain decreases lung function by precluding deep inspirations [27]. Furthermore; patients undergoing surgical procedures associated with opening the pleura will have increased rates of atelectasis [28], pleural effusions [29], postoperative hypoxemia [30] and postoperative pain especially in the early postoperative period [1, 31-33].

CPB can lead to the activation of different inflammatory and coagulation pathways and alters redox balance due to the passage of blood through the circuit (contact activation) and ischemia and reperfusion injury [1, 34-37]. Inflammatory activation and cytokine release have been correlated with harm following cardiac surgery [38] and pulmonary function 24 hrs after CPB is inversely associated with plasma IL 10 concentrations [39, 40]. Inflammatory markers raised in the lung following

CPB include $\text{TNF}\alpha$, IL-1 β , IL-18, IL-6, IP-10 and IL-8 and IL-10 [41]. CPB is also associated with increased caspase 3 activity, a marker of apoptosis (6). In addition, the p38 mitogen activated protein kinase (MAPK) and nuclear factor kappa beta (NF- κ B) pathways [42], which are intracellular signalling cascades associated with inflammation, are activated by CBP. Therefore, suppression of activation of inflammatory mediators during CPB is associated with a reduction in pulmonary dysfunction [43].

Vascular EC dysfunction during CPB due to changes in blood flow patterns, shear stress, ischaemia and reperfusion, and circulating cytokines result in the activation of multiple proinflammatory and apoptotic pathways [44-47] while suppressing the ability to produce vaso-protective mediators [47-50]. EC activation is known to initiate a leukocyte adhesion cascade through the expression of members of the selectin family that are responsible for the initial attachment of leukocytes from circulation. Transmigration of leukocytes through ECs (a crucial step for leukocytes recruitment to tissue) follows leukocyte attachment and is mediated by the up-regulation of different adhesion molecules such as platelet endothelial cell adhesion molecule (PECAM)-1, ICAM-1, and very late antigen-4 (VLA)-4 [51-54].

CPB is traditionally associated with inadequate lung perfusion as there is no flow through the pulmonary arteries during periods of cross clamping and when the heart is not ejecting, thus blood supply is limited to the bronchial arteries [55]. Pulmonary physiology alteration during CPB can result in disturbing the balance in the blood gas barrier due to the alteration of the different forces affecting the parenchyma thus abolishing gas exchange by passive diffusion at the blood–gas

barrier level and leading to a ventilation/perfusion mismatch and impaired pulmonary compliance [13, 14]. Moreover; ischaemia during CPB is associated with reduced alveolar blood supply resulting in alveolar ischaemia and hypoxic pulmonary vasoconstriction [56]. Pulmonary vascular EC dysfunction and activated neutrophil sequestration into lung parenchyma during periods of reperfusion can increase lung tissue permeability, elevate vascular resistance and pulmonary surfactant changes leading to alveolar protein accumulation, pulmonary oedema and increasing ROS and cytokine production [57-59]. Together this results in abnormal gas exchange, poor lung mechanics, increased pulmonary shunt fraction and reduced functional residual capacity and carbon monoxide transfer factor [59-61].

Blood and blood product use after surgery can be associated with the production of excessive amounts of ROS and systemic and pulmonary inflammation. Receiving blood that had been stored for longer than two weeks before transfusion can increase the risk of respiratory insufficiency and the need for prolonged ventilation [37, 62-65].

II.1.3 OXIDATIVE STRESS AND PULMONARY DYSFUNCTION

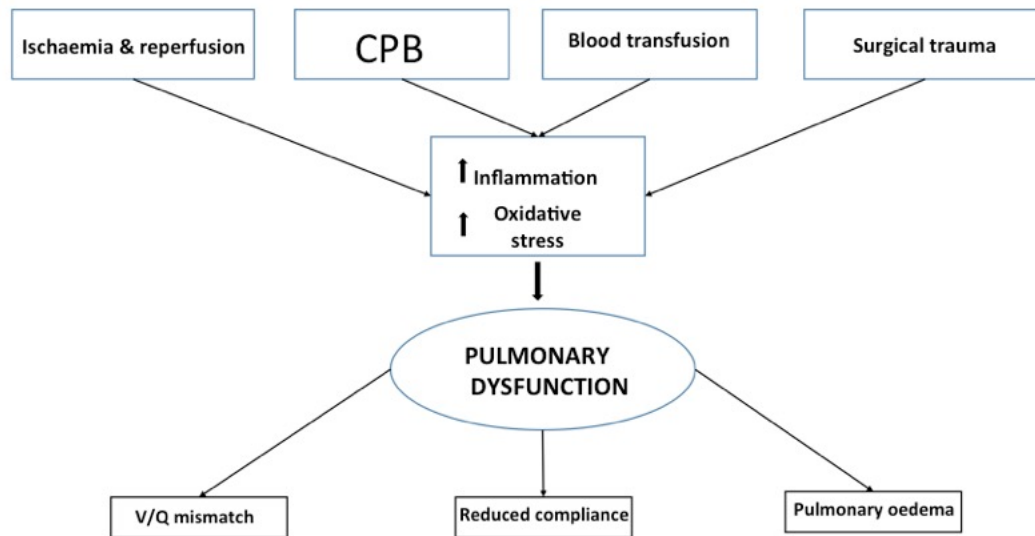
The cascade of proinflammatory chemokine and cytokine release activated by lung inflammation along with increased metabolic rate may upregulate oxidative stress and ROS (Figure II.1)

The term “oxidative stress” is frequently used to describe the imbalances in redox couples such as those reduced to oxidized glutathione (GSH/GSSG) or

NADPH/NADP⁺ ratios [66]. ROS are thought to be the major factor responsible for the alteration of macromolecules and their function during oxidative stress [67]. ROS are generated as by-products of cellular metabolism, primarily in the mitochondria, and include free radicals such as superoxide anion ($O_2^{\cdot -}$), perhydroxyl radical ($HO_2^{\cdot}$), hydroxyl radical ($\cdot OH$), NO, and other species such as hydrogen peroxide (H_2O_2), singlet oxygen (O), hypochlorous acid (HOCl), and peroxynitrite ($ONOO^-$) [1, 66, 68, 69].

To prevent damage from ROS, cells possess several antioxidant enzymes such as superoxide dismutases MnSOD and Cu/ZnSOD, which are located in the mitochondria and the cytosol, respectively, where they convert superoxide into hydrogen peroxide [70]. The reactive intermediates, produced by oxidative stress, can alter the membrane by layers and cause the lipid peroxidation of polyunsaturated fatty acids (PUFA) leading to the formation of lipoperoxyl radical ($LOO^{\cdot}$), which, in turn, reacts with a lipid to yield a lipid radical and a lipid hydroperoxide (LOOH). LOOHs are unstable: they generate new peroxy and alkoxy radicals and decompose to secondary products [66]. The lipid peroxidation and the breakage of lipids with the formation of reactive compounds can lead to changes in the permeability and fluidity of the membrane lipid bilayer and can dramatically alter cell integrity [1, 66, 71] This results in a feed-forward cycle, leading to more lipid peroxidation products, increased DNA damage and enhanced protein modification. These changes trigger a vicious cycle, and further exaggerate lung inflammation [1].

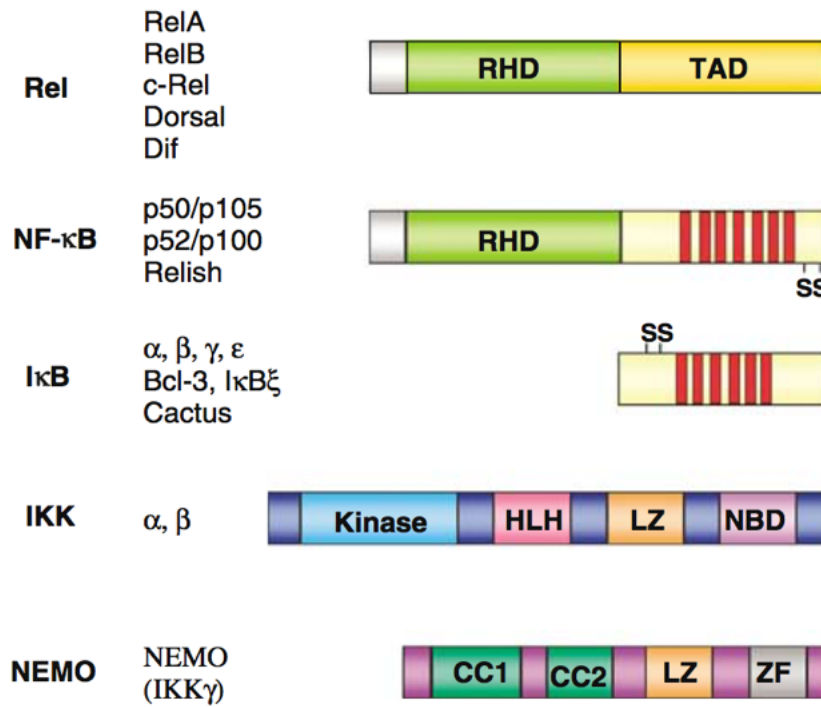
Figure II.1: Mechanisms involved in pulmonary dysfunction during cardiac surgery and its consequences. Taken from reference [1].



II.1.4 NUCLEAR FACTOR-KAPPAB (NF- κ B) AS A BIOMARKER IN LUNG DYSFUNCTION

The NF- κ B transcription factor family in mammals consists of five proteins, p65 (RelA), RelB, c-Rel, p105/p50 (NF- κ B1), and p100/52 (NF- κ B2) [72-74]. These transcription factors are related through an N-terminal DNA-binding/dimerization domain, called the Rel homology domain, allowing them to form homodimers and heterodimers, which can bind to a variety of target DNA sequences called κ B-RE to modulate gene expression [75] (Figure II.2).

Figure II.2: The structure of core NF- κ B signalling proteins. Taken from reference [74].



The generalised structures of the two subfamilies (Rel and NF- κ B) of NF- κ B transcription factors are shown at the top of Figure. II.2. All have a conserved DNA-binding/dimerization domain called the Rel homology domain (RHD), which also has sequences important for nuclear localization and I κ B inhibitor binding. The C-terminal halves of the Rel proteins have transcriptional activation domains. The C-terminal halves of the NF- κ B subfamily proteins have ankyrin repeat-containing inhibitory domains (Fig. II.2; red bars), which can be removed by proteasome-mediated proteolysis. As with the C-terminal domains of the NF- κ B proteins, the independent I κ B proteins consist mainly of ankyrin repeats, and several (I κ B α , I κ B β , I κ B ϵ and I κ B γ) have two N-terminal serine residues (S) that inhibit κ B kinase (IKK) phosphorylation sites, which signal the protein for ubiquitination and degradation. The generalised structures of IKK α and β (kinase domain; HLH, helix-loop-helix; LZ, leucine zipper; NBD, NF- κ B essential modulator binding domain) and of NEMO/IKK γ (CC, coiled coil; LZ, leucine zipper; ZF, zinc finger) are also shown in Figure. II.2 [74].

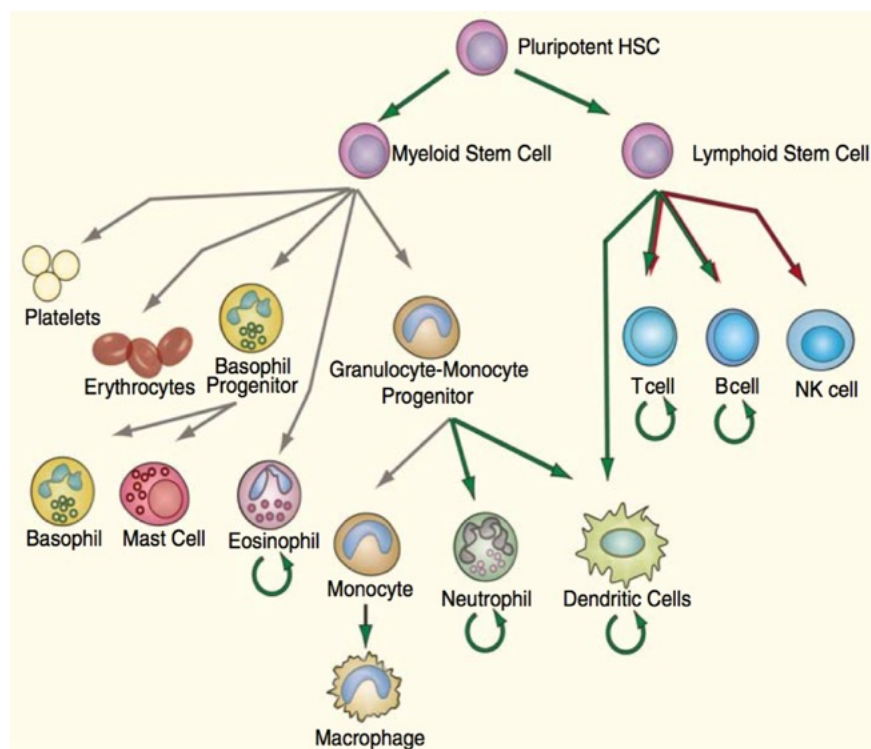
II.1.4.1 Origin and function of NF- κ B

Sen and Baltimore [76] first identified NF- κ B as a regulator of κ light chain expression in mature B and plasma cells, and discovered that it was induced in response to exogenous stimuli in various cell types. NF- κ B executes critical cell-autonomous functions within lymphocytes as well as within supportive cells (such as antigen-presenting cells or epithelial cells). Indeed, it is now known as a critical signalling mediator of most inflammatory processes. It is vital for developing lymphocytes during maturation and has anti-apoptotic functions that are vital for lymphocytes health even after they mature [77]. Adoptive transfer of hematopoietic stem cells compromised in their ability to activate NF- κ B fail to reconstitute lymphocytes in lethally irradiated hosts [78, 79].

NF- κ B is expressed in almost all cell types and tissues, and specific κ B-REs are present in the promoters/enhancers of many genes. As a result, the NF- κ B controls the transcription of cytokines and antimicrobial effectors and genes that regulate cellular differentiation, survival and proliferation [75, 80]. Hence, they regulate various aspects of both the innate and adaptive immune responses. NF- κ B also contributes to the development and survival of immune cells and tissues [75] (Figure II.3) and particularly in the development and function of primary and secondary lymphoid tissues. Primary (central) lymphoid organs include the bone marrow and thymus whereas secondary (peripheral) lymphoid organs include lymph nodes (LNs), Peyer's patches, mucosal-associated lymphoid tissue (MALT) and the spleen [75]. Therefore, NF- κ B is essential for many of the responses of

mature lymphocytes to invading pathogens and plays an important function in shaping the immune system to generate adaptive responses to pathogens [77]. This is in addition to its role in controlling the inflammatory response and cellular function of many structural cells and in cancer [81].

Figure II.3: Schematic of NF- κ B function in hematopoiesis. Taken from reference [75].



Red arrows (Figure. II.3) indicate the stages in which NF- κ B activation is thought to contribute negatively and green arrows indicate a positive function in the development of the indicated lineages. Curved arrows indicate examples in which NF- κ B contributes to the survival of the mature cell population, either in the resting state or during immune responses. Gray arrows indicate developmental events for which NF- κ B plays no role or for which the role of NF- κ B has not been clearly demonstrated [75].

It is now accepted that NF- κ B plays a critical role in mediating responses to a remarkable diversity of external stimuli and is a pivotal factor in multiple immune, physiological and pathological processes including transcription of adaptive immune response genes, hematopoiesis, lymphoid organogenesis and innate immunity [72, 75].

II.1.4.2 The role of NF- κ B in inflammation

The immune function and defence mechanism against microbes' invasion hence is crucial element in the recovery period post cardiac surgery. NF- κ B is the major pathway linking pathogen recognition to proinflammatory cytokine production. Epithelial or stromal cells of infected tissue or tissue resident hematopoietic cells such as mast cells or dendritic cells recognise an inflammatory stimulus (e.g. bacterial and viral infections) and propagate proinflammatory signals through the recognition of microbial or viral products by Toll-like receptors [75]. These signals lead to the recruitment and activation of effector cells, initially neutrophils and later macrophages and other leukocytes, resulting in the tissue changes characteristic of inflammation such as redness, heat, pain, swelling and loss of function [75]. NF- κ B is activated in response to Toll-like receptors activation and is responsible for the transcription of genes encoding many proinflammatory cytokines and chemokines, cell adhesion molecules, factors of the complement cascade and acute phase proteins [82]. One consequence of this inflammatory cascade, the expression of adhesion molecules on leukocytes and EC, enables the extravasation of leukocytes from the circulation to the site of infection [83].

Moreover, NF- κ B is a required element for the survival of these cells which must function to maintain the immune defence mechanism [84].

In contrast, NF- κ B is also essential for the production of the enzymes that generate prostaglandins and ROS e.g. iNOS and COX-2 and may, in a feed-forward mechanism, be involved in the signalling induced by prostaglandins [85]. Thus, NF- κ B activation leads to ROS production which, in turn, increases the production of NF- κ B. Furthermore, NF- κ B activation can be induced further upon physical or oxidative stresses resulting from cardiac surgery using CPB, UV- or γ -irradiation, physiological (ischemia and hyperosmotic shock) or by numerous inflammatory cytokines and chemokines [72].

II.1.4.3 NF- κ B pathways

NF- κ B signalling is primarily regulated by inhibitor of κ B (I κ B) proteins and the IKK complex through two major pathways: the canonical (classical) and non-canonical (alternative) NF- κ B pathways.

The classical or canonical pathway of NF- κ B is activated by the stimulation of a variety of cell membrane receptors such as the tumour necrosis factor (TNF) receptor (TNF-R), the IL-1 receptor, Toll-like receptors (TLRs), the TCR and the BCR. Receptor activation leads to a phosphorylation cascade resulting in phosphorylation, ubiquitination, and proteasomal degradation of I κ B [73, 86-88] (Figure II.4). In the canonical NF- κ B pathway, NF- κ B dimers such as p50/RelA (p65) are kept in the cytoplasm by interaction with I κ B molecules (often I κ B α).

Binding of a receptor ligand to a cell surface receptor such as TNF-R or TLRs leads to receptor rearrangement and the recruitment of receptor adaptor proteins such as TNF receptor-associated factors and receptor interacting protein to the cytoplasmic domain of the receptor (Figure. II.4).

In turn, these adaptors can recruit an IKK complex containing the α and β catalytic subunits and two molecules of the regulatory scaffold, NEMO, directly onto the cytoplasmic adaptors via utilizing the K63-ubiquitin-binding activity of NEMO. This clustering of molecules at the receptor activates the IKK complex. IKK then phosphorylates $\text{I}\kappa\text{B}\alpha$ at two serine residues (S32 and S36), which results in K48 ubiquitination and $\text{I}\kappa\text{B}\alpha$ degradation by the proteasome. Loss of $\text{I}\kappa\text{B}\alpha$ from the complex reveals the NF- κ B nuclear localization signal allowing NF- κ B to enter the nucleus and switch on target genes. The auto-regulatory aspect of the canonical pathway, wherein NF- κ B activates the expression of the $\text{I}\kappa\text{B}\alpha$ gene that leads to re-sequestration of the complex in the cytoplasm by the newly synthesized $\text{I}\kappa\text{B}$ protein is not shown [74].

The alternative NF- κ B pathway proceeds via phosphorylation and proteolytic processing of NF- κ B2 (p100) and most commonly leads to activation of the heterodimer RelB/NF- κ B2 (p52) [88, 89] (Figure II.5).

The alternative pathway usually activates p100/RelB complexes during B-cell and organ development. This pathway differs from the canonical pathway in that only certain receptor signals such as Lymphotoxin B, B-cell activating factor and CD40 activate this pathway and because it proceeds through an IKK complex that

contains two IKK α subunits but not NEMO. In the non-canonical pathway, receptor binding leads to activation of NF- κ B-inducing kinase (NIK), which phosphorylates and activates an IKK α complex, which in turn phosphorylates two serine residues (S866 and S870) adjacent to the ankyrin repeat C-terminal I κ B domain of p100, leading to its partial proteolysis and liberation of the p52/RelB complex (Figure. II.5) [74].

II.1.4.4 The termination of NF- κ B activity

In most unstimulated cells, NF- κ B dimers are retained in an inactive form in the cytosol through interaction with I κ B proteins. Degradation of I κ B upon their phosphorylation by the relevant IKK complex leads to NF- κ B nuclear translocation and target-gene induction [72, 75]. The best studied and accepted mechanism for termination of the NF- κ B response involves the synthesis of I κ B proteins induced by activated NF- κ B. Newly synthesized I κ B α can enter the nucleus, remove NF- κ B from DNA and enable export into the cytosol [73, 90]. Other inhibitors of the NF- κ B pathway are also induced by NF- κ B including A20 and Cezanne [91].

Figure II.4: The classical NF- κ B pathway. Taken from reference [74]

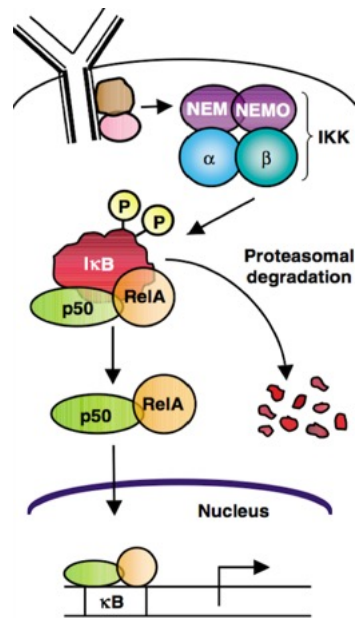
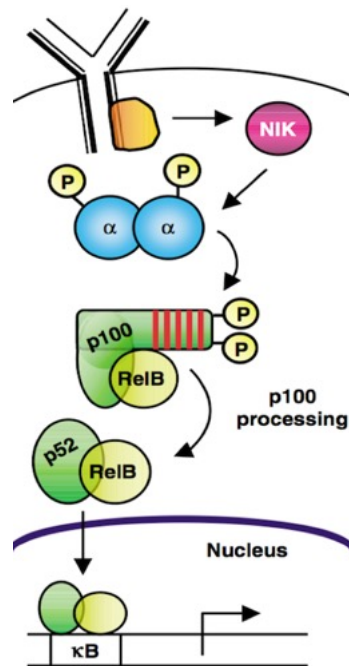


Figure II.5: The alternative NF- κ B pathway. Taken from reference [74]



II.2 Pulmonary protection strategies in cardiac surgery

II.2.1 PHARMACOLOGICAL INTERVENTIONS

Since inflammation and oxidative stress play a pivotal role in the development of pulmonary dysfunction, multiple studies have been performed on modulating these events by the administration of vasodilators, antioxidants and anti-inflammatory medications in animal models or in patients undergoing cardiac surgery [1].

Prostacyclins can induce relaxation of vascular smooth muscle cells by the release of intracellular adenosine 3',5' cyclic monophosphate resulting in pulmonary and systemic vasodilation [92-95]. Moreover, they can have an inhibitory effect on platelet aggregation and leukocyte and monocyte activity [96]. The use of inhaled prostacyclins (Epoprostenol or Flolan) decreased pulmonary arterial EC dysfunction induced by CPB in experimental studies [97-99]. Furthermore, when administered before CPB, they can reduce pulmonary hypertension and may result in a lower rate of re-intubation in high-risk postoperative cardiac patients [100, 101].

Nonselective phosphodiesterase inhibitors such as pentoxifylline can result in elevated levels of intracellular adenosine 3',5' cyclic monophosphate and vasodilatation. Although pentoxifylline has been used classically for claudication symptoms in peripheral vascular disease [102], it is an antioxidant that reduces inflammation, resulting in the modulation of acute lung injury [103-105]. Increased pulmonary vascular resistance and right ventricular selective phosphodiesterase

inhibitors such as nebulized and inhaled milrinone can be an advantageous therapeutic strategy for cardiac surgery patients with respiratory failure. It can cause selective pulmonary vasodilation and potentiate the vasodilation effects of inhaled prostacyclin [106, 107].

NO plays a pivotal role in vascular EC homeostasis and in the regulation of oxidative stress and inflammatory responses [108]. Ischaemia and reperfusion injury during surgery is associated with significant loss in NO and NO preconditioning has been suggested to reduce perioperative pulmonary dysfunction and its sequelae [109-111]. The protective effects may be due to reversal of post ischemic lung hypo-perfusion and a reduction of lung neutrophil sequestration. The administration of NO to patients with severe left ventricular dysfunction can lead to pulmonary vasodilatation and may augment left ventricular filling [112-114]. The timing of administration and/or concentration of inhaled NO during ischaemia or reperfusion periods is an important determinant of its effect as NO is toxic early in reperfusion, due to its interaction with superoxide which may lead to alveolar type 2 cells damage [115, 116].

Many other drugs have been used with various degrees of success. Aprotinin (serine proteases) had shown to reduce neutrophil elastase, malondialdehyde and proinflammatory cytokines levels in bronchoalveolar lavage fluids of patients undergoing cardiac surgery [117]. The use of Aprotinin can result in improved lung function and reduced reperfusion lung injury [118]. The administration of corticosteroids before CPB may reduce the activation of multiple pro-inflammatory mediators. The translation of proinflammatory mediator changes into clinical

outcomes remains controversial and most of the evidence in the literature originates from small randomised clinical trials or observational studies with biomarkers as primary end points [119, 120].

II.2.2 CPB MODIFICATION

Different strategies have been attempted over the years to minimise pro-inflammatory activation and oxidative stress during CPB such as coating the circuit with biocompatible material (heparin, poly-2-methoxyethyl acrylate, synthetic protein and phosphorylcholine), removal of leukocytes (by adding special filters to the CPB), ultrafiltration and reduced haemodilution [1].

Heparin is thought to reduce the inflammatory responses linked to platelets and leukocytes by reducing the release of IL-6, IL-8, E-selectin, lactoferrin, myeloperoxidase, integrin, selectin, and platelet thromboglobulin, and decreasing the production of oxygen free radicals [121-123]. It has been suggested that compared with conventional circuits (poly-2-methoxyethyl acrylate, synthetic protein and phosphorylcholine), the heparin-coated circuit may improve lung compliance and pulmonary vascular resistance and thus reduce intrapulmonary-shunt although intubation time and cardiac intensive care unit stay were not affected [1, 124, 125].

The use of leukocyte filtration mechanisms can modulate proinflammatory cytokines and oxidative stress [126-128]. A clinical study compared the effectiveness of leukocyte filter depletion with a common arterial filter in CABG patients reported better oxygenation indices and shorter duration of postoperative

mechanical ventilation in the leukocyte depletion filter group [1, 128]. Another study suggested that leukocyte depletion filters, preferentially remove activated leukocytes. Improvement in lung function was evident only in the early postoperative phase, however, this did not lead to decreased mortality or better clinical outcomes [1, 129]. The use of ultrafiltration or modified ultrafiltration techniques at the end of surgery may reduce postoperative oedema, specifically in the lungs, resulting in better oxygenation and improved lung compliance postoperatively. Furthermore, ultrafiltration may remove proinflammatory mediators from the circulation such as IL-6, and IL-8, although this did not result in significant clinical improvement [37]. Controlled haemodilution to regulate oncotic pressure can reduce priming volumes and result in better haemodynamic parameters such as vascular resistance and higher oxygen delivery and reduce hospital-stay significantly [130, 131].

Understanding the problems associated with pulmonary ischaemia and reperfusion has resulted in attempts to provide continues pulmonary perfusion during CPB. Experimental animal models of pulmonary perfusion demonstrated reduced inflammatory and apoptosis pathway activation with such a strategy [56, 132, 133]. Moreover; pulmonary perfusion was found to improve lung compliance, oxygenation, and vascular resistance in patients undergoing CABG [134, 135]. Furthermore, pulmonary artery perfusion during CPB can be effective in reducing postoperative right ventricular dysfunction in high-risk patients undergoing left ventricular assist device placement [136]. The translation of such changes into better clinical outcomes remains unclear and may be restricted to a specific group

of patients as demonstrated by a recent trial in patients with chronic obstructive pulmonary disease undergoing CPB where no significant protective effect on lungs was documented [137].

The deleterious effects of surface contact activation, as discussed previously has led to the development of the minimised CPB circuit (mini-CPB). This is characterised by reduced surface area and priming volume and the prevention of air-blood contact. The utilisation of mini-CPB has been shown to be associated with attenuated production of pro-inflammatory cytokines and complement activation and blunted leukocytes activation compared to a conventional circuit. Markers of oxidative stress tend to be reduced in patients undergoing surgery using a mini-CPB compared to a conventional circuit [138, 139]. Additionally, mini CPB reduces organ damage and results in better postoperative gas exchange and lower lung injury scores [140, 141]. Unfortunately, most of the clinical trials investigating the role of mini-CPB have evaluated diverse technologies of varying complexity and degree of miniaturisation, which, not unexpectedly, have given rise to heterogeneity in the findings [1]. The lack of definition of the components used, the management of the system and the small sample sizes involved make it difficult to be certain of the validity any benefits that have been attributed to these devices.

II.2.3 SURGICAL STRATEGIES

It has been suggested that maintaining a degree of lung ventilation during CPB may be better than the usual standard of no lung inflation [1]. The use of CPAP

during CPB may result in less shunt and better gas exchange [142], however, it seems that such effect is dependent on the airway pressure used. Using LFV along with CPAP during CPB to reduce lung injury has been evaluated in an experimental pig model [143]. This study showed that the use of LFV is associated with significantly better pulmonary gas exchange, higher ATP, lower lactate dehydrogenase levels and reduced histological damage in lung biopsies as well as lower DNA levels in bronchoalveolar lavage, compared to the collapsed lung control group. However, a clinical study in patients undergoing cardiac surgery compared the effect of low volume ventilation to the conventional strategy (no ventilation) did not show significant changes in PVRI, $\text{PaO}_2/\text{FiO}_2$ ratio, postoperative length of stay and postoperative pulmonary complications [1, 144]. Furthermore, a meta-analysis of 814 cases across 16 randomised clinical trials looking at three lung protective strategies in patients during CPB including CPAP, low-volume ventilation, or VC manoeuvres during CPB showed that effects are probably short lived with a questionable impact on long-term clinical outcomes [145].

Off pump coronary artery bypass (OPCAB) or "beating heart" surgery seems to provide better lung protection by eliminating ischemia-reperfusion injury through maintaining lung ventilation and avoiding CPB machinery. Many studies consistently reported better early and midterm outcomes in OPCAB when compared with conventional on pump CABG: fewer respiratory complications, shorter intubation time and cardiac ICU stay, reduced incidence of pneumonia, and overall shorter hospital stay [146-148]. Nevertheless, we have to consider the

limitations of OPCAB including being technically more difficult, the potential of fewer grafts and incomplete revascularization and, in addition, the risks of conversion to on-pump surgery include ischemia, inability to visualize a target vessel or hemodynamic instability [149].

II.2.4 PHYSIOTHERAPY

Preoperative prophylactic physiotherapy with inspiratory or expiratory muscle training can be used as a preventative measure for lung protection [150, 151]. Physiotherapy is used prophylactically after cardiac surgery. Different breathing techniques can be utilised during this period to improve ventilation-perfusion inequalities, increase pulmonary compliance and help re-inflate collapsed alveoli.

These techniques include deep breathing exercises, slow maximal inspirations with an inspiratory hold, intermittent deep breathing exercises with and without the use of incentive spirometer and deep breathing exercises with expiratory resistance [1, 152-155].

II.2.5 POST-OPERATIVE NIV

NIV refers to the administration of ventilatory support without using an invasive artificial airway (endotracheal tube or tracheostomy tube). NIV exerts its main effects on the pulmonary and on the cardiovascular system through the application of PEEP, with or without a pressure support during inspiration, NIV restores lung volumes by opening atelectatic areas, increases alveolar ventilation and reduces the work of breathing [1, 156-158].

CPAP aims to maintain a level of positive airway pressure in a spontaneously breathing patient. It is functionally similar to PEEP, except that PEEP is an applied pressure against exhalation and CPAP is a pressure applied by a constant flow. The ventilator does not cycle during CPAP, no additional pressure above the level of CPAP is provided, and patients must initiate all of their breaths. To avoid drying of the respiratory mucosa, there has been general agreement that the application of humidified CPAP helps to recruit the lungs by increasing functional residual capacity, increases the surface area of lung, decrease intra-pulmonary shunt and improves oxygenation [1, 159-161].

BiPAP is a CPAP with pressure supported breaths. It delivers a pre-set inspiratory positive airway pressure (IPAP) during inspiration and EPAP. BiPAP can be described as CPAP with a time-cycled or flow-cycled change of the applied pressure level [162]. BiPAP senses patients breathing efforts by monitoring air flow in the patient's circuit and adjusts its output by assisting inspiration. Therefore its physiological effects can benefit the patient in both phases of respiration [26, 163, 164]. BiPAP can only be applied to conscious, co-operative and hemodynamically stable patients who can breathe spontaneously, have an adequate gag and cough reflex and who can remove the mask when required. BiPAP reduces pulmonary complications and overall length of hospital stay after cardiac surgery [165-167]. Furthermore; the prophylactic use of BiPAP after early extubation is safe and effective [167, 168]. A better tolerance was noted when BiPAP settings were commenced at low levels and gradually adjusted to achieve

the therapeutic target. Radiological improvement of atelectasis after cardiac surgery has been achieved on maintaining 8-10ml/kg of TV with BiPAP [169].

II.3 Research hypotheses, aims and objectives

II.3.1 THE BiPAP TRIAL: A RANDOMISED CONTROLLED TRIAL EXPLORING THE EFFECT OF ADDING POSTOPERATIVE NON-INVASIVE VENTILATION TO USUAL CARE TO PREVENT PULMONARY COMPLICATIONS IN PATIENTS WHO HAVE UNDERGONE CABG.

We hypothesised that BiPAP after CABG would shorten the duration of post-operative hospital stay by improving lung physiology and gas exchange and reducing basal atelectasis.

The aims and objectives were to compare the efficacy of NIV with BiPAP added to usual care versus usual care alone in patients undergoing CABG. A randomised clinical trial was therefore, designed to quantify the effect of the addition of NIV with BiPAP compared to usual care alone on the speed of recovery in patients undergoing after isolated CABG for the first time.

II.3.2 THE VENTILATE TRIAL: A RANDOMISED CONTROLLED TRIAL EXPLORING THE EFFECT OF LOW FREQUENCY VENTILATION (LFV) DURING CARDIOPULMONARY BYPASS FOR PULMONARY PROTECTION IN PATIENTS UNDERGOING CABG.

We hypothesised that using LFV during CPB would result in reduced inflammatory activation, lung dysfunction, and ischemia/reperfusion injury compared with no ventilation of the lungs during CPB.

The aims and objectives were to investigate the effects of LFV during CPB on markers of inflammatory activation (ischemia/reperfusion injury) in the lung and peripheral blood.

II.3.3 STRUCTURE AND FUNCTION OF ISOLATED HUMAN PULMONARY ARTERY PRE- AND POST-CPB SURGERY

In a subgroup of patients from the Ventilate RCT described above, the structure and function of pulmonary arteries isolated from lung biopsies were investigated. Intralobular small arteries were dissected, cannulated, pressurized and imaged using confocal microscopy. Vasoconstriction, vasodilation and smooth muscle cell distribution and viability were compared in pre- and post-CPB biopsy samples. The aim was to compare structural and functional assessments of human pulmonary arteries before and after CPB in patients undergoing CABG.

**CHAPTER III. THE BIPAP TRIAL: A RANDOMISED
CONTROLLED TRIAL OF EFFECT OF ADDING
POSTOPERATIVE NON-INVASIVE VENTILATION
TO USUAL CARE TO PREVENT PULMONARY
COMPLICATIONS IN PATIENTS UNDERGOING
CORONARY ARTERY BYPASS GRAFTING [168]**

III.1 Abstract

Objective: To compare the efficacy of NIV with BiPAP added to usual care versus usual care alone in patients undergoing CABG.

Design: Two-group parallel randomised controlled trial.

Setting: Cardiac surgery department.

Participants: Patients undergoing elective CABG.

Intervention: NIV using BiPAP immediately after extubation and usual care (chest physiotherapy, nebulised bronchodilators, coughing exercises, nebulised saline, mobilisation and incentive spirometry).

Comparator: Usual care alone.

Main outcome measures: The primary outcome was time until fit-for-discharge. Secondary outcomes were the partial pressure of carbon dioxide in arterial blood (PaCO_2) and FEV1, atelectasis, adverse events, duration of intensive care stay and actual postoperative stay.

Results: 129 patients recruited between February 2008 and February 2011 were randomly allocated to BiPAP (66) or usual care (63). Three patients allocated to BiPAP withdrew. The median duration of BiPAP was 16 hrs (IQR, 11 to 19, range 0 to 22). The median duration of hospital stay until fit-for-discharge was 5 days for the BiPAP group (IQR, 4-6) and 6 days for the usual care group (IQR, 5-7; hazard ratio of 1.68, 95% CI 1.08 to 2.31, $p=0.019$). There was no significant difference in the duration of intensive care or post-operative stay. There was no difference in

mean percentage predicted FEV₁ on day 3 (BiPAP group, mean 43%; usual care group, mean 40%; adjusted mean difference 4%, 95%CI -3% to 12%, p=0.24). Mean PaCO₂ was significantly reduced 1 hour after BiPAP application but there was no overall difference between the groups up to 24 hrs (BiPAP group, mean 4.91 kPa; usual care group, mean 4.95 kPa; adjusted mean difference -0.06 kPa, 95%CI -0.25 to 0.13, p=0.54). Basal atelectasis occurred in 15 (24%) patients in the usual care group and 2 (3%) patients in the BiPAP group. Overall, 30% of patients in the BiPAP group experienced an adverse event compared to 59% in the usual care group.

Conclusions: BiPAP at extubation improved recovery time in patients undergoing elective CABG surgery.

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III.2 Introduction

The long-term benefits of CABG for patients with multi-vessel coronary heart disease are well established but they still need to be weighed against the possible iatrogenic harm from surgery. CABG interferes with pulmonary function, with basal atelectasis detected in as many as 94% of patients in the first 48h after surgery [24, 170]. Only a minority of these complications can be attributed to the pre-disposing factors of chronic obstructive pulmonary disease, smoking and obesity. Basal atelectasis is associated with increased intrapulmonary shunting, increased PA-a O₂, decreased lung compliance, a higher incidence of infection and prolonged hospital stay [24, 171].

Post-operative physiotherapy exercises to improve lung function, often incorporating feedback to patients about their respiratory effort (IS), are now a standard part of the care pathway after CABG surgery. The term "enhanced recovery" comprises various evidence-based individual components of perioperative care and is aimed at improving patient outcomes and recovery following surgery. During the past few years the concept of enhanced recovery [172] has become more accepted within anaesthetic and surgical circles. There is interest in the role of NIV after cardiac surgery given the benefits of NIV in respiratory failure in other clinical settings, e.g. exacerbations of chronic obstructive pulmonary disorder [173, 174], pneumonia [175, 176], cardiogenic pulmonary oedema [177-180], acute congestive heart failure (CHF) [181, 182] and in the perioperative period [183-185].

There is some evidence that NIV in the period after extubation may be beneficial. For example, preventive use of CPAP or BiPAP can reduce the degree of post-operative lung restriction and venous admixture [186]. BiPAP is a method of non-invasive ventilatory support developed from CPAP in the 1990s. It matches the patient's respiratory cycle by generating two different positive airway pressure levels [187] and can improve gas exchange and oxygenation [188, 189]. This improvement has been reported to be better with BiPAP than with CPAP due to improved alveolar recruitment and pulmonary compliance and a large decrease in the work of breathing [190]. Two recent trials of BiPAP after CABG have shown reductions in atelectasis but no improvement in other clinical measurements such as lung function [169, 191]. Also, intermittent NIV ventilation administered post-operatively for up to 3 hrs duration has been suggested as part of standard clinical practice despite the lack of substantial published evidence to support the practice [192].

A review aimed to assess the effects of IS to prevent postoperative pulmonary complications after CABG, identified trials that confounded the effects of IS with co-interventions, including NIV [193]. All previous trials were consistent and positive with respect to the reduction in atelectasis and all suggested a clinical benefit with BiPAP, yet extended treatment was thought to be infeasible due to presumed poor tolerance of patients to BiPAP masks. NIV was not started until 4 hrs after extubation and was applied for only 1h in every 3h [186]. NIPSV was applied four times a day for 30 min [169], and NIV was applied for 0.5 h periods every 6 h in the first postoperative day [191], intermittent 15 min bi-level positive airway pressure (7.3+/-3.6 times per patient) [189]. Hence, these trials did not

optimise the BiPAP strategy with extended treatment to maximise the number of hrs with BiPAP application.

I concluded that the major limitation of all the previous studies addressing the benefit of the NIV post-surgery has been the short or intermittent duration of NIV application. I therefore hypothesised that if short and sporadic BiPAP treatments are beneficial, then longer or continuous treatments could provide greater benefits.

III.3 Hypothesis

We hypothesised that BiPAP after CABG would shorten the duration of post-operative stay by improving lung physiology and gas exchange to a clinically important extent leading to reducing basal atelectasis and speeding recovery. A randomised clinical trial was designed to test this hypothesis.

III.4 Aims and objectives

To compare the efficacy of NIV with BiPAP added to usual care versus usual care alone in patients undergoing CABG.

III.5 Ethics committee approval and registration

This trial was approved by the Hammersmith and Queen Charlotte's & Chelsea research ethics committee (REC reference number 07/Q0406/79) on 04/09/2007. Further approval was obtained from the research and development department of the Imperial College Healthcare NHS Trust. This research complies with the Helsinki Declaration. This trial is registered as ISRCTN No: 85570732.

III.6 Trial design

The trial was conducted at a single centre with two groups; a parallel, pragmatic, and a randomised control trial (Figure III.1). A statistician who had no further role in the trial generated the random allocation sequence by computer (ratio 1:1). Randomisation was in blocks of four to minimise imbalance in the number of participants recruited to the two groups. EAJ enrolled participants and, after eligibility had been checked and written informed consent obtained, randomised them, usually immediately before the operation. Randomisation was performed by looking up the next numbered allocation in the randomisation schedule. Participants' random allocations were not concealed from the research team at the time of recruitment. The clinical staff was notified of the allocation when the patient was in theatre to allow time to prepare the equipment for participants allocated to the BiPAP group. The trial was registered as ISRCTN85570732.

III.7 Outcomes

III.7.1 THE PRIMARY OUTCOME

Time until fit-for-discharge (FFD) was analysed. The duration of the post-operative hospital stay, measured as the number of days from the operation until a participant was judged to be FFD. A judgment of FFD required: (a) normal BP, HR, temperature and oxygen saturation on air; (b) bowels open; (c) back to pre-operative mobility/ reasonable mobility; (d) satisfactory wound healing. FFD was chosen to reflect a participant's overall speed of recovery. It was considered an important measure for patients, clinicians and health services that would be unaffected by non-medical factors. Time until FFD and complications were

assessed by the clinical team. EAJ extracted the data from the notes on to the data collection forms. The clinical team was blinded to the random allocation.

III.7.2 THE SECONDARY OUTCOMES

Secondary outcomes measured included respiratory physiology (arterial pCO₂ and FEV₁), atelectasis scored clinically and radiologically, occurrence of complications, cardiac intensive care unit length of stay, and time until actual discharge. pCO₂ was measured immediately after extubation and subsequently after 1 hour, 12 hrs and 24h. FEV₁ was measured as early as practicable, i.e. on day 1 if possible, and on days 2 and 3 after the operation. Details of participants at randomisation, date when FFD, date of actual hospital discharge and other secondary outcomes were recorded on custom data collection forms by the clinical staff in the cardiac ICU and wards.

Spirometry was performed on days 1, 2 and 3 after surgery by the research fellow conducting the trial. However, due to sternotomy wound pain and despite several attempts to achieve acceptable manoeuvres, the ERS criteria for acceptable spirometry [194] was only met in a minority of patients. The values were markedly reduced in both groups postoperatively because of wound pain and the difficulty in performing forced manoeuvres in these patients [168].

Atelectasis was assessed on day 1 after surgery by the senior clinical team who were not involved in the trial but responsible for the patients' care in the cardiac CICU. The presence or absence of atelectasis was based on standard clinical and chest X-ray criteria including radiologists' reports on chest X-rays. The radiologists were blinded to the treatment allocation. The clinical research fellow conducting

the trial had no role in this decision-making process or in subsequent management decisions.

Post-operative complications other than atelectasis were recorded on the data collection form. Serious complications were itemised, and other complications were recorded as free text and classified at the analysis stage.

III.8 Materials and Methods

III.8.1 ANAESTHETIC PROTOCOL

Patients were premedicated with 20-30mg temazepam. Anaesthesia was induced with propofol and remifentanyl (5mg remifentanyl to 100ml propofol) using pancuronium 0.1 mg/kg for muscle relaxation. This was maintained by infusion of propofol and remifentanyl (5mg remifentanyl to 1g propofol), with isoflurane if required to keep the entropy value of the processed EEG below 55. The volume control settings for ventilation pre-CPB were: TV of 6-8 ml.kg⁻¹, I:E of 1:2, PEEP of 5cm H₂O and FiO₂ of 0.5 (range 45%-55% O₂). The ventilatory rate was set to keep the PaCO₂ between 4.5 and 5.5 kPa.

III.8.2 PERFUSION PROTOCOL

CPB and cardioplegic arrest was carried out as follows: a standard CPB circuit was used, primed with 1400ml of Hartmann's solution and 10000 IU of heparin. Non-pulsatile flow rates throughout bypass were maintained according to the Departmental standard operating procedure. The systemic temperature was maintained between 32°C and 35°C. Cardioplegic arrest was achieved with intermittent antegrade cold blood cardioplegia.

III.8.3 PARTICIPANTS

The reference population was patients referred to the Hammersmith Hospital for isolated first time CABG using CPB and mild hypothermia (32°C).

A participant may enter the study if ALL of the following apply

1. Age ≥ 40 and < 85 years
2. Undergoing any elective or urgent CABG with CPB and cardioplegic arrest.
3. Left ventricular ejection fraction $> 30\%$

A participant was excluded from the study if ANY of the following apply

1. Previous pulmonary embolism requiring long-term warfarin for ≥ 3 months
2. Previous cardiac surgery
3. Current congestive heart failure (NYHA class IV)/cardiogenic shock
4. Chronic renal failure requiring dialysis
5. Emergency or salvage operation
6. On corticosteroid or immunosuppressive treatment
7. Severe chronic obstructive pulmonary disorder, lung pathology and previous radiotherapy,
8. BMI > 35

III.8.4 EXTUBATION PROTOCOL

Patients were extubated when they met ALL the following criteria:

Temperature: normothermia (a core temperature range of 36.0°C to 37.0°C)

Cardiovascular: haemodynamically stable and blood loss < 50 mls/h

Respiratory: comfortable breathing pattern, good bilateral air entry (RR 10-20/min, TVs 8-10mls/kg, minimal tracheal suction)

Arterial Blood Gases: $\text{PaO}_2 > 10$ kPa on $\text{FiO}_2 < 50\%$, $\text{PCO}_2 < 7$ kPa, BE -5 to +5

Neurology: conscious, appropriately responsive, obeying command and able to move all limbs

III.8.5 MECHANICAL VENTILATION PROTOCOL

Immediately after the operation, all participants were ventilated using standard methods (Servo I and Servo 300 ventilators providing a TV of 7-10ml/kg). Synchronised intermittent mandatory ventilation (pressure controlled) was employed during weaning with PEEP support of +5 cm if the patient's BMI was less than 30, or PEEP support of +7.5 cm if the BMI was between 30 and 35 kg/m².

III.8.6 INTERVENTION AND COMPARATOR

Participants randomised to usual care alone received chest physiotherapy, nebulised bronchodilators (2.5mg salbutamol every six hrs), coughing exercises, nebulised saline (5ml every six hrs), mobilisation and IS, consistent with standard post-operative practice. The physiotherapy team oversaw this twice a day for the first 2-3 days after surgery. For participants in the BiPAP group, we aimed to maximise duration of BiPAP during the first 24 hrs post-extubation to the maximum number of hrs that each patient can tolerate.

III.8.7 NIV PROTOCOL

The BiPAP trial was conducted using Servo I and Servo 300 ventilators and a Performatrak full face mask (Phillips, Murrysville, USA). Correct selection, sizing and fitting of the mask interface is critical to the success of NIV. Time was spent on the sizing and fitting process and reassuring the patient, helped to maximise patient comfort and compliance. Fitting a face mask was easier when two people from the nursing team were available to assist.

BiPAP mask practical aspects:

1. Fitting Landmarks (Figure III.1)
 - i. Just below the junction of nasal bone and cartilage
 - ii. Corners of the mouth
 - iii. Below the lower lip with mouth open slightly
2. When in doubt, a smaller mask was used. Leaks occur when the mask is too large. Proper sizing results in the use of as many small masks as medium masks
3. We always used a sizing gauge. We either used the sizing gauge from the Performtrak pack or a separate plastic sizing gauge.
4. We removed the correctly sized mask from the pack and checked the headstraps, fully extending them if necessary. The lower swivel connectors were undone avoiding removing the headstrap from the mask. Although it may have been difficult to untangle it was also difficult to reattach correctly. The mask was held in one hand and head strap pulled the over the mask with the other hand.
5. The lower edge of the mask was placed gently against the patients face below the lower lip in the chin groove. The mask was rolled up until the top of the mask rested against the nose. The heads traps were taken backwards over the top of the patient's head and the lower swivel connectors attached.
6. At this stage, it was possible to connect the breathing circuit to the mask and turn on the ventilator. The patient was able to hold the mask in place him/herself before the straps were adjusted to fit.
7. The Velcro on the upper side straps were unfastened and adjusted to fit the patient's head by pulling both gently at the same time to ensure equal tension on both sides. The straps were fitted above the patient's ears. The straps were not overtightened as this caused both leaks and discomfort and reduced the compliance or tolerance to treatment. I ensured that the triangular part of the head strap was positioned centrally on the back of the patient's head.
8. Unfasten the Velcro on the lower side straps. Pulling both together to ensure equal tension on both sides, adjust to fit, ensuring that the back of the headgear fits snugly on the back of the patient's head. Do not overtighten the straps, as overtightening may cause or make leaks worse and may cause discomfort for the patient, reducing compliance or tolerance of therapy. The upper and lower side straps should lie above and below the patient's ears as in the diagram above.
9. Once upper and lower straps are secured, squeeze the adjustment tabs on the forehead support arm. The mask will tip forward so that pressure at the top of the nose is reduced and transferred to the foam forehead support. Ensure the whole area of the forehead support is in contact with the forehead of the patient.
10. The straps and forehead support were adjusted to achieve minimal leaks. In addition, we ensured that there was no eye leak which may have caused eye irritation or injury. BiPAP ventilators are highly leak-tolerant, so masks did not need to be strapped on too tightly. It should be possible to insert a finger between the

lower edge of the mask and the patient's chin. We aimed for a leak rate of 6 to 20 l/m.

11. Once the straps were adjusted comfortably, the whole mask was gently pulled away from the patient's face before being resealed against the face. The outer soft flexible layer of the mask cushion inflated slightly when air was blown into the mask by the ventilator. This further enhanced the mask's seal and minimised the leaks.
12. The straps were readjusted as necessary to minimise leaks as the patient changed position (Figure III.2).

Figure III.1: BiPAP mask fitting landmarks

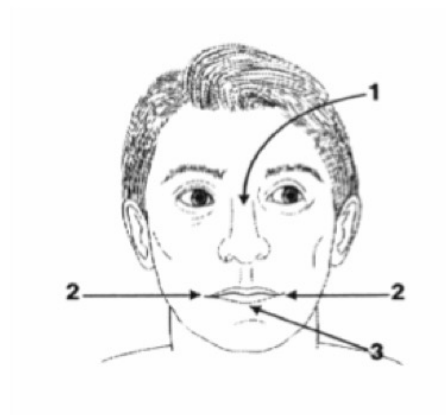
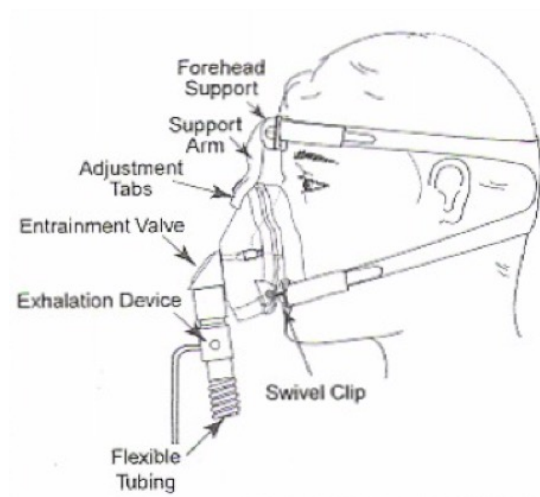


Figure III.2: BiPAP Mask



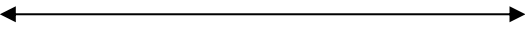
BiPAP mask technical aspects:

The BiPAP settings were pre-determined and the same for all study patients within certain weight categories i.e. -12cm H₂O and +5cm H₂O for inspiratory and expiratory pressure positive airway pressure respectively if the BMI was <30, and 17cm H₂O and 10cm H₂O if the BMI was ≥30. BiPAP was performed by the cardiac intensive care staff who received small group or individual training on 12 occasions before the study started.

III.8.8 RANDOMISATION AND DATA COLLECTION

Data were entered in a computerised database to allow the randomised allocation code to be generated for each study participant. The research clinician 'tracked' each patient and prompted staff to take the samples required at the designated times (Figure III.3) and was responsible for analysing the samples.

Figure III.3: The BiPAP Trial schedule of data collection

	Pre-assessment	Hospital stay										
	30 to -7 days	Admission	Day 0		Day 1			Days 2 to 5				
			Theatre	Extub.	1h	12 h	24 h	30 h	Day 2	Day 3	Day 4	Day 5
Hospital stay												
Inclusion/exclusion criteria	●											
Demographics	●											
Risk factors	●											
Height, weight and BMI	●											
Complete physical examination	●											
Details of study to patient	●											
Vital signs (BP , HR, RR, BT) and ECG	●	●	●	●	●	●	●	●	●	●	●	●
Lung function	●					●			●	●		
Informed Consent		●										
Clinical laboratory tests	●	●	●	●	●	●	●	●	●	●	●	●
Randomisation			●									
Blood Gas			●	●	●	●	●	●				
Operation details: Cross clamp & bypass time			●									
Chest X-ray	●					●					●	
Complications			●	●	●	●	●	●	●	●	●	●
Discharge from unit											●	

III.9 Statistical analysis

III.9.1 ANALYSIS

Primary analyses were by intention-to-treat. The analysis considered that time until FFD, cardiac ICU stay, and actual post-operative stay should be analysed as time-

to-event data. The analysis of repeated PaCO_2 used a mixed regression model to take into account the repeated measurements and to adjust for PaCO_2 at the time of extubation. Mean PaCO_2 levels were also analysed 1 hour after starting NIV to test the effectiveness of administration of BiPAP. Only the day 3 FEV_1 measurements was analysed, using multivariable regression to adjust for the baseline measurement. Power calculations are based on a mean hospital stay of 9 days with conventional management, which can be as long as 13 days in severe COPD [195].

Continuously scaled outcomes are described as means $\pm$ SD for each group. Treatment effects (mean differences between groups and 95% CIs) were estimated from the regression models. Binary/categorical outcomes are presented as frequencies. A 2-sided *P* value <0.05 was considered statistically significant. Statistical analyses were performed using STATA version 11 (Stata Corporation, Texas). Variables for analysis are summarised in Tables III.1, 2 & 3.

III.9.2 INTERPRETATION OF THE SAMPLE SIZE JUSTIFICATION

The target sample size was 126 participants (63 patient in each group). For time until FFD, the trial had 90% power at a 5% 2-tailed significance level to detect a standardised difference of ≥ 0.6 SD in log length of stay based on geometric mean times until FFD of 10 and 8.5 days. For PaCO_2 and FEV_1 , we estimated (retrospectively) that the trial had 90% power at a 5% 2-tailed significance level to detect a standardised difference of ≥ 0.38 SD.

Table III.1: The BiPAP trial baseline / prognostic variables

Baseline/prognostic	Measurement scale	Comment
Age	Continuous data	Calculated from date of birth and date of operation.
Height	Continuous data	
Weight	Continuous data	
BMI	Continuous data	Derived from height and weight
NYHA symptom classification	Categorical data	
Left Ventricular Function	Categorical data	
Pre-op co-morbidities	Categorical data	
Preoperative lung function	Continuous data	Measured by: FEV ₁ (predicted and absolute), VC, Peak Flow, TLC, RV, TLCO, KCO, VA, mouth pressure inspiration, mouth pressure expiration.
Preoperative Arterial Blood Gas	Continuous data	Measured by: pH, pO ₂ , pCO ₂ , BE, K, BM, FiO ₂ .
Peri-operative details	Continuous data	Measured in terms of Bypass and Cross-clamp time.
Duration of intubation	Continuous data	Strictly speaking, time to extubation.

Table III.2: The BiPAP Trial outcome

Outcome	Measurement scale	Comments
Postoperative Arterial Blood Gas	Continuous data	Measured by: pH, pO ₂ , pCO ₂ , BE, K, Hb, BM, FiO ₂ .
Postoperative lung function	Continuous data	Measured by: FEV ₁ (absolute), VC, FiO ₂ , SaO ₂ .
Postoperative blood values	Continuous data	Measured by: WBC, C-reactive protein, albumin.
CICU length of stay	Continuous data	
Adverse events	Categorical data	
Hospital length of stay	Continuous data	Measured from day of operation to fit-for-discharge-date.

Table III.3: The BiPAP Trial descriptor

Descriptor	Measurement scale	Comment
Hrs of BiPAP	Continuous data	duration of BiPAP during. 24 hrs post extubation.

III.10 Results

III.10.1 TRIAL POPULATION

A total of 134 patients were considered for inclusion in the study between February 2008 and February 2011 (Figure III.4).

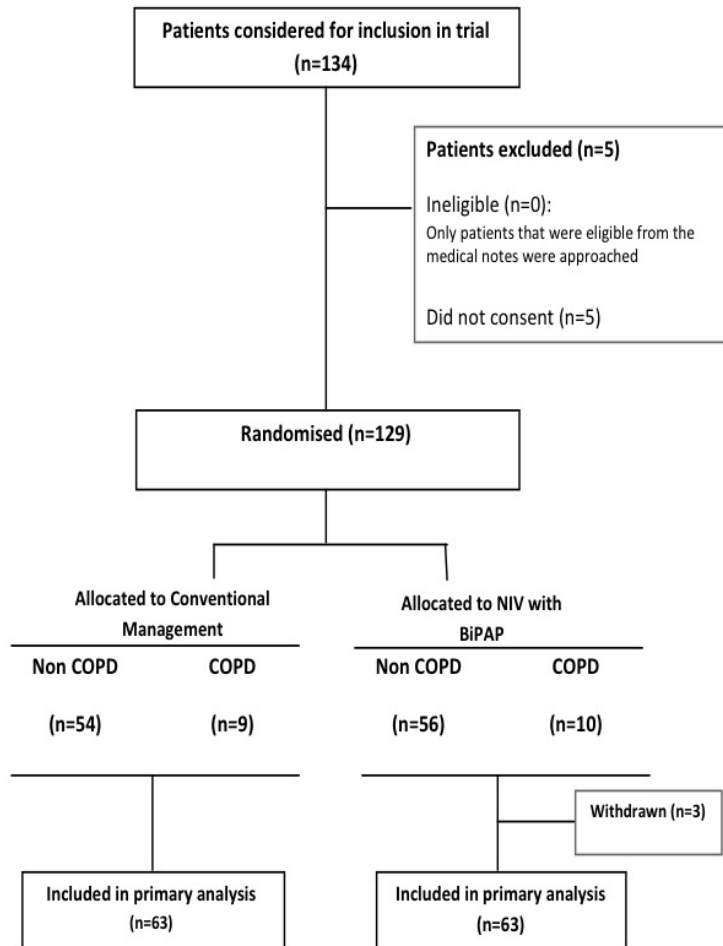
Figure III.4: The BiPAP trial consort chart

Figure III.4 shows all patients received conventional management. For 6 patients requiring noninvasive ventilation support, this included continuous positive air pressure. All received BLPAP but for varying duration (median time on BLPAP, 16 hrs [interquartile range, 11-19]). Three participants asked to be withdrawn because they could not tolerate BLPAP (duration of BLPAP, 0 hrs). After 5 patients were excluded for lack of consent, the trial recruited 129 participants; 66 were randomly allocated to BiPAP and 63 to usual care. Three participants withdrew from the BiPAP group because they were uncomfortable with the mask, giving a total of 126 patients in the intention-to-treat analysis, all of whom received their allocated management. Baseline characteristics, pre-operative co-morbidities and operation details were well balanced between the two groups as shown in (Table III.4) along with baseline respiratory measurements by group (Table III.5). Two patients died after surgery, one in each group (the patient in the BiPAP group died on day 2 and the patient in the usual care group on day 5). For trial participants with inclusion criteria and exclusion criteria please refer to section III.8.3.

III.10.2 DURATION OF VENTILATION

The maximum intubation time was 48h in the usual care group and 18h in the BiPAP group but the median was 6h for both groups (IQRs 5-8 and 4-10 respectively). The median time achieved on BiPAP in the BiPAP group was 16h (IQR, 11 to 19, range 0 to 22).

III.10.3 PRIMARY OUTCOME (TIME UNTIL FIT FOR DISCHARGE)

Follow up for the two patients who died in hospital was obviously stopped at the time of death. The median length of the hospital stay until fit-for-discharge (FFD) was 6 days for the control group (IQR, 5-7) and 5 days for the treatment group (IQR, 4-6), generating a hazard ratio of 1.68 (95% CI: 1.17 to 2.43, $p=0.005$) without adjustment for any covariates. Having observed the possible difference in intubation time between groups, we carried out a post-hoc analysis including intubation time as a baseline covariate, but this only changed the result for BiPAP vs. usual care slightly (median intubation time was 6hs in both groups, IQR of 4- for BiPAP VS 5-8 for usual care). (Figure III.5) shows the cumulative proportion of participants judged FFD with increasing time, with more patients in the BiPAP than in the usual care group judged FFD from day 3 onwards.

III.10.4 SECONDARY OUTCOMES

III.10.4.1 pCO₂

Mean pCO₂ levels at 1, 12 and 24h are shown in (Table III.6). pCO₂ at 1h was significantly different between groups (-0.41 kPa, 95%CI -0.73 to -0.09, $p=0.011$) when adjusted for pCO₂ at the time of extubation, providing evidence that BiPAP was administered efficaciously in the study. The mixed regression model

estimated the mean difference in $p\text{CO}_2$ between the BiPAP and usual care groups to be -0.06 kPa (95%CI -0.27 to 0.15 , $p=0.58$) when adjusted for $p\text{CO}_2$ at the time of extubation and pre-operatively. Adjusting for intubation time also did not appreciably change the effect estimate for BiPAP vs. usual care. Including an interaction term for group allocation by time change this effect estimate only slightly and the interaction term was not statistically significant.

III.10.4.2 FEV₁

Absolute values were markedly reduced in both groups post-operatively because of wound pain and difficulty performing forced manoeuvres (Table III.6). We estimated the mean difference in percent predicted FEV₁ at day 3 between BiPAP and usual care to be 4% (95%CI -3% to 12% , $p=0.24$) when including preoperative percent predicted FEV₁ as a covariate. Including intubation time as a covariate changed this effect estimate only slightly (Table III.6).

III.10.4.3 Cardiac intensive care unit length of stay

No patient died before being discharged from the cardiac intensive care unit. The median length of stay in the cardiac intensive care unit was 24 hrs for the usual care group (IQR: 18 to 42) and 20 hrs for the BiPAP group (IQR: 17 to 40). The hazard ratio for BiPAP vs. usual care was 1.20 (95% CI: 0.84 to 1.71, $p=0.306$) and this did not change appreciably when adjusted for intubation time (Figure III.6).

III.10.4.4 Time until discharge

Not considering the 2 patients who died before discharge, the median length of hospital stay (until discharge) was 6 days for both groups (IQR: 5 to 8 for both groups). The hazard ratio for BiPAP vs. usual care was 1.12 (95% CI: 0.78 to 1.60,

p=0.552) and this did not change appreciably when adjusted for intubation time (Figure III.7).

III.10.4.5 Post-operative complications

(Table III.7) describes the post-operative complications. One or more respiratory complications occurred in 23 (37%) patients in the usual care group and in 9 (14%) patients in the BiPAP group. As hypothesised, clinically defined atelectasis occurred less often in the BiPAP group (2 patients, 3%) than in the usual care group (15 patients, 24%). Non-respiratory complications (combined) occurred with similar frequency in both groups.

Table III.4: Demographic and clinical characteristics of BiPAP trial patients

BiPAP			Usual Care	
Patient Demographics	Mean	SD	Mean	SD
Age (years)	65.76	10.77	69.41	8.86
Height (cm)	169.17	7.89	168.03	7.69
Weight (kg)	81.82	18.29	80.39	14.68
BMI (kg/m2)	28.47	5.28	28.45	4.57
NYHA class	no. of patients	(%)	no. of patients	(%)
0	5	7.9	5	8
1	17	27	16	25.8
2	29	46	34	54.8
3	10	15.9	6	9.7
4	2	3.2	1	1.6
Left Ventricular Function				
Good (>50%)	53	84.1	52	82.5
Moderate (30%-50%)	10	15.9	11	17.5
Comorbidities				
Respiratory				
chronic obstructive pulmonary disorder *	10	15.9	9	14.3
Smoker/ Ex-smoker	29	46	30	47.6
Other	3	4.8	1	1.6
Cardiovascular				
Hypertension	58	92.1	54	85.7
Hypercholesterolemia	53	84.1	56	88.9
Other	4	6.4	3	4.8
Systemic				
Diabetes Mellitus	19	30.2	15	23.8
Other	1	1.6	1	1.6
Other				
Family History	16	15.9	15	14.3
Other	1	1.6	7	11.1
None	0	0	1	1.6
Operation details	median	IQR	median	IQR
Bypass (min)	71	63.5-93.5	80	60-92
Cross-clamp (min)	38	31-48	46	33-54
Intubation (hrs)	6	4-10	6	5-8

* Preoperative chronic obstructive pulmonary disorder was defined as FEV₁% (FEV₁/FVC ratio) < 70%.

Table III.5: Baseline respiratory measurements by group in the BiPAP Trial patients

Variable	BiPAP		Usual Care	
	Mean	SD	Mean	SD
% predicted FEV ₁ (litres)	0.84	0.19	0.88	0.22
Forced VC	3.26	0.89	3.26	0.93
PaCO ₂ (kPa)	5.38	0.70	5.14	0.78
PaO ₂	29.21	13.30	33.17	13.72
Inspiratory Mouth Pressure*	-84.28	24.08	-80.81	25.82
Expiratory Mouth Pressure*	136.87	47.65	128.59	35.58

*Inspiratory and expiratory mouth pressure measurements were missing for about 33% of the patients. This is because these patients had their lung function tests done at the referring hospital prior to their admission to the Hammersmith Hospital. In this case, lung function tests were not repeated when patients were admitted to Hammersmith Hospital.

Table III.6: PaCO₂ and percentage predicted FEV₁ in BiPAP trial patients

PaCO ₂ (kPa)					Percentage predicted FEV ₁ (litres)				
BiPAP		Usual Care			BiPAP		Usual Care		
Time	Mean	SD	Mean	SD	Time	Mean	SD	Mean	SD
Post-extubation	5.19	0.69	5.35	0.84					
1 hour	5.10	0.85	5.58	1.09	Day 1	0.30	0.19	0.37	0.23
12 hrs	4.95	0.72	5.13	0.60	Day 2	0.33	0.18	0.38	0.20
24 hrs	4.91	0.60	4.95	0.53	Day 3	0.43	0.22	0.40	0.20

Table III.7: Adverse events reported in the BiPAP trial

Adverse event	BiPAP		Usual Care	
	n	%	n	%
Respiratory				
CPAP	4	6.4	6	9.5
Re-intubation	1	1.6	2	3.2
Basal atelectasis	2	3.2	15	23.8
Bibasal collapse	0	0	1	1.6
Pneumonia	0	0	2	3.2
Pleural effusion	2	3.2	0	0
Cardiovascular				
Atrial Fibrillation	10	15.9	7	11.1
Sinus tachycardia	0	0	1	1.6
Ventricular tachycardia	0	0	1	1.6
Cardiac arrest	1	1.6	0	0
Re-sternotomy	1	1.6	3	4.8
Ischemia	0	0	1	1.6
Neurological				
Confusion	1	1.6	2	3.17
Transient Ischemic Attack	0	0	1	1.6
Renal				
Renal failure	4	6.4	7	11.1
Other				
Liver infarct	0	0	2	3.2
Diarrhoea	1	1.6	0	0
Sternal wound infection	1	1.6	1	1.6
Death	1	1.6	1	1.6
Other	0	0	1	1.6
None	44	69.8	26	41.3

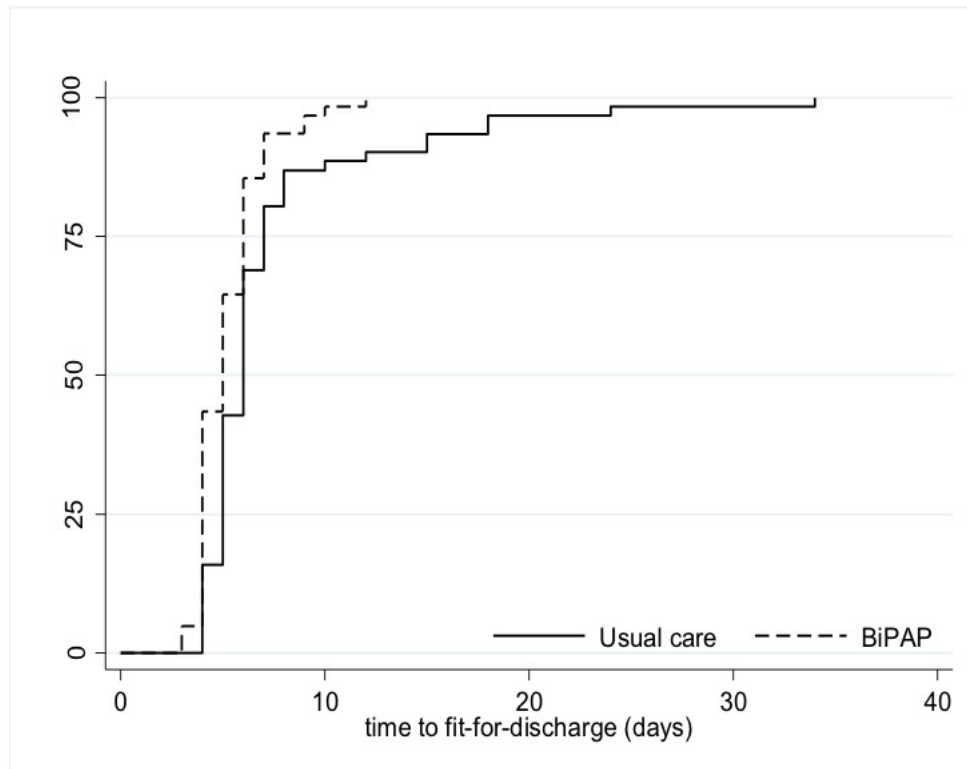
Figure III.5: Cumulative frequency of patients fit-for-discharge in the BiPAP trial

Figure III.6: Cumulative frequency of patients discharged from cardiac intensive care unit in the BiPAP Trial

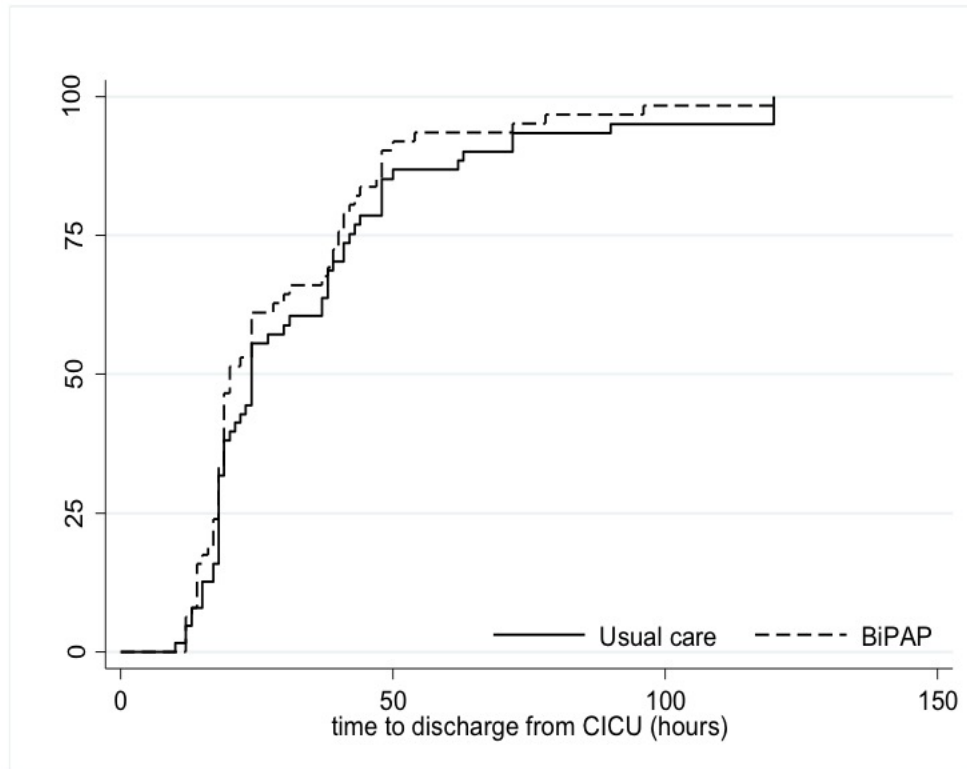
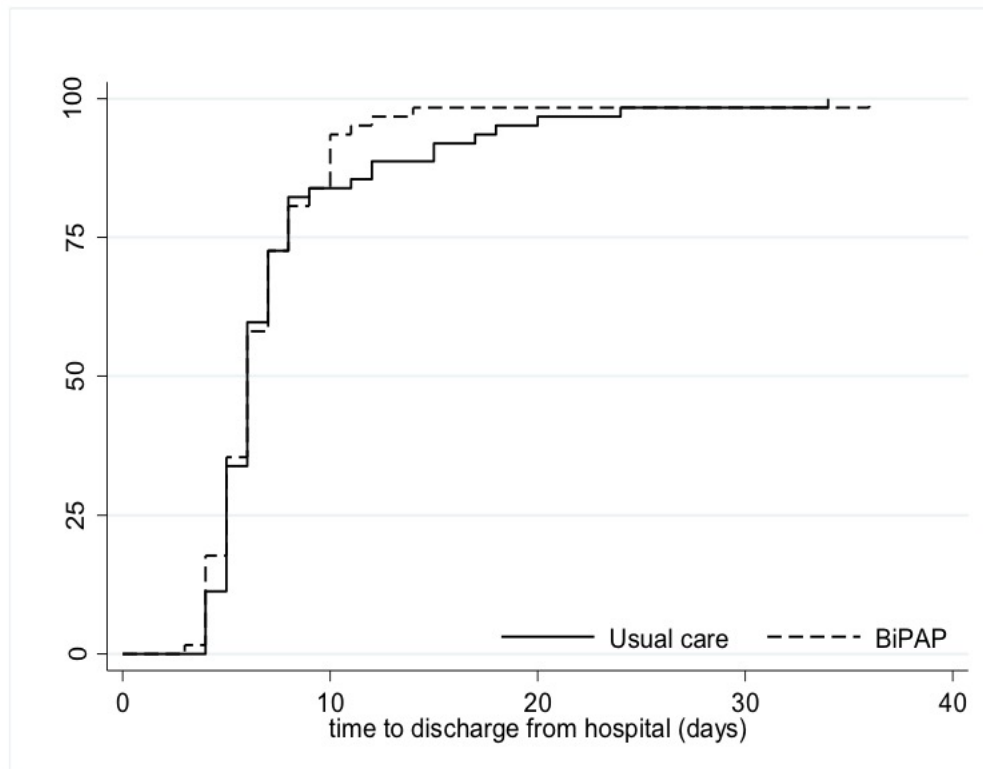


Figure III.7: Cumulative frequency of patients discharged from hospital in the BiPAP Trial



III.11 Discussion

There are three main findings from this trial. Firstly, we found that we could successfully implement BiPAP for extended periods. Among the participants allocated to BiPAP, the median duration of BiPAP was 16h and 75% had more than 10h. Only three participants rejected the BiPAP altogether. Implementation of BiPAP by cardiothoracic intensive care nursing staff was accomplished with limited training. This is important because continuous NIV for more than a few hrs at a time is often considered to be impractical and, for this reason, is often only implemented intermittently [186, 192, 196, 197].

Secondly, participants allocated to BiPAP were judged, on average, to be fit for discharge 1 day earlier than participants who had usual care alone. This is a clinically relevant finding for patients and important for health service providers since bed availability is a limiting factor on the capacity of cardiac surgery units in the UK.

Thirdly, overall respiratory complications and, in particular, atelectasis occurred less often in participants allocated to BiPAP. There were no differences between groups in FEV1 or pCO₂ overall. However, pCO₂ differed between groups one hour after extubation, which suggests improved alveolar ventilation resulting directly from the use of BiPAP.

III.11.1 STRENGTHS AND LIMITATIONS

To the best of our knowledge this is the first trial of preventive BiPAP in addition to usual care vs. usual care alone (including IS) in patients undergoing CABG. The primary outcome, time to medically fit for discharge, was chosen as a global

measurement of clinical importance because this outcome is a proxy for the successful recovery of patients. Actual discharge time is not as precise, because there may be cases where a patient is fit for discharge, but for practical reasons, remains for some time. The trial was designed with a sufficiently large sample size to detect a clinically important difference in the time until participants were fit for discharge. Our findings are applicable to most cardiac surgery settings; the trial was carried out in the UK NHS, where there were few exclusion criteria and the intervention was implemented by the nurses responsible for day-to-day staffing of the cardiac intensive care unit, who received pre-study training. Patients were not aware in advance of their allocation and we wrote a detailed analysis plan in advance of unblinding the data to enable the statistician to minimise the risk of within-study selective reporting [198, 199].

The trial had limitations. Primary analyses were by intention-to-treat. Allocation was not concealed from the research fellow responsible for recruiting participants and implementing randomisation. Given the nature of the intervention and the fact that the trial was set in a busy NHS cardiac intensive care unit, it was not feasible to blind participants, nurses and the research team to allocation. Therefore, the primary outcome was at risk of detection bias because the somewhat subjective criteria for judging a patient fit for discharge may have been influenced by a desire for the staff to "support" the treatment. Ascertainment of complications including clinical assessment of atelectasis from chest X-rays (reviewed by the cardiothoracic team on ward rounds), pCO₂ and FEV₁ measurements were expected to be less at risk of bias. Unfortunately, capturing reliable FEV₁ measurements proved difficult. When I was conducting the study, I had no proper

research team or statistical support before Professor Angelini's involvement. During my recruitment I had three patients randomised to the BiPAP group who withdrew from the study due to poor tolerance to the BiPAP mask. I regret that data collection was discontinued when the participants asked to be withdrawn. Later on when I discussed this with our statisticians, I came to know that I should have included data for the 3 patients randomised to the BiPAP group who withdrew from the study since this would have been more appropriate and would have reflected the intention-to-treat.

We did not plan to perform a formal health economic evaluation to quantify the cost-effectiveness of BiPAP in addition to usual care. The cost of a Level 3 *Intensive Care* bed average is £1932 per night [200-202], the average cost per hospital bed day is £222 [203] whereas the cost of the BiPAP consumables unit per patient is £60-£80. It seems therefore, reasonable to conclude that BiPAP by reducing the duration of the hospital stay has a significant economic importance for the health care system and is indeed cost effective.

III.11.2 RESULTS IN THE CONTEXT OF THE LITERATURE

Phase 1: at the time of publishing the study

The previous Cochrane review of IS (date of last search December 2004 although content assessed as up-to-date in April 2007) identified four relevant trials [193]. Two trials compared post-operative physical therapy (PPT) with post-operative physical therapy plus IS [196, 197]. One trial randomised patients into 3 groups, PPT plus IS vs. PPT plus NIV using CPAP vs. PPT plus NIV with two levels of pressure [186]; this latter intervention is similar to BiPAP but not as implemented

in our trial. The fourth trial compared PPT plus IS vs. PPT plus CPAP NIV vs. PPT plus NIV with intermittent positive pressure breathing [204]. Thus, although all of these trials included one group with IS, the comparator groups varied substantially. We used the same search strategy as the Cochrane review to identify additional recent relevant literature.

Matte et al. applied BiPAP differently, not starting the intervention until 4 hrs after extubation and applying it for only 1h in every 3h [186]. They reported physiological and lung function outcomes, cardiac intensive care unit stay and the proportion developing atelectasis on day 2 but reported changes between preoperative and day 1 measurements, and between day 1 and day 2 measurements, instead of comparing both day 1 and day 2 measurements to the pre-intervention baseline. The authors also combined CPAP and BiPAP groups to test the effect of NIV. A statistically significant difference in the mean change in VC and FEV₁ from day 1 to day 2 between the combined groups and usual care was reported but may have arisen from selective outcome reporting [198, 199]. There was a difference in the frequency of post-operative atelectasis (30% with usual care vs. 15% with CPAP or BiPAP combined, with blinded assessment) that was consistent with our findings.

In another trial, the benefits of using intermittent non-invasive pressure support ventilation (NIPSV, a form of BiPAP, applied four times per day) were compared to CPAP in 150 patients with evidence of post-operative radiological atelectasis [169]. The patients received either CPAP or NIPSV (the maximal pressure delivered was 30cm H₂O, PEEP was 5cm H₂O, the minimal trigger flow was selected) four times a day for 30 min. Compared to the CPAP group, the NIPSV

group had reduced atelectasis based on a radiological score. However, there were no statistically significant differences in lung function as a clinical outcome.

A third, four group trial evaluated the pulmonary effects of intermittent NIV with BiPAP (1h in every 6h), or recruitment manoeuvre (RM), compared to usual care, after open heart surgery in 25 patients per group [191]. Although this trial was factorial in its design, results were reported for the four groups separately. All three intervention groups had significantly reduced atelectasis scores (assessed by a radiologist blinded to allocation) with the lowest scores in the two BiPAP groups. However, no differences were observed in the duration of intensive care unit stay or the length of hospitalization.

Phase 2: at the time of submitting the thesis

To the best of our knowledge there are no relevant systematic review of NIV after cardiac surgery. A recent systematic review and meta-analysis included ten studies (n=1050 patients) and six studies respectively. Their results suggested that the use of NIV did not significantly reduce the risk for atelectasis, pneumonia, reintubation rate, and time spent in the cardiac intensive care unit [205]. Major limitations which prevent drawing a conclusion were the quality of some of the included studies, variations in physiotherapy techniques, resources and exercises used, time of intervention, time of application of NIV and frequency of examinations. The small number of studies and the limited patient sample sizes called for the need of appropriately designed randomised clinical trials [205].

III.11.2.1 Interpretation

Our finding, that BiPAP reduces atelectasis, is consistent with three previous trials. In the three previous trials, assessment of atelectasis was carried out by observers blinded to treatment allocation, preventing detection bias. This finding alone is important to patients and also for health service provision, since pulmonary complications and atelectasis are associated with longer duration of hospital stay following other types of thoracic surgery as well as cardiac surgery [206, 207]. Patients who stay in hospital longer than expected reduce the availability of beds for other patients awaiting elective surgery and decrease the capacity of cardiac surgery centres. The reduction in atelectasis with BiPAP probably explains our finding of a reduction in time until FFD.

Previous trials also found no significant effect of NIV interventions on other outcome measures such as $p\text{CO}_2$ and FEV_1 similar to that observed here for BiPAP. Our failure to observe differences is unlikely to be explained by a lack of power, since these outcomes are measured on continuous scales and, in the case of $p\text{CO}_2$, repeated measurements were analysed. We also considered that these measures would be less at risk of detection bias and, as much as they are intermediary measures of improving respiratory function and more likely to show a benefit. However, practical difficulties in this pragmatic setting, with lack of absolute standardisation, for example with respect to the fraction of inspired O_2 during BiPAP, makes them less robust.

In trying to reconcile the findings of the primary and secondary outcomes, we are faced with choosing between an explanation in terms of bias (which could explain the difference in FFD but not in the frequency of atelectasis) and an explanation

in terms of the intrinsic variability of post-operative measures of $p\text{CO}_2$ and FEV_1 . The latter were not robust in the trial setting and they are subject to large individual differences arising from variation in patients' pain thresholds, respiratory effort in the early post-operative period and in their adherence to instructions for lung function tests. These variations would be expected to contribute noise to the measurement of $p\text{CO}_2$ and FEV_1 .

The consistency of the findings across four trials with respect to the reduction in atelectasis strengthens our results and provides a mechanism for the clinical benefit we observed with BiPAP. However, we accept that the case for implementation of routine BiPAP after extubation in patients who have had CABG is not conclusively proven and we are exploring the feasibility of doing a larger multi-centre trial with features to reduce the risk of bias. It may be also possible to further optimise the application of BiPAP.

**CHAPTER IV. THE VENTILATE TRIAL: A RANDOMISED
CONTROLLED TRIAL OF LOW FREQUENCY
VENTILATION DURING CARDIOPULMONARY
BYPASS FOR PULMONARY PROTECTION IN
PATIENTS UNDERGOING CORONARY ARTERY
BYPASS GRAFTING [208]**

IV.1 The Ventilate Trial Abstract

Objective: To compare the efficacy of LFV versus conventional no ventilation during CPB in patients undergoing CABG.

Design: A two group parallel randomised controlled trial.

Setting: A cardiac surgery department.

Participants: Patients undergoing elective CABG.

Intervention: In the control group, as is standard practice, the lungs were allowed to collapse completely with no ventilation during CPB. In the treatment group (LFV during CPB the respiratory rate was maintained at 5 bpm with a fresh gas flow to air using minimal oxygen ($FiO_2 \cong 0.25$). The same ventilator protocol was used post CPB and used pre-CPB with a $PaO_2/FiO_2 >50$ required on first post-bypass gas (i.e. $PaO_2 >25kPa$).

Comparator: Standard care, with lungs allowed to collapse completely with no ventilation.

Main outcome measures: The primary outcome was inflammation measured by NF- κ B p65 activation in pre- and post-CPB lung biopsies. The pre-CPB biopsy provided a within-subject control for the second, post-CPB biopsy, taken after the intervention. Secondary outcomes were inflammatory markers in both biopsy tissue and blood, pulmonary gas exchange, pulmonary function tests (PFTs) and adverse events.

Results: Our data show that CPB had no effect on NF- κ B p65 activation in the lung biopsies at the time points studied. However, CPB significantly enhanced the expression of inflammatory mediator mRNA and protein in lung biopsies. Neither standard care nor LFV had a significant effect on NF- κ B p65 activation (post-CPB adjusted for pre-CPB level, difference between groups 0.101, 95% CI -0.022 to 0.226, $p=0.104$). The mean level of p38 MAPK analysis by Western blotting (WB) and by phospho-tracer analysis in the biopsies before chest closure (adjusted for pre-CPB concentration) was lower in the LFV group compared to the standard care group but again this did not reach significance (difference between groups -0.173, 95% CI -0.723 to 0.376, $p=0.525$). CPB negatively affects lung function and pulmonary gas exchange.

Conclusions: There was a significant induction of the expression inflammatory mediators (mRNA and protein) post CPB. LFV during CPB for patients undergoing CABG showed little effect on inflammation or clinical features compared to the standard of care.

IV.2 Introduction

Pulmonary dysfunction is a common complication for patients after cardiac surgery using CPB [209], with severity ranging from mild atelectasis with sub-clinical functional changes that go unnoticed to life threatening acute lung injury or adult respiratory distress syndrome requiring prolonged postoperative ventilation [19-21, 210]. Atelectasis is the end result of general anaesthesia leading to spontaneous cessation of airway patency and is determined by time and oxygen concentration [211, 212]. Mechanisms behind the development of atelectasis includes lung compression, depletion of alveolar space, and malfunctioning surfactant [213]. There is strong correlation between atelectasis and ALI [213-215]. Morphologically apparent ALI occurs in isolated, non-perfused lungs, ventilated with no or low PEEP and subjected to atelectasis [214].

Presumed causative factors for atelectasis and ALI, include inflammation, prolonged lung collapse, pulmonary ischemia and related reperfusion injury, blood contact with the surface of the heart-lung machine, endotoxemia, surgical trauma, blood loss and transfusion [216, 217]. Inflammatory activation and cytokine release have been correlated with outcome after cardiac surgery [218]. Pulmonary function 24 hrs after CPB is associated with raised plasma levels of inflammatory cytokines and reduced levels of anti-inflammatory cytokines [40]. The inflammatory response from the lung during CPB and mechanical ventilation originates at the alveolar membrane as a result of collapse, ischemia, reperfusion injury and mechanical stress [14, 39, 219]. Suppression of activation of

inflammatory mediators during CPB is associated with a reduction in pulmonary dysfunction [43].

We know that using LFV along with CPAP during CPB to reduce lung injury has been evaluated in an experimental pig model [143]. This study showed that the use of LFV is associated with significantly better pulmonary gas exchange, higher ATP, lower lactate dehydrogenase levels and reduced histological damage in lung biopsies as well as lower DNA levels in bronchoalveolar lavage, compared to the collapsed lung control group. However, a clinical study in patients undergoing cardiac surgery compared the effect of low volume ventilation to the conventional strategy (no ventilation) on particularly reperfusion injury of the pulmonary arterial tree as demonstrated by preservation of vasorelaxation to acetylcholine ACh [144]. This study didn't show significant changes in PVRI, $\text{PaO}_2/\text{FiO}_2$ ratio, postoperative length of stay and postoperative pulmonary complications but the authors didn't look for the effect of ventilation on atelectasis and the resultant ALI [1, 144]. Furthermore, a meta-analysis of 814 cases across 16 randomised clinical trials looking at three lung protective strategies in patients during CPB including CPAP, low-volume ventilation, or VC manoeuvres during CPB. They concluded that it might be impossible to advise an optimal or best-evidence strategy of lung preservation during CPB from available evidences [145].

Harmful effects of CPB on pulmonary function persist despite advances in anaesthetic techniques [220]. Pulmonary dysfunction after cardiac surgery also affects clinical outcomes with increasing morbidity, mortality and delaying discharge from the hospital, leading to increase in the health care resources used and their associated cost [19, 221-223].

Therefore, we believe that ameliorating the harmful effect of atelectasis and post-operative pulmonary dysfunction is vital for cardiac surgery patients. Nevertheless, prevention of atelectasis with subsequent ALI is challenging during long cardiac operations using CPB. Current clinical practice does not include any specific strategy for lung protection during CPB. When CPB is commenced, both lungs are disconnected or remain connected to the ventilator but are left collapsed for the entire CPB duration. Here, we report an evaluation of the efficacy of ventilating the lungs at low frequency during CPB in patients having CABG in an attempt to alleviate to the harmful effect of atelectasis as a leading cause for post-operative pulmonary dysfunction.

IV.3 Hypothesis

Observations suggest that keeping the lungs ventilated may be beneficial for achieving adequate alveoli perfusion [135, 136, 195-200]. Our trial is the first RCT to investigate the effects of LFV in patients undergoing CABG with CPB by measuring inflammatory markers directly in lung biopsies and blood. We hypothesised that using LFV during CPB would result in reduced inflammatory activation, lung dysfunction, and ischemia/reperfusion injury compared with not ventilating the lungs during CPB.

IV.4 Aims and objectives

The aim of the trial was to investigate the effects of LFV during CPB on markers of inflammatory activation (ischemia/reperfusion injury) in the lung and peripheral blood. Our long-term objective is to improve the standard of care to patients following CPB by reducing lung inflammation and dysfunction.

IV.5 Ethics committee approval and registration

This trial was approved by the NRES committee London-Camden and Islington (Research Ethics Committee reference number 12/LO/0458) on 25/04/2012. Further approval was obtained from the research and development department of the Imperial College Healthcare NHS Trust. This research complies with the Helsinki Declaration. This trial is registered as ISRCTN No: 34428459

IV.6 Trial design

This study was a single-centre, two-group parallel randomised controlled trial. Patients undergoing elective CABG in the department of cardiothoracic surgery at the Hammersmith hospital were recruited as part of a randomised controlled clinical trial comparing the strategy of ventilating the lungs during CPB, with the current standard practice of no ventilation, which allows the lungs to collapse (Figure VI.1). Patients were randomly assigned in a 1:1 ratio using a secure concealed internet-based randomisation system. Cohort minimisation was used to achieve balance between groups with respect to baseline lung function ($\geq 60\%$ predicted FEV₁). Patients returned to hospital for a follow up visit 6-8 weeks following the operation. No data were collected thereafter.

Participants, ward nurses, clinicians and radiologists not involved in the surgery, and research personnel responsible for data collection were masked to the treatment group to which each participant was assigned. Those involved in the surgery were unmasked.

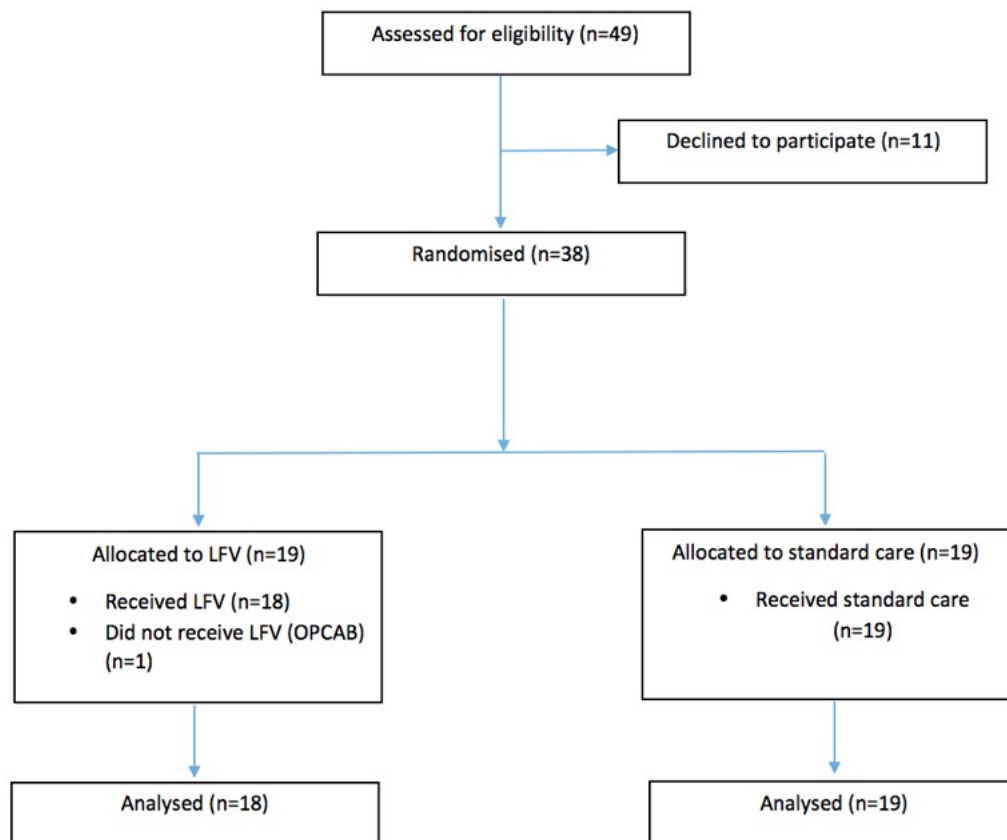
Figure IV.1: The Ventilate trial consort chart

Figure IV.1 shows the consort diagram for the randomised clinical trial with two arms: LFV (ventilating the lungs) during CPB vs. the current standard practice (collapsing the lungs). Forty-nine patients were considered for inclusion in the trial. Eight declined for personal reasons and 3 favour the standard procedure. Thirty-eight patients were recruited to the study. Six additional patients were recruited over the initial sample size calculation because of the learning process associated with harvesting biopsies. Nineteen were randomly allocated to LFV and 19 to usual care. One participant allocated to the LFV group was withdrawn because the operation was performed using an off-pump technique, resulting in the patient not receiving the intervention or standard care. Data were not subsequently collected for this patient. Therefore, we had data for 18 patients in the LFV group and 19 patients in the standard of care group. Baseline characteristics, pre-operative co-morbidities, operation details and baseline respiratory measurements were balanced. For trial participants with inclusion criteria and exclusion criteria please refer to section III.8.3.

IV.7 Outcomes

IV.7.1 THE PRIMARY OUTCOME

The primary outcome is inflammation measured by NF- κ B p65 activation in pre- and post- CPB lung biopsies. NF- κ B p65 was selected as a primary outcome biomarker in lung dysfunction because it is a critical signalling mediator of most inflammatory processes [77] and controls the transcription of cytokines and antimicrobial effectors and genes that regulate cellular differentiation, survival and proliferation [75, 80]. It hence regulates various aspects of both the innate and adaptive immune responses [75] (more details previously discussed II.1.4 Nuclear Factor-kappaB (NF- κ B)).

IV.7.2 THE SECONDARY OUTCOMES

Secondary outcomes included additional inflammatory markers in both lung biopsy tissue and blood were selected to add more information to our study including: -

IV.7.2.1 Lung tissues

1. p38 MAPK phosphorylation by ELISA and Western blotting.

p38 MAPK is associated with many types of chronic inflammation within the airways and various essential cellular functions including the regulation of TNF- α production (and the activation and release of other proinflammatory cytokines/chemokines), NADPH oxidase activation (i.e. oxidative stress), neutrophil migration and DNA-damage-induced cell cycle arrest which are all involved in the molecular pathogenesis of acute lung injury [224, 225].

2. Expression of $\text{TNF}\alpha$, IL-1 β , IL-18, IL-6, IP-10 and IL-8 and IL-10 by real-time PCR and ELISA.

IL-1 β , IL-18 and caspases play a role in the acute response to inflammasome activation [226-228]. $\text{TNF}\alpha$, IL-6 and IL-8/CXCL8 are classic ROS-sensitive inflammatory readouts of NF- κ B activation [42, 229-232]. IP-10/CXCL10 is an NF- κ B-inducible chemokine important for the recruitment of T-cells, dendritic cells and monocytes to the site of inflammation. It has pro-inflammatory properties and acts as a modulator of angiogenesis in conditions such as wound healing and ischemia [233, 234]. IL-10 is a key anti-inflammatory molecule that can regulate immune functions and whose expression increases during CPB in infants [40].

3. Caspase 3 as a marker of apoptosis.

IV.7.2.2 Blood biomarkers

The following inflammatory markers were measured in monocytes and polymorphonuclear cells from peripheral blood samples:

Blood leukocyte levels and/or activation status alters with CPB [1, 128, 210, 235]. Indeed, there was better graft function and less inflammatory response if leukocytes were removed using a filter in a rat EVLP model [236].

1. ROS levels using a redox-sensitive fluorescent probe (2',7'-dichlorofluorescein) followed by flow cytometry and oxidative stress was measured by Redox SYSTM Diagnostic System,

2. Phosphorylation of p38 MAPK (Thr180/Tyr182) and NF- κ B p65 (Ser529) phosphorylation by intracellular staining using specific antibodies followed by flow cytometry,

These pro-inflammatory ROS-driven signaling pathways are increased following ischemia-reperfusion [237-239].

3. Heme assay.

Heme can act as an alarmin or a Danger-Associated Molecular Pattern (DAMP) [240, 241].

IV.7.2.3 Blood samples timing

1. After anaesthetic induction and pre-sternotomy
2. 10 min after the end of CPB
3. 2, 6 & 24 hrs after the end of CPB.

IV.7.2.4 Pulmonary function tests

Carried out pre-operatively and at 6-8 weeks post-surgery.

IV.7.2.5 Pulmonary gas exchange

Assessed by $(PAO_2 - PaO_2)$ (28) and the respiratory index $[(PAO_2 - PaO_2) / (PaO_2)]$ measured at:

1. Post-induction and pre-sternotomy
2. 10 min following CPB weaning
3. 2h post CPB
4. 4h post CPB
5. First gas post extubation
6. 12h post CPB
7. 24h before removal of the arterial line

IV.7.2.6 Adverse events

Adverse events were collected during hospital stay and at the follow up visit. At this visit, participants underwent a pulmonary function test and any documented occurrence of adverse events in the period between the operation and the follow up visit day were recorded.

IV.8 Materials and Methods

IV.8.1 ANAESTHETIC PROTOCOL

Please refer to Section III.8.1

IV.8.2 PERFUSION PROTOCOL

Please refer to Section III.8.2

IV.8.3 PARTICIPANTS

Please refer to Section III.8.3

IV.8.4 EXTUBATION PROTOCOL

Please refer to Section III.8.4

IV.8.5 INTERVENTION AND COMPARATOR

Pre-CPB the lungs were ventilated with a TV of 6-8 ml.kg⁻¹, I:E ratio of 1:2, PEEP of 5cm H₂O and FiO₂ of 0.5 (range 45%-55% O₂). The ventilatory rate was set to keep the PaCO₂ between 4.5 and 5.5 kPa.

In the control group, at the onset of CPB, the lungs were allowed to collapse completely with no ventilation during CPB. In the treatment group (LFV during CPB) the respiratory rate was maintained at 5 bpm using oxygen enriched air (FiO₂ 0.25). PEEP was turned off whilst the TV and I:E ratio were unchanged. At the end of the CPB: all patients had a lung recruitment manoeuvre using an FiO₂ of 0.5 and holding the lungs inflated for 15sec at 30cm H₂O before the lungs were placed back on the ventilator. The same ventilator protocol was used post-CPB and used pre-CPB with a PaO₂/FiO₂ >50 required on first post-bypass gas (i.e. PaO₂>25kPa). If PaO₂/FiO₂ <50 then the recruitment manoeuvre was repeated

IV.8.6 BLOOD SAMPLING PROTOCOL

Blood samples were obtained from in situ venous/arterial lines at five time points: i) after anaesthetic induction and pre-sternotomy, ii) 10min after the end of CPB, and at iii) 2, 6 and 24h after the end of CPB. Each sample was labelled and identified by unique patient study number, patient initials, date of birth, trial acronym, and the time point of the sample, in accordance with local standard operating procedures.

IV.8.7 LUNG SAMPLES PROTOCOL

The pre-CPB biopsy provided a within-subject baseline control for the second, post-CPB biopsy, taken after the intervention. NF-κB nuclear localization and

activation was assessed by testing nuclear lysates by DNA-binding ELISA (TransAm Assay).

To measure inflammatory markers in the lung (Outcomes), two lung biopsies (1cm x 1cm) were taken using the LigaSure Impact™ instrument (Figure IV.2) (LF4318, Covidien, Watford, Hertfordshire, UK). This works exclusively with the ForceTriad™ energy platform and combines TissueFect™ sensing technology with hand activation and an integrated cutting mechanism to create a sealing system that provides a consistent, controlled effect on tissue. The first (pre-CPB) biopsy was taken from the left upper lobe immediately after sternotomy and before harvesting of the left internal mammary artery whereas the second was taken from the left lower lobe prior to weaning from CPB.

Figure IV.2: LigaSure Impact™ instrument



IV.8.8 BLOOD BIOMARKERS

Oxidative stress in Leukocytes measured by FACS

Table IV.1: Oxidative stress materials

Company	Product:
BD Pharmingen (San Jose, CA, USA)	BD FACS Lysing Solution 10X Concentrate (Cat No. 349202)
	Lyse/Fixation Buffer 5X (Cat No. 558049)
	Permeabilisation Buffer III (Cat No. 558050)
	Stain Buffer (BSA) (Cat No. 554657)
	p38 MAPK PE-Cy TM 7 Mouse anti-phospho-p38 MAPK (pT180/pY182) (Cat No. 560241)
	p65 PE Mouse anti-phospho-NF-κB p65 (pS529) (Cat No. 558423)
BioLegend Inc (San Diego, CA, USA)	p38 MAPK PE/Cy7 Mouse IgG1, k Isotype Control (Cat No. 400125)
	p65 PE Mouse IgG2b, k Isotype Control (Cat No. 400313)

a) Sample preparation

All analysis of blood samples was performed in triplicate and compared to a blank PBS control. 50µl of blood was added to an Eppendorf tube containing 450µl PBS and 1µl APF solution to give a final DCF concentration of 10µM. After incubation in the dark for 30min at room temperature, samples were transferred to FACS - falcon tubes (polystyrene) and 3ml PBS added before centrifugation at 300 x g for 5min. The pellet was re-suspended in 1ml ice-cold freshly prepared 1x lysis buffer and incubated at 37°C for 20min before addition of 4ml of PBS. The sample was re-pelleted by centrifuging as above and each pellet resuspended in 200µl PBS. Samples thus prepared were ready for FACS analysis (Figures IV.4. & IV.5).

b) Detection of activated phospho-p38 MAPK and NF- κ B p65 sub-units by intracellular staining using FACS

Sample Preparation

500 μ l of blood was added to 9.5ml fixation buffer in a 15ml falcon tube and incubated at 37°C for 20min in a dark room. 4mls of PBS was added to each sample, followed by centrifugation 300 x g for 5 min. The pellet was re-suspended in 10ml PBS and placed on ice in the cold room until ready to analyse by flow cytometry. At this time, the sample was centrifuged as above and resuspended in 0.5ml Permeabilisation buffer (Perm buffer3, stored at -20°C) by short vortexing before storing on ice for 30min.

Flow cytometric analysis

Background: -

Fluorescence-activated cell sorting (FACS) is a technique that enables sorting of a heterogeneous mixture of biological cells, one cell at a time, by gating using the specific light scattering and fluorescent characteristics of each cell type [242-246] (Figure IV.4). Gates, or regions, are drawn on fluorescence scatter plots and histograms to selectively focus on populations of interest. These gates can then be applied to view and analyze specific populations. The first step in gating is typically to distinguish the cells based on their light scattering properties. The different light scatter signals of granulocytes, monocytes, and lymphocytes allow them to be distinguished from one another and from cellular debris. Dead cells tend to have lower forward scatter and higher side scatter than living cells. Gating

is commonly applied to assist in the analysis of whole blood cell samples and using this approach (Figure IV.5).

Data presentation: -

Overall, data can be analyzed as a) histograms or b) in two-parameter dot or density plots.

- a) Histograms display a single measurement parameter e.g. relative fluorescence or light scatter intensity on the x-axis and the number of events (cell count) on the y-axis. The histograms can be useful for evaluating the total number of cells in a sample that possesses the physical properties selected for or which express the marker of interest. Ideally, flow cytometry will produce a single distinct peak, however, flow analysis performed on a mixed population of cells often results in several histogram peaks. In order to identify the positive dataset, flow cytometry is repeated in the presence of a control.
- b) Density plot, each dot or point represents an individual cell that has passed through the instrument. The plots in (Figure IV.5) are colour intensity plots with the red/yellow/green/blue hot spots indicate increasing numbers of events from discrete populations of cells.

Assay method: -

Antibodies were prepared in stain buffer (Figures IV.3, IV.4 & IV.5) and 10µl of antibody was added per well (180µl of each antibody was prepared and 150µl used for each experiment). Antibody stained cells were separated and analysed with flow cytometry (FACS) (Figures IV.4 & IV.5,). p38 MAPK & p38i (Isotype

control) was diluted 1:100 (1.8µl Ab and 178.2µl stain buffer), and p65 & p65i (Isotype control) was diluted 1:125 (1.44µl Ab and 178.6µl stain buffer).

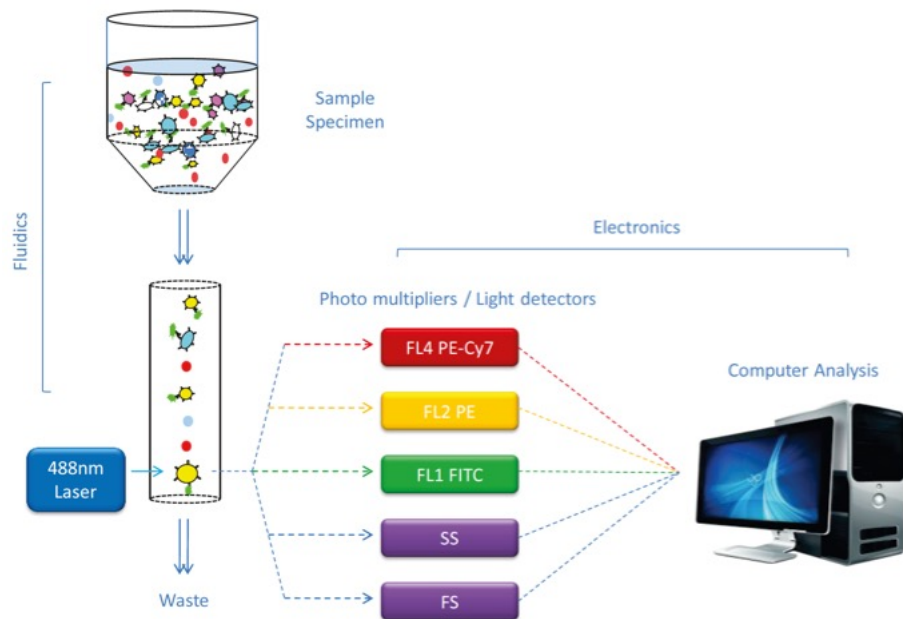
Leukocytes were loaded with APF or permeabilised and stained with a directly conjugated fluorescent antibody (or the relevant matched isotype controls for the target molecule). Samples were then re-suspended in PBS for analysis.

As dyed cells pass through the beam, the light deflection gives values of forward (FS) and side scatter (SS) indicating cell size and intracellular granularity respectively. Forward and side scatter were used to gate the cell populations (Figure. IV.4). The appropriate fluorescence channel was used to measure each cell individually according to the APF specific for the antibody used. The geometric mean of each population was calculated for comparison.

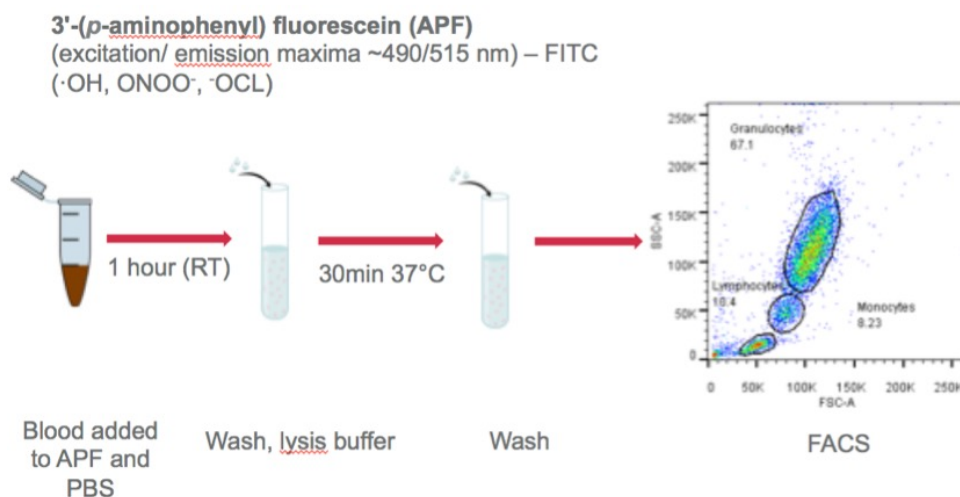
Figure IV.3: Flow chart of 96 well plate preparation.

	p38			p38i			p65			p65i		
Baseline												
10 min												
2 hrs												
6 hrs												
24 hrs												
Blanks (No Ab)												
	0	10m	2hr	6hr	24hr							

Each coloured cell represents one well. Coloured rows are differentiated by time and columns by antibody (in triplicate). White cells were empty.

Figure IV.4: Schematic representation of the flow cytometer channels

The schema is for of a column of cells, which are dispensed individually prior to excitation with a blue 488nm laser. FS = forward scatter. SS = side scatter.

Figure IV.5: Blood Protocol (oxidative stress in patient no. 36).

Representative FACS analysis of APF staining in granulocytes, lymphocytes and monocytes in peripheral blood cells gated on forward and side scatter. The colour intensity (red/yellow/green/blue) indicates the increasing numbers of events from the discrete cell populations.

In the analysis of p38 MAPK and NF- κ B phosphorylation the monocytes often overlapped with lymphocytes due to the fixing/permeablising method used. Therefore, these cells were treated as one sample in subsequent analysis. In total, 195 FACS traces were generated per patient (A total of 65 FACS plots were obtained per patient 4 antibodies x 3 tubes = 12 tubes +1 blank x5 time points = 65 plots per cell type X 3 cell types).

The geometric mean of each sample was calculated for comparisons. This data is summarised in (Tables IV.9 & 10).

Oxidative stress measured by Redox SYSTM

Given that oxidation is the loss of electrons and reduction is the gaining of electrons, the ability of a substance to gain or lose electrons in solution can be detected and recorded as the oxidation reduction potential (ORP). A high positive redox potential is associated with a strong oxidizing agent. It is important to note that the ORP value does not reflect a concentration but an activity measure. Oxidative stress is the result of an excess of ROS or a shortage of protective antioxidants. In clinical situations, redox-induced signalling pathways may lead to tissue damage and is therefore an important indicator for this study.

The RedoxSYS Diagnostic System

To measure oxidative stress, I used the Redox SYSTM Diagnostic System (Luoxis, Englewood, CO, USA). Collection tubes containing blood were centrifuged at 1,000 xg for 10min to separate the plasma. 30 μ l plasma was then applied to the sample application port on the inserted RedoxSYS Sensor (Fig. IV.6).

Figure IV.6: The Redox SYSTM Diagnostic System

The Redox SYS™ Diagnostic System is a simple, rapid test that delivers results in less than four minutes

The Redox SYS™ Diagnostic System is appropriate for both clinical and research settings.



Heme assay

Heme is a powerful pro-oxidant that is released actively by cells after injury, causing further tissue damage and inflammation. Heme may also be released passively as a result of sterile cellular injury after ischemia to cause oxidative damage and inflammation [241]. Heme can also act as an alarmin or a DAMP [240, 241]. Heme is an important building block for a variety of molecules and enzymes, including hemoglobin and as such, Heme is commonly measured for studies in blood health. The QuantiChrom™ Heme Assay Kit (DIHM-250, BioAssay Systems, Hayward, USA) was used for the colorimetric determination of total heme according to the manufacturer's instructions. In this kit, absorbance of 400nm is directly proportional to heme concentration in the sample.

Procedure using 96-well plate:

Calibration: 50 μ l water (Blank) and 50 μ l "Calibrator 1" solution (BioAssay Systems) were pipetted into wells of a 96-well plate. Then, 200 μ l of water was added to each well. The calibrator wells were equivalent to 62.5 μ M heme. Serum and plasma samples were assayed undiluted, while blood samples were diluted 100-fold in distilled water. 50 μ l samples were transferred into wells, avoiding bubble formation, and 200 μ l reagent was added and mixed by lightly tapping the plate. All samples were incubated for 5min at room temperature. The optical density (OD) was read between 380 and 420nm with a peak at 400nm. The final heme concentration was determined by subtracting the OD of the blank from the OD of the sample, and this value divided by the OD of the blank. This value was then multiplied by 62.5 x the dilution factor (either 1 for serum or plasma or 100 for blood) to obtain the concentration of heme in μ M.

IV.8.9 PROTEIN EXTRACTION

A small piece of lung tissue (1-2 mm in diameter) was taken in a Petri-dish on dry ice (one Petri-dish for each sample was used at time of cutting and the knife was washed with water and 70% ethanol). The biopsy was then placed in a Pegg lab tube with sterile ceramic beads (1-4mm) and 500 μ l of 1x hypotonic buffer (part 100505, Active Motif Nuclear extract kit part 40010) which contains protease and phosphatase inhibitors. The sample was kept on ice. Tissue was lysed and homogenised in a Precellys homogeniser for 15sec. The homogenate was transferred to a new tube on ice and 20 μ l of detergent (provided in the Active Motif kit) was added. Cells were pelleted by centrifugation at 7000rpm for 5min at 4°C before the cytoplasmic supernatant fraction was transferred to a new tube and

stored at -80°C until used in ELISAs. The pellet was then re-suspended in 100µl buffer (15mM Tris HCl, pH7.9, 450mM NaCl, 0.25mM EDTA, 10mM β-mercaptoethanol (β-ME), 10% glycerol with double amount of complete protease inhibitor cocktail tablet added just before using [1 tablet in 5ml]). Samples were kept on ice on a rocking platform for 30min in the cold room before pellets were lysed by gently scratching and 100µl of buffer B (buffer A without NaCl) was added. The sample was centrifuged at 14000rpm for 10min at 4°C and the supernatant collected and the resulting nuclear fraction aliquoted into 50µl/tube for further analysis.

Bradford protein assay

Protein concentrations of whole cell, cytoplasmic and nuclear extracts were determined with a Bradford assay. This assay is based on an absorbance shift of Coomassie brilliant blue dye; the red form of the dye is converted into the blue form with the addition of proteins. Bradford reagent (200µl/well) was dispensed in a 96-well microtitre plate. A standard curve, using a stock of 2mg/ml BSA standard solution was constructed and 1µl of the unknown sample was added in duplicate to additional wells. After 5 min, the absorbance was read at 595nm using a microtitre plate reader and a standard curve generated using GraphPad prism (GraphPad Software, La Jolla, CA, USA) and this was used to determine the concentrations of the extracts.

Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis

Protein extracts (30-50µg) were transferred into new tubes and gently mixed with the correct volume of 4X NuPAGE® LDS sample buffer containing 4% β-

Mercaptoethanol. The samples were then boiled for 5min at 95°C to denature the proteins. The samples were then loaded onto a 10% Bis-Tris Novex® precast mini-gel along with Novex® Sharp pre-stained protein standard to allow for band size comparisons. The precast gels were placed in electrophoresis gel tanks and filled with either 1X NuPAGE® MES SDS running buffer, for small molecular weight proteins, or 1X NuPAGE® MOPS SDS running buffer, for larger molecular weight proteins. The tank was connected to a power supply and the gel was electrophoresed for 35-55min at 200V. All materials were from ThermoFisher.

Semi-dry transfer

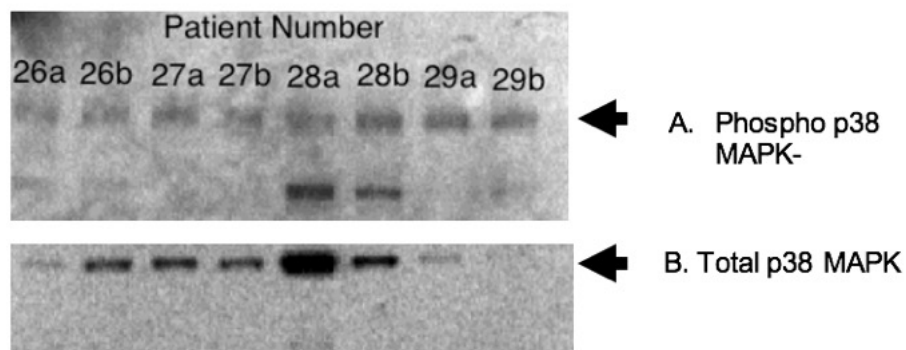
After electrophoresis, the proteins were transferred from the polyacrylamide gel onto a nitrocellulose membrane by a semi-dry method using the iBlot™ Dry Blotting system (ThermoFisher). Briefly, the gel cassettes were opened with a gel knife and the gel was transferred to the nitrocellulose membrane on the 'bottom' stack of the iBlot™ gel transfer stacks. This 'bottom' stack contains a copper anode (positive) and a nitrocellulose membrane. Filter paper soaked in deionised water was then placed on top of the gel before a 'top' stack was added, to the copper cathode (negative). The sandwich of layers was placed on the iBlot™ device in addition to a sponge and tightly closed. A mini roller was used to remove any bubbles between all the layers. The iBlot™ device was used on program 3 at 20V and run for 7min. After transfer, the sandwich of layers was disassembled and the nitrocellulose membrane, on which the proteins had now transferred, was cut to size, and rinsed in TBS-Tween (0.1% Tween) before immunodetection.

Immunodetection

Membranes were incubated in blocking buffer, 5% milk in TBS-Tween, for at least 1h at room temperature on a rocker to prevent non-specific binding of proteins. Membranes were then incubated on a rocker in a suitable concentration of primary antibody (Table IV.2) diluted in 5% milk in TBS-Tween for 1h at room temperature or at 4°C overnight. After incubation, the membranes were washed three times in TBS-Tween for 5min on a rocker, followed by 1h incubation with a secondary antibody (Table IV.2) diluted in 5% milk in TBS-Tween. The secondary antibodies were conjugated with alkaline phosphatase (AP). The membranes were again washed three times in TBS-Tween for 10min each and then gently blotted to remove excess wash buffer. The blotted dry membranes were placed on Saran wrap, protein side up and ECF substrate solution pipetted to cover them (24 μ l/cm²). They were incubated for 1min in the dark before being blotted to remove excess substrate and then placed between plastic sheets and scanned on a storm phosphoimager. Membranes were re-probed for the detection of different proteins by incubating in reBlot™ stripping buffer for 15-20 min. The membranes are then re-blocked in 5% milk in TBS-Tween and the process repeated with the new primary antibody (Table IV.2 & Figure IV.7). The gels were compared according to the ratio of the phospho/total non-phospho protein bands detected (Figure IV.7).

Table IV.2: Primary and secondary antibodies for Western blotting

Company (Catalogue #)	Primary antibody (Dilution)	Secondary antibody (Dilution)	MW (kDa)
Cell Signalling (cat. No. 4511s)	Phospho-p38 MAPK Rabbit Monoclonal (1:1000)	Polyclonal Goat anti- Rabbit IgG- HRP (1:2000)	43kD
Cell signalling (cat. No. 9212s)	Total p38 MAPK Rabbit Polyclonal (1:1000)	Polyclonal Goat anti- Rabbit IgG- HRP (1:2000)	43kD

Figure IV.7: Representative Western blot of phospho p38 MAPK in lung biopsy.

Expression of phospho p38 MAPK- α /pT180/Y182 (A. top row at arrow) & total p38 MAPK (B. at arrow), in lung biopsies. (A) Represents first lung biopsy pre-CBP. (B) Represents second lung biopsy post-CBP for the same 4 patients, in the trial (26, 27, 28, and 29).

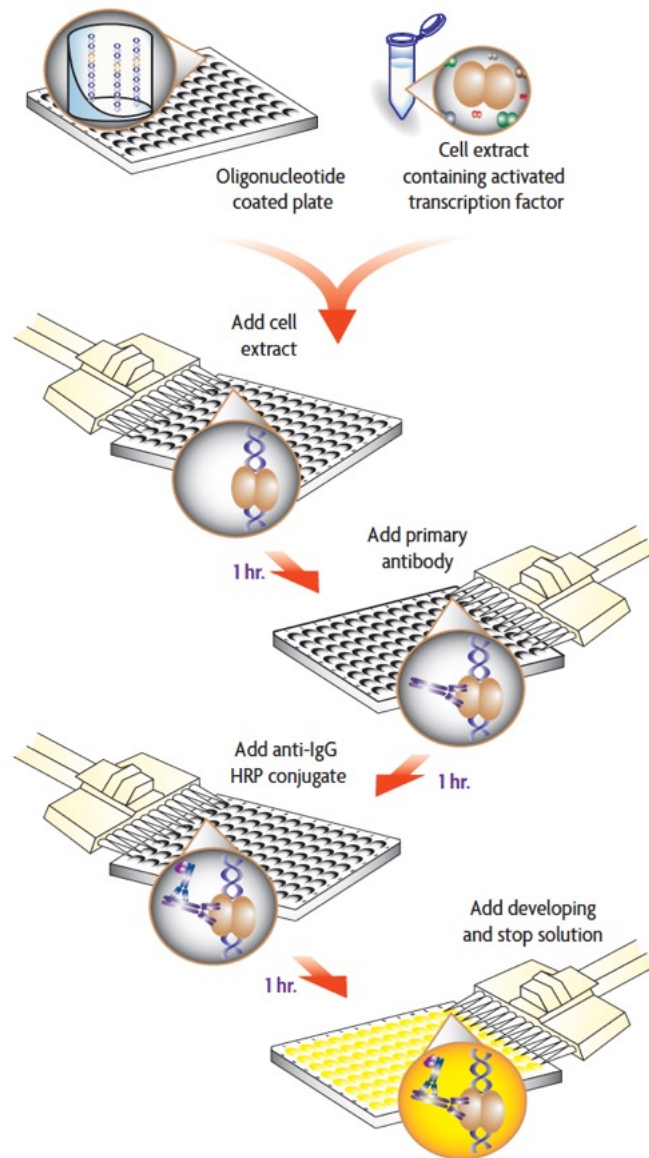
Band quantitation

Band density was quantified using ImageQuant TL software (Fisher Scientific, Loughborough, UK) and the rolling ball method used for background subtraction. Rolling ball [247, 248] can be effective for subtracting background from images that have smooth and continuous backgrounds. The image background is identified by traversing a ball along the bottom surface of the pixel intensity histogram. The highest point where the ball can fit underneath the surface is considered the threshold for background subtraction. Densitometric data of phosphorylated proteins was normalised to total protein and levels of endogenous control β -actin.

IV.8.10 NF- κ B TRANSCRIPTION FACTOR ACTIVATION ASSAY

Nuclear protein extracts were collected as for Western blot analysis but were used to detect NF- κ B activity using a TransAm kit. This is like an ELISA kit except that the capture Ab is replaced by a specific DNA sequence. Activated NF- κ B, from the nuclear extracts, binds to the DNA and can be detected by the detection Ab. Nuclear extracts from lung tissue were used following determination of protein concentration by Bradford assay. Nuclear translocation of NF- κ B reflects its activity as inactive NF- κ B remains in the cytoplasm. The Trans AMTM NF- κ B kit (Active Motif Cat. No. 40096) was used exactly according to the manufacturer's instructions (Figure IV.8). Assays were carried out in a 96 well plate (Figure IV.8). Each well of a 96-well plate was immobilised with an oligonucleotide containing an NF- κ B p65 consensus site (5'-GGGACTTTCC-3') to which activated p65 binds. 30 μ l of complete binding buffer (2 μ M DTT, 10ng/ μ l Salmon sperm DNA in binding buffer AM3) was added to each well followed by 20 μ l of nuclear proteins (15 μ g)

diluted in complete lysis buffer (0.2µl protease inhibitor cocktail in lysis buffer AM1, 1mM DTT) and incubated for an hour with mild agitation. The plate was then incubated with 100µl/well anti-p65 primary antibody and HRP-conjugated secondary antibody for an hour at room temperature. The plate was washed with washing buffer after each incubation. The developing solution was then added and left until a blue colour developed. Stop solution was added to each well to stop the reaction. Chemiluminescence was read at 450nm against a reference wavelength of 655nm within 5min.

Figure IV.8: Showing the flow chart of the process of NF- κ B activation

IV.8.11 CASPASE-3 ACTIVITY ASSAY (CYSTEINE-ASPARTIC ACID PROTEASE)

Caspases (cysteine-aspartic proteases, cysteine aspartases) are a family of protease enzymes essential for programmed cell death (apoptosis, necroptosis and pyroptosis) which regulate the inflammatory response. Caspase and caspase-like proteases (termed metacaspases) are present in multicellular and unicellular eukaryotes, as well as several prokaryotes [249]. The role of these enzymes in programmed apoptotic programmed cell death (PCD) has been extensively investigated [250]. However, caspases, are involved in various non-apoptotic processes including cell differentiation, cell cycle progression, proteostasis and cellular remodelling [249]. Caspase 3 is usually present in cells as an inactive dimer as is activated upon proteolytic cleavage of both monomers at loops containing specific cleavage sites [251, 252]. Activity can be detected using antibodies that recognize caspase proforms and/or cleavage fragments [251].

Activation of the IL-1 β converting enzyme (ICE) family of proteases/caspases initiates mammalian cell apoptosis. The Caspase-3 Fluorometric Protease Assay Kit (R&D Systems, Minneapolis, USA) detects the presence of caspase 3 and related caspases that recognise the amino acid sequence DEVD in a biological sample. The assay is based on detection of the cleavage product of AFC (7-amino-4-trifluoromethyl coumarin)-modified substrate (DEVD-AFC). DEVD-AFC emits blue light and upon cleavage of the substrate free AFC emits a yellow-green fluorescence which can be quantified using a fluorometer or a fluorescence microtiter plate reader. Protein extracts from apoptotic cells can be used as a positive control.

Extraction of protein samples

To release whole protein from the biopsies and make it available for detection, a small piece of lung tissue was placed in a petri dish on dry ice and washed as described above (Section IV.9.9). The tissue was then placed in a PEGlab tube containing 300µl RIPA buffer (Santa Cruz Inc, Santa Cruz, CA, USA) and homogenised in a Precellys homogeniser for 15sec. The homogenate was transferred to a new Eppendorf tube placed on ice for 30min during which samples were agitated and vortexed (2x 10sec) followed by centrifuging at 14000rpm for 20min at 4°C. The supernatant was transferred to a new tube prior to conducting the assay.

Caspase-3 activity assay for apoptosis

25µl of each sample corresponding to 0.5-4mg/ml protein, as determined by Bradford assay, was mixed with 200µl of substrate in a well of the black 96-well plate. Relative Fluorescent Units (RFU) were measured on a fluorescence plate reader at 0, 1, 16 and 24h in the dark at 37°C. Relative Fluorescent Units were read immediately using excitation at 380nm and emission at 420-460nm and the change in fluorescence was plotted over time.

IV.8.12 SEMI- QUANTITATIVE MEASUREMENT OF P38 MAPK- α P-T180/Y182 AND TOTAL P38 MAPK USING PHOSPHO-TRACER ASSAY

For each phospho-tracer assay both the analyte (cellular fraction) and assay reagents were added at the same time to the microplate following the manufacturer's protocol (Abcam, ab119638 and ab119639). The antibody mix was then added and incubated at 37°C for 1h. p38 MAPK in the sample binds to both

the antibodies attached to the plate and to those in solution. The unbound assay reagents and analytes were removed by washing the plate leaving the immune-complexes containing both antibodies ready for detection. The microplate was read at 450nm in the plate reader.

IV.8.13 DETERMINATION OF MRNA EXPRESSION

The determination of mRNA expression was performed by extracting total RNA from the cells. Since mRNA is single-stranded, and PCR requires double-stranded molecules, double-stranded complementary DNA (cDNA) was prepared by reverse transcription (RT). The amount of cDNA is then directly proportional to the original mRNA, and the starting concentration was calculated through real-time quantitative PCR (RT-qPCR).

RNA Extraction

Total RNA was isolated from lung tissue (1-2mm in diameter) using the RNeasy Mini Kit (Qiagen) at room temperature. Cell lysis was achieved by the addition of RLT buffer containing 1% β -mercaptoethanol which immediately inactivated RNases and maintained the integrity of RNA. Homogenisation of the lysate was performed using QIAshredder spin columns (Qiagen) followed by centrifugation of the samples for 2min at 12,000rpm. An equal volume of 70% ethanol was added to the homogenised lysate and the sample transferred to RNeasy spin columns. The columns were centrifuged for 15sec at 10,000rpm; spin columns were washed with Buffer RW1, and then washed twice with Buffer RPE, after the final wash step the remaining RPE buffer was removed by centrifugation for 2min at 10,000rpm. Total RNA was eluted from the spin column in 50 μ l RNase-free water by

centrifugation for 1min at 10,000rpm. Total RNA was then stored at -80°C until use. Quantification and purity was measured using a Nanodrop™ Lite (ThermoFisher). The RNA concentration was given in ng/μl and purity was determined by ratio of absorbance at 260nm and 280nm (A260/A280). All mRNA used in this thesis had an A260/A280 value ≥ 1.6 and ≤ 2 .

Reverse Transcription (RT)

Total RNA (0.5μg) was re-suspended in 10μl of RNase free water and was denatured at 70°C for 5min. Single stranded cDNA was then synthesised by addition of a master mix containing relevant components to complete the RT reaction, shown in Table IV.3. Samples were then incubated at 42°C for 1h followed by enzyme inactivation at 90°C for 4min. After the RT reaction, 40μl of DEPC-treated water was added to the RT-product to dilute the magnesium present in sample. cDNA was stored at -20°C until use.

Table IV.3: Master mix components for reverse transcription (RT) reaction

Reagent	Volume/Reaction (μl)	Final Concentration
5x AMV buffer	4	1x
dNTPs	2	1mM
Random primers	1	0.5μM
RNasin	1	40U
AMV RTase	1	10U
H ₂ O-DEPC	1	N/A
cDNA product	Make up to 10μl in H ₂ O	

Real time quantitative polymerase chain reaction (qPCR)

Gene transcript levels of specific genes (Table IV.4) were quantified by RT-qPCR using either QuantitTect SYBR® Green (Qiagen, Table IV.5) using a Rotor-Gene 3000 machine and Rotor-Gene 6 software (Corbett Research; Cambridge, UK) or TaqMan® (Table IV.6) and performed on an ABI systems model and analysed using 5700 software (Figure IV.9). The total reaction volume was 20µl for both protocols; see (Tables IV.3 & IV.4) for reagents and concentrations. To account for variations within the protocol, gene transcript levels of 18S RNA, a housekeeping gene, was also quantified and used for normalisation (Figure IV.9). Primer sequences and optimal annealing temperatures used in the SYBR® green method according to the manufacturer's instructions (Qiagen). For the SYBR® green method, the reaction involved an initial heat step of 95°C for 15min to activate the DNA polymerase followed by 30-40 cycles of denaturation (94°C, 15sec), annealing (55-60°C, 30sec) and extension (72°C, 30sec). Similarly, the TaqMan® protocol (Applied Biosystems through ThermoFisher), consisted of an initial 2min at 50°C step followed by 10min at 95°C and 40 cycles of 95°C for 15sec and a 1min at 60°C acquisition step; the commercial probes are all designed to anneal at 60°C. DNA is quantified by using the delta-delta CT method:

$$\Delta\Delta CT = (2^{\Delta CT_{\text{target gene}}}) / (2^{\Delta CT_{\text{Reference gene}}})$$

The threshold cycle (Ct) in which the fluorescence crossed the background fluorescence value was recorded for each PCR reaction and compared to the 18S rRNA qPCR Ct value from the same sample to generate the $\Delta\Delta Ct$ values (Figs. IV.8).

Table IV.4: TaqMan PCR probes for real-time RT-PCR.

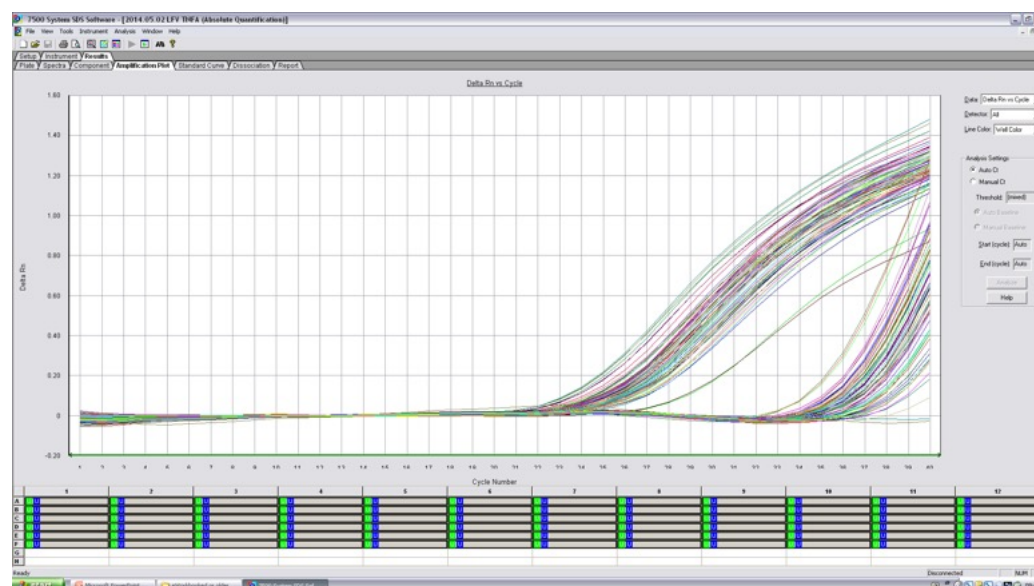
Taqman probe	Primer catalogue number
18S	4310893E
GAPDH	431088E
CXCR3 (IP10)	Hs 01847760_s1
IL-6	Hs00985639_m1
IL-18	Hs01038788_m1
IL-10	Hs 00961622_m1
TNF-α	Hs01113624_g1

Table IV.5: Components for Sybr[®] green qRT-PCR master mix.

Component	Volume
2x QuantiTect SYBR Green PCR Master Mix	1X
Forward primer	1 μ l
Reverse primer	1 μ l
RNase-free water	3 μ l
Total master mix reaction volume	15 μ l
cDNA product	5 μ l
Total volume	50 μ l

Table IV.6: Components for TaqMan[®] qRT-PCR master mix.

Component	Volume
TaqMan[®] Master Mix	10 μ l
18S Probe	1 μ l
Gene of interest Probe	1 μ l
RNase-free water	3 μ l
Total master mix reaction volume	15 μ l
cDNA product	5 μ l
Total volume	35 μ l

Figure IV.9: Representative example of real-time qPCR data.

Representative Taqman qPCR graph, illustrating changes in TNFA gene expression. After an initial baseline period in which fluorescence was undetectable the level of fluorescence in the PCR reaction increased exponentially.

IV.8.14 ELISA

Human sandwich ELISA was performed using DuoSet assays as described by the Manufacture (R&D, Minneapolis, USA). A list of capture and detection antibodies used is provided in (Table IV.10).

The 96-well microtitre plates were coated with 100µl of capture antibody (Table IV.7) at the working concentration in PBS for 16 hrs at room temperature. The capture antibody was aspirated off and the plate washed three times with 300µl of wash buffer (PBS-Tween) using an ELISA plate washer. The plate was then blocked with 300µl blocking buffer (1% BSA in PBS) for at least 1 hour followed by a further three washes as described above. For protein lysates, 1µg of total protein was diluted into 100µl of reagent diluent. Eight recombinant protein standards

including a blank were prepared by a 2-fold serial dilution of the stock protein standard in reagent diluent buffer. 100µl of these (in duplicate) were then dispensed into the wells along with 100µl of diluted sample supernatant in triplicate. The plates were incubated at room temperature for 2 hrs followed by another wash step. Detection antibody diluted to the working concentration (Table IV.7) in reagent diluent (100µl) was dispensed into the wells and incubated for a further 2h at room temperature. The plate was again washed and 100µl of Streptavidin-HRP solution, diluted to the working concentration in reagent diluent, was added to the wells and incubated for 20 min at room temperature avoiding direct light. A final wash step was performed before 100µl of the substrate solution, prepared by mixing equal volumes of colour reagents A (H_2O_2) and B (Tetramethylbenzidine), were added to wells and incubated for 10 min to allow colour to develop. The substrate reaction was stopped by the addition of 50µl of stop solution ($2\text{N H}_2\text{SO}_4$). The absorbance of each well was measured at 450nm and 540nm (correction) using a microtitre spectrophotometer. A four-parameter logistic standard curve was generated using GraphPad Prism (GraphPad Software) and the cytokine concentrations of each sample calculated from this, the concentrations were corrected for dilution by multiplying by the dilution factor.

Table IV.7: List of antibody pairs and standard ranges used in ELISA assays

Cytokine	Capture Antibody	Detection Antibody	Standard Range	DuoSet #
IL-1β	4 μ g/ml mouse anti-human IL-1 β	200ng/ml biotinylated goat anti-human IL-1 β	3.9 - 250pg/ml rhIL-1 β	DY 201
IL-18BPα	2 μ g/ml mouse anti-human IL-18 BP α	200ng/ml biotinylated goat anti-human IL-18BP α	93.8 - 6,000pg/ml rhIL-18BP α	DY119
IL-10	2 μ g/ml mouse anti-human IL-10	75ng/ml biotinylated goat anti-human IL-10	31.2 - 2,000ng/ml rhIL-10	DY217B
IP-10	2 μ g/ml mouse anti-human IP-10	12.5ng/ml biotinylated goat anti-human IP-10	31.2 - 2,000ng/ml rhIP-10	DY266
CXCL-8	4 μ g/ml mouse anti human IL-8	20ng/ml biotinylated goat anti human IL-8	31.2 - 2000ng/ml rhIL-8	DY208

IV.8.15 RANDOMISATION AND DATA COLLECTION

Data were entered in a computerised database to allow the randomised allocation code to be generated for each study participant (Figure IV.10). The research clinician tracked each patient and prompted staff to take the samples required at the designated times and was responsible for analysing the samples in the laboratory.

Figure IV.10: The Ventilate trial schedule of data collection

	Inflammatory Markers (tissue sample)	Inflammatory Markers (blood sample)	Pulmonary function Tests	Pulmonary gas exchange
Pre-op			✓	
Pre-induction				
Post-induction		✓		✓
Before CPB	✓			
10mins post CPB		✓		✓
Before chest closure	✓			
First gas post extubation				✓
2hr post CPB		✓		✓
4hr post CPB				✓
6 hr post CBP		✓		
12hr post CPB		✓		✓
Before removal of arterial line				✓
24hr post CPB		✓		
5 days post-op			✓	
6-8 weeks post surgery			✓	

IV.9 Statistical analysis

IV.9.1 SAMPLE SIZE JUSTIFICATION

Without taking the baseline biopsy into account, it was calculated that a sample size of 32 patients would be able to detect a standardised difference of 1.0 with 80% power and 5% significance (2-tailed). Assuming that the baseline biopsy improves the relative efficiency of the comparison (with an estimated correlation of 0.5 between measures of the primary outcome for the pre/upper lobe and post/lower lobe), the trial either had more power (88%) or was able to detect a smaller target difference (0.9SD). These standardised differences (1.0 or 0.9) represent large differences between groups; however, the primary outcome was chosen to assess the biomarker and site i.e. the lungs that we hypothesised would

be most directly influenced by the intervention and we considered a difference of this magnitude to be plausible.

This target sample size was able to detect a standardised difference of 0.75 in biomarkers measured in the serum with 80% power and 5% significance (2-tailed), assuming a correlation of 0.5 between pre and post-intervention measures and a correlation of 0.7 between the four repeated post-intervention measures. The study was not powered to detect differences between the groups in pulmonary function or adverse events. Data about pulmonary function (and adverse events) have been collected but differences between groups were not formally tested. Specific adverse events were too infrequent to be able to detect differences between groups. Frequencies of these adverse outcomes are tabulated, in line with guidelines for reporting adverse events in trials.

IV.9.2 PRIMARY ANALYSES

Primary analyses were by intention-to-treat. The final analyses were performed after all subjects completed the study, after the database was locked and the statistical analysis plan was approved. The statistical software STATA (version 13.2) was used to analyse the data as well as to generate tables, figures, and listings.

The majority of the data measured continuously scaled outcomes which are summarised as means and SD, at each time point if measured more than once, in each treatment group. If distributions were non-normal, appropriate transformations were used. The analysis was carried out on the transformed data and the findings were transformed back to the original scale where possible, e.g.

if a logarithmic transformation was used then the results are presented as geometric means (GM).

For inflammatory markers in biopsy samples, measures at each time point were analysed, using linear regression to adjust for the baseline level. The model only estimated the main effect of group allocation (LFV vs. conventional management) and the baseline covariate.

For the inflammatory markers in monocytes and polymorphonuclear cells from peripheral blood samples and pulmonary gas exchange expressed as *A-a gradient*, mixed regression models were fitted. These models estimated coefficients for group allocation and the interaction term for group allocation by time. If the interaction was significant ($p < 0.05$), then a group comparison is reported at each time point. All results are presented as differences between, or ratios of, the means for the two groups with 95% CIs.

The measures of inflammation more conventionally as absolute or relative levels before and after CPB in both groups as bar charts or dot plots showing the mean values $\pm$ sem were also reported.

ANCOVA (Analysis of Covariance) in general is used where the outcome variable is continuous and one of the at least two predictors is categorical, hence ANCOVA divides Between Group (BG) variation, into treatment and covariate, whereas ANOVA (Analysis of Variance) attributes Between Group (BG) variation to treatment and is used for at least 3 continuous variables to compare their means (average scores). In our study we used ANCOVA as we wished to analyse the effect of LFV treatment in the 2 populations whilst considering that other variables

or covariates may have an effect e.g. pre-CPB levels of mediators. This was not possible with ANOVA.

IV.10 Results

IV.10.1 STUDY POPULATION

Forty-nine patients were considered for inclusion in the trial (Figure IV.1). Eight declined for personal reasons and 3 declined in favour of the standard procedure. Thirty-eight patients were recruited to the study between December 2012 and February 2014. In all, 6 additional patients were recruited over the initial sample size calculation because of the learning process associated with harvesting biopsies that would be suitable for the laboratory analysis The LigaSure Impact™ instrument (Figure IV.2) (LF4318, Covidien, Watford, Hertfordshire, UK) used for the lung biopsy uses ForceTriad™ energy platform. This high energy results in a burn over the edges of the lung biopsies. When the biopsies were small this resulted in poor quality samples which were not suitable for analysis). Data were recorded as they became available. Nineteen were randomly allocated to LFV and 19 to usual care. One participant allocated to the LFV group was withdrawn because the operation was performed using an off-pump technique, resulting in the patient not receiving the intervention or standard care. Data were not subsequently collected for this patient. Therefore, we had data for 18 patients in the LFV group and 19 patients in the standard of care group. Baseline characteristics, pre-operative co-morbidities, operation details and baseline respiratory measurements were balanced (Figure IV.1 and Table IV.8).

Table IV.8: Patient population characteristics and operative details

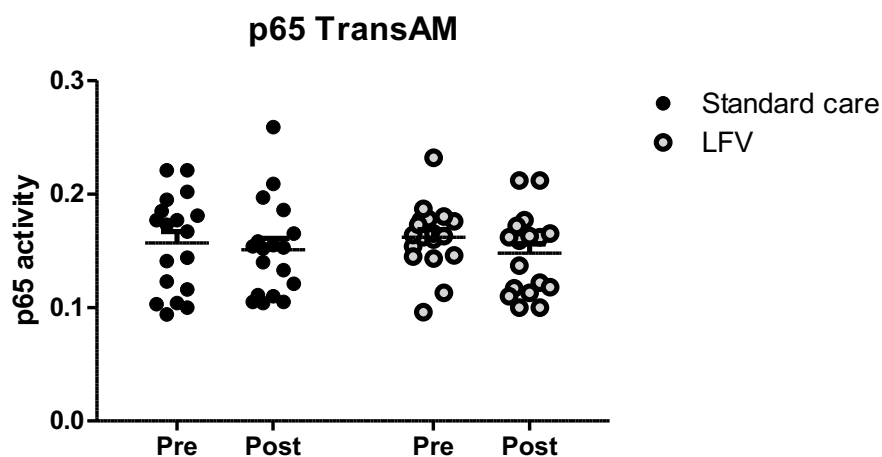
Variable*	LFV (N=18)	Standard care (N=19)
Age (years) – mean (SD)	65.39 (12.09)	62.86 (10.08)
Height (m) - mean (SD)	1.70 (0.09)	1.69 (0.06)
Weight (kg) - mean (SD)	85.72 (15.49)	80.84 (15.29)
BMI (kg/m ²) - mean (SD)	29.69 (4.14)	28.15 (4.82)
NYHA class – no. of patients (%)		
0	0	0
1	4 (22%)	9 (47%)
2	12 (67%)	9 (47%)
3	2 (11%)	1 (5%)
4	0	0
Left Ventricular Function – no. of patients (%)		
Poor (<30%)	0	0
Moderate (30-50%)	4 (22%)	4 (21%)
Good (>50%)	14 (78%)	15 (79%)
Smoker/ex-smoker – no. of patients	14 (78%)	12 (63%)
Asthma – no. of patients (%)	4 (22%)	0
COPD – no. of patients (%)	0	1 (5%)
Operation details		
Bypass, min - median (IQR)	87.5 (68-97)	69 (54-79)
Cross-clamp, min - median	44.5 (37-50)	35 (30-43)
Intubation, hrs - median (IQR)	8.7 (7.1-10.3)	7 (6.4-10)
Time to discharge, days - median	6 (6-7)	6 (5-7)

*The median and interquartile ranges are reported for the variables whose distribution is skewed. Baseline characteristics, pre-operative co-morbidities, operation details and baseline respiratory measurements were balanced.

IV.10.2 PRIMARY OUTCOME. NF- κ B ACTIVITY IN LUNG BIOPSIES.

The mean concentration of NF- κ B p65 in the biopsies before chest closure (adjusted for pre-CPB concentration) was higher in the LFV group compared to the routine CPB group but this was not significant (Figure IV.11) and (Table 9); group difference 0.101, 95% CI -0.022 to 0.226, $p=0.104$). The activity of p65, although reduced, was not significantly affected by LFV or standard care in lung biopsies (Figure IV.11).

Figure IV.11: NF- κ B p65 activation in the LFV compared to the Standard care



The activity of p65 NF- κ B in lung biopsies was determined by TransAm assay and the intensity units plotted against LFV or standard care pre- and post-CPB.

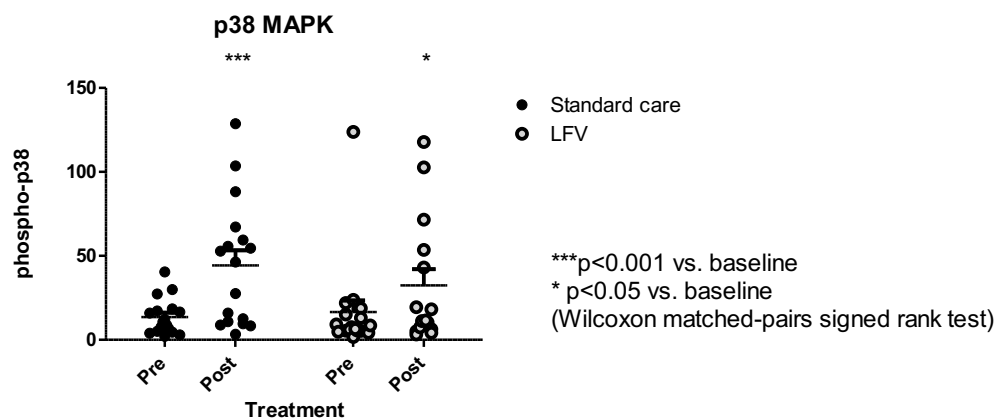
IV.10.3 SECONDARY OUTCOMES

IV.10.3.1 Biopsy markers

Phospho p38 MAPK

CPB was associated with an increase in p38 MAPK activation in all subjects (Figure IV.12). The mean concentration of p38 MAPK in the **biopsies** before chest closure (adjusted for pre-CPB concentration) was lower for the LFV group compared to the standard care CPB group but this was not significant (group difference -0.173, 95% CI -0.723 to 0.376, $p=0.525$) (Figure IV.12) and (Table 9).

Figure IV.12: p38 MAPK activation in the LFV compared to the Standard care.

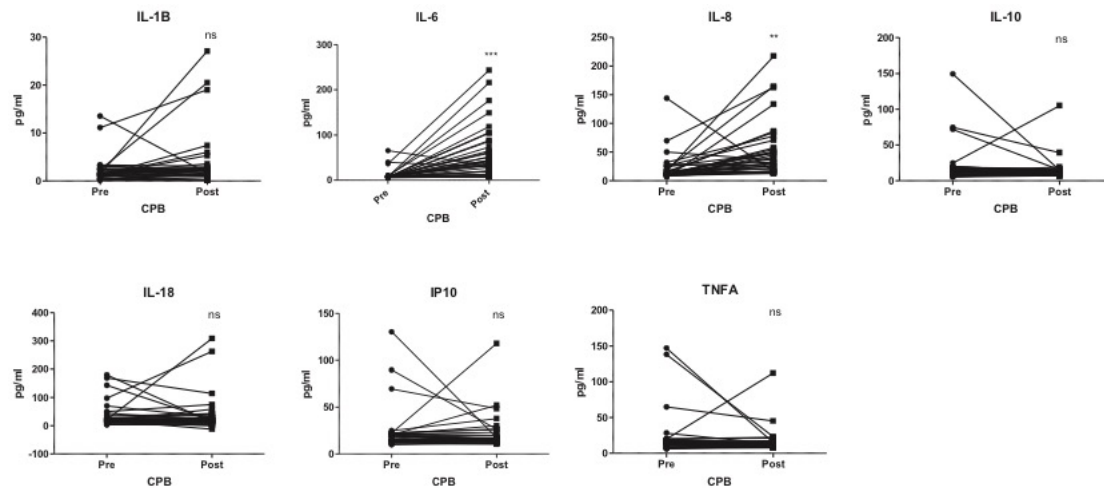


p38 MAPK phosphorylation status determined by Western blotting in lung biopsies plotted against LFV or standard care pre- and post-CPB. Phosphorylated p38 MAPK expression was higher post CPB in both groups but there was no treatment effect. * $p<0.05$, *** $p<0.001$ compared with respective pre-CPB levels.

As a whole IL-6 and IL-8 protein expression was enhanced in lung biopsies post-CPB compared with pre-CPB biopsies (Figure IV.13). The levels of IL-1 β , although elevated in some subjects post CPB, did not reach significance.

Inflammatory mediator expression in lung biopsies

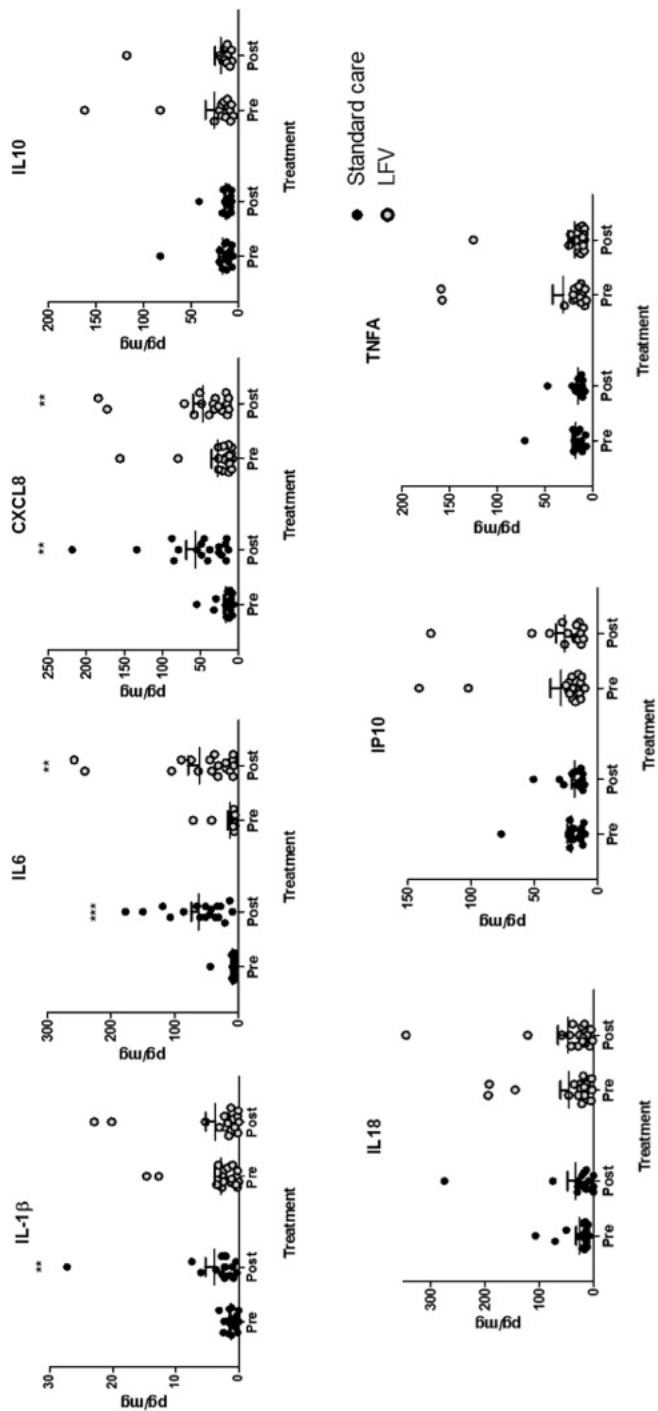
Figure IV.13: Inflammatory mediator protein expression in lung tissue.



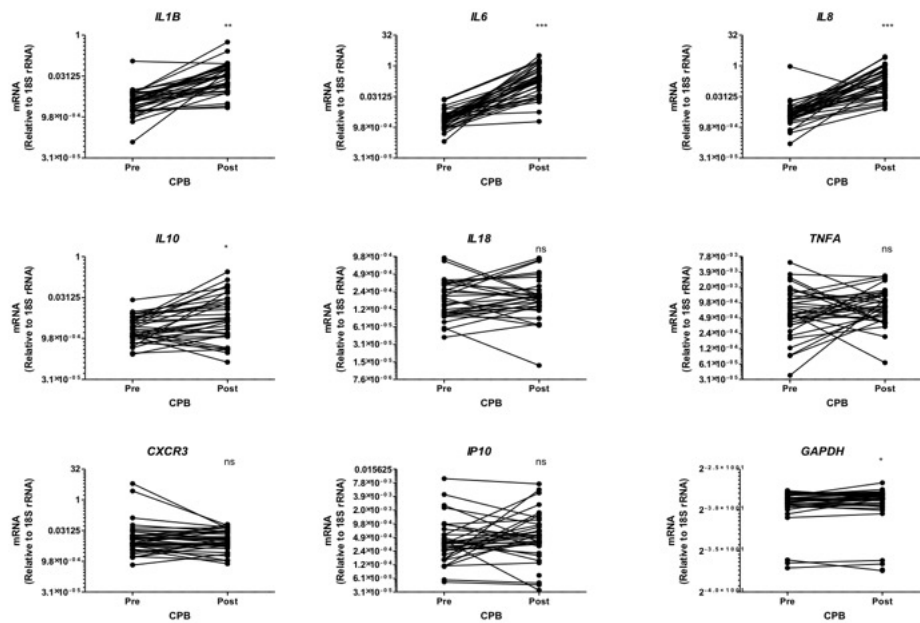
Expression of inflammatory mediator protein in lung tissue pre- and post-CPB (IL-1 β , IL-6, IL-8, IL-10, IL-18, IP10, TNFA). Data are expressed as pg/ml and data for individual patients are connected by lines.

The expression of TNF α ($p=0.127$), IL-1 β ($p=0.012$), IL-6 ($p=0.005$), IP10 ($p=0.533$) and IL-8 ($p=0.073$) were also lower in the LFV group compared to the standard care group but this was significant only for IL-1 β and IL-6 when the pre-CPB levels were taken into account (Figure IV.14). The expression of IL-18 ($p=0.244$) and IL-10 ($p=0.929$) were lower in the LFV group compared to the routine CPB group but these differences were not significant (Figure IV.14).

Figure IV.14: Inflammatory mediator protein expression in lung tissue according to treatment.



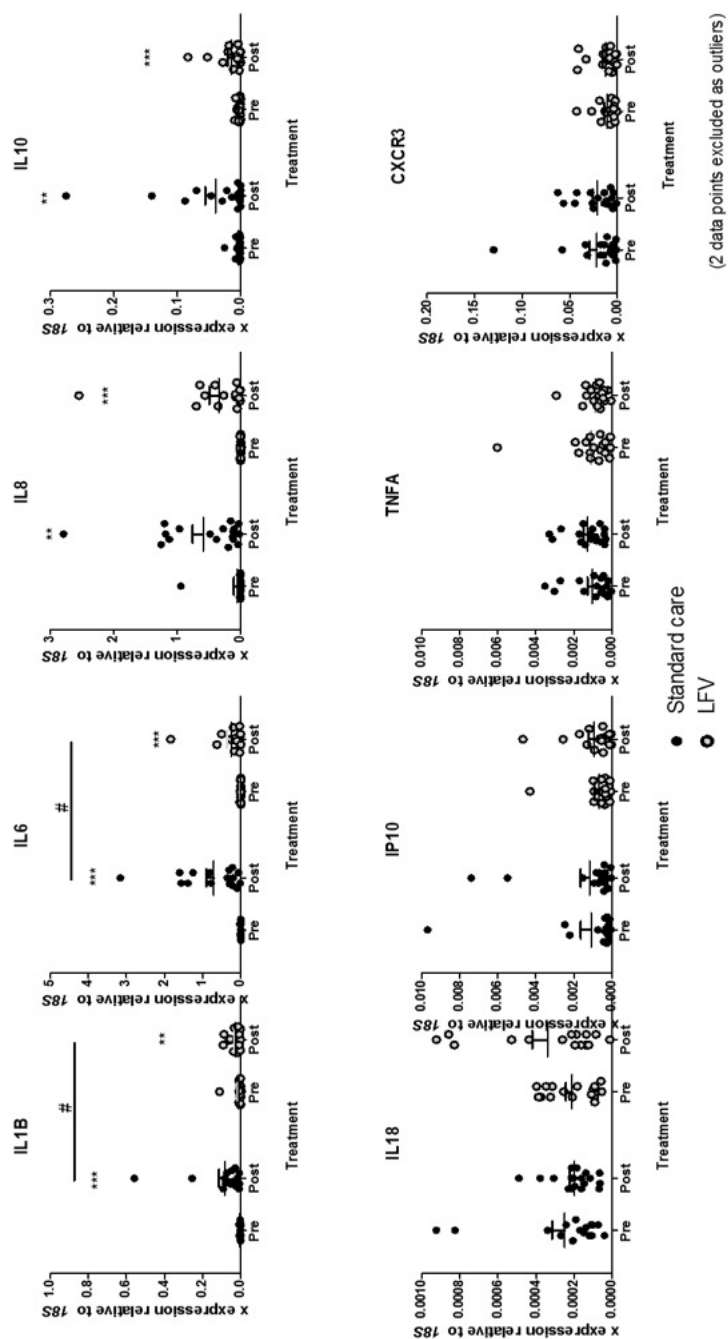
Similar results were observed for inflammatory mediator mRNA in lung biopsies (Figure IV.15). This showed a significant up-regulation of IL-1 β , IL-6, IL-8 and IL-10 mRNAs whilst CPB had no effect on IL-18, TNF α , CXCR3 and IP10 mRNAs.

Figure IV.15: Expression of inflammatory mediator mRNA in lung tissue.

Expression of inflammatory mediator mRNA in lung tissue pre- and post-CPB for IL-1 β , IL-6, IL-8, IL-10, IL-18, TNFA, CXCR3, IP10 and GAPDH. Data are expressed as individual connected points per patients. mRNA levels were calculated by the $\Delta\Delta C_t$ method and presented as relative amounts compared to the 18S RNA housekeeping gene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

When separated according to treatment group (Figure IV.16). There was a significant reduction in the expression of IL-1 β and IL-6 mRNAs with LFW compared with standard care ($p < 0.05$) (Figure IV.16).

Figure IV.16: Expression of inflammatory mediator mRNA in lung tissue in Patients treated with LFV compared to Standard care.



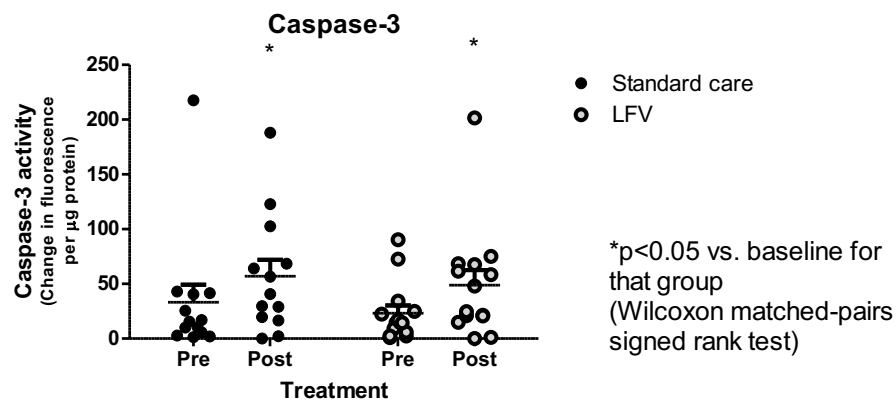
Expression of IL-1 β , IL-6, IL-8, IL-10, IL-18, TNFA, CXCR3, IP10 and GAPDH mRNA in lung according to treatment group. mRNA levels were calculated by the $\Delta\Delta C_t$ method and presented as relative amounts compared to the 18S RNA housekeeping gene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. # $p < 0.05$ between treatment groups.

The results indicate that CPB significant up-regulates the expression of IL-1 β , IL-6, IL-8 and IL-10 mRNAs but had no effect on that of IL-18, TNF α , CXCR3 and IP10 mRNAs. LFV significantly reduced the expression of IL-1 β and IL-6 mRNA expression compared with standard care.

Caspase 3 activity in lung biopsies

Caspase 3 activity in lung biopsies was enhanced post-CPB but this increase was not affected by LFV or standard care (Figure IV.17).

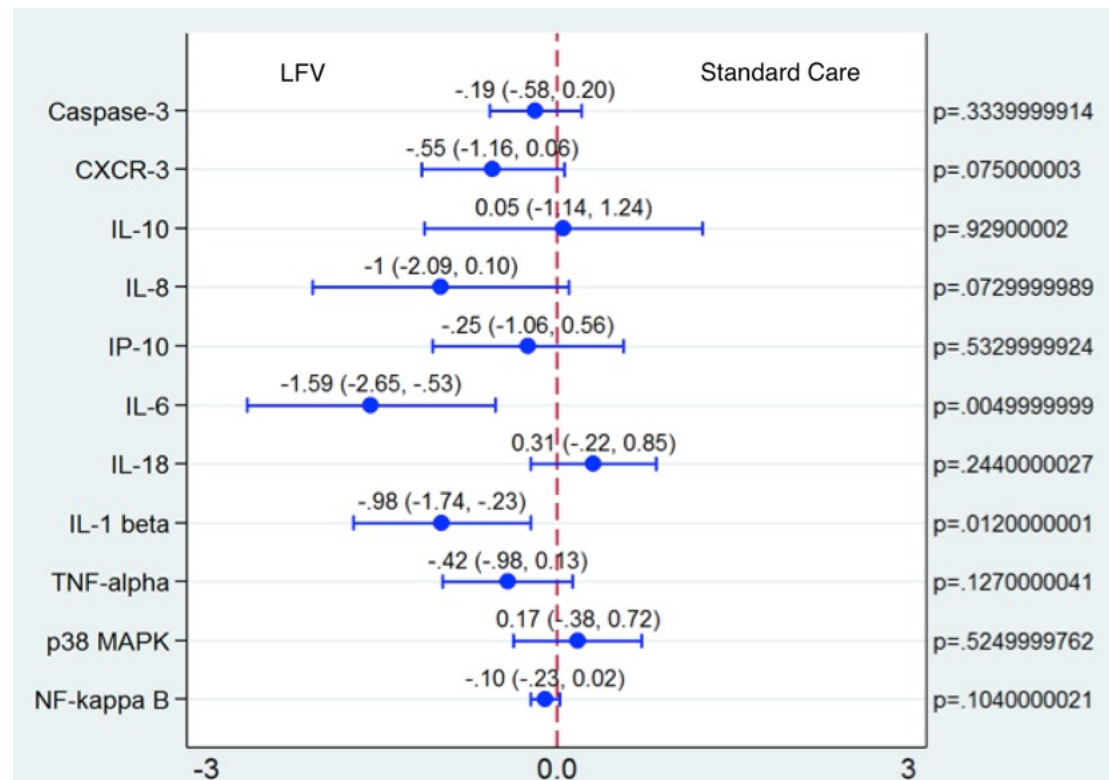
Figure IV.17: Caspase 3 activity in lung biopsies showing effect of LFV compared to Standard care post CPB.



Caspase 3 activity reported as a change in fluorescence was enhanced by CPB. There was no effect of treatment with LFV compared to standard care. *p<0.05 compared with pre-CPB levels.

A summary of the effects of LFV compared with standard care on primary and secondary endpoints in lung biopsies is shown below in (Figure IV.18).

Figure IV.18: Data summary for the main effect for primary and secondary endpoints (standard care vs. LFV).



Results of the statistical inference summary: The expression of IL-1 and IL-6 are significantly reduced in the LFV treatment group compared with the standard care group. Although LFV is favoured compared with standard for some of the other outputs, these did not reach significance. A positive value indicates that standard care was better than LFV whilst a negative value indicates an improved response to LFV.

Table IV.9: Summary of the main effects for primary and secondary endpoints in inflammatory markers in the lung tissue (LFV vs. Standard care)

	Event	LFV vs Standard Care		p value
		Treatment effect	95% CI	
In Biopsy	NF- κ B p65	0.102	-0.022 0.226	0.104
	p38 MAPK	-0.173	-0.723 0.376	0.525
	TNF α	0.425	-0.128 0.977	0.127
	IL-1 β	0.985	0.228 1.742	0.012
	IL-18	-0.310	-0.845 0.224	0.244
	IL-6	1.59	0.528 2.654	0.005
	IP-10	0.251	-0.567 1.06	0.533
	IL-8	0.995	-0.098 2.088	0.073
	IL-10	-0.053	-1.244 1.138	0.929
	CXCR3	0.551	-0.058 1.159	0.075
	Caspase 3	0.186	-0.204 0.576	0.334

Results of the statistical inference summary showing the main effect for primary and secondary end points in standard care vs. LFV. Cytokines increased following surgery. A positive value indicates that standard care was better than LFV whilst a negative value indicates an improved response to LFV.

IV.10.3.2 Blood markers

Leukocytes were separated into monocytes, granulocytes and lymphocytes by forward and side scatter. The permeabilised monocytes and granulocytes, when measuring p38 MAPK and NF- κ B p65, activation showed significant overlap by this method and were therefore treated as a single group.

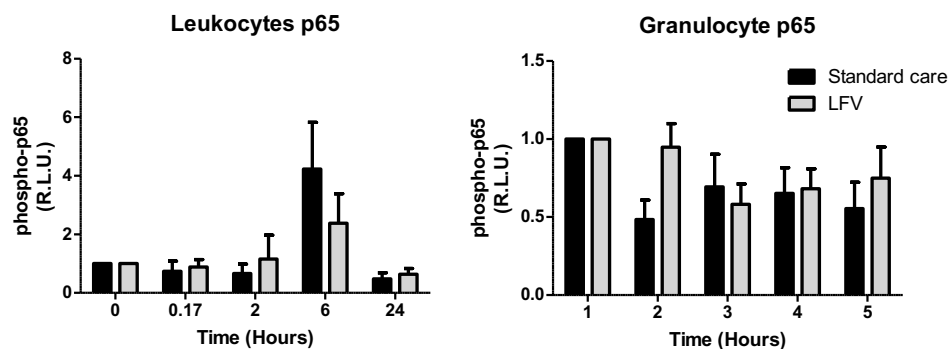
NF- κ B p65 activation

NF- κ B p65 levels in combined monocytes and granulocytes were lower in the LFV group compared to the routine CPB group but these were not significant (median group difference -0.125, 95% CI -0.433 to 0.182, $p=0.424$). NF- κ B p65 levels in lymphocytes were higher in the LFV group compared to the routine CPB group but also not significant (median group difference 0.023 (95% CI -0.420 to 0.467, $p=0.918$)). (Figure IV.28).

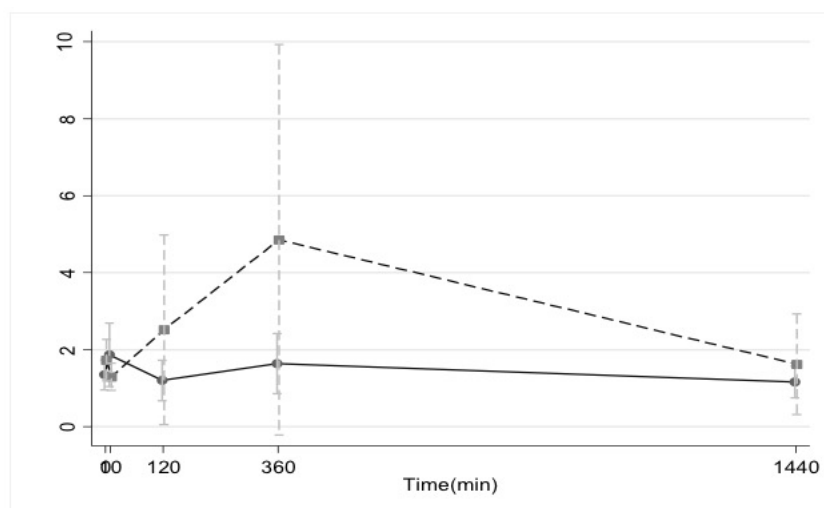
Phospho p38 MAPK expression

p38 MAPK activation as determined by phosphorylation status in combined monocytes and granulocytes and lymphocytes were lower in the LFV group compared to the routine CPB group but this was not significant (monocytes/granulocytes: median group difference -0.029, 95% CI -0.284 to 0.226, $p=0.823$ and lymphocytes: median group difference -0.146, 95% CI -0.524 to 0.232, $p=0.448$)). (Figure IV.28).

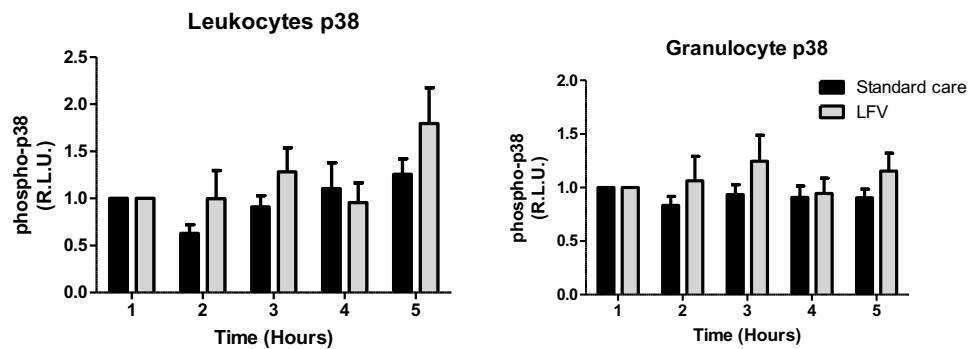
Time was the only significant variable for both p65 (Figures IV.19 & 20) and p38 MAPK activation (Figures IV.21 & 22). When time was fitted in the model, we found that p38 MAPK activation status in lymphocytes were significantly lower in the LFV group at 2, 6 and 24 hrs compared to 10 min. The interaction of intervention x time was not significant at any time point for any of the blood markers.

Figure IV.19: Time is only significant variable for p65 activation

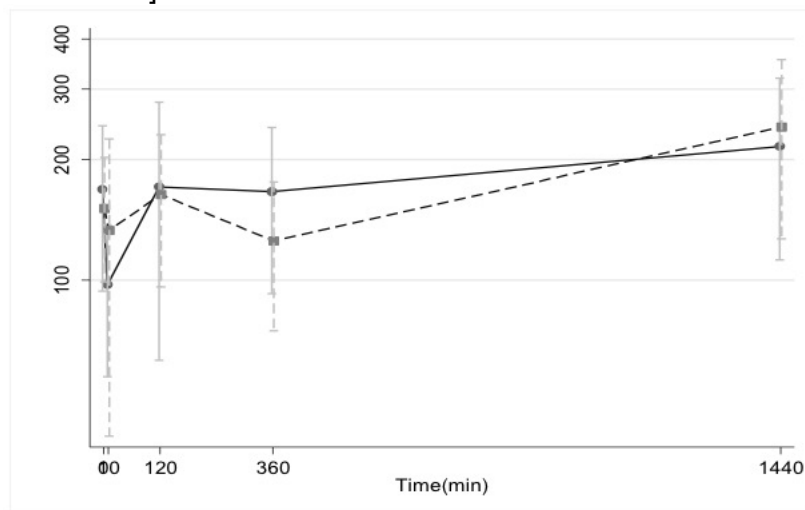
The activity of p65 NF- κ B in blood cells as determined by FACs analysis. The intensity units are plotted against time post CPB according to treatment with LFV or standard care. Results are presented as mean $\pm$ sem for leukocytes (left panel) and granulocytes (right panel).

Figure IV.20: P65 raw data [solid line=LFV, dash line=standard care]

NOTE: y axis is not in the log scale because most values are close to 1. Results of the statistical inference illustration of the raw data on a log scale for y axis.

Figure IV.21: Time is only significant variable for p38 MAPK activation

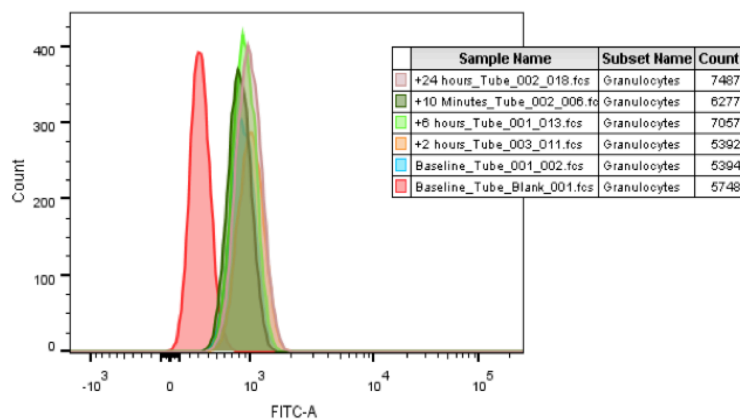
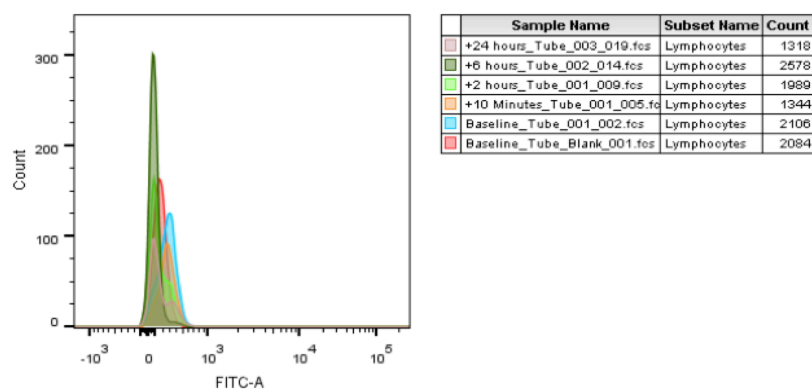
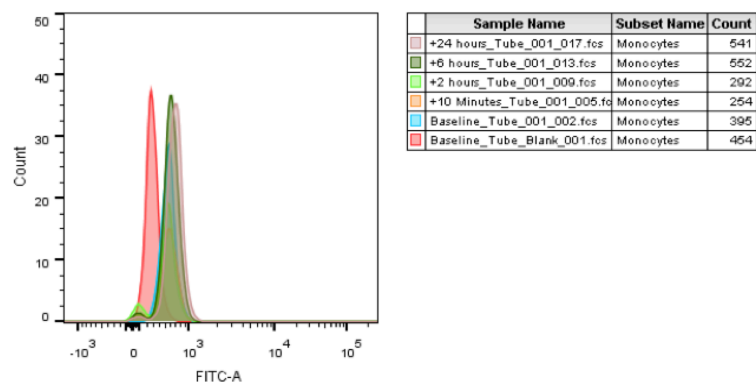
The activity of p38 MAPK in blood cells as determined by FACs analysis. The intensity units are plotted against time post CPB according to treatment with LFV or standard care. Results are presented as mean $\pm$ sem for leukocytes (left panel) and granulocytes (right panel).

Figure IV.22: p38 raw data on log scale (for y axis) [solid line=LFV, dash line=standard care]

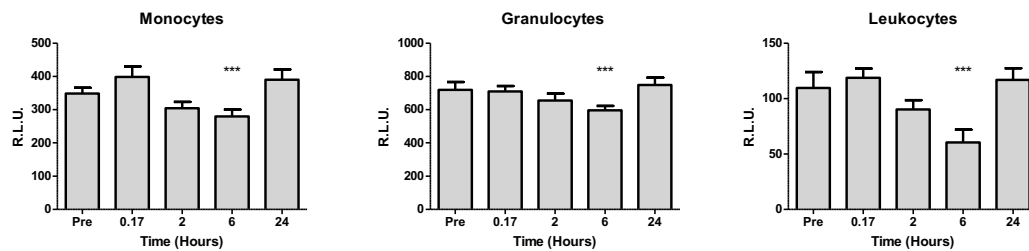
Results of the statistical inference illustration of the raw data on a log scale for y axis.

Oxidative stress levels in blood samples

ROS levels in monocytes, granulocytes and lymphocytes were lower in the LFV group compared to the routine CPB group but these were not significant (monocytes: between treatment difference -0.1 (95% CI -0.353 to 0.152, $p=0.436$), granulocytes: between treatment difference -0.01 (95% CI -0.196 to 0.176, $p=0.913$) and lymphocytes: between treatment difference -0.213 (95% CI -0.585 to 0.159, $p=0.262$). (Figures IV.23 & IV.24)

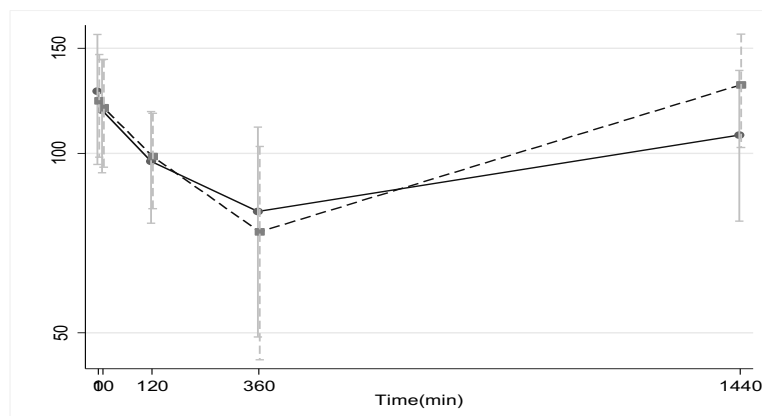
Figure IV.23: Inflammatory Markers in blood APF (oxidative stress assay).**1****2****3**

4



The above histogram plots a single parameter (FITC intensity, horizontal axis) against the number of events detected (vertical axis). We have 4 histogram panels representing FACS data for cellular oxidative stress (APF) (1) granulocytes, (2) monocytes and (3) lymphocytes. (4) Grouped shows the pooled data. Suggesting a reduction in blood cells at 6hrs and recovery at 24hrs hence time is the only significant factor.

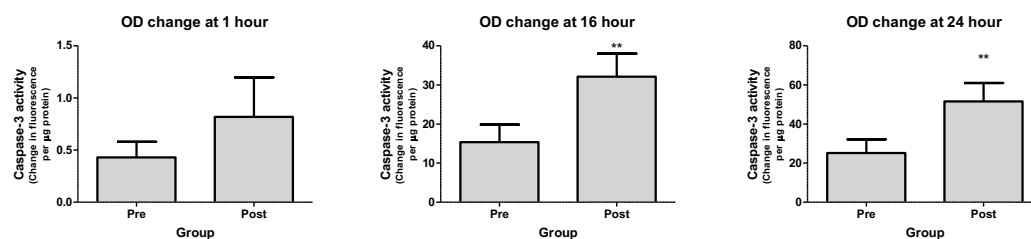
Figure IV.24: ROS raw data on log scale (for y axis) [solid line=LFV, dash line=standard care]



Caspase 3 activity in blood

Caspase 3 activity was significantly increased by CPB in the blood at 1, 16 & 24 hrs post CPB (Figure IV.25). There was no difference between the effect of LFV and standard care for blood caspase 3 over time (Table IV.9).

Figure IV.25: Caspase 3 activity over time



The activity of caspase 3 in blood cells was not affected by the LFV or standard care but was significantly increased by CPB in the blood. The intensity units are plotted against time post CPB. Results are presented as mean $\pm$ sem for pre CPB (left bar) and post CPB (right bar) at 1, 16 & 24 hrs post CPB.

Heme levels in blood after LFV or standard care

When time was fitted to the model, we found that heme levels were significantly lower in the LFV intervention group at 2, 6 and 24 hrs compared to 10 min. However, the interaction of intervention x time was not significant at any time point for any of the blood markers (Figures IV.26 & IV.27).

Figure IV.26: Heme raw data on log scale (for y axis) [solid line=LFV, dash line=standard care]

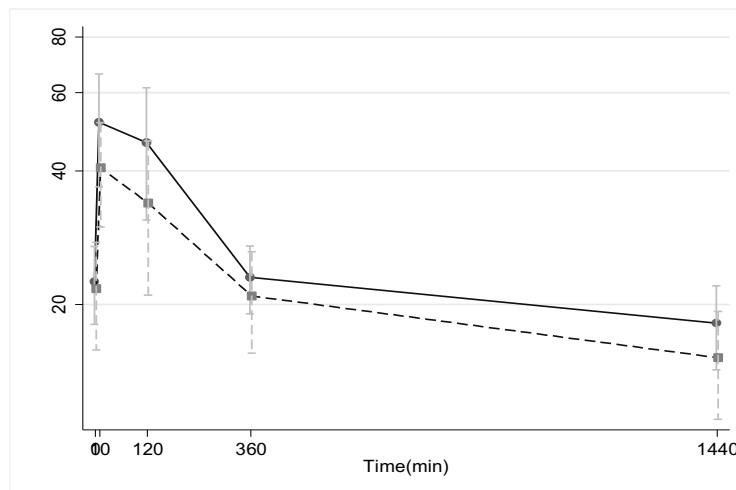
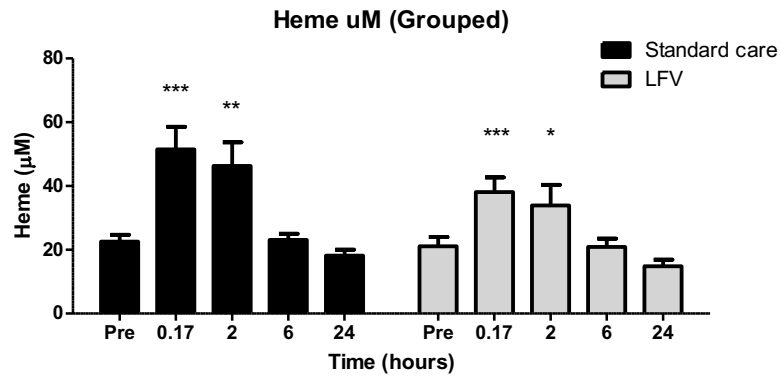


Figure IV.27 LFV treatment trended towards reduced serum heme

Source of Variation	% of total variation	P value
Interaction	1.49	0.2102
Time	27.23	< 0.0001
Treatment	2.31	0.1662
Subjects (matching)	36.8982	< 0.0001

The activity of Heme in blood showed that levels were significantly lower in the LFV intervention group compared to pre CPB. Results are presented as mean $\pm$ sem against time pre & post CPB according to treatment with LFV (right column) or standard care (left column) at pre, 2, 6 and 24 hrs in relation to CPB.

Summary of LFV effects on blood inflammatory parameters compared with standard care.

A summary of the effects of LFV and standard care on blood markers is provided below in (Table IV.10 and Figure IV.28) and a summary of the comparative effect is reported in (Table IV.11).

Table IV.10: Summary of the comparison between standard care and low LFV on blood inflammatory markers.

Inflammatory Marker Mean(SD)*	LFV					Standard care				
	Post-induction	10 min post CPB	2 h	6 h	24 h	Post-induction	10 min post CPB	2 h	6 h	24 h
<i>Leukocytes</i>										
NF-κB p65	0.16 (0.54)	0.40 (0.63)	-0.03 (0.80)	0.08 (0.99)	-0.30 (1.26)	0.44 (0.44)	0.10 (0.67)	0.11 (1.22)	0.21 (1.56)	-0.05 (0.94)
p38 MAPK	4.84 (0.80)	4.22 (1)	4.57 (1.26)	4.79 (1)	4.90 (1.19)	4.75 (0.83)	4.41 (1.07)	4.72 (1)	4.63 (0.77)	5.00 (1.11)
ROS**	4.43 (1.48)	4.46 (1.33)	4.41 (0.76)	3.80 (1.60)	4.35 (1.13)	4.71 (0.51)	4.69 (0.45)	4.51 (0.46)	3.79 (1.24)	4.75 (0.54)
<i>Monocytes/Granulocytes***</i>										
NF-κB p65	0.03 (0.60)	0.32 (0.41)	0.37 (0.85)	0.21 (0.63)	-0.07 (0.94)	0.38 (0.88)	0.60 (0.73)	0.28 (1.22)	0.43 (0.76)	0.60 (1.02)
p38 MAPK	6.63 (0.55)	6.38 (0.48)	6.50 (0.59)	6.42 (0.59)	6.46 (0.72)	6.42 (0.60)	6.25 (0.64)	6.44 (0.62)	6.21 (0.54)	6.40 (0.76)
<i>Monocytes</i>										
ROS**	5.54 (1.11)	5.52 (1.58)	5.47 (1.45)	5.38 (0.91)	5.78 (0.89)	5.73 (0.24)	5.87 (0.45)	5.58 (0.49)	5.55 (0.51)	5.82 (0.46)
<i>Granulocytes</i>										
ROS**	6.25 (1.07)	6.27 (1.38)	6.21 (1.27)	6.17 (0.93)	6.42 (0.94)	6.52 (0.36)	6.48 (0.33)	6.44 (0.31)	6.36 (0.21)	6.54 (0.38)
Heme (μM)	3.05 (0.38)	3.79 (0.57)	3.65 (0.64)	3.08 (0.34)	2.81 (0.43)	2.95 (0.51)	3.58 (0.50)	3.35 (0.56)	2.93 (0.48)	2.60 (0.49)

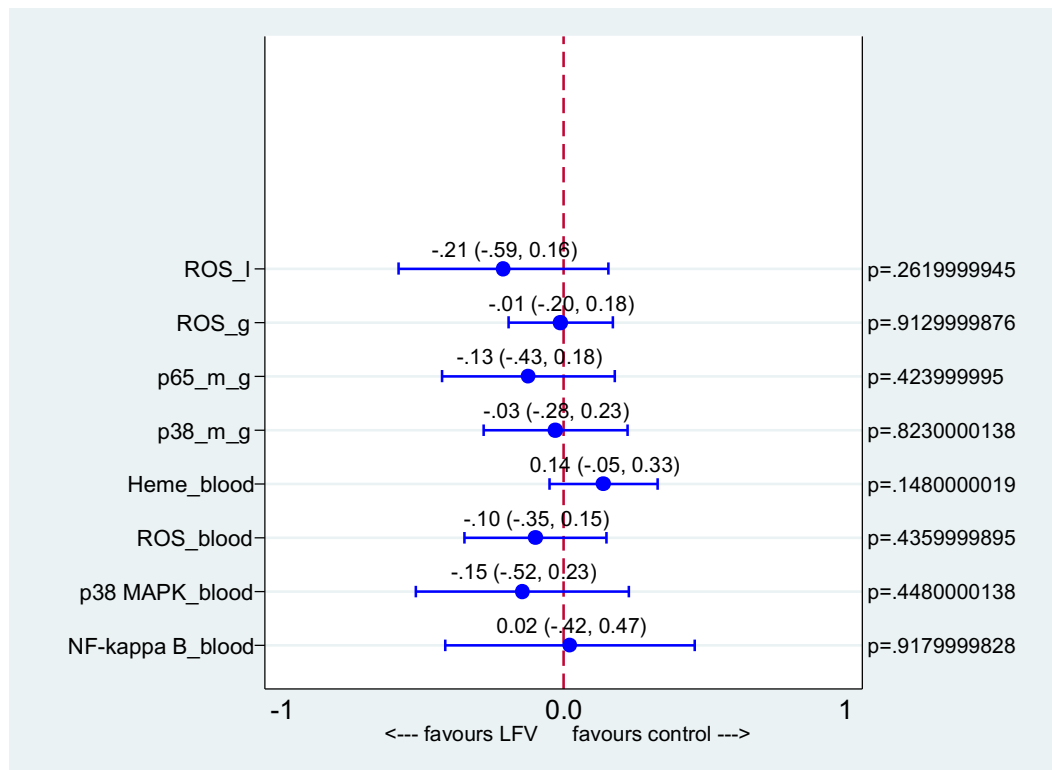
*Data are transformed because their distribution is not normal, the mean of the transformed data is tabulated.

**Negative data for ROS was set to missing. Most time points and cell type had only one value set to missing. ROS in Leukocyte at baseline and 2 hrs had three missing values, ROS in Leukocyte at 6 hrs had 6 missing values.

***Monocytes overlapped with granulocytes for NF-κB p65 and p38 MAPK

A summary of the effects of LFV compared with standard care on primary and secondary endpoints in the blood is shown below in (Figure IV.28).

Figure IV.28 Summary of the effects of LFV compared with standard care on primary and secondary endpoints in the blood



Results of the statistical inference summary: A positive value indicates that standard care was better than LFV whilst a negative value indicates an improved response to LFV.

Table IV.11: Summary of the main effect for primary and secondary endpoints in inflammatory markers in the blood (LFV vs. Standard care)

	Event	LFV vs Standard Care		p value
		Treatment effect	95% CI	
In Blood	NF-κB p65 leukocytes	0.023	-0.420 0.467	0.918
	NF-κB p65 monocytes_granulocyte	-0.125	-0.433 0.182	0.424
	p38 MAPK leukocytes	-0.146	-0.524 0.232	0.448
	p38 MAPK monocytes_granulocyte	-0.029	-0.284 0.226	0.823
	ROS monocytes	-0.100	-0.353 0.152	0.436
	ROS granulocyte	-0.010	-0.196 0.176	0.913
	ROS leukocytes	-0.213	-0.585 0.159	0.262
	Heme	0.142	-0.050 0.334	0.148

Results of the statistical inference summary showing the main effect for primary and secondary end points in LFV vs standard care.

The effect of LFV versus standard care on clinical parameters.

The alveolar-arterial oxygen gradient (A-a gradient) is the difference between alveolar oxygen level (PAO_2) and arterial oxygen level (PaO_2) and is calculated by the equation: Alveolar to arterial (A-a) oxygen gradient = $PAO_2 - PaO_2$. [253]. The A-a oxygen gradient indicates the integrity of the alveolo-capillary membrane and degree of gas exchange. Pathological conditions at the alveolo-capillary level will increase the gradient. Hence as example hypoxemia due to V/Q mismatch and diffusion limitation will have bigger gradient, but hypoxemia due to hypoventilation would have normal gradient [253]. In our data, the A-a gradient had no significant difference in the LFV group compared to the standard CPB group (19.325, 95% CI -12.507 to 51.157, $p=0.234$). When time was fitted in the model, we found that there was a significant reduction in the A-a gradient in time compared to 10 min in

the LFV group. However, the interaction of intervention x time was not significant at any time point. (Tables IV.12-14).

Overall, we have marked increase in the activity of most inflammatory biomarkers in the lung and blood as a result of the CPB including the primary outcome NF- κ B p65 in the lung biopsies and moving towards the secondary outcome measures in the lung and in the blood. The primary end point (NF- κ B p65 activation) was not reached but despite that we don't have statistical significance, we observed a lower trend for various inflammatory biomarkers towards an improvement with the LFV (in the blood including NF- κ B p65 & p38 MAPK) and (in lung biopsies including p38 MAPK, IL-18, IL-10, TNF α , IL-1 β , IL-6, IP10, IL-8, chemokine receptor CXCR3).

Table IV.12: Lung function parameters (Standard care vs LFV)

Lung Function test Mean(SD)*	LFV		Standard care	
	Pre-op	Follow up	Pre-op	Follow up
FEV1 (% predicted) litres - mean (SD)	90.94 (21.58)	82.06 (19.86)	97.22 (18.83)	91 (20.20)
FVC (% predicted) litres - mean (SD)	93.24 (20.73)	83.00 (18.37)	97.11 (18.51)	91.25 (16.55)
MEF75(% predicted) litres/sec - mean (SD)	84.13 (42.32)	85.31 (39.01)	87.87 (30.83)	87.00 (30.37)
MEF25(% predicted) litres/sec - mean (SD)	68.27 (36.25)	60.56 (25.77)	70.47 (25.82)	67.33 (23.94)
TLC(% predicted) litres - mean (SD)	97.40 (14.53)	88.94 (11.76)	103.27 (14.11)	90.08 (13.57)
RV (% predicted) litres - mean (SD)	112.73 (25.60)	98.75 (24.47)	116.33 (34.67)	95.17 (21.44)
TL _{co} (% predicted) (mmol/Kpa/min) - mean (SD)	89.79 (18.13)	84.75 (18.05)	84.14 (12.98)	76.92 (18.13)
K _{co} (% predicted) (mmol/Kpa/min) - mean (SD)	103.71 (17.29)	108.56 (17.53)	96.00 (12.17)	97.75 (15.36)
FEV1(measured) litres - mean (SD)	2.51 (0.91)	2.36 (0.81)	2.75 (0.81)	2.58 (0.89)
FVC(measured) litres - mean (SD)	3.27 (1.07)	3.04 (0.99)	3.47 (0.95)	3.30 (0.99)
MEF75 (measured) litres/sec - mean (SD)	5.40 (3.32)	5.87 (2.93)	5.93 (2.41)	6.09 (2.45)
MEF25 (measured) litres/sec - mean (SD)	1.40 (2.02)	1.17 (1.57)	0.97 (0.51)	0.97 (0.55)
TLC(measured) litres - mean (SD)	5.87 (1.19)	5.59 (1.07)	6.15 (1.07)	5.58 (1.16)
RV (measured) litres - mean (SD)	2.57 (0.61)	2.29 (0.7)	2.61 (0.87)	2.17 (0.51)
TL _{co} (average) (mmol/Kpa/min) - mean (SD)	7.63 (1.93)	7.29 (1.8)	7.53 (2.56)	6.69 (2.40)
K _{co} (average) (mmol/Kpa/min) - mean (SD)	1.39 (0.26)	1.46 (0.25)	1.29 (0.19)	1.31 (0.27)
Percentage O ₂ saturation - mean (SD)	96.33 (1.05)	96.88 (1.58)	97.07 (1)	98 (0.85)

*If data were not normally distributed and log transformed then the mean of the transformed data is being tabulated.

Table IV.13: Pulmonary gas exchange parameters (LFV vs Standard care)

Pulmonary gas exchange Median(IQR)	LFV						
	Post-induction	10 min post CPB	2 h	4h	Post extubation	12 h	24 h
PaO₂ (mmhg)	215.25 (144.7-344.3)	168 (145.5-264)	133.9 (103.5-203)	119.25 (105-130.5)	110.25 (84.7-117.8)	102.35 (94.5-122.3)	99 (86.3-114.8)
A-a Gradient	148.19 (33.18 - 204.96)	176.74 (121.39 - 233.55)	114.89 (108.5 - 150.53)	116.26 (91.63 - 126.95)	113.63 (77.56 - 133.00)	97.56 (80.36 - 116.88)	104.04 (81.97 - 132.83)

Pulmonary gas exchange Median(IQR)	Standard care						
	Post-induction	10 min post CPB	2 h	4h	Post extubation	12 h	24 h
PaO₂ (mmhg)	229.55 (164.3-293.3)	211.5 (117-328.5)	129 (117-184.5)	132 (106-5-157.5)	114.8 (94.5-148.5)	122.3 (105.8-146.3)	97 (90.8-111.8)
A-a Gradient	111.59 (85.05 - 168.65)	178.21 (40.63 - 336.49)	113.78 (95.1 - 146.83)	90.63 (62.75 - 144.45)	105.95 (58.43 - 122.40)	62.68 (35.86 - 99.70)	67.98 (46.47 - 114.63)

Table IV.14: Main effect for primary and secondary endpoints in A-a gradient

	Event	Standard Care vs. LFV	
		Treatment effect	95% CI
Pulmonary gas exchange	A-a gradient	-19.32464	-51.15657 12.50729

IV.10.4 ADVERSE EVENTS

The frequency of adverse events are summarised in (Table IV.15).

Table IV.15: Adverse events

Adverse event	LFV n (%)		Standard Care n (%)	
	In hospital	At follow up	In hospital	At follow up
Respiratory				
Re-intubation/Ventilation	0	0	0	0
Mask CPAP	1 (5.6%)	0	2 (10.5%)	0
Tracheostomy	0	0	0	0
Prolonged ventilation (24 h)	0	0	0	0
ARDS	0	0	0	0
Pneumothorax / Effusion	0	0	0	0
Cardiovascular				
MI	0	0	0	0
Cardiac arrest	0	0	0	0
Arrhythmias	9 (50%)	0	7 (36.8%)	0
Haemodynamic support	16 (89%)	0	9 (47.4%)	0
Neurological				
Permanent Stroke	0	0	0	0
TIA	0	0	0	0
Renal				
Hemofiltration / dialysis	0	0	0	0
Other				
GI complications	0	0	0	0
Thromboembolic	0	0	0	0
Bleeding complications	0	0	0	0
Wound complications	0	0	0	1 (5.3%)
Infective complications	4 (22.2%)	3 (17%)	4 (21.1%)	3 (15.8%)
Reoperation	0	0	0	0

Mask CPAP was necessary for one patient in the LFV group and 2 in the routine CPB group. Arrhythmia occurred in 50% of patients in the LFV group and 37% of patients in the routine CPB group. The biggest difference in groups for occurrence of adverse events was for need for haemodynamic support, 89% of patients in the LFV group vs 47% in the routine CPB group. Hospital and post discharge infective complications were similar between groups.

IV.11 Discussion

IV.11.1 SUMMARY OF EXISTING EVIDENCE

This trial investigated the possibility of reducing the inflammatory response associated with CABG by a technique of ventilating of the lungs at low frequency during CPB. The change in inflammatory response was measured in both tissue biopsy and blood using NF- κ B p65 in lung biopsies and other inflammatory markers in both tissue and blood. Levels of all markers apart from p38 MAPK, IL-18 and IL-10 measured in the lung biopsy tissue were lower in the LFV group compared to the routine CPB group. However, only the decreases in IL-6 and IL-1 β were statistically significant. There was no significant difference in the blood inflammatory markers between LFV and standard care during CPB although there was a trend towards a reduction in both NF- κ B p65 and p38 MAPK activation in peripheral blood leukocytes. For p38 MAPK this reached significance over the time series 2, 6 and 24 hrs compared to the levels at 10 min post CPB. Time had no effect on any of the inflammatory and oxidative stress parameters studied.

We observed a marked increase in the expression of a number of NF- κ B-induced inflammatory markers at both the protein and mRNA level in lung tissue following CPB. The failure to observe any change in NF- κ B p65 activation in tissue may reflect the rapid early nature of NF- κ B activation compared to the time under CPB (in the LFV group median of 71min, IQR 63.5-93.5 and in the Standard care group median of 80min, IQR 60-92). However, LPS stimulation can lead to waves of NF- κ B activation over time [254] and we may have missed a second round of NF- κ B activation at the time points analysed chosen. However, we detected a marked

effect on p38 MAPK activity in both lung tissue and in peripheral blood following CPB and the effect in blood reached significance over the time series and may have been greater if subsets of cells were analysed. It is possible that CPB is a greater activator of inflammation in response to oxidative stress than NF- κ B and further studies would be required in disease models to test this.

The blood–gas barrier is strikingly strong, despite its thinness, allowing it to tolerate prevailing stresses and maintain structural integrity under physiological conditions [10-12]. This equilibrium is dramatically altered by the iatrogenic, prolonged lung collapse/atelectasis induced during CPB [13]. These conditions during CPB may affect gas exchange by passive diffusion at the blood–gas barrier [9-15]. This means that any disruption to the respiratory passage and circulating volume can lead to the ventilation-perfusion mismatch, increased PA-a O₂, increased extravascular lung water and impaired lung compliance. They may also induce poor alveolar blood supply with high potential for marked alveolar ischemia triggering hypoxic pulmonary vasoconstriction mediated by arachidonic acid metabolites and endothelin [16].

The degree of atelectasis/lung collapse and intrapulmonary shunt has been reported to be associated with gas exchange impairment [14, 16]. This is the most common cause of acute respiratory failure (ARF) after open heart surgery [23-25]. Midline sternotomy incisions affect respiratory mechanics by abolishing the integrity of the chest wall leading to impaired respiratory effort. Postoperative pain has been shown to be associated with decreased lung function by precluding deep inspirations. Furthermore; patients undergoing surgical procedures associated

with opening the pleura will have increased rates of atelectasis, pleural effusions and postoperative pain especially in the early postoperative period [31-33].

CPB can lead to the activation of different inflammatory and coagulation pathways and alters redox balance due to the passage of blood through the circuit (contact activation) and ischaemia and reperfusion injury [34-37]. Vascular EC dysfunction during CPB due to changes in blood flow patterns, shear stress, ischaemia and reperfusion and circulating cytokines will result in the activation of multiple proinflammatory and pro-apoptosis pathways [44-47] while suppressing its ability to produce vaso-protective mediators [47-50]. EC activation is known to initiate a leukocyte adhesion cascade by the expression of members of the selectin family which are responsible for the initial attachment of leukocytes from the circulation. Transmigration of leukocytes through EC, the crucial step for leukocytes recruitment to tissue, follows leukocyte attachment and is mediated by the up-regulation of adhesion molecules such as PECAM-1, ICAM-1 and VLA-4 [51-54].

CPB is traditionally associated with inadequate lung perfusion as there is no flow to the pulmonary artery during periods of cross clamping and when the heart is not ejecting, thus blood supply is limited to the bronchial arteries [55]. Pulmonary physiology alteration during CPB can result in disturbing the balance in the blood gas barrier due to the alteration of the different forces affecting the parenchyma thus abolishing gas exchange by passive diffusion at the blood–gas barrier level and leading to ventilation/perfusion mismatch and impaired pulmonary compliance [13, 14]. Moreover; ischaemia during CPB is associated with reduced alveolar blood supply resulting in alveolar ischaemia and hypoxic pulmonary vasoconstriction [56]. Pulmonary vascular EC dysfunction and activated

neutrophils sequestration into lungs parenchyma during period of reperfusion can increase lung tissue permeability, elevate vascular resistance and pulmonary surfactant changes leading to alveolar protein accumulation, pulmonary oedema and driving more ROS and cytokines production [57-59]. This will manifest as abnormal gas exchange, poor lung mechanics, increased pulmonary shunt fraction and reduced functional residual capacity and carbon monoxide transfer factor [59-61].

IV.11.2 FINDINGS IN THE CONTEXT OF EXISTING LITERATURE

A clinical study reported the use of CPAP during CPB in 14 elective cardiac surgery patients. Seven patients received CPAP at 10cm H₂O during CPB, and in the other seven patients, the lungs were open to the atmosphere (control). Measurements were taken before and after CPB and 4h after CPB in the intensive care unit. CPAP at 10cm H₂O resulted in significantly more perfusion of lung areas with a normal ventilation/perfusion distribution (V/Q) and significantly less shunt and low V/Q perfusion 4h after CPB in comparison with the control group. Consequently, arterial oxygen partial pressure was significantly higher and PA-a O₂ was significantly smaller. They concluded that CPAP at 10cm H₂O during CPB is a simple manoeuvre that improves postoperative gas exchange and resulted in significantly more perfusion of lung areas with a normal ventilation/perfusion distribution (V/Q) and significantly less shunt [142].

The efficacy of both LFV and CPAP during CPB to reduce post-CPB lung injury have been evaluated recently in an established experimental pig model on 18 Yorkshire pigs subjected to 120min of CPB (one hour of cardioplegic arrest) followed by 90min of recovery before being sacrificed [143]. Six animals served as

controls with the endotracheal tube open to the atmosphere during CPB. The remaining animals were divided into two groups of six; one received CPAP of 5cm H₂O and the other LFV (5/minute) with air (21% oxygen) at a TV of 6-8 ml/kg during CPB. Lung tissue biopsy and bronchoalveolar lavage samples were obtained serially for measurement of adenine nucleotides (ATP, ADP, AMP), lactate dehydrogenase, DNA levels and histology as markers of cell death and tissue damage. Hemodynamic data and arterial blood gases were also collected throughout the study [143].

This study strongly suggested that the use of LFV is associated with significantly better pulmonary gas exchange, higher adenine nucleotide, lower lactate dehydrogenase levels and reduced histological damage in lung biopsies as well as lower DNA levels in bronchoalveolar lavage compared to the control group. The CPAP group showed fewer differences compared to the control group, with a significant reduction only in lactate dehydrogenase levels compared to control. The rationale for this experimental study was to maintain some degree of ventilation during CPB to prevent persisting lung collapse and complete loss of gas exchange by passive diffusion at the blood–gas barrier. A similar concept was used by Reis Miranda and colleagues who studied 62 patients post cardiac surgery, randomly assigned to three groups: (1) conventional mechanical ventilation (CV), (2) Open Lung Concept (OLC) started after arrival on the ICU and (3) OLC started directly after intubation. They observed an increase in functional residual capacity, reduced risk of hypoxemia and lower levels of IL-10 and IL-8 release, hence concluded that OLC ventilation leads to an attenuated

inflammatory response, presumably by reducing additional lung injury after cardiac surgery [255].

Previous studies have shown that oxygen in the alveolar space helps maintain physiological levels of tissue adenine nucleotides and prevents histopathological damage in the lungs during ischemia [256-260]. A recent study was undertaken to examine the effect of maintaining ventilation during bypass compared with discontinued ventilation upon a number of parameters that may be indicative of lung injury. Twenty-three elective patients for coronary artery surgery were randomised to either ventilation (VB) (n=12) or non-ventilation on bypass (NVB) (n=11). The post-bypass extravascular lung fluid was significantly smaller in the VB group compared to the NVB group (530 $\pm$ 50 ml vs. 672 $\pm$ 32 ml; P=0.028). Extubation time was also significantly shorter in the VB group (3.6 $\pm$ 0.3 h vs. 4.8 $\pm$ 0.4 h; P=0.038) [261], hence this study showed the benefits of maintaining ventilation during CPB on post-CPB oxygenation including shorter mechanical ventilation [261].

In contrast, a small study of Fifty-nine patients prospectively randomised to continuous ventilation and no ventilation, during CABG on CPB, looking at inflammatory markers showed there was no statistically significant difference in most of inflammatory makers (IL-6, IL8, IL-10 & lactate). Yet the alveolar–arterial oxygen gradient was higher in the non-ventilated group after induction, and immediately after and at one hour after discontinuation of CPB ($p < 0.001$). Overall this study concluded that there was no evidence that ventilation during CPB attenuated the inflammatory response [262]. A recent meta-analysis for patients undergoing cardiac surgery and received ventilation during CPB, included

seventeen trials with 1162 patients, showed that ventilation during CPB significantly increased post-CPB $\text{PaO}_2/\text{FiO}_2$ ratio, but there was insufficient evidence to show that ventilation during CPB could influence the long-term prognosis of these patients [263].

Previous studies did not draw a conclusion on the potential beneficial or detrimental effects of LFV during CPB. We therefore, conducted this study, first RCT to investigate the effects of LFV in patients undergoing CABG with CPB by measuring inflammation directly in lung biopsies aiming at drawing a final conclusion to support or reject the use of LFV during CPB.

This chapter showed that CPB produced a pronounced increase in lung and systemic inflammation as well as elevated levels of oxidative stress biomarkers. The study was powered on NF- κ B activation in the lung to examine whether LFV during CPB would reduce inflammation as observed in a pig model. We did not show any effect of CPB on NF- κ B activity in lung tissue which may reflect the single time point studied as downstream inflammatory and oxidative stress markers were elevated. Alternatively, it is possible that these events are driven by p38 MAPK to a greater extent than by NF- κ B as we provided evidence for p38 MAPK activation in lung tissue and blood cells. The use of selective inhibitors in disease models may help in differentiating the driver of inflammation in CPB.

In addition, we did not see a clear beneficial effect of LFV during CPB on any inflammatory readouts compared to standard care although the effect on IL-1 β and IL-6 reached significance compared with the standard care group. Future studies should look at other time points or power the study on other inflammatory

mediators such as p38 MAPK activity. Alternatively, the primary outcome could have been on clinical features.

A limitation to this study was the failure to use the same readouts as detected in the pig model. The use of nucleotides, LDH and DNA levels together with histology provided evidence that LFV attenuated CPB-induced tissue damage. We did not directly measure tissue damage here but samples should be re-analysed to measure these analytes where possible. However, follow up of these patients did not show any improvement in clinical parameters and so although there were suggestions that LFV may have an effect on inflammatory markers it is unlikely that this approach will be continued.

IV.11.3 CONCLUSION

Contrary to our initial hypothesis LFV during CPB did reduce pulmonary or systemic inflammation compared to standard of care and may in fact increase the levels of specific inflammatory cytokines. We saw an effect of CPB on lung and circulatory inflammation. In order to determine whether alterations in the pulmonary vasculature may drive this effect it would be useful to examine the pathology and pharmacology of pulmonary vessels isolated before and after CPB. This is examined in the next chapter.

**CHAPTER V. CONTRIBUTIONS TO DORA ET AL'S STUDY
ON ISOLATED HUMAN PULMONARY ARTERY
STRUCTURE AND FUNCTION PRE- AND POST-
CARDIOPULMONARY BYPASS SURGERY [223]**

V.1 Abstract

The effects of CPB on pulmonary arterial function have been understudied. Using samples from the ventilate study, Dora and colleagues [207] compared pre- and post-CPB lung biopsies from 12 patients. Intralobular small arteries were dissected, cannulated, pressurized and imaged using confocal microscopy. U46619 resulted in vasoconstriction in 100% and 75% of pre- and post-CPB arteries, respectively. Bradykinin stimulated vasodilation in 45% and 33% of arteries pre- and post-CPB respectively. Smooth muscle cells were aligned circumferentially in most arteries. 100% of smooth muscle cells and 90% of EC had intact membranes and were considered viable before CPB, but viability was significantly reduced after CPB.

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V.2 Introduction

Although respiratory distress affects up to 25% of patients after CPB [264], studies of the effects of CPB on pulmonary arteries in humans are sparse. Understanding what happens to these arteries is important for the development of treatments to improve patient outcomes. A full understanding of how pulmonary arteries are impacted by cardiac surgery with CPB has not been possible because fresh lung tissue has not been available for study. Our work in the ventilate trial solved this problem by providing Dora and colleagues [207] with samples to study pulmonary artery structure and function before and after CPB.

V.3 Objectives and Hypothesis

To compare structure and function of human pulmonary arteries before and after CPB in patients undergoing CABG. Each patient served as their own control with paired biopsies taken before and after CPB. We hypothesised that CABG alters the structure and function of isolated and cannulated human pulmonary arteries.

V.4 Information provided previously

Ethics approval information was reported in Section VI.4, and Dora et al. [207]. Trial population is given in Section VI.6. Materials and methods are fully described in Dora et al. [207]. Anaesthetic, perfusion, surgical and extubation protocols are provided in Section III.1.1, 1, 2, 1.3 and 1.4. The intervention and comparator is described in Section III.2.5. Lung sampling protocols are given in Section IV.2.7. Patients' characteristics are given in Dora et al. [223]. Details of the response to bradykinin and U46619 are given in Dora et al. [207], as were the details of the microscopic structural studies.

V.5 Results

Twenty-four biopsies were performed, one before, and one after CPB for each of 12 patients. Five failed the leak test, and we were unable to assess dilation on two others. These failures were non-overlapping, so that of 12 patients, full data were collected on only five (Table V.1). Details are given in Dora et al [223].

Table V.1: Patient Characteristics.

Total number of patients	12
Patient age, y	71.3±7.1
Sex (male/female)	11/1
BMI, kg/m ²	28.3±3.7
Diabetic	58%
Previous T.I.A.	0%
Hypertensive	100%
Hypercholesterolemia	100%
Asthma	17%
Aspirin	83%
Clopidogrel	33%
Warfarin	8%
β-Blockers	83%
Calcium channel blockers	42%
Oral nitrate	25%
Statins	92%
ACE inhibitors	58%
Angiotensin II antagonists	17%
Diuretics	8%
Insulin	17%
Oral anti-diabetic	33%

Data presented as the mean±SD or as a percentage of the whole patient group. ACE indicates angiotensin-converting enzyme; BMI; T.I.A., transient ischemic attack.

Functional responses of cannulated arteries to the vasoconstrictor, U46619, and the vasodilator, bradykinin, showed no significant differences between pre- and post-CPB. U46619 (3μmol/l) caused at least some contraction in all the pre-CPB arteries but only 75% of post-CPB arteries. This contraction can be seen in the Fig. 1A in Dora et al (Isolated human pulmonary artery structure and function pre- and post-CPB surgery) [223]. U46619 caused a significant reduction in the % maximum constriction, without affecting the EC₅₀ value. Bradykinin (1μmol/l) did

not produce any noticeable relationships between the magnitudes of vasoconstriction and vasodilation, pre- and post-CPB (Table V.2).

Table V.2: Summary data showing functional responses to the vasoconstriction and vasodilation in human pulmonary arteries

Artery Characteristics	Pre-CPB		Post-CPB	
		n		n
Maximum internal diameter, μm	240 $\pm$ 23	11	246 $\pm$ 30	8
Number responding to vasoconstrictor (%)	11 (100)	11	6 (75)	8
Maximum constriction, μm	68 $\pm$ 16	11	71 $\pm$ 35	8
Percentage maximum constriction	26 $\pm$ 5	11	27 $\pm$ 8	8
Maximum constriction to U46619, μm	59 $\pm$ 14	11	67 $\pm$ 32	8
Percentage maximum constriction to U46619	23 $\pm$ 4	11	25 $\pm$ 10	8
Paired artery U46619 EC ₅₀	-7.4 $\pm$ 1.6	7	-6.5 $\pm$ 0.8	7
Number responding to endothelium-dependent vasodilator (%)	5 (45)	11	2 (33)	6
Percentage maximum dilation to bradykinin	35 $\pm$ 14	11	29 $\pm$ 19	6
Percentage live smooth muscle cells	100 $\pm$ 0	7	60 $\pm$ 21	5
Percentage live endothelial cells	90 $\pm$ 5	7	58 $\pm$ 19	5

Summary data showing functional responses to the vasoconstrictor U46619 and endothelium-dependent vasodilator bradykinin in human pulmonary arteries. No significant differences were observed between pre- and post-CPB. Values are the mean $\pm$ s.e.m. 100% maximum constriction equates to a lumen diameter of 0 μm ; 100% maximum dilation equates to full reversal of U46619 tone; CPB.

Endothelium-dependent vasodilation from 1 $\mu\text{mol/l}$ bradykinin pre- (n=11) and post-CPB (n=6) showed that differences were not significant. A scatter plot is shown in Fig. 2A in Dora et al (Isolated human pulmonary artery structure and function pre- and post-CPB surgery) [223]. The mean dilation was lower post CPB, but the variance was high, and it did not show a significant difference in the 6 subjects studied. Comparisons of vasoconstriction and vasodilation in both pre- and post-CPB showed responses going in both directions (Table V.2).

Staining arteries with 4',6-Diamidino-2-Phenylindole (nuclear stain), and phalloidin (F-actin stain) revealed inconsistent cell orientation and density as shown in Fig.3

in the Dora et al (Isolated human pulmonary artery structure and function pre- and post-CPB surgery) [223]. Sometimes the pattern was uniform, but occasionally, the smooth muscle structure was disparate in both pre- and post-CPB biopsies, with elastin-filled gaps between smooth muscle cells. In some areas, elastin ran through the artery from the external layer to the endothelium. Biopsies revealed that CPB killed about half the cells but with only 5 observations, this was not statistically significant.

V.6 Discussion

Structural assessment showed that some arteries had altered smooth muscle cells both pre- and post-CPB, perhaps as a characteristic of this group of patients, who were not healthy to start with, prior to surgery. Therefore, we could not link altered smooth muscle cells orientation and density to CPB. Before CPB, smooth muscle cells were alive, but only half were alive after CPB. CPB may cause decreased arterial contraction due to damage to smooth muscle cells in the arterial wall and this may account for the increased inflammation, oxidative stress and the production of DAMPs reported in Chapter IV. Hypoxia is known to provoke an increased immunological response through ischemia reperfusion injury and mechanical stress, causing atelectasis and lung dysfunction [39, 55, 219]. CPB carried out in rabbits caused an increase in leakage of intravenous Evans blue dye, which was inhibited by an antibody against $\text{TNF}\alpha$ [265]. Therefore, macrophage and neutrophils released during CPB could cause $\text{TNF}\alpha$ -induced apoptosis in pulmonary smooth muscle cells, similar to that seen in Dora et al [223]. However, we did not report a difference in $\text{TNF}\alpha$ protein or mRNA expression following CPB in Chapter IV.

Endothelium-dependent vasodilation decreases after CPB. We found less than half the arteries were capable of endothelium-dependent vasodilation, with an average dilation less than 50%. After CPB, dilation to bradykinin was measurable in only one of five, consistent with a previous finding in porcine coronary arterioles of a slight decrease in vasorelaxation after CPB, which was associated with increased EC adhesion molecules and myeloperoxidase leading to cell death [266]. Our structural studies, which showed a smaller percentage of live cells in arteries from post-CPB biopsies compared to pre-CPB biopsies, further supports this hypothesis.

EC orientation, as for smooth muscle cells, was atypical. Irrespective of the timing of biopsies in relation to CPB, ECs were often arranged at various angles instead of running in line with blood flow. This finding has previously been associated with altered shear stress associated with peripheral vascular diseases [267, 268].

CHAPTER VI. THESIS DISCUSSION, CONCLUSION AND FUTURE DIRECTION

VI.1 Discussion and Conclusion

It is well documented in the literature that cardiac surgery is responsible for the various degree of lung dysfunction observed post operatively [19-25]. This results in prolong intensive care and hospital stay particularly in patients with existing comorbidities and after complex cardiac operations [24, 171]. Historically the standard care is about treating complications and improving poor oxygenation, rather than attempting to prevent complications and alleviating the surgical side effects on lung function. During my training looking after patients in the ITU was the stimulus to explore other ways for lung protection in cardiac surgery

After researching the literature I realised that NIV is probably considered the gold standard adjunct to improve lung function in respiratory failure and various other clinical settings [173-176, 183-185]. I also found few previous studies looking at the benefits of NIV post-surgery [169, 186, 189, 191]. I concluded that the major limitation of all the previous studies addressing the benefit of the NIV post-surgery has been the short or intermittent duration of NIV application. NIV was not started until 4 hrs after extubation and was applied for only 1h in every 3h [186]. NIPSV was applied four times a day for 30 min [169], and NIV was applied for 0.5 h periods every 6 h in the first postoperative day [191], intermittent 15 min bi-level positive airway pressure (7.3+/-3.6 times per patient) [189]. Hence all previous trials were consistent and positive with respect to the reduction in atelectasis and all suggested a clinical benefit with BiPAP, yet extended treatment was thought to be infeasible due to presumed poor tolerance of patients to BiPAP masks. It is however, logical to assume that if short and sporadic BiPAP treatments are beneficial, then longer or continuous treatments could provide greater benefits.

My thoughts and research were further developed when I met Dr Rob Shiner and Dr Philip Ind (consultant respiratory physicians). My first trial was designed based on the hypothesis that NIV with BiPAP after CABG would shorten the duration of post-operative stay by improving lung physiology, gas exchange and reducing basal atelectasis to a clinically important extent. Our RCT was novel in that it was the first study to attempt continuous NIV for long durations within the first 24h post extubation in cardiac surgery.

We demonstrated that the elective use of NIV with BiPAP for patients following CABG is feasible and safe. Participants in our study who received NIV with BiPAP in addition to usual care were, on average, judged to be medically fit for discharge one day earlier than participants who had only the usual care. If this reduction in hospital stay could be confirmed in a larger multicentre trial, it would represent an important benefit to patients and for health services provision, reducing the cost of an admission for CABG and increasing the capacity of cardiac surgery units. We conclude that BiPAP should be considered an important part of an enhanced recovery programme for patients undergoing CABG.

Nevertheless, there are a few limitations: choosing a subjective clinical end point as FFD, despite its major importance to clinical managers from a cost saving point of view, it is of less interest to researchers. Greater value would have been added to the study if an unbiased biomarker could have been determined that differentiates between the groups in the study. Ideally, these inflammatory biomarkers would be derived from lung tissue with an accessible correlate in blood.

In addition, we only included patients in the trial who had good lung function and FEV1>70%. We excluded patients with severe COPD, with lung pathology and who had received previous radiotherapy. This patient selection criterion was driven by the need to standardise the trial population, yet I think that patients with pre-existing poor lung conditions, may theoretically benefit the most from applying the NIV. Hence, any future study should contain a subgroup of patients with poor lung function could show even greater benefits to patients.

Furthermore, one could argue that the pulmonary function tests (spirometry), conducted for each patient on day 1, 2 & 3 post extubation, were probably not that informative about lung function status due to the sternotomy incision pain, sedation and overall weakness that patients experience in the cardiac ITU setting after cardiac surgery.

Also, it is not easy to have the study fully blinded due to the nature of the intervention; hence our randomisation was unblinded to the research team, nursing team and patients. In retrospect, possibly we could have tried to consider the BiPAP masks to all patients but only apply the NIV for those in the treatment arm of the study. This will not be fully blinding to nursing or clinical research teams but could have been sufficient to blind the clinical team who were not directly looking after patients and for the patients. I do also recognise the issue of the potential bias inherent in using a pre-randomisation step.

Moreover, the duration of the recruitment process across three years might suggest a possible recruitment bias. Hence it is important to elaborate on the circumstances of that period. I started in February 2008 with the first patient in the trial and by the end of the year I had (39 patients) patients recruited to the study.

Most of the study patients (82 patient) were recruited during 2009 and 2010 with (5 patients) recruited early in 2011. When I started the study, I was a full time senior house officer and I had no research team to support me, hence I had to deal with all the tasks involved in the study. The process wasn't simple as I also had to negotiate with the sponsoring body (Respironics) the donation of four new BiPAP machines and organise 12 training session to our nursing staff. There were also some issues concerning consumable replacement.

One more important factor to consider was that the CICU in the Hammersmith hospital underwent complete refurbishment and was shut between 2009 and 2010. This introduced some constraints on patient recruitment during the study. The cardiac surgery unit utilised the general ITU at the Hammersmith hospital in order to accommodate postoperative patients but only four dedicated beds were available. Hence, the flow of patients was dramatically reduced and the number of patients who matched the recruitment criteria was reduced as more urgent unwell patients had priority and these were not suitable for our trial. This considerably affected the speed of recruitment. Furthermore, not all surgeons agreed to have their patients enrolled into the study. Also, the volume of CABG cases in the unit was relatively low with a significant number of patients undergoing off pump coronary artery bypass surgery. Finally, there were limitation on the use of BiPAP in term of the numbers of machines and trained personnel available.

The BiPAP study introduced me to research and fuelled my desire to continue exploring this area of research. Hence, I utilised the knowledge obtained from this first study to further extend our understanding of the factors that may affect patient outcome following an intervention post-surgery. We knew that the major insult to

lung function happens during surgery on CPB. So we explored ideas and interventions that can be applied during surgery on CPB to protect lung function. To have better understanding of these events, I had to understand the pathophysiology of lung dysfunction related to atelectasis and acute lung injury (ALI) resulting from surgery.

Atelectasis is the end result of general anaesthesia leading to spontaneous cessation of airway patency and is determined by time and oxygen concentration [211, 212]. Mechanisms behind the development of atelectasis includes lung compression, depletion of alveolar space, and malfunctioning surfactant [213]. It known that there is relation between atelectasis and intrapulmonary shunt [269]. Atelectasis reduces lung volumes by forcing breathing cycles to the lower curve of the FRC, this increases intrapulmonary pressure to maintain a given TV, with the end result being a significant increase in the respiratory effort [270] and the development of ALI [213]. In addition, atelectasis, with the resulting insufficient ventilation of the lung, may lead to lower oxygenation in the blood and hypoxia inside the affected parts of the lung. This can drive hypoxic pulmonary vasoconstriction with raised alveolar and mixed venous tension [270]. The latter can even results in right heart failure [271].

The term acute respiratory distress syndrome (ARDS) was first used in 1967 [272]. Years later, the American-European Consensus Conference Committee recommended the adoption of a consensus definition for ALI/ARDS allowing for the first time to quantify the severity of lung injury. This was defined by the acute onset of diffuse bilateral pulmonary infiltrates by chest radiograph; a $\text{PaO}_2/\text{FiO}_2 \leq 300$ for ALI and ≤ 200 for ARDS; and a pulmonary artery wedge pressure (PAWP)

≤18 or no clinical evidence of left atrial hypertension [273]. Morphologically apparent ALI occurs in isolated, non-perfused lungs, ventilated with no or low PEEP and subjected to atelectasis [214]. This explains the correlation between atelectasis and ALI [213, 214].

Prevention of atelectasis with subsequent ALI is challenging during long cardiac operations such as CPB. In addition, there is currently no means of preventing atelectasis although there is evidence suggests that positive pressure ventilation with PEEP, rather than spontaneous ventilation, might be the best option during invasive cardiac surgery [269, 270]. This concept was established in a rat experimental model in 1974 when Webb and colleagues determined that maintaining lung volume is important in preventing lung injury and suggested that PEEP ameliorated the development of lung injury induced by a high TV [274].

However, it is important to determine another intervention during CPB that could prevent lung dysfunction with atelectasis and ALI as a result of the general anaesthetic and the associated long duration of the operations.

Professor Angelini, showed me an experimental study he had performed using LFV during CPB in a pig model and this suggested an ideal intervention to explore in human subjects [143]. We hypothesised that using LFV during CPB would reduce inflammatory activation, lung dysfunction and ischemia/reperfusion injury compared with conventional non-ventilation of the lungs during CPB. We further extended the study to add greater novelty by examining inflammatory and oxidative stress biomarkers and by examine these biomarkers in lung biopsies from patients undergoing cardiac surgery on CPB. The study was based on sound pre-clinical models and the recruitment of patients was good.

Our results showed the deleterious effects of CPB on lung function and pulmonary gas exchange. CPB itself significantly enhanced the expression of inflammatory and oxidative stress markers. However, LFV did not result in a significant reduction in inflammation as determined by the expression of inflammatory mediators (mRNA and protein) post CPB compared to standard of care. As the primary end point (NF- κ B p65 activation) was not reached and the concentration of most of the other inflammatory markers in the lung and blood, despite being lower in the LFV compared to the standard care group but this was significant only for IL-1 β and IL-6 in the lung biopsies, we conclude that there was no anti-inflammatory effect of intervention with LFV compared to standard care. This study indicates the importance of performing human intervention studies in a well-controlled experimental setting as well as highlighting some of the limitations of the pig model experiment. Further understanding of the strengths and limitations of this model will allow additional translation into effective treatments in humans. Among the main limitations of this study is the small sample size because of the relatively invasiveness of the lung biopsy.

Furthermore, the use of PEEP in the intraoperative mechanical ventilation was associated with a reduction of atelectasis in postoperative period as reported by studies using high PEEP level (10 cm H₂O) [275-277]. Overall the role of PEEP in surgery has been extensively studied with positive impression [263, 269, 270, 278]. In cardiac surgery particularly as the chest cavity is open, the lungs are arbitrarily exposed to atmospheric pressure, rather than normal negative intrathoracic pressure. Hence the transpulmonary pressure (airway pressure minus intrathoracic pressure) becomes abnormally low at end-expiration

leading to collapse of the lungs, if we do not apply PEEP of at least 3-5 cm H₂O. Nevertheless, in our treatment group we applied the LFV without PEEP. This is probably a major limitation that we could have avoided by adding PEEP to our LFV group. Also, regarding the respiratory rate and keeping it maintained at 5 bpm using oxygen enriched air ($F_{I_{O_2}}$ 0.25), is rather an educated guess coming from our anaesthetic team at time of the project design. We don't have any evidence to support or reject the selection of these figures.

Moreover, our selection for NF- κ B as a primary end point was because its activation can be induced upon physical or oxidative stresses resulting from cardiac surgery using CPB [72]. With all the previously discussed pathophysiological pathways in the lung as a result of cardiac surgery on CPB, NF- κ B seems to be a reasonable end point reflecting the adverse effect of the inflammation on the lungs. We could argue that it might not be very specific to lung tissue but rather systemic inflammatory status as it can be also produced by, physiological changes including ischemia and hyperosmotic shock, or by numerous inflammatory cytokines and chemokines [72]. Our choice to measure NF- κ B in the lung tissue with a control sample as the primary end-point can be justified as CPB enhanced lung and systemic inflammation but in hindsight earlier time points to examine NF- κ B activation should have been examined (CPB in the LFV group median of 71min, IQR 63.5-93.5 and in the Standard care group median of 80min, IQR 60-92). Samples have been collected for future transcriptomic and proteomic analysis and this may help provide insight into possible pathways driving CPB. We believe that further studies with increased numbers of patients and more detailed investigation of inflammatory markers

should be performed to provide a conclusive answer as to the effect of LFV in CPB.

During the early part of my PhD I established a collaboration with the team of Professor Kim Dora in Oxford. Professor Dora is an expert in the functional and structural anatomy of pulmonary vessels. I was able to provide the Oxford team with lung samples from patients pre- and post-CPB and provided a unique opportunity to link my in vivo data with an analysis of vascular changes ex vivo.

The study was the first to combine pressure myography, pharmacology and dyes to characterise both the structure and function of human pulmonary arteries. It was also the first study to use live and fixed isolated, cannulated and pressurized human pulmonary arteries. It provided proof of principle of the feasibility of using biopsies in a clinical trial to evaluate the effects of an intervention on pulmonary artery function. It was also the first study to investigate the effects of cardiac surgery and CPB on the structure and function of isolated human arteries obtained from lung biopsy in patients undergoing elective CABG. We have shown that CPB is detrimental to both smooth muscle cells and EC function in pulmonary arteries and that this is potentially a consequence of cell death. The ability to distinguish changes that are likely to be attributable to CPB suggests that these methods could be used to evaluate the effects of interventions to reduce the adverse consequences of CPB on the lungs. Further studies investigating pulmonary vessels ex vivo in relation to potential therapies are warranted.

It is however important to state my role in this study. Although I did not undertake training in the techniques or perform any of the laboratory experiments, the study was made possible by the provision of the tissues from the Ventilate trial and the

lung biopsies. I had a major input into the study design, contributed to the ethics approval and tissue transport paperwork and most importantly, was fully engaged in the writing and critical analysis of the data obtained.

VI.2 Future Directions

Managing pulmonary dysfunction needs to be a multistep process involving more than one modality for each step of the anesthetic, cardiopulmonary and surgical pathway. Pulmonary protection strategies have seen various degrees of success. The main weakness of most studies is that they are often observational, have a small sample size or focus on a single intervention. A better understanding of available modalities and/or combinations will help reduce pulmonary dysfunction and hence improve early outcome and costs after cardiac surgery [1, 2]. Further research is necessary to clarify and influence clinical decision in patients undergoing cardiac surgery.

With the work of this thesis, I hope to have contributed even if in small part to a better understanding of the problem of pulmonary dysfunction after CABG. I have also acquired a better knowledge, which hopefully will drive my future research in this area. Possible development of this work may involve: -

- I. To consider investigating modalities before surgery rather than after the intervention. We have evidence that pulmonary rehabilitation is recommended, in chronic respiratory condition [279]. Hence, I would recommend investigating the effectiveness of pulmonary rehabilitation before cardiac surgery for high risk group of patients with severe chronic obstructive pulmonary disorder and interstitial lung disease. We know from clinical practice that this group of patients is most likely to experience higher incidence of lung complication after cardiac surgery.

- II. Given the positive findings of The BiPAP trial, it would be reasonable to try to validate our results in a large multicentre randomised clinical trial on the use of NIV with BiPAP in addition to usual care for patients following CABG.
- III. The Ventitale trial provided evidence on the deleterious effects of CPB on lung function and pulmonary gas exchange, Adding PEEP to our LFV group would deal with one of the potential limitation of the study. In relation to biomarkers, we should measure NF- κ B at an earlier time points due to the relatively lengthy overall time on CPB (CPB in the LFV group median of 71min, IQR 63.5-93.5 and in the Standard care group median of 80min, IQR 60-92).
- IV. We have achieved a major first on the feasibility and characterisation of both the structure and function of human pulmonary arteries in patients undergoing CABG with CPB. This is very likely to be of value for future research in the field. This may include pharmacological studies on vascular function which may reveal important targets for novel preservation of lung function.
- V. The majority of biochemical reactions at cellular level are regulated by highly specific proteins, acting as prime mediators of the cellular phenotype. The identification, quantitation and characterization of all proteins in a cell are of extreme importance to understand the molecular processes that mediate cellular physiology [280]. Transcriptomics techniques are used to study organism's transcriptome, the amount of a RNA transcripts, recorded in the DNA and expressed through transcription. So, a transcriptome captures a snapshot in time of the total transcripts present in cells [281]. A Broad analysis of gene expression in its whole permits detection of trends which cannot be distinguished by more targeted tests. Therefore, I would suggest performing

bioinformatic analysis of proteomics and transcriptomics obtained with samples from the ventilate trial to determine whether this provides greater insight into the key pathways regulated by CPB and which may be targeted by novel therapeutic approaches.

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APPENDIX

Research Presentations

MY RESEARCH LIFE STORY (PULMONARY PROTECTION IN CARDIAC SURGERY)

E Al Jaaly. Oral Presentation, Bristol Royal Infirmary, Grand Round 12/04/2016

PULMONARY PROTECTION IN CARDIAC SURGERY (PHD RESEARCH).

E Al Jaaly. Oral Presentation, NHLI Postgraduate Research Day 30/06/2014

EFFECT OF POSTOPERATIVE NON-INVASIVE VENTILATION IN PATIENTS UNDERGOING CORONARY ARTERY BYPASS GRAFTING CABG.

E Al Jaaly, G Angelini. Oral presentation, Vascular away Day 15/11/2013, NHLI, Imperial, London

EFFECT OF POSTOPERATIVE NON-INVASIVE VENTILATION IN PATIENTS UNDERGOING CORONARY ARTERY BYPASS GRAFTING CABG.

E Al Jaaly, F Fiorentino. O Mangoush, G Angelini. Oral presentation, ACTA/SCTS Annual meeting & Cardiothoracic forum. Manchester 18TH-20TH APRIL 2012

Research Publications

AL JAALY E, FRANCESCA F, DURHAM A L, ADCOCK I M, LOCKWOOD G, ROGERS C, ASCIONE R, REEVES B C AND ANGELINI G D, LOW-FREQUENCY VENTILATION DURING CARDIOPULMONARY BYPASS FOR LUNG PROTECTION: A RANDOMIZED CONTROLLED TRIAL. J CARD SURG, 2019.

DURHAM AL, **AL JAALY E**, FIORENTINO F, BROOK F, HEESOM K, LAVENDER P, ANGELINI GD & ADCOCK IM. NON-BIASED INVESTIGATION INTO CARDIO-PULMONARY BYPASS LUNG INJURY (LSC ABSTRACT). EUROPEAN RESPIRATORY JOURNAL 2016 48. PP224.

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Copies of Research Publications

Low-frequency ventilation during cardiopulmonary bypass for lung protection: A randomized controlled trial

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Abstract

Objective: Pulmonary dysfunction is a common complication in patients undergoing heart surgery. Current clinical practice does not include any specific strategy for lung protection. To compare the anti-inflammatory effects of low-frequency ventilation (LFV), as measured by nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) p65 pathway activation, for the entire cardiopulmonary bypass (CPB) vs both lungs left collapsed in patients undergoing coronary artery bypass grafting (CABG).

Methods: Two groups parallel randomized controlled trial. The primary outcome was inflammation measured by NF- κ B p65 activation in pre- and post-CPB lung biopsies. Secondary outcomes were additional inflammatory markers in both biopsy tissue and blood.

Results: Thirty-seven patients were randomly allocated to LFV (18) and to both lungs left collapsed (19). The mean concentration of NF- κ B p65 in the biopsies before chest closure (adjusted for pre-CPB concentration) was higher in the LFV group compared to both lungs left collapsed group but this was not significant (0.102, 95% confidence interval, -0.022 to 0.226, $P = 0.104$). There were no significant differences between groups in the other inflammatory markers measured in tissue and blood.

Conclusions: In patients undergoing elective CABG, the use of LFV during CPB when compared to both lungs left collapsed does not seem to reduce inflammation in lung biopsies and blood.

KEYWORDS

cardiopulmonary bypass, low-frequency ventilation, lung biopsy, lung protection, nuclear factor κ -light-chain-enhancer of activated B cells

Abbreviations: ALI, acute lung injury; ARDS, adult respiratory distress syndrome; CA, cardioplegic arrest; CABG, coronary artery bypass grafting; CPAP, continuous positive airway pressure; CPB, cardiopulmonary bypass; CV, conventional mechanical ventilation; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; FVCR, forced vital capacity ratio; GM, geometric means; LFV, low frequency ventilation; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; OLC, open lung concept; $(\text{PAO}_2 - \text{PaO}_2)/(\text{PaO}_2)$, respiratory index; PEEP, positive end-expiratory pressure; PFTs, pulmonary function tests; PaO₂, partial pressure of oxygen; SD, standard deviation; V/Q, ventilation/perfusion distribution.

Francesca Fiorentino and Emad Al Jaaly are first authors.

1 | INTRODUCTION

Pulmonary dysfunction is a common complication for patients after cardiac surgery using cardiopulmonary bypass (CPB).¹ Severity ranges from mild atelectasis to life-threatening acute lung injury (ALI) or respiratory failure requiring prolonged postoperative ventilation² or adult respiratory distress syndrome (ARDS).^{3–5} Harmful effects of CPB on pulmonary function persist despite advances in anesthetic techniques.⁶ Pulmonary dysfunction after cardiac surgery also affects clinical outcomes with increased morbidity, mortality and delayed discharge from hospital, leading to increasing in the health care resources used and their associated cost.^{3,7–13}

Presumed causative factors for atelectasis and ALI, include inflammation, prolonged lung collapse, pulmonary ischemia, and related reperfusion injury, blood contact with the surface of the heart-lung machine, endotoxemia, surgical trauma, blood loss, and transfusion.^{14,15} Inflammatory activation and cytokine release have been correlated with outcome after cardiac surgery.¹⁶ Pulmonary function 24 hours after CPB is associated with raised plasma levels of inflammatory cytokines and reduced levels of anti-inflammatory cytokines.¹⁷ The inflammatory response from the lung during CPB and mechanical ventilation originates at the alveolar membrane as a result of the collapse, ischemia, reperfusion injury, and mechanical stress.^{18–20} Suppression of activation of inflammatory mediators during CPB is associated with a reduction in pulmonary dysfunction.²¹

Current clinical practice does not include any specific strategy for lung protection during CPB. When CPB is started, often both lungs are left collapsed for the entire CPB duration. We recently provided evidence in an experimental pig model that low-frequency ventilation (LFV) during CPB reduces post-CPB lung injury.²²

Here, we report an evaluation of the effect of ventilating the lungs at low frequency during CPB comparing to collapsing the lungs in patients having coronary artery bypass graft surgery (CABG) with respect to inflammation measured by nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) p65 pathway activation and postoperative pulmonary dysfunction. We used a primary outcome measure that would give an early indication of inflammatory changes in the lungs and would allow detection of a large effect in a relatively small trial.

2 | MATERIALS AND METHODS

This study was a single-center, two-group parallel randomized controlled trial. Patients were randomly assigned in a 1:1 ratio, using a secure concealed internet-based randomization system (Sealed Envelope, <https://www.sealedenvelope.com/>). Cohort minimization was used to achieve balance between groups with respect to baseline lung function ($\geq 60\%$ predicted FEV₁). Patients returned to the hospital for a follow-up visit 6 to 8 weeks following the operation.

Study period between 07 January 2013 to 27 June 2014. Trial registration ISRCTN-34428459, protocol approved by the NRES London-Camden and Islington (REC-12/LO/0458). Protocol published at <http://www.isrctn.com/ISRCTN34428459>.

2.1 | Trial population

Patients having elective or urgent CABG with CPB and cardioplegic arrest (CA) at the Hammersmith Hospital (Table 1).

2.1.1 | Inclusion criteria

- Age greater than or equal to 40 and less than 85 years
- Left ventricular ejection fraction greater than 30%

2.1.2 | Exclusion criteria

- Previous pulmonary embolism requiring long term warfarin for more than or equal to 3 months
- Previous cardiac surgery
- Current congestive heart failure (NYHA class IV)
- Chronic renal failure
- Emergency or salvage operation

TABLE 1 Patient population characteristics and operative details

Variables ^a	LFV (N = 18)	Lungs left collapsed (N = 19)
Age, y, mean (SD)	65.39 (12.09)	62.86 (10.08)
Height, m, mean (SD)	1.70 (0.09)	1.69 (0.06)
Weight, kg, mean (SD)	85.72 (15.49)	80.84 (15.29)
BMI, kg/m ² , mean (SD)	29.69 (4.14)	28.15 (4.82)
NYHA class, n (%)		
0	0	0
1	4 (22%)	9 (47%)
2	12 (67%)	9 (47%)
3	2 (11%)	1 (5%)
4	0	0
Left ventricular function, n (%)		
Poor (<30%)	0	0
Moderate (30%-50%)	4 (22%)	4 (21%)
Good (>50%)	14 (78%)	15 (79%)
Smoker/ex-smoker, n (%)	14 (78%)	12 (63%)
Asthma, n (%)	4 (22%)	0
COPD, n (%)	0	1 (5%)
Operation details		
Bypass, min, median (IQR)	87.5 (68-97)	69 (54-79)
Cross-clamp, min, median (IQR)	44.5 (37-50)	35 (30-43)
Intubation, h, median (IQR)	8.7 (7.1-10.3)	7 (6.4-10)
Time to discharge, d, median (IQR)	6 (6-7)	6 (5-7)

Abbreviations: BMI, body mass index; COPD, chronic obstructive pulmonary disease; IQR, interquartile range; LFV, low-frequency ventilation.

^aThe median and interquartile range are reported for the variables whose distribution is skewed.

- On corticosteroid or immunosuppressive treatment
- Severe chronic obstructive pulmonary disease, lung pathology, previous radiotherapy,
- Body mass index more than 35

2.1.3 | Ventilation protocol

Before starting CPB, for all participants, the lungs were ventilated with a tidal volume of 6 to 8 mL/kg, tidal volume and inspiration to expiration (I:E) ratio of 1:2, positive end-expiratory pressure (PEEP) of 5 cm H₂O and FiO₂ of 0.5 (range, 0.45-0.55 O₂). The ventilatory rate was set to keep the PaCO₂ between 4.5 and 5.5 kPa.

In the comparator group (both lungs left collapsed), at the onset of CPB, the lungs were disconnected from the ventilator and allowed to collapse completely for the duration of CPB.

In the treatment group (LFV), the respiratory rate was maintained during CPB at 5 bpm with (FiO₂ $\cong$ 0.25). PEEP was turned off during LFV but the I:E ratio was maintained at 6 to 8 mL/kg and 1:2. At the end of the CPB, patients in both groups had a lung recruitment maneuver using an FiO₂ of 0.5 and holding the lungs inflated for 15 seconds at 30 cm H₂O, before the lungs were reconnected to the ventilator. The recruitment maneuver was repeated if necessary, though this event was not recorded on the data. No other variation in ventilation was permitted or necessary. The same ventilator protocol was used after CPB as before CPB with a PaO₂/FiO₂ greater than 50 required on first postbypass gas (ie, partial pressure of oxygen [PaO₂] > 25 kPa). If PaO₂/FiO₂ less than 50 then the recruitment maneuver was repeated.

2.1.4 | Anesthetic protocol

Following premedication with temazepam (dose, 20-30 mg), anesthesia was induced with propofol and remifentanyl, using pancuronium 0.1 mg/kg for muscle relaxation. This was maintained by infusion of propofol and remifentanyl (5 mg remifentanyl to 1 g propofol), with isoflurane if required to keep the entropy value of the processed electroencephalogram (EEG) below 55. At chest closure, 7 μ g/kg fentanyl was given in combination with plain propofol, which was switched to a propofol/remifentanyl mixture for the transfer to the cardiac intensive care unit. It is important to mention that mixing remifentanyl in propofol was at the time of our study common practice and was used in every subject of both groups. O'Connor's work²³ was published shortly after recruitment to our study had finished, and in any case, may not be relevant because our syringes were kept horizontal throughout the procedure.

2.1.5 | Perfusion protocol

A standard CPB was used, primed with 1400 mL of Hartmann's solution and 10 000 IU of heparin. The systemic temperature was between 32°C and 35°C. Cardioplegic arrest was achieved with intermittent antegrade cold blood cardioplegia.

2.1.6 | Lung biopsy protocol

To measure inflammatory markers in the lung, two lung biopsies (1 cm x 1 cm) were taken using the LigaSure Impact instrument (LF4318; Covidien, Minneapolis, MN). The first (pre-CPB) biopsy was taken from the left upper lobe immediately after sternotomy and the second from the left lower lobe before weaning from CPB after lung recruitment maneuver (see above).

The criteria for extubation were: normothermia (a core temperature range of 36.0°C-37.0°C); haemodynamical stability and blood loss less than 50 mL/h; comfortable breathing with good bilateral air entry (Respiratory rate [RR], 10-20 per minutes, tidal volumes 8-10 mL/kg, minimal tracheal suction) and arterial blood gases with parameters PaO₂ greater than 10 kPa on FiO₂ less than 0.5, PCO₂ less than 7 kPa, Bases excess (BE) -5 to +5.

Blood samples were taken: (i) after anesthetic induction and pre-sternotomy, (ii) 10 minutes after the end of CPB, and (iii) 2, 6, and 24 hours after the end of CPB. No blood sample was taken during CPB as our end points were to look into the effect before and after CPB.

2.1.7 | Outcomes

The primary outcome was inflammation measured by NF- κ B p65 activation in pre- and post-CPB lung biopsies. This outcome measure was chosen because exposure of a cell to a cytokine or an infectious agent leads to binding to a cell surface receptor and activation of a kinase cascade resulting in the nuclear translocation of the master proinflammatory transcription factor NF- κ B. NF- κ B is known to drive the expression of most inflammatory genes.^{24,25}

Secondary outcomes included additional inflammatory markers in both biopsy tissue and blood. Namely, p38 MAPK phosphorylation, expression of tumor necrosis factor- α (TNF- α), interleukin 1 β (IL1 β), IL18, IL6, IP10, IL8, IL10, chemokine receptor CXCR3, and caspase-3 measurements of apoptosis in biopsies. Reactive oxygen species (ROS) levels, phosphorylation of p38, and NF- κ B p65 in blood.

2.1.8 | Laboratory analysis

Blood samples were taken: (i) after anesthetic induction and pre-sternotomy, (ii) 10 minutes after the end of CPB, and (iii) 2, 6, and 24 hours after the end of CPB.

Leukocytes were fixed and lysed with BD Phosflow Lyse/Fix buffer, (BD Biosciences, Oxford, UK). Samples were stained using a redox-sensitive fluorescent probe 3'-(p-aminophenyl) and stained with antibodies raised against phosphorylated p38 (Thr180/Tyr182) (Cell Signaling #6908, Danvers, MA) and NF- κ B p65 (Ser529) (Cell Signaling #4887). Samples were analyzed by flow cytometry compared to unstained controls.

2.2 | Biopsies

The pre-CPB biopsy provided a within-subject control for the second, post-CPB biopsy.

NF- κ B p65 nuclear localization and activation was assessed by immunofluorescent staining followed by confocal microscopy and by testing nuclear lysates by DNA-binding enzyme-linked immunosorbent assay (ELISA) (TransAm Assay, Carlsbad, CA).

Measurements of p38 MAPK phosphorylation in biopsies were carried out using Western blot analysis and were analyzed and expressed as the ratio of phosphorylated p38 and total p38. Expression of TNF- α , IL-1 β , IL-18, IL-6, IP-10, IL-8, IL-10, and chemokine receptor CXCR3 were done using ELISA and quantitative polymerase chain reaction (qPCR).

For RNA extraction biopsies were homogenized in RLT buffer (QIAGEN, Hilden, Germany), containing β -mercaptoethanol (Sigma-Aldrich, St Louis, MO). RNA was quantified using NanoDrop and reverse-transcribed to (ThermoFisher Scientific, Waltham, MA).

Gene expression was measured using TaqMan qPCR and normalized to 18S rRNA using the $\Delta\Delta C_t$ method.

For protein extraction biopsies were homogenized in either radioimmunoprecipitation assay (RIPA) (Sigma-Aldrich) buffer or Lysis buffer AM1 (Active Motif).

Protein concentration was determined using the Bicinchoninic Acid Assay (ThermoFisher Scientific). ELISA was used to measure the expression of TNF- α , IL-1 β , IL-18, IL-6, IP-10, CXCL-8, and IL-10 in the cytoplasmic fraction. Caspase-3 activity was measured by Colorimetric Assay Kit (Abcam, Cambridge, UK), p38 phosphorylation by ELISA and Western blot analysis.²⁶

Other secondary outcomes included pulmonary function tests (PFTs), pulmonary gas exchange, and adverse events.

Pulmonary gas exchange was assessed by the respiratory index $[(PAO_2 - PaO_2)/(PaO_2)]$ measured (i) postinduction and presternotomy, (ii) 10 minutes following CPB weaning, (iii) 2 hours post CPB, (iv) 4 hours post CPB, (v) first gas postextubation, (vi) 12 hours post CPB, and (vii) before removal of the arterial line.

PFTs were carried out preoperatively and at 6 to 8 weeks postsurgery. PFTs included forced vital capacity (FVC), forced vital capacity ratio (FVCR), forced expiratory volume in 1 second (FEV₁), and FEV to FEV₁ ratio. Pulmonary function was assessed by a combination of the following tests: spirometry, gas diffusion, and thoracic gas volume.

During hospital stay and at the follow-up visit patients underwent a pulmonary function test and any documented occurrence of adverse events were recorded.

2.3 | Statistical analysis

Without taking the baseline biopsy into account, it was calculated that a sample size of 32 patients would be able to detect a standardized difference of 1.0 with 80% power and 5% significance (two-tailed). If the baseline biopsy improves the relative efficiency of the comparison (with an estimated correlation of 0.5 between measures of the primary outcome for the pre/upper lobe and post/lower lobe), the trial either had more power (88%) or was able to detect a smaller target difference (0.9 SD). These standardized differences (1.0 or 0.9) represent large differences between groups;

we justified the plausibility of this large target difference on the grounds that the primary outcome was chosen to assess the biomarker and site (ie, the lungs) which we hypothesized would be most directly influenced by the intervention.

This target sample size was able to detect a standardized difference of 0.75 in biomarkers measured in the serum with 80% power and 5% significance (two-tailed), assuming a correlation of 0.5 between preintervention and postintervention measures and a correlation of 0.7 between the four repeated postintervention measures.

The study was not powered to detect differences between the groups in pulmonary function or adverse events. Specific adverse events were too infrequent to be able to detect differences between groups. Frequencies of these adverse outcomes are tabulated, in line with guidelines for reporting adverse events in trials. The trial was an "early phase" and it aimed at identifying an intervention worth taking forward to late phase three trials quickly and relatively inexpensively, hence the choice of a primary outcome and an effect size that would allow a small sample size.

Primary analyses were by intention-to-treat. The final analyses were performed after the database had been locked and the statistical analysis plan approved. The statistical software STATA (version 13.2 www.stata.com) was used to analyze the data as well as to generate tables, figures, and listings.

Most of the data measured continuously scaled outcomes which are summarized as means and SD, at each time point if measured more than once, in each treatment group. If distributions were nonnormal, appropriate transformations were used. Analyses were carried out on the transformed data and the findings were transformed back to the original scale where possible, for example, if a logarithmic transformation was used then the results are presented as geometric means (GM).

For inflammatory markers in biopsy samples, where only one postintervention measure was collected, models were fitted using linear regression to adjust for the baseline level. Each model estimated the main effect of group allocation (LFV vs conventional management) and the baseline covariate.

For inflammatory markers in monocytes and polymorphonuclear cells from peripheral blood samples and pulmonary gas exchange expressed as *A-a gradient*, mixed regression models were fitted. These models estimated coefficients for group allocation and the interaction term for group allocation by time. If the interaction was significant ($P < 0.05$), then a group comparison is reported at each time point. All results are presented as differences between, or ratios of, the means for the two groups with 95% confidence intervals (CIs).

3 | RESULTS

3.1 | Study population

Forty-nine patients were eligible and were invited to take part in the trial between January 2013 and June 2014; 38 gave written informed

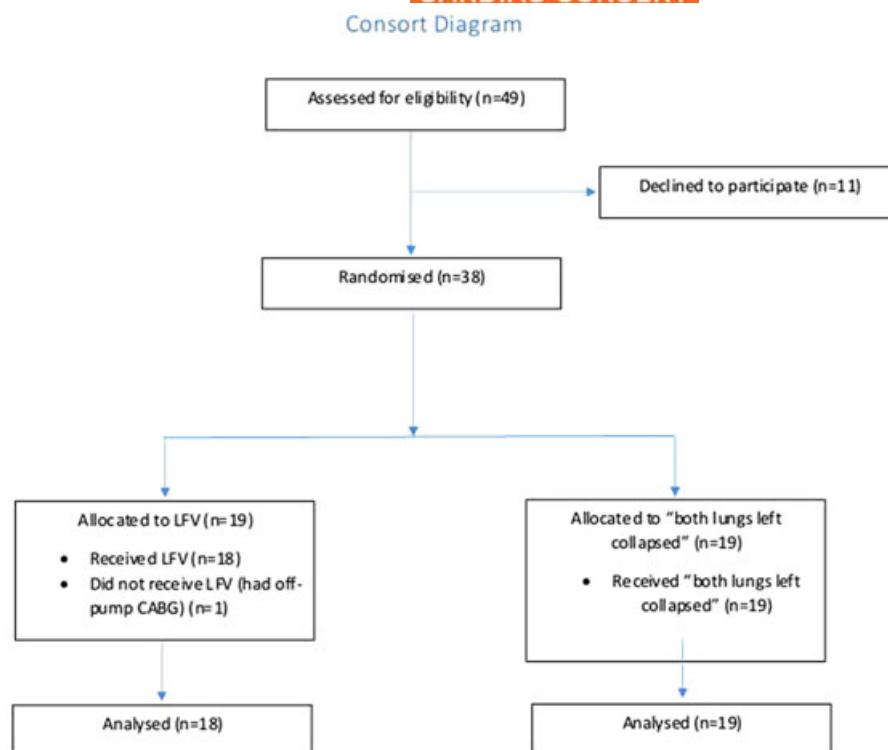


FIGURE 1 Consort diagram. CABG, coronary artery bypass grafting; LFV, low-frequency ventilation

consent, eight declined for personal reasons, and three preferred the standard procedure. One patient became ineligible (had off-pump CABG) and was withdrawn (see consort diagram, Figure 1); available data for the remaining 37 patients were analyzed (18 in the LFV group and 19 in both lungs left collapsed CPB group). Baseline characteristics, preoperative comorbidities, operation details, and baseline respiratory measurements were balanced (Table 1). Means and standard deviations (SDs) for all the markers are tabulated in (Tables 3–5). Adverse events during the hospital stay and at follow up are tabulated in (Table 2), Figure 2 illustrates the treatment effect and (Figure 3) illustrates the raw data on a log scale (for y axis).

3.2 | Primary outcome

The mean concentration of NF- κ B p65 in the biopsies before chest closure (adjusted for pre-CPB concentration) was higher in the LFV group compared to both lungs left collapsed CPB group but this was not significant (Tables 3,5), (Figures 2a and 3a); (0.102, 95% CI, -0.022-0.226 $P = 0.104$).

3.3 | Secondary outcomes

Biopsy markers analysis results are summarized in (Table A2) and (Figure 2a).

The mean concentration of p38 MAPK in the biopsies before chest closure (adjusted for pre-CPB concentration) was lower for the LFV group compared to both lungs left collapsed CPB group but this was not significant. Expression of *IL18* and *IL10* were also lower in the LFV group compared to both lungs left collapsed CPB group but not by statistically significant amounts.

Gene expression of *TNFA*, *IL1B*, *IL6*, *IP10*, *IL8*, and *CXCR3*, as well as caspase activity, were higher in the LFV group compared to the standard CPB group. These differences were statistically significant for *IL1B* and *IL6*. There were no significant differences in cytokine levels between each group.

Blood markers analysis results are summarized in (Tables A1b and A2) and (Figure 2b).

Leukocytes were separated into monocytes, granulocytes, and lymphocytes by forward and side scatter. The permeabilized monocytes and granulocytes, when measuring p38 MAPK and NF- κ B p65, showed significant overlap by this method and were therefore treated as a single group.

There were no statistically significant differences in NF- κ B, MAPK, ROS, or A-a gradients between groups.

NF- κ B p65 and p38 MAPK levels in combined monocytes and granulocytes were lower in the LFV group compared to both lungs collapsed CPB group, but these differences were not statistically significant. NF- κ B p65 levels in lymphocytes were higher in the LFV group compared to both lungs left collapsed CPB group but not significantly so. ROS levels were not significantly lower in all cell types in the LFV group compared to both lungs left collapsed group.

When time was fitted in the model, we found that p38 MAPK in lymphocytes levels were significantly lower in the LFV group at 2, 6, and 24 hours compared to 10 minutes. The interaction of intervention $\times$ time was not significant at any time point for any of the blood markers.

The A-a gradient was higher in the LFV group compared to both lungs left collapsed CPB group, but this was not significant Tables A3a. When time was fitted in the model, we found that there was a significant reduction in A-a gradient in time compared to 10 minutes in the LFV group. However, the interaction of intervention $\times$ time was

TABLE 2 Adverse events

Adverse event	LFV (N = 18), n (%)		Lungs left collapsed (N = 19), n (%)	
	In hospital	At follow-up	In hospital	At follow-up
Respiratory				
Reintubation/ventilation	0	0	0	0
Mask CPAP	1 (5.6)	0	2 (10.5)	0
Tracheostomy	0	0	0	0
Prolonged ventilation (24 h)	0	0	0	0
ARDS	0	0	0	0
Pneumothorax/effusion	0	0	0	0
Cardiovascular				
MI	0	0	0	0
Cardiac arrest	0	0	0	0
Arrhythmias	9 (50)	0	7 (36.8)	0
Haemodynamic support	16 (89)	0	9 (47.4)	0
Neurological				
Permanent Stroke	0	0	0	0
TIA	0	0	0	0
Renal				
Hemofiltration/dialysis	0	0	0	0
Other				
GI complications	0	0	0	0
Thromboembolic complications	0	0	0	0
Bleeding complications	0	0	0	0
Wound complications	0	0	0	1 (5.3)
Infective complications	4 (22.2)	3 (17)	4 (21.1)	3 (15.8)
Reoperation	0	0	0	0

Abbreviations: ARDS, adult respiratory distress syndrome; CPAP, continuous positive airway pressure; GI, geometric means; MI, myocardial infarction; TIA, transient ischemic attack.

not significant at any time point. No difference was found in lung functions between the two groups (Table A3b)

Frequency of adverse events is summarized in (Table 2).

Mask CPAP was necessary for one patient in the LFV group and two in both lungs left collapsed CPB group. Arrhythmia occurred in 50% of patients in the LFV group and 37% of patients in both lungs left collapsed CPB group. The biggest difference in groups for the occurrence of adverse events was in relation to the need for

hemodynamic support, 89% of patients in the LFV group vs 47% in both lungs left collapsed CPB group. Hospital and postdischarge infective complications were similar between groups.

4 | DISCUSSION

This trial investigated the possibility of reducing the inflammatory response associated with CABG by a technique of ventilating of the lungs at low frequency during CPB. The change in inflammatory response was measured in both tissue biopsy and blood using NF- κ B p65 in lung biopsies and other inflammatory markers in both tissue and blood.

Our selection for NF- κ B as a primary end point was because its activation can be induced upon physical or oxidative stresses resulting from cardiac surgery using CPB.²⁷ NF- κ B seems to be a reasonable end point reflecting the adverse effect of the inflammation on the lungs. We could argue that it might not be very specific to lung tissue but rather systemic inflammatory status as it can also be produced by, physiological changes including ischemia and hyperosmotic shock, or by numerous inflammatory cytokines and chemokines.²⁷ Our choice to measure NF- κ B in the lung tissue with a control sample as the primary end-point can be justified as CPB enhanced lung and systemic inflammation. Samples have been collected for future transcriptomic and proteomic analyses and this may help provide insight into possible pathways driving CPB.

Results showed levels of all markers apart from p38 MAPK, *IL18*, and *IL10* measured in the lung biopsy tissue were higher in the LFV group compared to both lungs left collapsed CPB group. However, only the increases in *IL6* and *IL1B* gene expression were statistically significant and these differences were counter to our working hypothesis that LFV would reduce inflammation following surgery. We observed a marked increase in the expression of a number of NF- κ B-induced inflammatory markers at both the protein and messenger RNA level in lung tissue following CPB. The failure to observe any change in NF- κ B p65 activation in tissue may reflect the rapid early nature of NF- κ B activation compared to the time under CPB (in the LFV group median of 71 minutes, interquartile range [IQR], 63.5-93.5 and in both lungs left collapsed group median of 80 minutes, IQR, 60-92). Gene stimulation can lead to waves of NF- κ B activation over time²⁸ and we may have missed the second round of NF- κ B activation at the time points analyzed chosen. However, we detected a marked effect on p38 MAPK activity in both lung tissue and in peripheral blood following CPB and the effect in blood reached significance over the time series and may have been greater if subsets of cells were analyzed. It is possible that CPB is a greater activator of inflammation in response to oxidative stress than NF- κ B and further studies would be required in disease models to test this hypothesis. Overall, there was no significant difference in the inflammatory markers measured in the blood between LFV and both lungs left collapsed CPB group.

A clinical study reported the use of continuous positive airway pressure (CPAP) during CPB in 14 elective cardiac surgery patients.

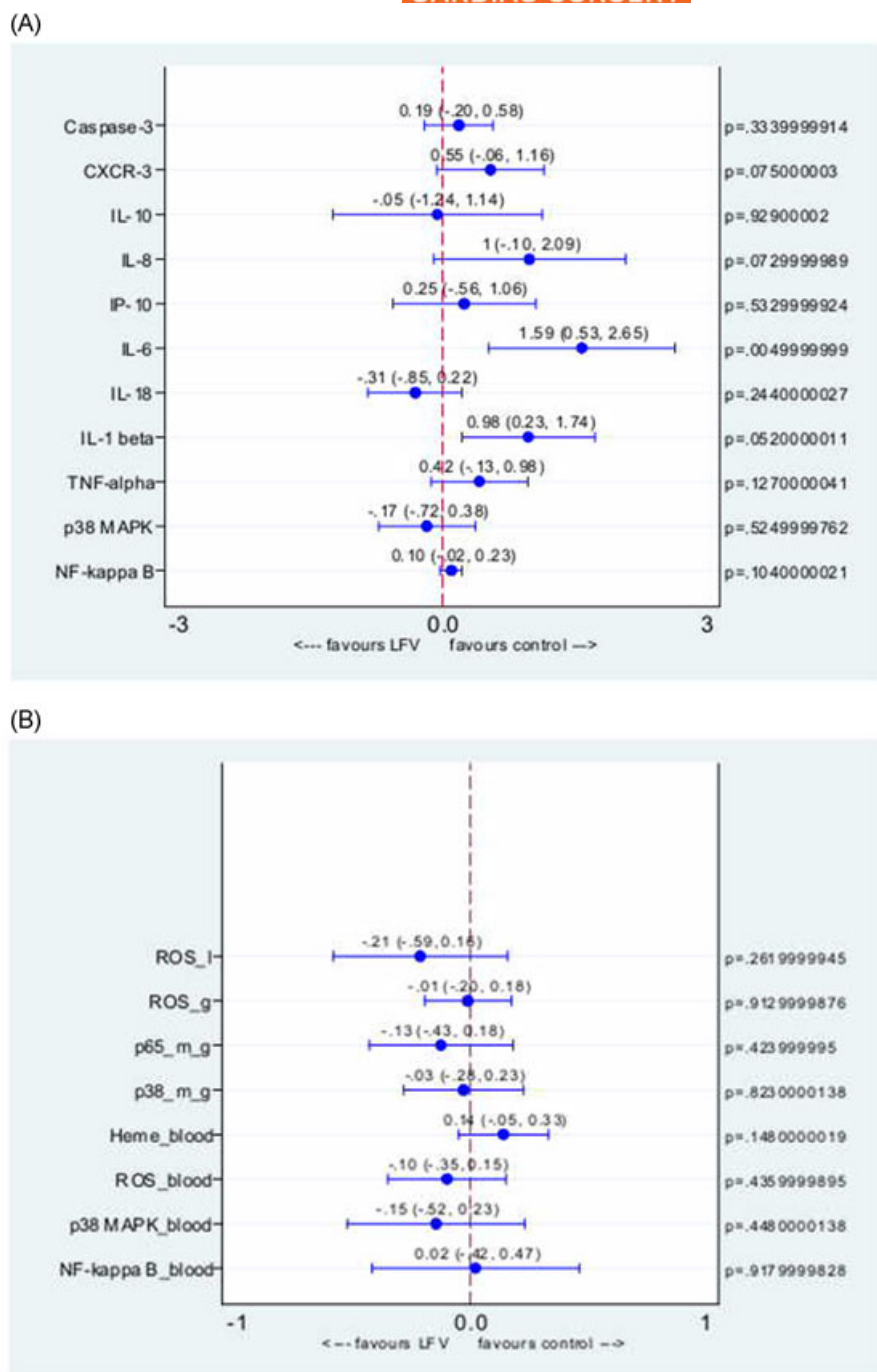


FIGURE 2 Forest plot illustrating the treatment effect for each inflammatory marker measured (A) in the lung tissue biopsies and (B) in the blood samples. IL, interleukin; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; ROS, reactive oxygen species; TNF- α , tumor necrosis factor- α

Seven patients received CPAP at 10 cm H₂O during CPB, and in the other seven patients, the lungs were open to the atmosphere (control). CPAP at 10 cm H₂O resulted in significantly more perfusion of lung areas with a normal ventilation/perfusion distribution (V/Q) and significantly less shunt and low V/Q perfusion 4 hours after CPB in comparison with the control group. The authors concluded that CPAP at 10 cm H₂O during CPB is a simple maneuver that improves postoperative gas exchange and resulted in significantly more perfusion of lung areas with a normal ventilation/perfusion distribution (V/Q) and significantly less shunt.²⁹

The effect of both LFV and CPAP during CPB to reduce post-CPB lung injury have been evaluated in an established experimental pig model.²² This study strongly suggested that the use of LFV is associated with the significantly better pulmonary gas exchange, higher adenine nucleotide, lower lactate dehydrogenase levels and reduced histological damage in lung biopsies, as well as lower DNA levels in bronchoalveolar lavage, compared with the control group. The rationale for this experimental study was to maintain some degree of ventilation during CPB to prevent persisting lung collapse and complete loss of gas exchange by passive diffusion at the blood-gas barrier. A similar concept was used by Reis Miranda et al³⁰ who

studied 62 patients postcardiac surgery, randomly assigned to three groups: (1) conventional mechanical ventilation (CV), (2) open lung concept (OLC) started after arrival on the ICU, and (3) OLC started directly after intubation. They observed an increase in functional residual capacity, reduced risk of hypoxemia and lower levels of IL-10 and IL-8 release, hence concluded that OLC ventilation leads to an attenuated inflammatory response, presumably by reducing additional lung injury after cardiac surgery.

A recent study was undertaken to examine the effect of maintaining ventilation during bypass compared with discontinued ventilation upon several parameters that may be indicative of lung injury. Twenty-three elective patients for CABG were randomized to either ventilation (VB) ($n = 12$) or nonventilation on bypass (NVB) ($n = 11$). The postbypass extravascular lung fluid was significantly smaller in the VB group compared to the NVB group and extubation time was significantly shorter,³¹ hence this study has shown the benefits of maintaining ventilation during CPB on post-CPB oxygenation and included shorter mechanical ventilation.³¹ In contrast, a small study of fifty-nine patients prospectively randomized to continuous

ventilation and no ventilation, during CABG on CPB, showed there was no statistically significant difference in most of the inflammatory makers (IL-6, IL8, IL-10, and lactate).³² A recent meta-analysis for patients undergoing cardiac surgery and received ventilation during CPB included seventeen trials with 1162 patients, showed that ventilation during CPB significantly increased post-CPB $\text{PaO}_2/\text{FiO}_2$ ratio, but there was no sufficient evidence to show that ventilation during CPB could influence long-term prognosis of these patients.³³

The discordant conclusion from all previous studies on the effect of LFV during CPB on lung function was what prompted us to design and conduct this study. This is the first RCT to investigate the effects of LFV in patients undergoing CABG with CPB by measuring inflammation directly in lung biopsies and blood samples.

4.1 | Strengths and limitations

Our trial to the best of our knowledge is the first to report the effects of LFV on pulmonary inflammation in the blood and directly in lung biopsy in patients undergoing CABG with CPB. Random allocation

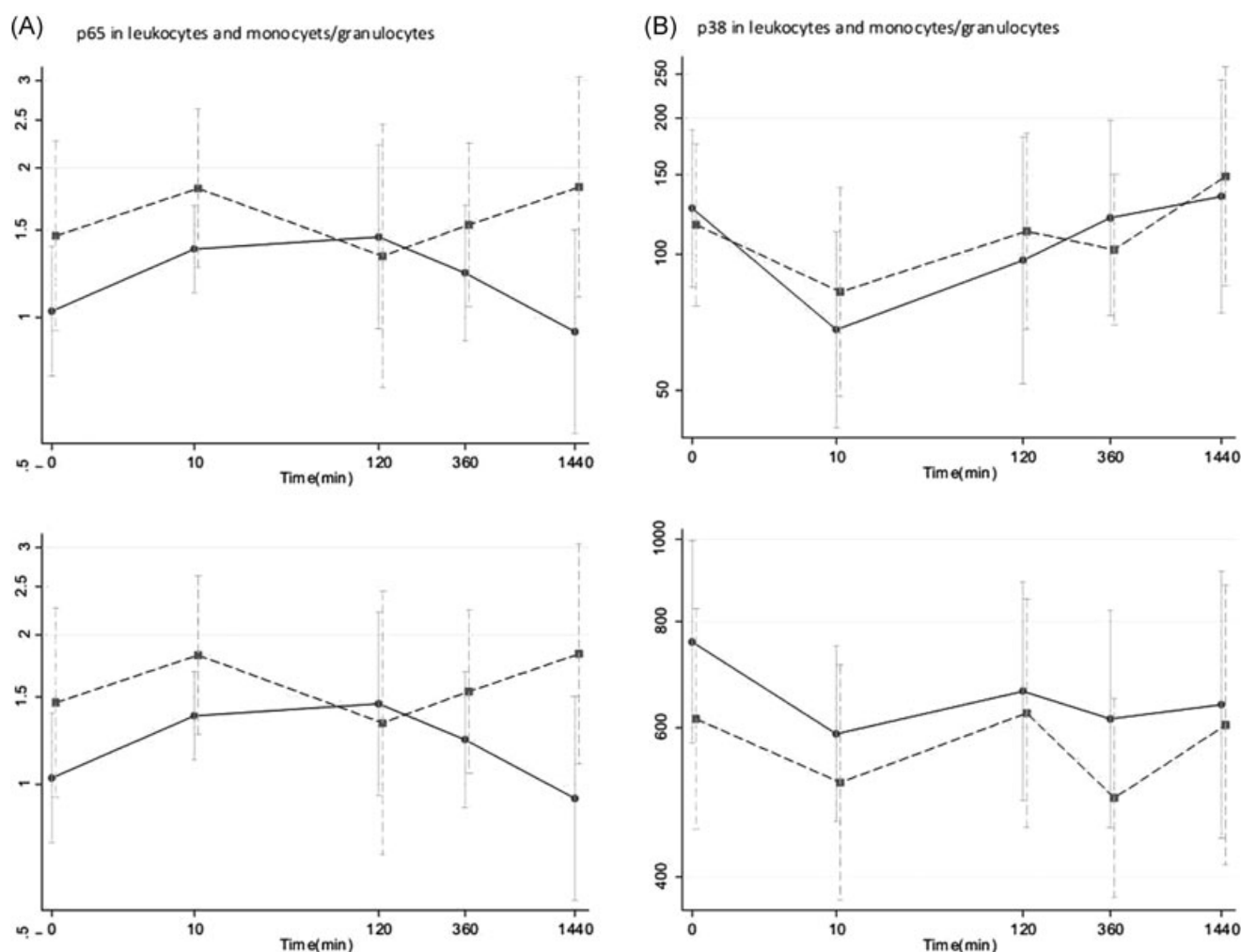
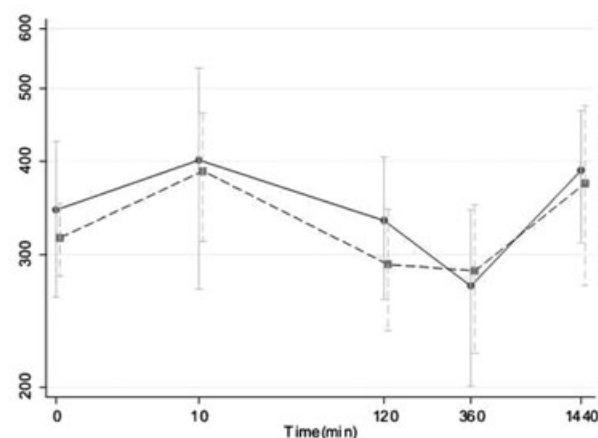
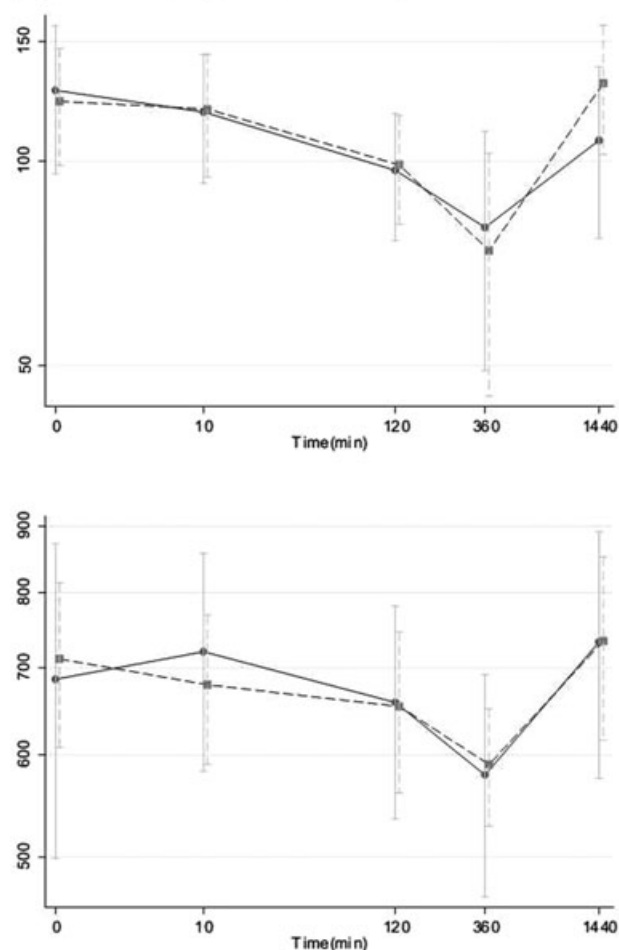
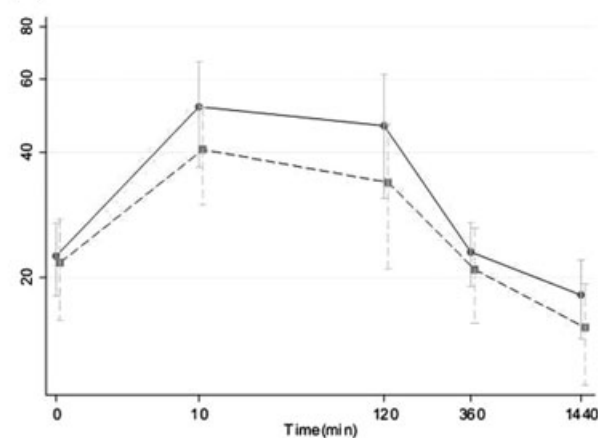


FIGURE 3 Raw data on log scale (for y and x axis) (solid line = LFV, dash line = both lungs left collapsed) for p65, p38, ROS, and HEME in blood. LFV, low-frequency ventilation, ROS, reactive oxygen species

(C) ROS in leukocytes, granulocytes and monocytes



(D) Heme

**FIGURE 3** Continued

was concealed, retention was good, data collection was blinded, and analyses were carried out and reported in accordance with a prespecified analysis plan. Therefore, the trial was at low risk of bias.³⁴ Although the sample size was small, there was no suggestion of any benefit from the adoption of LFV.

The surgical team could not be blinded, and we cannot rule out the possibility that this led to variations in surgical technique by the group. The use of PEEP in the intraoperative mechanical ventilation has been associated with a reduction of atelectasis in postoperative period as reported by studies using high PEEP level (10 cm H₂O).^{35–37} Overall the role of PEEP in surgery has been extensively studied with positive impression.^{33,38–40} In cardiac surgery particularly as the chest cavity is open, the lungs are arbitrarily exposed to atmospheric pressure, rather than normal negative intrathoracic pressure. Hence the transpulmonary pressure (airway pressure minus intrathoracic pressure) becomes abnormally low at end-expiration leading to collapse of the lungs, if we do not apply PEEP of at least 3 to 5 cm H₂O. Nevertheless, in our treatment group, we applied the LFV without PEEP. This is probably a major limitation that we could have avoided by adding PEEP to our LFV group.

5 | CONCLUSION

Contrary to our working hypothesis LFV during CPB has not been demonstrated to reduce pulmonary or systemic inflammation compared to both lungs left collapsed and may, in fact, increase the levels of specific inflammatory cytokines.

ACKNOWLEDGMENTS

We wish to thank the patients who participated and the staff in the cardiothoracic unit, who made this study possible. The study was jointly supported by the British Heart Foundation (BHF) and the National Institute for Health Research (NIHR) Bristol Biomedical Research Center. Neither the BHF nor the NIHR had any role in the design, conduct, analysis, or reporting of the trial.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

KEY QUESTION

Does low-frequency ventilation (LFV) during cardiopulmonary bypass (CPB) improve inflammatory markers and lung function compared to both lungs left collapsed in patients undergoing CABG?

KEY FINDINGS

There were no significant differences between groups in inflammatory markers measured in the lung tissue and blood.

TAKE-HOME MESSAGE

LFV during CPB when compared with both lungs left collapsed does not reduce inflammation in lung biopsies and blood.

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APPENDIX A

TABLE A1a Comparison between LFV and both lungs left collapsed on lung tissue inflammatory markers

Inflammatory marker	LFV (N = 18), mean (SD)NaN		Lungs left collapsed (N = 18), mean (SD)NaN	
	Before CPB	Before chest closure	Before CPB	Before chest closure
NF- κ B p65	-1.92 (0.27)	-1.86 (0.32)	-1.87 (0.18)	-1.91 (0.22)
p38 MAPK	-1.12 (1.09)	-1.19 (1.05)	-1.26 (1.05)	-1.08 (0.75)
TNF- α	-7.36 (1.12)	-6.91 (0.73)	-7.34 (1.12)	-7.33 (0.86)
IL-1 β	-5.72 (1.05)	-3.15 (1.13)	-5.64 (1.16)	-4.10 (1.17)
IL-18	-8.63 (0.81)	-8.67 (0.56)	-8.67 (0.71)	-8.42 (1.09)
IL-6	-0.99 (1.31)	-6.12 (1.10)	-2.56 (1.75)	-6.03 (0.87)
IP-10	-7.86 (1.27)	-7.54 (1.24)	-7.84 (1.01)	-7.66 (1.33)
IL-8	-5.45 (1.77)	-1.30 (1.39)	-5.64 (0.78)	-2.30 (1.72)
IL-10	-6.03 (1.05)	-5.03 (2.30)	-6.32 (1.09)	-5.26 (1.63)
CXCR3	-4.22 (1.77)	-4.28 (1.07)	-4.59 (1.95)	-4.98 (1.20)
Caspase-3	-2.46 (0.38)	-2.60 (0.47)	-2.56 (0.60)	-2.50 (0.64)

Abbreviations: MAPK, mitogen-activated protein kinases; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; ROS, reactive oxygen species.

^aData are transformed because their distribution is not normal, the mean of the transformed data is tabulated.

TABLE A1b Comparison between LFV and both lungs left collapsed on blood inflammatory markers with time fitted in the model

Inflammatory marker	LFV, mean (SD)NaN				Lungs left collapsed, mean (SD)NaN					
	Postinduction	10 min post-CPB	2 h	6 h	24 h	Postinduction	10 min post-CPB	2 h	6 h	24 h
Leukocytes										
NF-κB p65	0.16 (0.54)	0.40 (0.63)	-0.03 (0.80)	0.08 (0.99)	-0.30 (1.26)	0.44 (0.44)	0.10 (0.67)	0.11 (1.22)	0.21 (1.56)	-0.05 (0.94)
p38 MAPK	4.84 (0.80)	4.22 (1)	4.57 (1.26)	4.79 (1)	4.90 (1.19)	4.75 (0.83)	4.41 (1.07)	4.72 (1)	4.63 (0.77)	5.00 (1.11)
ROSNaN	4.43 (1.48)	4.46 (1.33)	4.41 (0.76)	3.80 (1.60)	4.35 (1.13)	4.71 (0.51)	4.69 (0.45)	4.51 (0.46)	3.79 (1.24)	4.75 (0.54)
Monocytes/granulocytesNaN										
NF-κB p65	0.03 (0.60)	0.32 (0.41)	0.37 (0.85)	0.21 (0.63)	-0.07 (0.94)	0.38 (0.88)	0.60 (0.73)	0.28 (1.22)	0.43 (0.76)	0.60 (1.02)
p38 MAPK	6.63 (0.55)	6.38 (0.48)	6.50 (0.59)	6.42 (0.59)	6.46 (0.72)	6.42 (0.60)	6.25 (0.64)	6.44 (0.62)	6.21 (0.54)	6.40 (0.76)
Monocytes										
ROSNaN	5.54 (1.11)	5.52 (1.58)	5.47 (1.45)	5.38 (0.91)	5.78 (0.89)	5.73 (0.24)	5.87 (0.45)	5.58 (0.49)	5.55 (0.51)	5.82 (0.46)
Granulocytes										
ROSNaN	6.25 (1.07)	6.27 (1.38)	6.21 (1.27)	6.17 (0.93)	6.42 (0.94)	6.52 (0.36)	6.48 (0.33)	6.44 (0.31)	6.36 (0.21)	6.54 (0.38)
Heme, μM	3.05 (0.38)	3.79 (0.57)	3.65 (0.64)	3.08 (0.34)	2.81 (0.43)	2.95 (0.51)	3.58 (0.50)	3.35 (0.56)	2.93 (0.48)	2.60 (0.49)

Abbreviations: MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; ROS, reactive oxygen species.

^aData are transformed because their distribution is not normal, the mean of the transformed data is tabulated.

^bNegative data for ROS was set to missing. Most time points and cell type had only one value set to missing. ROS in leukocyte at baseline and 2 h had three missing values, ROS in Leukocyte at 6 h had six missing values.

^cMonocytes overlapped with granulocytes for NF-κB p65 and p38 MAPK.

TABLE A2 Summary of the main effects for primary and secondary endpoints in inflammatory markers (LFV vs Lungs left collapsed)

		LFV vs lungs left collapsed		P value
Event		Treatment effect	95% CI	
In biopsy	NF- κ B p65	0.102	-0.022, 0.226	0.104
	p38 MAPK	-0.173	-0.723, 0.376	0.525
	TNF- α	0.425	-0.128, 0.977	0.127
	IL-1 β	0.985	0.228, 1.742	0.012
	IL-18	-0.310	-0.845, 0.224	0.244
	IL-6	1.59	0.528, 2.654	0.005
	IP-10	0.251	-0.567, 1.06	0.533
	IL-8	0.995	-0.098, 2.088	0.073
	IL-10	-0.053	-1.244, 1.138	0.929
	CXCR3	0.551	-0.058, 1.159	0.075
	Caspase-3	0.186	-0.204, 0.576	0.334
In blood	NF- κ B p65 leukocytes	0.023	-0.420, 0.467	0.918
	NF- κ B p65 monocytes_granulocyte	-0.125	-0.433, 0.182	0.424
	p38 MAPK leukocytes	-0.146	-0.524, 0.232	0.448
	p38 MAPK monocytes_granulocyte	-0.029	-0.284, 0.226	0.823
	ROS monocytes	-0.100	-0.353, 0.152	0.436
	ROS granulocyte	-0.010	-0.196, 0.176	0.913
	ROS leukocytes	-0.213	-0.585, 0.159	0.262
	Heme	0.142	-0.050, 0.334	0.148

Abbreviations: CI, confidence interval; IL, interleukin; LFV, low frequency ventilation; MAPK, mitogen-activated protein kinases; NF- κ B, nuclear factor κ -light-chain-enhancer of activated B cells; ROS, reactive oxygen species; TNF- α , tumor necrosis factor- α .

Results of the statistical inference summary showing the main effect for primary and secondary end points in standard care vs LFV. Cytokines increased following surgery. A positive value indicates that standard care was better than LFV whilst a negative value indicates an improved response to LFV.

TABLE A3a Pulmonary gas exchange parameters (LFV vs lungs left collapsed)

Pulmonary gas exchange	LFV						Lungs left collapsed							
	Postinduction	10 min post CPB	2 h	4 h	Post extubation	12 h	24 h	Postinduction	10 min post-CPB	2 h	4 h	Postextubation	12 h	24 h
PaO ₂ , mm Hg	215.25 (144.7-344.3)	168 (145.5-264)	133.9 (103.5-203)	119.25 (105-130.5)	110.25 (84.7-117.8)	102.35 (94.5-122.3)	99 (86.3-114.8)	229.55 (164.3-293.3)	211.5 (117-328.5)	129 (117-184.5)	132 (106.5-157.5)	114.8 (94.5-148.5)	122.3 (105.8-146.3)	97 (90.8-111.8)
A-a Gradient	148.19 (33.18-204.96)	176.74 (121.39-233.55)	114.89 (108.5-150.53)	116.26 (91.63-126.95)	113.63 (77.56-133.00)	97.56 (80.36-116.88)	104.04 (81.97-132.83)	111.59 (85.05-168.65)	178.21 (40.63-336.49)	113.78 (95.1-146.83)	90.63 (62.75-144.45)	105.95 (58.43-122.40)	62.68 (35.86-99.70)	67.98 (46.47-114.63)

Abbreviations: CPB, cardiopulmonary bypass; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; IQR, interquartile range; LFV, low frequency ventilation; PaO₂, partial pressure of oxygen.

TABLE A3b Lung function parameters (LFV vs lungs left collapsed)

Lung function test	LFV, mean (SD)NaN		Lungs left collapsed, mean (SD)NaN	
	Pre-op	Follow-up	Pre-op	Follow-up
FEV1 (% predicted), L	90.94 (21.58)	82.06 (19.86)	97.22 (18.83)	91 (20.20)
FVC (% predicted), L	93.24 (20.73)	83.00 (18.37)	97.11 (18.51)	91.25 (16.55)
MEF75 (% predicted), L	84.13 (42.32)	85.31 (39.01)	87.87 (30.83)	87.00 (30.37)
MEF25 (% predicted), L/s	68.27 (36.25)	60.56 (25.77)	70.47 (25.82)	67.33 (23.94)
TLC (% predicted), L	97.40 (14.53)	88.94 (11.76)	103.27 (14.11)	90.08 (13.57)
RV (% predicted), L	112.73 (25.60)	98.75 (24.47)	116.33 (34.67)	95.17 (21.44)
TL _{CO} (% predicted), mmol/kPa/min	89.79 (18.13)	84.75 (18.05)	84.14 (12.98)	76.92 (18.13)
K _{CO} (% predicted), mmol/kPa/min	103.71 (17.29)	108.56 (17.53)	96.00 (12.17)	97.75 (15.36)
FEV1 (measured), L	2.51 (0.91)	2.36 (0.81)	2.75 (0.81)	2.58 (0.89)
FVC (measured), L	3.27 (1.07)	3.04 (0.99)	3.47 (0.95)	3.30 (0.99)
MEF75 (measured), L/s	5.40 (3.32)	5.87 (2.93)	5.93 (2.41)	6.09 (2.45)
MEF25 (measured), L/s	1.40 (2.02)	1.17 (1.57)	0.97 (0.51)	0.97 (0.55)
TLC (measured), L	5.87 (1.19)	5.59 (1.07)	6.15 (1.07)	5.58 (1.16)
RV (measured), L	2.57 (0.61)	2.29 (0.7)	2.61 (0.87)	2.17 (0.51)
TL _{CO} (average), mmol/kPa/min	7.63 (1.93)	7.29 (1.8)	7.53 (2.56)	6.69 (2.40)
K _{CO} (average), mmol/kPa/min	1.39 (0.26)	1.46 (0.25)	1.29 (0.19)	1.31 (0.27)
Percentage O ₂ saturation	96.33 (1.05)	96.88 (1.58)	97.07 (1)	98 (0.85)

Abbreviations: CPB, cardiopulmonary bypass; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; IQR, interquartile range; LFV, low frequency ventilation; PaO₂, partial pressure of oxygen; MEF75, maximal expiratory flow at 75%; RV, residual volume; TLC, total lung capacity; TL_{CO}, transfer factor of the lungs for carbon monoxide; K_{CO}, transfer coefficient for carbon monoxide.

^aIf data are transformed because of the distribution is nonnormal, then the mean of the transformed data will be tabulated.

Non-biased investigation into cardio-pulmonary bypass induced lung injury

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BACKGROUND:

Heart disease is currently the world's leading cause of death. Many heart conditions can only be treated through surgery, during which cardio-pulmonary bypass (CPB) is often used. During CPB the heart is stopped and the blood supply diverted from the heart and lungs to allow the operation to proceed in a largely blood-free environment. CPB is still associated with severe systemic inflammation and tissue damage and post-operative lung dysfunction of various degrees occurs in 30% of patients.

METHODS:

Biopsies were taken from 36 patients undergoing surgery involving cardio-pulmonary bypass (CPB), at the beginning of surgery and immediately before reperfusion. Biopsies were homogenised in either RLT buffer (RNA) or RIPA buffer with protease inhibitors (Protein). RNA was extracted using Qiagen RNeasy spin columns. Following isolation RNA integrity was confirmed, the RNA converted to cDNA and gene expression analysed using Affimetrix microarray. Protein was isolated and the samples from all patient pooled into either pre or post surgery samples. Subsequently, the changes in protein levels were measured using Tandem Mass Tagging (TMT).

TABLE 1

Significantly induced genes following surgery:

ProbeSet ID	p-value(Post vs. Pre)	Fold-Change(Post vs. Pre)	Gene
ADAMT1	2.20E-14	3.46079	A disintegrin and metalloproteinase with thrombospondin motifs 1
ALDH1A3	2.05E-08	2.33175	Aldehyde and lipid metabolism
ADUO1	7.62E-16	3.42286	Aim 1 (upregulated - down in lung cancer)
BHLHE22	1.27E-13	2.47507	Cell differentiation
CCL2	5.10E-20	5.02006	Produced smooth muscle, chemotactant
CCL8	9.21E-11	2.20899	Monocyte chemoattractant protein 2
CDKN1A	2.13E-16	2.96273	Cyclin-dependent kinase inhibitor 1
CEBPD	1.48E-12	2.29829	CEBPD is involved in regulation of apoptosis and cell proliferation. Linked to inflammation and macrophages
CHY1	2.26E-09	2.15588	Cell surface protein, including cell proliferation and recognition, extracellular matrix deposition, and morphogenesis
CRP	6.38E-10	2.6509	C-reactive protein
CCL22	3.47E-13	2.47795	Cytokine-rich secretory proteins - reproduction and smooth muscle inhibition
CYR1	9.05E-08	2.43778	Macrophage inflammatory protein 2 alpha (MIP2) alpha
EGFR	7.63E-07	2.21634	Dual Specificity Phosphatase 1
DUSP5	1.01E-14	2.45719	Dual Specificity Phosphatase 5
EGR1	6.78E-17	4.57514	Early growth response protein 1
EGFR	1.25E-13	2.28143	Early growth response protein 3
ELL2	9.38E-10	2.13815	Elongation Factor, RNA Polymerase II, 2
ERBB1	7.92E-10	2.21222	ERBB receptor feedback inhibitor 1
FOS	3.75E-09	2.19214	FBJ Murine Osteosarcoma Viral Oncogene Homolog (AP1 component)
FOSB	9.85E-10	2.10451	FBJ Murine Osteosarcoma Viral Oncogene Homolog B (AP1 component)
FOSL2	1.28E-13	2.11281	Fos related antigen 2 (AP1 component)
GADD45A	1.40E-14	2.10184	Growth arrest and DNA-damage-inducible protein GADD45 alpha
GADD45B	4.21E-15	3.11502	Growth arrest and DNA-damage-inducible protein GADD45 beta
IER3	3.18E-17	4.08872	Radiation-inducible immediate-early gene IEX-1, protection from apoptosis.
IL6	1.54E-14	4.59538	Interleukin 6
LOC441019	4.89E-12	2.27893	Hypothetical gene
MDN	0.01E-10	2.20029	Mitotin, neurogenesis
MR1	2.24E-09	2.10673	mRNA - tumour suppressors and cardiac disease
MT1A	4.47E-10	2.90983	Metallothionein 1A
MT2A	2.60E-12	3.15905	Metallothionein 2
MTE	1.97E-09	2.35834	Retinol-binding Protein-Binding Zinc Finger Protein
MYC	1.82E-15	2.8184	C-myc
NAMPT	2.55E-16	3.43475	Nicotinamide phosphoribosyltransferase (aka pre-B-cell colony-enhancing factor 1 (PBEF1) or vitamin)
NF1	1.39E-15	2.88338	Nicotinamide N-methyltransferase is an enzyme that in humans is encoded by the NAMPT gene.[1][2]
NM1	3.81E-09	2.06021	Nuclear receptor subfamily 4, group A, member 3
NM2	3.74E-14	2.77508	Nuclear receptor related 1 protein (NURR1)
NM3	5.06E-14	2.88338	Nuclear receptor subfamily 4, group A, member 3
ORF22A	7.07E-10	2.02236	Single stranded DNA (ssDNA) binding protein
P81	1.76E-13	2.56386	Protein oncogene serine/threonine kinase Pim-1
P1G52	2.79E-13	3.14254	Prostaglandin-endoperoxide synthase 2 (aka Cox-2)
RASD1	5.52E-07	2.12228	Deaminase-induced Ras-related protein 1
RGS2	2.93E-14	2.83021	Regulator of G-protein signaling 2
RNF122	3.02E-12	2.34752	RING finger protein 122
SOK	9.65E-07	2.07768	Serine/threonine-protein kinase SGK
SRK1	4.42E-15	2.59714	Serine/threonine-protein kinase SRK1
SLC2A3	8.96E-13	2.32146	Solute carrier family 2, facilitated glucose transporter member 3
SFRB1	9.27E-09	2.04286	SPRY domain-containing SOCS box protein 1
THBS1	4.71E-11	3.59339	Thrombospondin 1
TRB1	3.44E-08	1.15923	Tribbles homolog 1 (MAPK regulator)
ZFP36	1.55E-17	3.79832	zinc finger protein 36 homolog

TABLE 3

Significant changes in protein levels following surgery:

Q70L17	14.876	Type II hair keratin 2 OS-Homo sapiens GN-KRT18B2
Q8B175	14.741	Keratin-associated protein 2-2 OS-Homo sapiens GN-HKTP2-2
B4MU05	8.276	cDNA FLJ36312, highly similar to Tubulin beta' chain OS-Homo sapiens
Q70L17	7.140	Keratin, type I cuticular HaB OS-Homo sapiens GN-HKTP36
Q8B175	4.627	Keratin, type I cuticular HaB-1 OS-Homo sapiens GN-HKTP3B
Q8B175	3.857	KRT18B protein OS-Homo sapiens GN-KRT18B
Q8B175	3.627	Glutathione S-transferase P1 (Fragment) OS-Homo sapiens GN-HGSTP1
Q8B175	3.266	Uncharacterized protein NCBP2-AS2 OS-Homo sapiens GN-NCBP2-AS2
A4F000	2.885	SVT2 protein (Fragment) OS-Homo sapiens GN-SVT2
B4MU05	2.860	cDNA FLJ26887, highly similar to Cell cycle control protein 50A OS-Homo sapiens
H0U002	2.740	Taxilin beta, isoform CRA_1 OS-Homo sapiens GN-TXLNB
Q8B175	2.683	Keratin-associated protein 2-2 OS-Homo sapiens GN-HKTP2-2
Q70L17	2.555	Nebulette OS-Homo sapiens GN-NEBL
F8WJ44	2.521	Dystonin OS-Homo sapiens GN-DST
Q70L17	2.519	Type II hair keratin 2 OS-Homo sapiens GN-KRT18B2
Q8B175	2.107	TMP1 protein (Fragment) OS-Homo sapiens GN-TMP1
Q8B175	2.097	Rheumatoid factor RF-ET6 (Fragment) OS-Homo sapiens
B7Z682	2.430	Uncharacterized protein 2 OS-Homo sapiens
B7Z682	2.379	cDNA FLJ25754, highly similar to Voltage-dependent L-type calcium channel subunit alpha-1D OS-Homo sapiens
Q6Q528	2.342	CCDC91 protein (Fragment) OS-Homo sapiens GN-CCDC91
B3K556	2.327	cDNA FLJ35559 fs, clone SPLN200009, highly similar to GRP and coiled-coil domain-containing protein 1 OS-Homo sapiens
Q8B175	2.313	Phospholipase A2, membrane associated OS-Homo sapiens GN-PLA2G5A
H0Y882	2.291	Zinc finger protein 782 (Fragment) OS-Homo sapiens GN-ZNF782
Q8B175	2.297	UHRF1 protein (Fragment) OS-Homo sapiens GN-UHRF1
B2R8C8	2.263	Ubiquitin-like protein ATG12 OS-Homo sapiens
Q1L7X3	2.241	Serine/threonine-protein kinase TAO1 OS-Homo sapiens GN-TACK1
Q1S323	2.168	Keratin, type I cuticular HaB OS-Homo sapiens GN-HKTP31
B3K556	2.107	cDNA FLJ12840 fs, clone NT284400184, highly similar to Timeless homolog OS-Homo sapiens
P14505	2.101	Phospholipase A2, membrane associated OS-Homo sapiens GN-PLA2G5A
B0YQV0	2.088	BTB/POZ domain-containing protein 3 (Fragment) OS-Homo sapiens GN-HBTB03
P14505	2.082	Eosinophil peroxidase OS-Homo sapiens GN-EPX
P46109	2.003	Cxk-like protein OS-Homo sapiens GN-CXKL
Q8B175	0.497	Hemoglobin alpha-2 (Fragment) OS-Homo sapiens GN-HBA2
Q13938	0.485	Calcyonin OS-Homo sapiens GN-CCAPS
E8P689	0.450	Ubiquitin OS-Homo sapiens GN-UBC1A1
A8B867	0.448	Glutathione S-transferase OS-Homo sapiens
P09115	0.442	Carbonic anhydrase 1 OS-Homo sapiens GN-CA1
Q8B175	0.429	Hemoglobin beta chain (Fragment) OS-Homo sapiens GN-HBB
H3BML9	0.397	Myosin regulatory light chain 2, skeletal muscle isoform (Fragment) OS-Homo sapiens GN-HMYLFP
Q8B175	0.384	Densin (Fragment) OS-Homo sapiens GN-DEN
Q8B175	0.380	Hemoglobin subunit delta OS-Homo sapiens GN-HBD
Q8B175	0.380	Zinc finger protein 648 OS-Homo sapiens GN-ZNF648
Q8B175	0.346	Protein S100A2 OS-Homo sapiens GN-S100A2
Q8B175	0.270	Hemoglobin beta chain variant 10 (Fragment) OS-Homo sapiens GN-HBB
B4E114	0.257	cDNA FLJ35881, highly similar to Mucin-5B OS-Homo sapiens
B2R726	0.148	cDNA FLJ35674 OS-Homo sapiens
B7Z682	0.121	cDNA FLJ25754, highly similar to Voltage-dependent L-type calcium channel subunit alpha-1D

DISCUSSION:

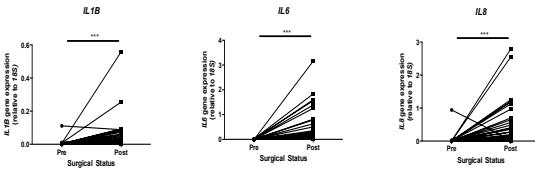
Lung ischemia during cardio-pulmonary bypass surgery (before reperfusion) causes significant changes to gene expression and protein expression in lung tissue. These include inflammatory mediators (*IL6* & *IL8*) and signalling molecules. Pathway analysis show increase in stress response genes and proteins that are involved in biogenesis and morphogenesis. One of the most highly up-regulated proteins in the lung biopsies post-surgery is keratin. At present further work is needed to confirm that keratin expression is found in the lungs and increased by surgery or whether this is due to sample contamination.

CONCLUSIONS:

Cardio-pulmonary bypass surgery causes significant changes to gene and protein expression in lung tissue.

FIGURE 1

Gene expression of inflammatory cytokines *IL1B*, *IL6* and *IL8* are significantly increased following bypass surgery:



Data shows cytokine gene expression levels, measured by Taqman qPCR, normalised against 18S rRNA, analysed by T-test (**p<0.001)

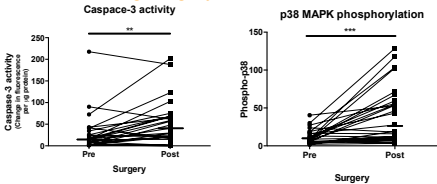
TABLE 2

Statistically over-represented pathways, identified by PANTHER:

	Homo sapiens (REF)		Client Text Box Input			
PANTHER GO-Slim Biological Process	#	#	expected	Fold Enrichment	+/-	P value
response to stress	659	8	1.39	> 5	+	1.43E-02
immune system process	1391	13	2.94	4.42	+	8.45E-04
cellular process	6708	26	14.18	1.83	+	4.88E-02

FIGURE 2

p38 MAPK phosphorylation and Caspase-3 activity are increased following surgery:



Data shows individual data points and the median, analysed by Wilcoxon matched-pairs ranked test (**p<0.01, ***p<0.001)

TABLE 4

Statistically over-represented pathways, identified by PANTHER:

PANTHER GO-Slim Biological Process	Homo sapiens (REF)		Client Text Box Input				
	#	#	expected	Fold Enrichment	+/-	P value	
	596	7	0.94	> 5	+	7.73E-03	
	1206	9	1.91	4.71	+	1.75E-02	
	1316	9	2.09	4.31	+	3.41E-02	

Isolated Human Pulmonary Artery Structure and Function Pre- and Post-Cardiopulmonary Bypass Surgery

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Background—Pulmonary dysfunction is a known complication after cardiac surgery using cardiopulmonary bypass, ranging from subclinical functional changes to prolonged postoperative ventilation, acute lung injury, and acute respiratory distress syndrome. Whether human pulmonary arterial function is compromised is unknown. The aim of the present study was to compare the structure and function of isolated and cannulated human pulmonary arteries obtained from lung biopsies after the chest was opened (pre-cardiopulmonary bypass) to those obtained at the end of cardiopulmonary bypass (post-cardiopulmonary bypass) from patients undergoing coronary artery bypass graft surgery.

Methods and Results—Pre- and post-cardiopulmonary bypass lung biopsies were received from 12 patients undergoing elective surgery. Intralobular small arteries were dissected, cannulated, pressurized, and imaged using confocal microscopy. Functionally, the thromboxane mimetic U46619 produced concentration-dependent vasoconstriction in 100% and 75% of pre- and post-cardiopulmonary bypass arteries, respectively. The endothelium-dependent agonist bradykinin stimulated vasodilation in 45% and 33% of arteries pre- and post-cardiopulmonary bypass, respectively. Structurally, in most arteries smooth muscle cells aligned circumferentially; live cell viability revealed that although 100% of smooth muscle and 90% of endothelial cells from pre-cardiopulmonary bypass biopsies had intact membranes and were considered viable, only 60% and 58%, respectively, were viable from post-cardiopulmonary bypass biopsies.

Conclusions—We successfully investigated isolated pulmonary artery structure and function in fresh lung biopsies from patients undergoing heart surgery. Pulmonary artery contractile tone and endothelium-dependent dilation were significantly reduced in post-cardiopulmonary bypass biopsies. The decreased functional responses were associated with reduced cell viability.

Clinical Trial Registration—URL: <http://www.isrctn.com/ISRCTN34428459>. (*J Am Heart Assoc.* 2016;5:e002822 doi: 10.1161/JAHA.115.002822)

Key Words: bradykinin • cardiopulmonary bypass • lung • thromboxane • vasoconstriction • vasodilation

The pulmonary circulation receives the total cardiac output; yet in humans the characterization of pulmonary

arteries is sparse. Most research into the structure of pulmonary arteries has been carried out using animal models or cast models from humans¹ with rare studies of arterial wall thickness and morphology in sectioned tissue samples biopsied from patients.^{2,3}

In addition to the paucity of studies looking at the structure of human pulmonary arteries, there is no report in the literature on the physiological behavior of these arteries. An understanding of structure and function is important in order to characterize dysfunction and to investigate potential interventions to remedy derangements. A relevant context is cardiac surgery with cardiopulmonary bypass (CPB) where respiratory distress or dysfunction is one of the most common complications, affecting up to 25% of patients.⁴ Presumed causative factors include inflammation, prolonged lung collapse, pulmonary ischemia and related reperfusion injury, blood contact with the surface of the heart–lung machine, endotoxemia, surgical trauma, blood loss, and transfusion.^{5,6}

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Accompanying Videos S1 through S3 are available at <http://jaha.ahajournals.org/content/5/2/e002822/suppl/DC1>

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These factors have been associated with in-hospital mortality, morbidity, and increased hospital costs.^{7–9} Current strategies to reduce the respiratory dysfunction/edema associated with cardiac surgery have been the following: technical modifications of CPB, hemodilution, postoperative administration of steroids, and a range of lung ventilation protocols.¹⁰ However, these modifications have had moderate or short-lived therapeutic success. A better understanding of the circulatory changes responsible for the lung dysfunction associated with cardiac surgery with CPB may help devise strategies aimed at reducing postoperative pulmonary complications, but to date this has not been possible due to a lack of fresh lung tissue.

We took advantage of a randomized trial to obtain lung biopsies for studies of structure and function. The aims of the present study were 2-fold: first, to demonstrate proof-of-principle that structural and functional assessments of human pulmonary arteries from lung biopsies are possible; and, second, to compare structural and functional assessments of human pulmonary arteries before and after CPB in patients undergoing coronary artery bypass graft (CABG).

Methods

Patients undergoing CABG in the department of cardiothoracic surgery at the Hammersmith Hospital were recruited, after giving written informed consent, to a randomized controlled clinical trial comparing low-frequency ventilation during CPB to standard care; the trial is registered as ISCTRN 34428459. Biopsy samples for a subgroup were transported to Oxford under a material transfer agreement. The study completed recruitment and follow-up in June 2014. A favorable Research ethics opinion approval was granted by the Camden & Islington NRES Research Ethics Committee London (reference 12/LO/0458) in April 2012 and amended in April 2013 to allow the use of some biopsy tissue for this substudy. This research complies with the Helsinki Declaration.

Study Participants

Patients aged ≥ 40 and < 85 years and having elective or urgent CABG with CPB and cold blood cardioplegic arrest for CABG were eligible. Exclusion criteria were the following: left ventricular ejection fraction $\leq 30\%$, previous pulmonary embolism requiring warfarin for ≥ 3 months, previous cardiac surgery, NYHA class IV, cardiogenic shock, chronic renal failure requiring dialysis, treatment with corticosteroid or immunosuppressive drug, severe chronic obstructive pulmonary disease, lung pathology, previous radiotherapy, or body mass index > 35 .

Surgical, Anesthetic, and Cardiopulmonary Techniques

Operations were carried out following standard protocols for the Hammersmith Hospital. Anesthetic, cardiopulmonary, and surgical techniques were as previously reported.^{6,11} Briefly, after premedication with temazepam, anesthesia was induced with a combination of propofol and remifentanyl; muscle relaxation was achieved using vecuronium. Anesthesia was maintained by infusion of propofol and remifentanyl (5 mg remifentanyl to 1 g propofol) to keep the entropy value of the processed electroencephalogram below 55. After intubation, all participants were ventilated mechanically until starting CPB. Mechanical ventilation used volume control at the following settings: tidal volume of 6 to 8 mL·kg⁻¹, I:E ratio of 1:2, positive end-expiratory pressure of 5 cm H₂O, FiO₂ of 0.5, and ventilatory rate as required to keep the PaCO₂ between 4.5 and 5.5 kPa. CPB was conducted with systemic temperature between 32°C and 35°C. Cardioplegic arrest was achieved with antegrade cold blood cardioplegia.

Two lung biopsies were taken from each patient using the LigaSure Impact™ instrument (LF4318) connected to a ForceTriad™ energy platform (Covidien, Boulder, CO). The system combines TissueFect™ sensing technology with hand activation and an integrated cutting mechanism to achieve multifunctional tissue sealing. The pre-CPB biopsy was taken from the left upper lobe after sternotomy and the post-CPB biopsy was taken from the left lower lobe prior to weaning from CPB. Samples were placed in ice-cold Ca²⁺-free MOPS buffer solution (mmol/L: NaCl 145, KCl 4.7, MgSO₄·7H₂O 1.17, MOPS 2.0, NaH₂PO₄·H₂O 1.20, glucose 5.0, pyruvate 2.0, EDTA 0.02, and NaOH 2.75 with pH adjusted to 7.40 $\pm$ 0.02), transferred from surgery to the laboratory on ice, and dissection commenced within 2 to 4 hours. Once in the laboratory, samples were placed in Ca²⁺-containing MOPS (composition as above with the addition of 2.0 mmol/L CaCl₂·2H₂O) and an intralobular artery was dissected free from surrounding tissue. The artery was cannulated onto glass micropipettes, heated to 36.5 $\pm$ 0.4°C, pressurized to 40 mm Hg, and left to equilibrate for 30 minutes in a 2 mL chamber (RC-27 chamber, PH-6 platform; Warner Instruments, Hamden, CT), as previously described using other tissue.¹²

Confocal Microscopy Studies

In all studies, arteries were visualized using Olympus linescan confocal microscopes (FV500 and FV1000). When studying function, arteries were imaged with transmitted light using a $\times 10$ Olympus objective and recorded using Fluoview software (Olympus, Tokyo, Japan) at 1 Hz. The structure of arteries was studied with imaging using a $\times 40$ (1.15 NA, 0.25 mm WD)

water immersion Olympus objective, obtaining z-stacks through the arterial wall in 0.5 or 1 μm steps to observe each cell type, in turn. Fluorescent dyes were used to stain cells with calcein (excitation 488 nm), and nuclei with Hoechst 33342 (excitation 405 nm) and propidium iodide (PI, excitation 543 nm), and postfixation, smooth muscle actin with phalloidin (excitation 543 nm), nuclei with DAPI (excitation 405 nm), and elastin with Alexa Fluor[®] 633 hydrazide (AF-633, excitation 633 nm).

Functional Studies

Each artery was tested for leaks, assessed as an ability to hold diameter to >90% of original for a minute during a sealed pressure test. Small side-branches were common and detected by rapid deflation of arteries during this test, and these arteries were therefore not used for experiments. On occasion, no arteries within a biopsy passed this leak test, causing the number of samples with data to be less than the maximum of 12, with incomplete pairing between pre- and post-CPB biopsies for some patients.

To test contractile function of the arteries, concentration response curves were determined by varying the concentration of the thromboxane mimetic U46619 from 0.03 to 3 $\mu\text{mol/L}$. From a state of submaximal contraction to U46619, endothelial function (dilation of the arteries) was assessed using a single concentration of bradykinin (1 $\mu\text{mol/L}$). Finally, maximum contraction was measured at the end of the experiment using a combination of 3 $\mu\text{mol/L}$ U46619 and 45 mmol/L KCl.

Structural Studies

Studies of smooth muscle cell (SMC) orientation and density were carried out by fixing cannulated arteries with 2% (wt/vol) paraformaldehyde for 10 minutes at $36.5 \pm 0.4^\circ\text{C}$, washing with PBS, then incubating arteries overnight at 4°C with phalloidin-TRITC (6.4 $\mu\text{mol/L}$); nuclei were stained with DAPI (1.5 $\mu\text{mol/L}$). The elastin present throughout the arterial wall was visualized with AF-633 hydrazide (250 nmol/L), and the integrity of inter-endothelial cell (EC) tight junctions assessed by immunohistochemistry (mouse monoclonal anti-human ZO-1, aa 334-634, 1:200, Life Technologies 339100; Alexa Fluor[®] 488 chicken anti-mouse IgG 1:200, Life Technologies A-21200) using established methods.¹²

Once it was clear that cell orientation and density per se did not relate to cell function, an additional protocol was applied at the completion of functional experiments, in unfixed tissue. Live cells were classified according to their ability to uptake and de-esterify the fluorescent dye calcein AM, and classification was confirmed by comparing staining with cell permeant and impermeant nuclear dyes. This live cell

staining analysis of SMCs and ECs was carried out by perfusing the artery lumen with calcein AM (1 $\mu\text{mol/L}$), Hoechst 33342 (Hoechst, cell-permeant nuclear dye, stains live and dead cells, 16 $\mu\text{mol/L}$), together with PI (cell-impermeant nuclear dye, stains dead cells, 10 $\mu\text{mol/L}$) at 8 $\mu\text{L/min}$ for 30 minutes using a Beehive[®] syringe pump system. Cells were classified as live (calcein in cytoplasm; nuclei stained with Hoechst but not PI) or dead (nuclei stained with both Hoechst and PI; no calcein); live cells were expressed as a percentage of all cells. The elastin was not specifically stained in this protocol, but the autofluorescence of elastin was observed.

Materials

NaCl, KCl, and D-glucose were purchased from Fisher Scientific (Loughborough, UK); MOPS, EDTA, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pyruvate, PBS sachets, Tween20, and bovine serum albumin were purchased from Sigma (Poole, UK); and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NaOH, and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ were purchased from VWR (Leicestershire, UK). Bradykinin and U46619 were purchased from

Table 1. Patient Characteristics

Total number of patients	12
Patient age, y	71.3 $\pm$ 7.1
Sex (male/female)	11/1
BMI, kg/m ²	28.3 $\pm$ 3.7
Diabetic	58%
Previous T.I.A.	0%
Hypertensive	100%
Hypercholesterolemia	100%
Asthma	17%
Aspirin	83%
Clopidogrel	33%
Warfarin	8%
β -Blockers	83%
Calcium channel blockers	42%
Oral nitrate	25%
Statins	92%
ACE inhibitors	58%
Angiotensin II antagonists	17%
Diuretics	8%
Insulin	17%
Oral anti-diabetic	33%

Data presented as the mean $\pm$ SD or as a percentage of the whole patient group. ACE indicates angiotensin-converting enzyme; BMI, body mass index; T.I.A., transient ischemic attack.

Table 2. Protocols Performed on Arteries Isolated From Each Biopsy Obtained Pre- and Post-CPB for Patients in the Study

Patient (n)	1		2		3		4		5		6		7		8		9		10		11		12	
CPB	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+	—	+
Leak test	#	+	+	+	+	+	+	+	+	#	+	#	+	#	+	+	+	+	+	+	+	+	+	#
Contraction	—	+	+	+	+	+	+	+	+	—	+	—	+	—	+	+	+	+	+	+	+	+	+	—
Dilation	—	+	+	+	+	+	+	§	+	—	+	—	+	—	+	§	+	+	+	+	+	+	+	—
Phalloidin	—	—	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—
Live/dead	—	—	+	+	—	—	+	+	—	—	+	—	+	—	+	+	+	+	+	+	—	—	—	—
ZO-1	—	+	+	+	—	—	+	+	—	—	+	—	—	—	—	—	—	—	+	+	—	—	—	—

Arteries from 5 biopsies did not pass the leak test (marked #). In those arteries where contraction was not observed, the dilation could not be assessed (marked §). On 2 occasions the complete protocol was performed on arteries isolated from both pre- and post-CPB biopsies (patients 2 and 10, labeled yellow and cyan, respectively, in all figures). —, protocol not performed; +, protocol performed; CPB, cardiopulmonary bypass.

Tocris (Bristol, UK). The structural dyes Hoechst (H3570), PI (P1304MP), DAPI (D3571), and AF-633 (A30634) were purchased from ThermoFisher Scientific (Paisley, UK) and phalloidin-TRITC from Sigma-Aldrich (Dorset, UK). Paraformaldehyde was purchased from Electron Microscope Sciences (Hatfield, PA).

Statistical Analysis

All images were analyzed offline, measuring inner diameters of arteries and staining using Imaris[®] software (version 7.7 Bitplane). Decreases in diameter to U46619 are expressed either as % constriction (100% is equivalent to 0 μ m; this established the ability of each artery to contract to U46619) or as % maximum constriction (100% is the constriction to

3 μ mol/L U46619 plus 45 mmol/L KCl; used for concentration response curves to U46619). The dilation evoked by bradykinin is expressed as a reversal of the pre-imposed 3 μ mol/L U46619 tone, with 100% equivalent to the maximum inner diameter of arteries. Percentage contraction and percentage dilation are summarized as the mean $\pm$ SEM, with n representing the number of arteries studied. No experiments were discarded from the study but, for most pairs of biopsies, the entire protocol could not be successfully performed on arteries from both biopsies from a patient (see Functional Studies above); therefore, means are based on denominators that are less than 12 for most outcomes. Every effort was made to pair pre-CPB and post-CPB data where possible; statistical power could not be improved due to completion of trial. Descriptive statistics are used to summarize

Table 3. Summary Data Showing Functional Responses to the Vasoconstrictor U46619 and Endothelium-Dependent Vasodilator Bradykinin in Human Pulmonary Arteries

Artery Characteristics	Pre-CPB		Post-CPB	
		n		n
Maximum internal diameter, μ m	240 $\pm$ 23	11	246 $\pm$ 30	8
Number responding to vasoconstrictor (%)	11 (100)	11	6 (75)	8
Maximum constriction, μ m	68 $\pm$ 16	11	71 $\pm$ 35	8
Percentage maximum constriction	26 $\pm$ 5	11	27 $\pm$ 8	8
Maximum constriction to U46619, μ m	59 $\pm$ 14	11	67 $\pm$ 32	8
Percentage maximum constriction to U46619	23 $\pm$ 4	11	25 $\pm$ 10	8
Paired artery U46619 EC ₅₀	−7.4 $\pm$ 1.6	7	−6.5 $\pm$ 0.8	7
Number responding to endothelium-dependent vasodilator (%)	5 (45)	11	2 (33)	6
Percentage maximum dilation to bradykinin	35 $\pm$ 14	11	29 $\pm$ 19	6
Percentage live smooth muscle cells	100 $\pm$ 0	7	60 $\pm$ 21	5
Percentage live endothelial cells	90 $\pm$ 5	7	58 $\pm$ 19	5

No significant differences were observed between pre- and post-CPB. Values are the mean $\pm$ SEM. 100% maximum constriction equates to a diameter of 0 μ m; 100% maximum dilation equates to full reversal of U46619 tone; CPB, cardiopulmonary bypass.

unpaired data for pre- and post-CPB arteries. Formal statistical comparisons on paired data first tested for Gaussian distributions (D'Agostino and Pearson omnibus normality test), which confirmed nonparametric tests should be used. For U46619 concentration response curves, individual EC_{50} values, and live cell experiments the Wilcoxon matched-paired signed-rank test was used (pre- versus post-CPB paired biopsies).

Results

Pre- and post-CPB lung biopsies were taken from 12 patients. Patient characteristics, comorbidities, and medications are summarized in Table 1. It should be noted that all patients enrolled in this study were hypertensive, and suffered from hypercholesterolemia, and most were diabetic. For analyses based on paired biopsies when each patient served as their

own control, alterations in arterial function or structure from arteries pre-CPB to post-CPB are at least partly attributable to the effects of the surgery and/or the use of CPB. Table 2 outlines the protocols performed on each of the arteries isolated from the 24 biopsies. Table 3 shows the summary data for arterial characteristics and the experimental protocols.

Functional Analysis

The average internal diameter of pre-CPB arteries was $240 \pm 23 \mu\text{m}$ ($n=11$), which was not different from that for post-CPB arteries $246 \pm 30 \mu\text{m}$ ($n=8$). U46619 caused at least some contraction in 100% of arteries from pre-CPB biopsies. However, only 75% of arteries from post-CPB biopsies contracted to U46619 (Table 3, Figure 1B). Contractile responses averaged across available pre- and post-CPB

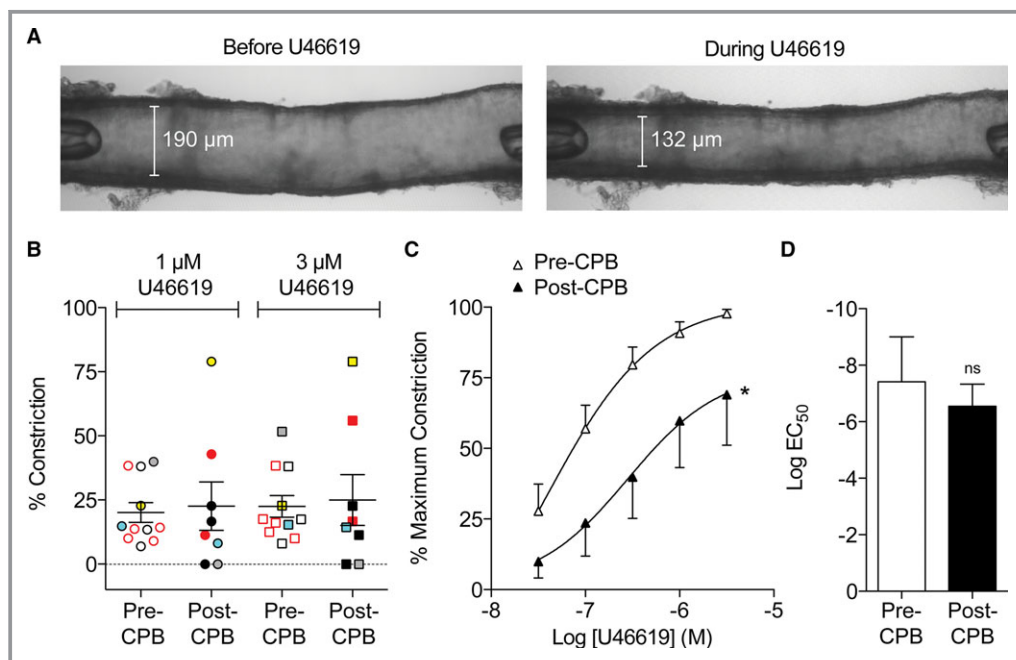


Figure 1. Constriction of human pulmonary arteries. A, Brightfield image showing a cannulated and pressurized human pulmonary artery before (left) and after contraction to 3 $\mu\text{mol/L}$ U46619 (right). B, Contraction of human pulmonary arteries using 1 $\mu\text{mol/L}$ and 3 $\mu\text{mol/L}$ U46619 before (pre-CPB, $n=11$) and after CPB (post-CPB, $n=8$), expressed as a percentage constriction (100% equates to constriction to 0 μm). Scatter plots show individual data points for all biopsies for which measurements could be made; comparisons were made using 1-way ANOVA with Bonferroni post hoc test, no significant difference between concentrations (1 or 3 $\mu\text{mol/L}$) or biopsy time (Pre/Post-CPB). Yellow and blue-filled symbols correspond to Patients 2 and 10, respectively; gray-filled symbols indicate contractile responses from the artery shown in (A) (Patient 8); red symbols represent responses from patients taking calcium antagonist medication. C, Paired U46619 concentration response curves in human pulmonary arteries obtained from the same patients pre- and post-CPB, expressed as a percentage of the maximum contraction (determined using 3 $\mu\text{mol/L}$ U46619 and 45 mmol/L KCl). Values are the mean $\pm$ SEM ($n=7$), comparison of post vs pre-CPB arteries made using the Wilcoxon matched-paired signed rank test (* $P<0.0001$ post vs pre-CPB). D, pEC_{50} determined from (C); values are the mean $\pm$ SEM ($n=7$) and comparisons were made using the Wilcoxon matched-paired signed rank test; ns, not significant. CPB indicates cardiopulmonary bypass.

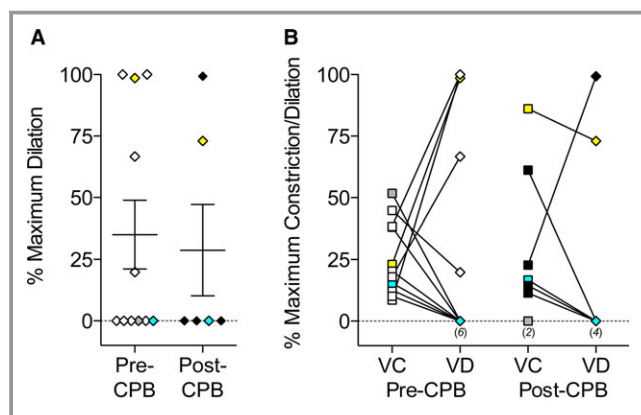


Figure 2. Dilation of human pulmonary arteries. A, Endothelium-dependent vasodilation to 1 $\mu\text{mol/L}$ bradykinin pre- ($n=11$) and post-CPB ($n=6$). Data shown as a scatter plot showing individual responses and the mean $\pm$ SEM with comparisons made using 1-way ANOVA with Bonferroni post hoc test (not significant). B, Relationship between vasoconstriction (VC, square symbols) and vasodilation (VD, diamond symbols) responses within a given artery. Yellow, gray, and blue-filled symbols correspond to Patients 2, 8, and 10, respectively. CPB indicates cardiopulmonary bypass; numbers in parentheses indicate the number of equal values.

arteries (unpaired) are shown in Figure 1B. When U46619 concentration response curves were compared in paired pre/post-CPB arteries, there was a significant reduction in the %

maximum constriction to U46619 (Figure 1C), without affecting the EC_{50} value (Table 3, Figure 1D).

Bradykinin (1 $\mu\text{mol/L}$) caused dilation in 35% of arteries from pre-CPB biopsies, compared to 29% of arteries from post-CPB biopsies (unpaired; Table 3, Figure 2A). The % maximum EC-dependent dilation in pre-CPB arteries was not different from that in post-CPB arteries. There were no noticeable relationships between the magnitudes of vasoconstriction and vasodilation (Figure 2B).

Structural Analysis

Staining to show the structure of fixed pulmonary artery SMC layers using DAPI (nuclear stain) and phalloidin (F-actin stain) revealed inconsistent cell orientation and density (Figure 3). In most cases a uniform pattern was observed; however, on occasion smooth muscle structure, instead of being oriented circumferentially, was at a range of angles. This misalignment was observed in some arteries from both pre- and post-CPB biopsies (Figure 3B). Furthermore, gaps were observed between SMCs (Figures 3 and 4). These gaps were often filled by elastin, confirmed by AF-633 staining, and in some areas it was elastin that ran the entire way through the artery from the external layer through to the endothelium (Videos S1 and S2).

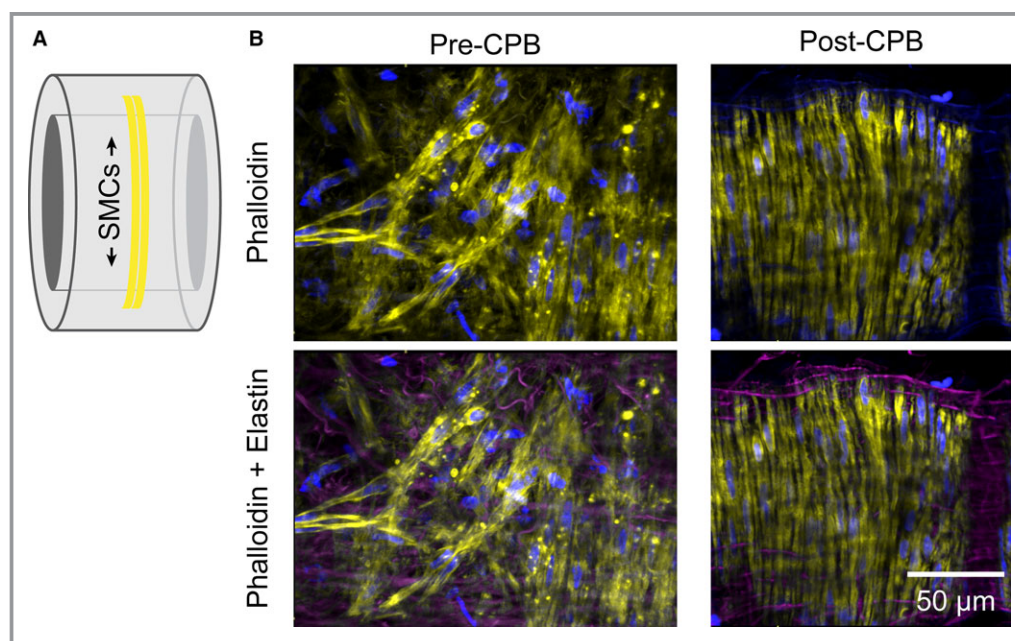


Figure 3. Orientation of cells in the arterial wall. A, Cartoon of arterial wall with circumferentially oriented smooth muscle cells (SMCs) vertically aligned. B, In most arteries fixed and stained with phalloidin (yellow) the SMCs were adjacent and vertically aligned, but in some cases the orientation was inconsistent and sparse. This misalignment occurred in arteries obtained both pre- and post-CPB, here shown in the pre-CPB artery; both arteries are from Patient 10. The nuclei were stained with propidium iodide (blue), and elastin with AF-633 (pink, lower panels). CPB indicates cardiopulmonary bypass. See Video S1 for a z-stack through the wall of an artery stained with AF-633.

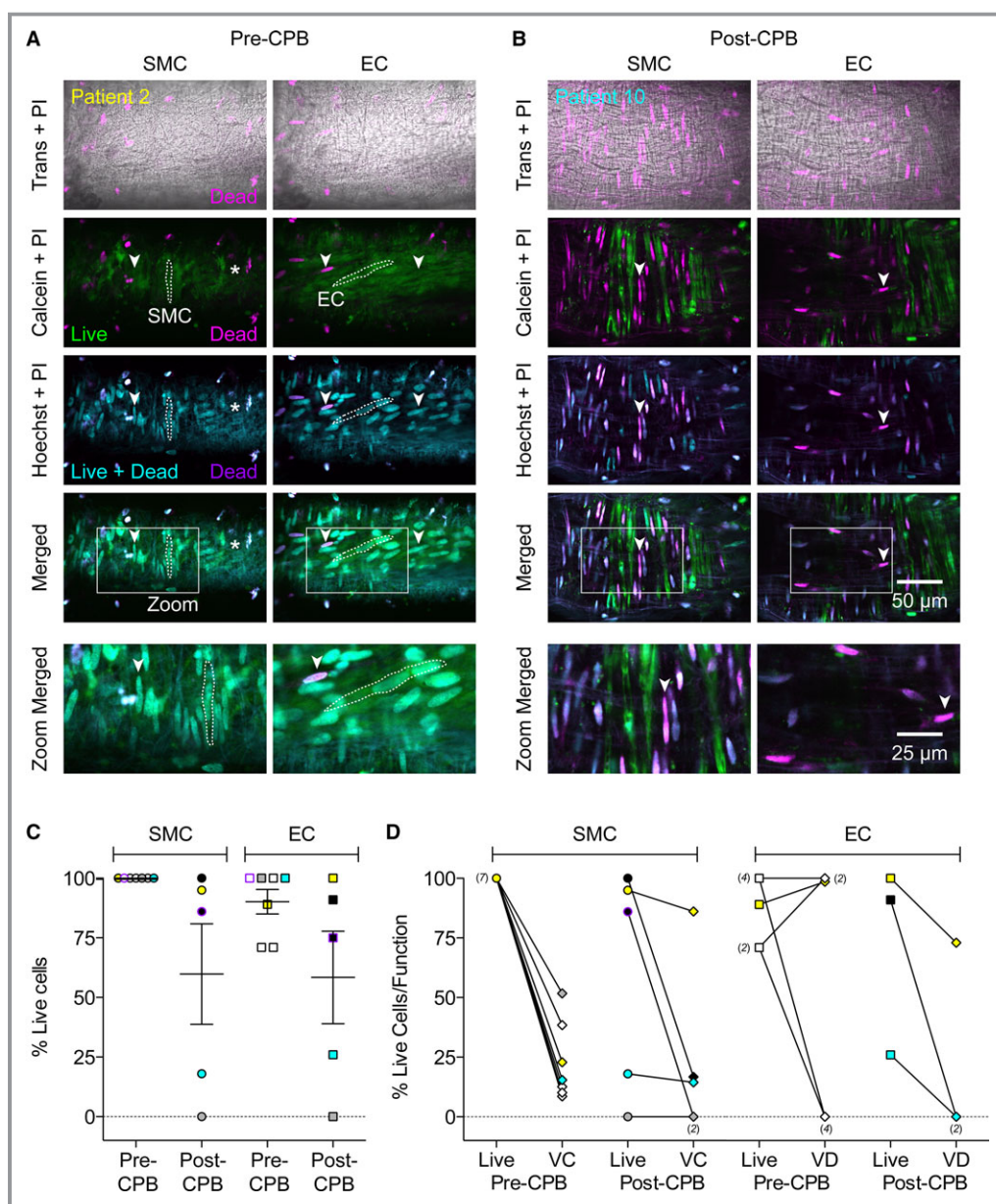


Figure 4. Live/dead staining in cannulated human pulmonary arteries. The cytosolic marker calcein AM is only de-esterified in live cells (Calcein, green). The cell permeant dye Hoechst 33342 (Hoechst, cyan) stains all nuclei, whereas the cell impermeant propidium iodide (PI, pink) only penetrates dead cell membranes, co-localized nuclear stains are purple and also reflect dead cells. Trans, transmitted light image. A, By focusing through the wall of the artery from a pre-CPB biopsy (Patient 2), it was clear that there were no dead smooth muscle cells (SMCs, left panels, vertical orientation, one cell outlined with dashed line, no SMCs at the right of the image indicated by asterisk); and very few dead endothelial cells (ECs, right panels, near-horizontal orientation, one cell outlined with dashed line). B, In contrast, the wall of the artery from a post-CPB biopsy (Patient 10) was littered with dead SMCs and no live ECs were present. C, Scatter plots showing individual data points of the percentage of cells taking up calcein and not PI (Live cells) from 7 pre-CPB biopsies, and 5 post-CPB biopsies along with the mean $\pm$ SEM. In paired comparisons there was no significant difference between pre vs post-CPB cells ($n=5$, Wilcoxon matched-paired signed-rank test). D, Overall, the staining for live SMCs tended to reflect the ability of U46619 to stimulate vasoconstriction (VC), whereas the presence of live ECs did not uniformly reflect an ability of bradykinin to stimulate vasodilation (VD). Arrowheads indicate the same position in all images within a column. See Videos S2 and S3 for z-stacks through these arteries. Yellow, gray, and cyan-filled symbols correspond to arteries from patients 2, 8, and 10, respectively (paired data in both C and D). Purple symbols correspond to arteries from patient 4 (paired data in C). CPB indicates cardiopulmonary bypass; numbers in parenthesis indicate the number of equal values.

In arteries from pre-CPB biopsies, live cell staining revealed that 100% of SMCs and 90% of ECs were viable (Table 3, Figure 4, Video S2). Conversely, in arteries from post-CPB lung biopsies, only about half of the SMCs and ECs were viable (Table 3, Figure 4, Video S3). Although there was a trend for the number of live SMCs and ECs to decrease after CPB, this was not statistically significant (Table 3, $n=5$). When the percentage of live cells was plotted against the percentage of maximum contraction and percentage of dilation (Figure 4D), there was no positive relationship between the amount of live cells and magnitude of response.

Discussion

This is the first study to combine pressure myography, pharmacology, and dyes to characterize both the structure and function of human pulmonary arteries. It provides proof of principle of the feasibility of carrying out such studies in the context of a clinical trial to evaluate the effects of an intervention on lung function. This is also the first study to investigate the effects of cardiac surgery and CPB on structure and function in isolated human arteries obtained from lung biopsy in patients undergoing elective CABG.

Functional studies revealed that the thromboxane mimetic U46619 caused vasoconstriction of all human pulmonary arteries isolated from pre-CPB biopsies but not all those from post-CPB biopsies. A paired comparison of the contractile responses of pre- and post-CPB arteries revealed a significant reduction in contraction to U46619 in arteries from post-CPB biopsies. Therefore, the inability of some arteries to contract may reflect the iatrogenic harm of cardiac surgery and CPB.

Previous structural analysis of human pulmonary arteries was last conducted in the 1970s using fixed and sectioned tissues taken from biopsies without isolating the arteries.^{1–3} This is the first study to report the structure of live and fixed isolated, cannulated, and pressurized human pulmonary arteries. The structural assessment carried out in this study shows that the interlobular human pulmonary arteries studied were partially muscular in structure, often showing incomplete coverage of the artery circumference. Furthermore, although SMC orientation was often typical (ie, wrapped around the circumference of the artery and perpendicular to flow), in some arteries this was not so, instead being oriented over a range of angles and in some places showed spiral structures similar to those seen in rat pulmonary arteries.¹³ Also, in the SMC layer there were nuclei of cells that did not appear to be SMC. Although the present study did not investigate the exact cell type, it is possible that these cells were fibroblasts or immune cells that are common components of the adventitia.¹⁴ It should also be noted that the gaps left in the SMC layer were often filled with AF-633 staining, likely to represent

the elastin component of the media.¹⁵ Altered SMC structure was found in arteries both pre- and post-CPB. Therefore, in the present study we cannot link altered SMC orientation and density to CPB; rather, this may be a characteristic of this group of patients prior to surgery.

We sought to establish which of the visible SMCs were viable, so we carried out a live/dead protocol in tissues after the functional studies to investigate the possibility that decreased contraction was caused by SMC death. We showed that all SMCs were alive in arteries isolated from biopsies taken pre-CPB, whereas only half the SMCs were alive in arteries from biopsies taken post-CPB. One explanation for these findings is that CPB causes decreased arterial contraction due to damage to SMCs in the arterial wall. During CPB, there is minimal blood perfusion of pulmonary arteries/arteries and decreased oxygenation; these conditions are known to provoke an increased immunological response through ischemia–reperfusion injury and mechanical stress, causing atelectasis and lung dysfunction.^{16–18} Indeed, a recent study has shown that CPB carried out in rabbits caused an increase in leakage of Evans blue dye administered intravenously, which could be inhibited by an antibody against tumor necrosis factor α .¹⁹ Therefore, macrophage and neutrophils released during CPB could cause tumor necrosis factor α -induced apoptosis in pulmonary SMCs, such as that seen in this study.

Functional studies of ECs suggested decreased endothelium-dependent vasodilation after CPB. In arteries from pre-CPB biopsies, less than half were capable of endothelium-dependent vasodilation, with an average dilation <50%. However, in arteries from biopsies taken after CPB, dilation to bradykinin was measurable in only 1 of 5. As with the contraction findings, this is consistent with a previous finding in porcine coronary arterioles of a slight decrease in vasorelaxation after CPB, which was associated with increased EC adhesion molecules and myeloperoxidase leading to cell death.²⁰ Our structural studies, which showed a smaller percentage of live cells in arteries from post-CPB biopsies compared to pre-CPB biopsies, further support this hypothesis.

EC orientation, as for SMC, was atypical. Irrespective of the timing of biopsies in relation to CPB, ECs were often arranged at various angles instead of running in line with blood flow. This finding has previously been associated with altered shear stress associated with some vascular disease.^{21,22}

In conclusion, we have demonstrated the feasibility of obtaining and studying the function and structure of arteries from human lung biopsies. We have shown that CPB is detrimental to both SMC and EC function in pulmonary arteries and that this is potentially a consequence of cell death. The ability to distinguish changes that are likely to be attributable to CPB suggests that these methods could be

used to evaluate the effects of interventions to reduce the adverse consequences of CPB on the lungs.

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Disclosures

None.

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Review Article

Pulmonary Protection Strategies in Cardiac Surgery: Are We Making Any Progress?

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Pulmonary dysfunction is a common complication of cardiac surgery. The mechanisms involved in the development of pulmonary dysfunction are multifactorial and can be related to the activation of inflammatory and oxidative stress pathways. Clinical manifestation varies from mild atelectasis to severe respiratory failure. Managing pulmonary dysfunction postcardiac surgery is a multistep process that starts before surgery and continues during both the operative and postoperative phases. Different pulmonary protection strategies have evolved over the years; however, the wide acceptance and clinical application of such techniques remain hindered by the poor level of evidence or the sample size of the studies. A better understanding of available modalities and/or combinations can result in the development of customised strategies for the different cohorts of patients with the potential to hence maximise patients and institutes benefits.

1. Introduction

Pulmonary dysfunction is a common complication of cardiac surgery that can impact patient's outcomes and health economics. It is recognised that many patients will have altered pulmonary mechanics after surgery which may appear in a wide range of clinical presentations, from mild atelectasis to life threatening acute lung injury (ALI) or adult respiratory distress syndrome (ARDS) [1–3].

Surgical incisions by abolishing the integrity of the chest wall affect respiratory mechanics leading to impaired respiratory effort. Postoperative pain has been shown to be associated with decreased lung function by precluding deep inspirations. Furthermore, patients undergoing surgical procedures associated with opening the pleura will have increased rates of atelectasis, pleural effusions, and postoperative pain especially in the early postoperative period [4–6].

Cardiopulmonary bypass (CPB) can lead to the activation of different inflammatory and coagulation pathways and alters redox balance due to the passage of blood through the circuit (contact activation) and ischaemia and reperfusion injury [7–10].

Vascular endothelial cells (EC) dysfunction during CPB due to changes in blood flow patterns, shear stress, ischaemia, and reperfusion and circulating cytokines will result in the activation of multiple proinflammatory and proapoptosis pathways [11–14] while suppressing its ability to produce vasoprotective mediators [14–17]. EC activation is known to initiate leukocytes adhesion cascade by the expression of members of the selectin family which are responsible for the initial attachment of leukocytes from circulation. Transmigration of leukocyte through EC (crucial step for leukocytes recruitment to tissue) follows leukocytes attachment and is mediated by the upregulation of different adhesion molecules such as platelet endothelial cell adhesion molecule-1 (PECAM-1), ICAM-1, and very late antigen-4 (VLA-4) [18–21].

CPB is traditionally associated with inadequate lung perfusion as there is no flow to the pulmonary artery during periods of cross clamping and when the heart is not ejecting blood, and thus blood supply is limited to the bronchial arteries [22]. Pulmonary physiology alteration during CPB can result in disturbing the balance in the blood gas barrier due to the alteration of the different force

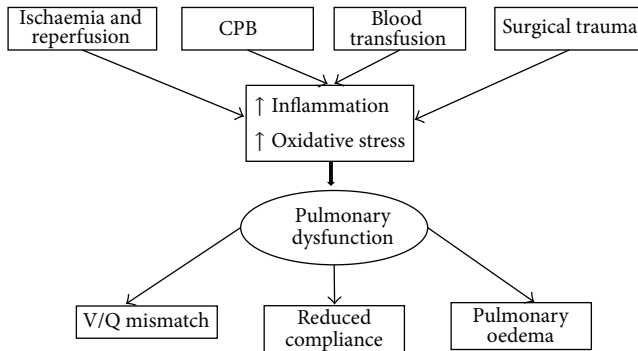


FIGURE 1: Mechanisms involved in pulmonary dysfunction during cardiac surgery and its consequences.

affecting the parenchyma thus abolishing gas exchange by passive diffusion at the blood-gas barrier level and leading to ventilation/perfusion mismatch and impaired pulmonary compliance [23, 24]. Moreover, ischaemia during CPB is associated with reduced alveolar blood supply resulting in alveolar ischaemia and hypoxic pulmonary vasoconstriction [25]. Pulmonary vascular endothelial cells dysfunction and activated neutrophils sequestration into lungs parenchyma during period of reperfusion can increase lung tissue permeability and elevate vascular resistance and pulmonary surfactant changes leading to alveolar protein accumulation and pulmonary oedema and driving more ROS and cytokines production [26–28]. This will be manifested as abnormal gas exchange, poor lung mechanics, increased pulmonary shunt fraction and reduced functional residual capacity, and carbon monoxide transfer factor [28–30] (Figure 1).

Blood and blood products usage after surgery can be associated with the production of excessive amount of ROS and systemic and pulmonary inflammation. It has been previously shown that the duration of blood storage before transfusion can influence adverse effect associated with transfusion as there are an increased risk of respiratory insufficiency and the need for prolonged ventilation in patients receiving blood stored for longer than two weeks [10, 31–34].

Here we review the most commonly used strategies to minimise pulmonary dysfunction after cardiac surgery.

2. Pulmonary Protection Strategies

2.1. Pharmacological Interventions. The fact that inflammation and oxidative stress play a pivotal role in the development of pulmonary dysfunction resulted in multiple studies aimed toward modulating such events by the administration of vasodilators and antioxidant and anti-inflammatory medications in experimental animal models or patients undergoing cardiac surgery.

Prostacyclins can induce vascular smooth muscle cells relaxation by the release of intracellular adenosine 3',5' cyclic monophosphate (cAMP) resulting in pulmonary and systemic vasodilation [35–38]. Moreover, they can have inhibitory effect on platelet aggregation and leukocytes and monocytes activity [39]. The use of inhaled prostacyclins

(Epoprostenol or Flolan) has been shown to decrease pulmonary arterial endothelial dysfunction induced by CPB in experimental studies [40–42]. Furthermore, when administered prior to CPB, they can have beneficial effect in the presence of pulmonary hypertension and may result in lower rate of reintubation in high risk postoperative cardiac patients [43, 44].

Phosphodiesterase inhibitors such as pentoxifylline (PTX) which is a known nonselective phosphodiesterase (PDE) inhibitor can result in elevated levels of intracellular cAMP and vasodilatation. Although PTX has been used classically for claudication symptoms in peripheral vascular disease [45], it has been shown to exert anti-inflammatory and antioxidative properties resulting in modulation of ALI [46–48]. More selective PDE inhibitors such as milrinone can be an advantageous therapeutic strategy for cardiac surgical patients with increased pulmonary vascular resistance (PVR) and right ventricular failure when nebulized and inhaled. It can cause selective pulmonary vasodilation and potentiate the vasodilation effects of inhaled prostacyclin [49, 50].

Nitric oxide (NO) is known to play a pivotal role in vascular endothelial cells homeostasis and regulation of oxidative stress and inflammatory responses [51]. Ischaemia and reperfusion injury during surgery is associated with significant loss in NO; thus NO preconditioning has been suggested to reduce perioperative pulmonary dysfunction and its sequels [52–54]. The protective effects may be due to reversal of postischemic lung hypoperfusion and reduction of lung neutrophil sequestration. The administration of NO in patients with severe left ventricular dysfunction can lead to pulmonary vasodilatation and may augment left ventricular filling [55–57]. The timing of administration and/or concentration of inhaled NO during ischaemia or reperfusion periods is a very important determinant of its effect as NO is toxic early in reperfusion, due to its interaction with superoxide which may lead to damage of alveolar type 2 cells [58, 59].

A large number of other drugs have been used with various degrees of success. Aprotinin (serine proteases) had been shown to reduce neutrophil elastase, malondialdehyde, and proinflammatory cytokines levels in bronchoalveolar lavage fluids of patients undergoing cardiac surgery [60]. The use of aprotinin can result in improving lung function and reducing reperfusion lung injury [61]. The administration of corticosteroids before CPB may reduce the activation of multiple proinflammatory mediators. The translation of proinflammatory mediator's changes into clinical outcomes remains controversial and most of the evidence in the literature originates from small RCTs or observational studies with biomarkers as primary end points [62, 63].

2.2. CPB Modification. Different strategies have been attempted over the years to minimise proinflammatory activation and oxidative stress when using CPB such as coating the circuit with biocompatible material (heparin, poly-2-methoxyethyl acrylate, synthetic protein, and phosphorylcholine), removal of leukocytes (by adding special filters to the CPB), ultrafiltration, and reduced haemodilution.

Heparin is thought to reduce the inflammatory responses linked to platelets and leukocytes by reducing the release of IL-6, IL-8, E-selectin, lactoferrin, myeloperoxidase, integrin, selectin, and platelet thromboglobulin and decreasing the production of oxygen free radicals [64–66]. It has been suggested that compared with conventional circuits (poly-2-methoxyethyl acrylate, synthetic protein, and phosphorylcholine), the heparin-coated circuit may improve lung compliance and pulmonary vascular resistance and thus reduce intrapulmonary shunt although intubation time and ICU stay were not affected [67, 68].

The use of leukocyte filtration mechanisms can modulate proinflammatory cytokines and oxidative stress [69–71]. A clinical study compared the effectiveness of leukocyte filter depletion with a common arterial filter in CABG patients who reported better oxygenation indices and less duration of postoperative mechanical ventilation in the leukocyte depletion filter group [71]. Another study suggested that leukocyte depletion filters preferentially remove activated leukocytes. Improvement in lung function was evident only in the early postoperative phase, but this did not lead to decreasing mortality or better clinical outcomes [72]. The use of ultrafiltration or modified ultrafiltration techniques at the end of surgery may reduce postoperative oedema specifically that of lungs resulting in better oxygenation and improved lung compliance postoperatively. Furthermore, ultrafiltration may remove proinflammatory mediators from the circulation such as IL-6 and IL-8 but it did not result in significant improvement of clinical outcomes [73]. Similarly, controlled haemodilution to regulate oncotic pressure can reduce priming volumes and result in better haemodynamic parameters such as vascular resistance and higher oxygen delivery and affect hospital stay significantly [74, 75].

Understanding problems associated with pulmonary ischaemia and reperfusion results in attempts to provide continuous pulmonary perfusion during CPB. Experimental animal models of pulmonary perfusion demonstrated reduced inflammatory and apoptosis pathways activation with such strategy [25, 76, 77]. Moreover, pulmonary perfusion was found to have favorable effect on lung compliance, oxygenation, and vascular resistance in patients undergoing CABG [78, 79]. Furthermore, pulmonary artery perfusion during CPB can be effective in reducing postoperative right ventricular dysfunction in high-risk patients undergoing LVAD placement [80]. The translation of such changes into better clinical outcomes remains unclear and may be restricted to selective group of patients as demonstrated by a recent trial in patients with COPD undergoing cardiac surgery using CPB where no significant protective effect on lungs was documented [81].

The deleterious effects of surface contact activation as discussed previously have led to the development of minimised cardiopulmonary bypass circuit (mini-CPB). This is characterised by reduced surface area and thus priming volume and prevention of air-blood contact. The utilisation of mini-CPB has been shown to be associated with attenuated production of proinflammatory cytokines and complement activation and blunted leukocytes activation compared to conventional circuit. Markers of oxidative stress tend to be

reduced in patients undergoing surgery using mini-CPB compared to conventional circuit [82, 83]. Additionally, mini-CPB reduces organ damage and results in better postoperative gas exchange and lower lung injury scores [84, 85]. Unfortunately most of the clinical trials investigating the role of mini-CPB have evaluated diverse technologies of varying complexity and degree of miniaturisation, which would be expected to give rise to heterogeneity in findings.

2.3. Surgical Strategies. It has been suggested that eliminating the usual standard of no lung inflation during CPB by maintaining a degree of lung ventilation may be beneficial. The use of continuous positive airway pressure (CPAP) during CPB may result in less shunt and better gas exchange [86]; however, it seems that such effect is dependent on the airway pressure used. Using low frequency ventilation (LFV) along with CPAP during CPB to reduce post-CPB lung injury has been evaluated in an experimental pig model [87]. This study showed that the use of LFV is associated with significantly better pulmonary gas exchange, higher adenine nucleotide, lower LDH levels, and reduced histological damage in lung biopsies as well as lower DNA levels in bronchoalveolar lavage (BAL) compared to the collapsed lungs control group. However, a clinical study in patients undergoing cardiac surgery compared the effect of low volume ventilation to conventional strategy of no ventilation and demonstrated no significant changes in PVRI, $\text{PaO}_2/\text{FiO}_2$ ratio, postoperative length of stay, and postoperative pulmonary complications [88]. Furthermore, a meta-analysis of 814 cases in 16 RCTs looking at three lung protective strategies in patients during CPB including CPAP, low-volume ventilation, and vital capacity manoeuvres during CPB showed that the effects of the designated techniques are probably short lived with a questionable impact on the long term clinical outcome of the treated patients [89].

Off pump coronary artery bypass (OPCAB) surgery seems to provide better lung protection by eliminating ischemia-reperfusion injury through maintaining lung ventilation and avoiding CPB. Many studies consistently reported better early and midterm outcomes in OPCAB when compared with conventional on-pump CABG: fewer respiratory complications, shorter intubation time and ITU stay, reduced incidence of pneumonia, and overall shorter hospital stay [90–92].

2.4. Physiotherapy. Preoperative prophylactic physiotherapy with inspiratory or expiratory muscle training can be used as a preventative measure for lung protection [93, 94]. Postoperative physiotherapy is used prophylactically in patients undergoing cardiac surgery. Different techniques can be utilised during this period to improve ventilation-perfusion inequalities, increase pulmonary compliance, and help re-inflate collapsed alveoli.

These techniques include deep breathing exercises, slow maximal inspirations with an inspiratory hold, intermittent deep breathing exercises with and without the use of incentive spirometer, and deep breathing exercises with expiratory resistance [95–98].

2.5. Postoperative Noninvasive Ventilation (NIV). NIV refers to the administration of ventilatory support without using an invasive artificial airway (endotracheal tube or tracheostomy tube). NIV exerts its main effects on the pulmonary and on the cardiovascular systems through the application of a positive end-expiratory pressure (PEEP); with or without a pressure support during inspiration, NIV restores lung volumes by opening atelectatic areas, increases alveolar ventilation, and reduces the work of breathing [99–101].

Continuous positive airway pressure (CPAP) aims to maintain a level of positive airway pressure in a spontaneously breathing patient. It is functionally similar to positive end-expiratory pressure (PEEP), except that PEEP is an applied pressure against exhalation and CPAP is a pressure applied by a constant flow. The ventilator does not cycle during CPAP, no additional pressure above the level of CPAP is provided, and patients must initiate all of their breaths. To avoid drying of the respiratory mucosa, there has been general agreement that the application of humidified CPAP helps to recruit the lungs by increasing functional residual capacity (FRC), increase the surface area of lung, decrease intrapulmonary shunt, and improve oxygenation [102–104].

Bilevel positive airway pressure (BLPAP) is a continuous positive airway pressure with pressure support breaths. It delivers a preset inspiratory positive airway pressure (IPAP) during inspiration and expiratory positive airway pressure (EPAP). BLPAP can be described as CPAP with a time-cycled or flow-cycled change of the applied pressure level [105]. BLPAP senses patients breathing efforts by monitoring air flow in the patient's circuit and adjusts its output by assisting inspiration. Therefore, its physiological effects can benefit the patient in both phases of respiration [106–108]. BLPAP application can only be commenced on conscious, cooperative, and hemodynamically stable patients who can breathe spontaneously, have an adequate gag and cough reflex, and are able to remove the mask when required. Several studies have demonstrated beneficial effects of BLPAP in reducing pulmonary complications and overall length of hospital stay after cardiac surgery [109–111]. Furthermore, the prophylactic use of BLPAP after early extubation has been shown to be safe and effective [111, 112]. A better tolerance was noted when BLPAP settings were commenced on low level and gradually adjusted to achieve the therapeutic target. Radiological improvement of atelectasis after cardiac surgery has been achieved on maintaining 8–10 mL/kg of tidal volume with BLPAP [113].

3. Conclusions

Pulmonary dysfunction is one of the most common and serious complications after cardiac surgery and can significantly impact on patient outcomes and health economics. The mechanisms involved in the development of pulmonary dysfunction are multifactorial and are related to the activation of different inflammatory and oxidative stress pathways. Clinical manifestation varies from mild atelectasis to severe respiratory failure. Managing pulmonary dysfunction postcardiac surgery is a multistep process that starts before surgery and continues during both the operative and postoperative

phases. Pulmonary protection strategies have evolved over the years with various degrees of success. The main weakness of the majority of studies is often being observational in nature, small sample size, or being concentrated on a single intervention. Managing pulmonary dysfunction needs to be a multistep process involving more than one modality for each step of the surgical pathway. A better understanding of available modalities and/or combinations will result in the development of customised strategies for the different cohorts of patients. This in turn will help reduce pulmonary dysfunction and hence improve early outcome and costs after cardiac surgery.

Conflict of Interests

No conflict of interests is declared by any of the authors.

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POSTOPERATIVE NONINVASIVE VENTILATION IN PATIENTS UNDERGOING CORONARY ARTERY BYPASS GRAFTING: A SMALL STEP WITH GREAT REPERCUSSIONS To the Editor:

Commonly, anesthesia, postoperative pain, and surgery induce respiratory modifications (hypoxemia, pulmonary volume decrease, and atelectasis) associated with a restrictive syndrome.¹ In some cases, postextubation acute respiratory failure is a serious complication after coronary artery bypass grafting. The role of noninvasive ventilation (NIV) in the postoperative period is growing as an alternative therapy to avoid reintubation and associated complications¹; however, pulmonary complications and NIV interactions still show a complex interaction with preoperative or intraoperative procedures.

In their recent article, Al Jaaly and colleagues² compared 2 strategies in the postoperative period: bilevel positive airway pressure (NIV) and usual care with oxygen therapy. Major findings of this study were that there were no significant differences in intensive care stay, postoperative stay, mean percentage of predicted forced expiratory volume in 1 second on days 1 and 3, and rate of pulmonary complications (except atelectasis).

We read this original article with interest; however, there are some interesting aspects that were not taken into account and could influence the

TABLE 1. Aspects related to atelectasis rate in postoperative surgery

Muscle paralysis
Intravenous or inhalational anesthetics
Body mass index*
Induction of anesthesia (use of a gas mixture)
Intraoperative fraction of inspired oxygen†
Surgery times
Sedation

*Although obese patients were not included in this analysis, there is no independent analysis of this factor between the groups. †This is an important element because it is associated with a greater reduction in the proportion of nitrogen and development of atelectasis. This aspect could explain the worse response in the group with standard oxygen versus noninvasive ventilation with respect to a different fraction of inspired oxygen; the intraoperative aspect was not evaluated in the study.⁵

interpretation of the outcome. (1) Small numbers of patients were enrolled in both arms of the study, and current smokers should have been grouped separately. Al Jaaly and colleagues² did not describe the preoperative baseline situation, and that is a crucial aspect.³ For assessment of postoperative acute respiratory failure, it would be relevant to know the baseline oxygenation index and fraction of inspired oxygen before and after extubation. (2) We do not have information regarding the use of any intraoperative recruitment maneuvers that have shown to have implications for the perioperative period.⁴ (3) Atelectasis rates were clearly different between groups. Atelectasis should be assessed by the radiologist, not the senior clinical team. The atelectasis was diagnosed on clinical basis, and bias may have been a factor. Chest radiography is not sensitive for detection of postoperative atelectasis. There are some factors in the preoperative and postoperative periods that need clarification because they may have influence the results observed⁵; we have summarized them in Table 1. Finally, limited training of the cardiac intensive care unit staff may have influenced the benefit of NIV. NIV was applied intermittently, rather than continuously for few hours.

We consider that further prospective randomized studies will encourage evaluating specific NIV strategies and how intra-operative factors are implicated in postoperative pulmonary complications, especially atelectasis.

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Reply to the Editor:

We thank Esquinas and colleagues for their interest in our article and for their comments. They make 3 points. (1) Our trial was small and did not include any subgroup analyses, in

The Editor welcomes submissions for possible publication in the Letters to the Editor section that consist of commentary on an article published in the Journal or other relevant issues. Authors should: • Include no more than 500 words of text, three authors, and five references. • Type with double-spacing. • See <http://jtcvs.ctsnetjournals.org/misc/fora.shtml> for detailed submission instructions. • Submit the letter electronically via jtcvs.editorialmanager.com. Letters commenting on an article published in the JTCVS will be considered if they are received within 6 weeks of the time the article was published. Authors of the article being commented on will be given an opportunity of offer a timely response (2 weeks) to the letter. Authors of letters will be notified that the letter has been received. Unpublished letters cannot be returned.

particular, current smokers versus former smokers and lifetime non-smokers. (2) Information on intraoperative recruitment maneuvers was not reported. Such maneuvers may have implications during the perioperative period. (3) Atelectasis was diagnosed clinically and not by a radiologist, and possible influences of preoperative and postoperative factors were not taken into account. We have responses to these points.

First, our trial was randomized. This created intervention and control groups that were balanced with regard to demographic, anthropometric, and respiratory factors. Randomization does not rule out effect modification (point 1); however, it does ensure that an overall treatment effect is not biased (point 3).

Second, subgroup analyses are controversial. International guidelines focus on prespecified subgroup analyses to avoid selective reporting.¹ Our trial was not powered to detect subgroup effects, and we did not specify any in advance other than preoperative chronic obstructive pulmonary disease (we originally aimed to stratify by chronic obstructive pulmonary disease, but there were insufficient patients with this comorbidity). If we were to carry out subgroup analyses, the findings would be very difficult to interpret. Failure to demonstrate statistical significance might well be due to low power, whereas observation of statistical significance might well be due to chance. For example, Esquinas and colleagues mention the potential influence of body weight: 17 patients with body mass index greater than 30 kg/m² had bilevel positive airway pressure, whereas 17 had usual care.

Third, our trial was designed pragmatically to estimate the effect of bilevel positive airway pressure in a real-life setting. Noninvasive ventilation was applied for a median of 11 hours. In the trial, as in usual care, "lung recruitment" (point 2) typically constituted lung reexpansion

and the application of positive end-expiratory pressure. Assessment of atelectasis by the senior clinical team was also pragmatic (point 3). From a specialist respiratory perspective, such assessment might be considered insensitive; however, it reflected accurately the information on which management decisions are made in practice. Moreover, we did show a substantial difference in atelectasis frequency; better sensitivity would be expected to increase, not decrease, the observed difference.

Finally, we would like to point out that their summary of the findings of our trial ignores our primary outcome (prespecified in our analysis plan) and our explanation of the reason for failing to observe differences between groups in predicted forced expiratory volume in 1 second and other outcomes that one would expect to differ. As we acknowledged and tried to explain in the article, we had expected the respiratory outcomes to parallel the clinical ones. Because this expectation was not met, we recommended a larger multicenter trial at the end of our Discussion section. This is one point on which we agree. Just as one swallow doesn't make a summer, one trial by itself very rarely brings about (or warrants) a change in practice. We recommended that a larger multicenter, randomized trial of bilevel positive airway pressure be carried out to decide whether the promise suggested in our trial can be substantiated.

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MITRAL VALVE REPLACEMENT IN THE PRESENCE OF MASSIVE CALCIFICATION

To the Editor:

I was pleasantly surprised to read the novel technique reported by Husain and colleagues.¹ I reported on the technique used at the All India Institute of Medical sciences way back in 1988,² which my group used when confronted with a heavily calcified mitral annulus. We used a piece of autologous pericardium to cover the raw surface of the brittle and sandy remnant after limited débridement of calcium and to prevent calcific emboli. This patient was followed up and seen again in 2001, at which time I reported the favorable result of 13 years free of embolism.³ He was last seen in 2009 and was well. Others⁴ have also reported the successful use of this technique.

Effect of adding postoperative noninvasive ventilation to usual care to prevent pulmonary complications in patients undergoing coronary artery bypass grafting: A randomized controlled trial

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Objective: We compared the efficacy of noninvasive ventilation with bilevel positive airway pressure added to usual care versus usual care alone in patients undergoing coronary artery bypass grafting.

Methods: We performed a 2-group, parallel, randomized controlled trial. The primary outcome was time until fit for discharge. Secondary outcomes were partial pressure of carbon dioxide, forced expiratory volume in 1 second, atelectasis, adverse events, duration of intensive care stay, and actual postoperative stay.

Results: A total of 129 patients were randomly allocated to bilevel positive airway pressure (66) or usual care (63). Three patients allocated to bilevel positive airway pressure withdrew. The median duration of bilevel positive airway pressure was 16 hours (interquartile range, 11-19). The median duration of hospital stay until fit for discharge was 5 days for the bilevel positive airway pressure group (interquartile range, 4-6) and 6 days for the usual care group (interquartile range, 5-7; hazard ratio, 1.68; 95% confidence interval, 1.08-2.31; $P = .019$). There was no significant difference in duration of intensive care, actual postoperative stay, and mean percentage of predicted forced expiratory volume in 1 second on day 3. Mean partial pressure of carbon dioxide was significantly reduced 1 hour after bilevel positive airway pressure application, but there was no overall difference between the groups up to 24 hours. Basal atelectasis occurred in 15 patients (24%) in the usual care group and 2 patients (3%) in the bilevel positive airway pressure group. Overall, 30% of patients in the bilevel positive airway pressure group experienced an adverse event compared with 59% in the usual care group.

Conclusions: Among patients undergoing elective coronary artery bypass grafting, the use of bilevel positive airway pressure at extubation reduced the recovery time. Supported by trained staff, more than 75% of all patients allocated to bilevel positive airway pressure tolerated it for more than 10 hours. (*J Thorac Cardiovasc Surg* 2013;146:912-8)

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The equipment for the BLPAP used in the study was donated by Philips Respironics (Chichester, UK). The study was partly supported by the National Institute for Health Research Bristol Cardiovascular Biomedical Research Unit. Neither Philips Healthcare nor the National Institute for Health Research had any role in the design, conduct, analysis, or reporting of the trial. The trial was sponsored by Imperial College London. The trial was designed, conducted, analyzed, and interpreted independently of the sponsor. The authors had full access to the data and are responsible for submitting the publication. The views expressed are those of the authors and not necessarily those of the NHS, the National Institute of Health Research, or the Department of Health.

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The long-term benefits of coronary artery bypass grafting (CABG) for patients with multivessel coronary heart disease are established but must be weighed against possible short-term iatrogenic harm. CABG interferes with pulmonary function, with basal atelectasis detected in up to 94% of patients in the first 48 postoperative hours.^{1,2} Basal atelectasis is associated with decreased lung compliance, a higher incidence of infection, and prolonged hospital stay.^{2,3}

Postoperative physiotherapy, often incorporating incentive spirometry, is now usual care after CABG. The term “enhanced recovery” comprises various evidence-based components of perioperative care and aims to expedite recovery after surgery. In recent years, the concept of enhanced recovery⁴ has become more accepted. There is interest in the role of noninvasive ventilation (NIV) after cardiac surgery given the benefits of NIV in respiratory failure in other clinical settings, for example, exacerbations

Abbreviations and Acronyms

BLPAP	= bilevel positive airway pressure
BMI	= body mass index
CABG	= coronary artery bypass grafting
CI	= confidence interval
CICU	= cardiac intensive care unit
CPAP	= continuous positive airway pressure
FEV ₁	= forced expiratory volume in 1 second
FFD	= fit for discharge
ICU	= intensive care unit
IQR	= interquartile range
NIPSV	= noninvasive pressure support ventilation
NIV	= noninvasive ventilation
Paco ₂	= partial pressure of carbon dioxide
PPT	= postoperative physical therapy
SD	= standard deviation

of chronic obstructive pulmonary disease,⁵ cardiogenic pulmonary edema, and pneumonia.

There is some evidence that NIV after extubation may be beneficial. For example, preventive use of continuous positive airway pressure (CPAP) or bilevel positive airway pressure (BLPAP) can reduce postoperative lung restriction and venous admixture.⁶ BLPAP developed from CPAP in the 1990s. It matches the respiratory cycle by generating 2 different positive airway pressure levels.⁷ BLPAP can improve gas exchange and oxygenation,⁸⁻¹⁰ even in the absence of hypercapnea, and BLPAP was significantly more effective than CPAP in treating postoperative atelectasis after CABG in a randomized comparison in 150 patients.¹¹ Another recent trial of BLPAP after CABG showed reductions in atelectasis but not in other clinical measurements.¹² However, these trials did not optimize BLPAP. It has been suggested that intermittent postoperative NIV for up to 3 hours should be part of standard clinical practice despite the paucity of evidence.¹³ There is no relevant systematic review of NIV after cardiac surgery. One review of incentive spirometry to prevent postoperative pulmonary complications after CABG identified trials that confounded incentive spirometry with co-interventions, including NIV.¹⁴

We hypothesized that BLPAP after CABG would reduce postoperative stay by improving lung physiology and gas exchange and reducing basal atelectasis. We conducted a randomized controlled trial to compare BLPAP added to usual care with usual care alone in patients undergoing isolated first-time CABG.

MATERIALS AND METHODS

Study Design

The study was a single-center, 2-group, parallel, pragmatic, randomized controlled trial. An independent statistician generated the random

allocation sequence by computer in blocks (ratio 1:1). E.A.J. enrolled and randomized participants, usually immediately before the operation, by looking up the next numbered allocation in the sequence. Participants' random allocations were not concealed during recruitment. The allocation was communicated to cardiac intensive care unit (CICU) staff during the operation so the BLPAP equipment could be prepared if required. The trial was registered as ISRCTN85570732.

This study was approved by the Hammersmith and Queen Charlotte's & Chelsea research ethics committee (reference number 07/Q0406/79) on April 9, 2007. Further approval was obtained from the research and development department of the Imperial College Healthcare NHS Trust. This research complies with the Helsinki Declaration.

Trial Population

The reference population was patients referred to the Hammersmith Hospital for isolated first-time CABG using cardiopulmonary bypass and mild hypothermia (32°C). Patients requiring emergency surgery, with a history of renal impairment (creatinine >200 µmol/L), with hepatic or hematologic disease, with poor left ventricular function, or with chronic infection (eg, tuberculosis, chronic bronchitis) were ineligible.

Intervention and Comparator

Immediately after surgery, all participants were mechanically ventilated using standard method (Servo I and Servo 300; Maquet Getinge Group, Wayne, NJ) ventilators providing a tidal volume of 7 to 10 mL/kg. Immediately after weaning and extubation, participants received BLPAP treatment in addition to usual care (BLPAP group) or usual care alone, as allocated.

We aimed to maximize the duration of BLPAP during the first 24 hours after extubation. BLPAP was administered through an oronasal mask, which was worn continuously except when a participant needed to eat or drink. BLPAP was discontinued after 24 hours or earlier if it was not tolerated by the participant. Inspiratory and expiratory positive airway pressure settings were predetermined and stratified by body mass index (BMI) (ie, -12 cm H₂O and +5 cm H₂O, respectively, if BMI <30, and 17 cm H₂O and 10 cm H₂O if BMI ≥30). BLPAP was performed by CICU staff who received training on 12 occasions before the study started.

Usual care alone included chest physiotherapy, nebulized bronchodilators (2.5 mg salbutamol every 6 hours), coughing exercises, nebulized saline (5 mL every 6 hours), mobilization, and incentive spirometry, overseen by physiotherapists twice per day for the first 2 to 3 days after surgery.

Outcomes

The primary outcome was days until fit for discharge (FFD), measured from the operation date. A judgment of FFD required (1) normal blood pressure, heart rate, temperature, and oxygen saturation on air; (2) bowels open; (3) back to preoperative mobility/reasonable mobility; and (4) satisfactory wound healing. FFD was chosen to reflect the overall speed of recovery. It was considered an important measure for patients, clinicians, and health services that would be unaffected by nonmedical factors. FFD and serious complications (postoperative morbid events considered to lengthen expected hospital stay) were judged by the clinical team, who were not blinded to allocation.

Secondary outcomes included arterial partial pressure of carbon dioxide (Paco₂) and forced expiratory volume in 1 second (FEV₁), atelectasis scored clinically and radiologically, complications, intensive care unit (ICU) length of stay, and time until actual discharge. Paco₂ was measured immediately after extubation and at 1, 12, and 24 hours later. FEV₁ was measured on days 1, 2, and 3 after surgery. However, because of sternotomy wound pain, acceptable measurements (European Respiratory Society criteria) were achieved in only a minority of patients.¹⁵

Atelectasis was assessed on day 1 after surgery by the senior clinical team, not involved in the trial but responsible for the patients' care, as present or absent on the basis of standard clinical and chest x-ray criteria. Radiologists reporting on x-rays were blinded to allocation.

Postoperative complications other than atelectasis also were recorded. Serious complications were itemized. Other complications were recorded as free text and classified subsequently.

Statistical Analysis

The target sample size was 126 participants. For time until FFD, the trial had 90% power at a 5% 2-tailed significance level to detect a standardized difference of 0.6 standard deviations (SDs) or more in log length of stay based on geometric mean times until FFD of 10 and 8.5 days (Online Data Supplement). For Paco_2 and FEV_1 , we estimated (retrospectively) that the trial had 90% power at a 5% 2-tailed significance level to detect a standardized difference of 0.38 SD or more.

Primary analyses were by intention to treat. The analysis plan (Online Data Supplement) specified that time until FFD, CICU stay, and actual postoperative stay should be analyzed as time-to-event data. The analysis of repeated Paco_2 used a mixed regression model to take into account the repeated measurements and to adjust for Paco_2 at the time of extubation. Mean Paco_2 levels also were analyzed 1 hour after starting NIV to test the effectiveness of administration of BLPAP. Only the day 3 FEV_1 measurement was analyzed, using multivariable regression to adjust for the baseline measurement.

Continuously scaled outcomes are described as means $\pm$ SD for each group. Treatment effects (mean differences between groups and 95% confidence intervals [CIs]) were estimated from the regression models. Binary/categorical outcomes are presented as frequencies. Statistical analyses were performed using STATA version 11 (StataCorp LP, College Station, Tex).

RESULTS

Study Population

Of 134 patients considered for the study between February 2008 and February 2011, the trial recruited 129 participants; 66 were randomly allocated to BLPAP, and 63 were randomly allocated to usual care (Figure 1). Three participants withdrew from the BLPAP group because they could not tolerate the mask, giving a total of 126 patients who all received their allocated management. Six participants in the usual care group required ventilatory support and received CPAP. Baseline characteristics, preoperative comorbidities, operation details, and baseline respiratory measurements were balanced (Tables 1 and 2). Two patients died; 1 in the BLPAP group died on day 2, and 1 in the usual care group died on day 5.

Duration of Ventilation

The median intubation time was 6 hours for both groups (interquartile range [IQR], 5-8 and 4-10, respectively). The median time achieved on BLPAP in the BLPAP group was 16 hours (IQR, 11-19; range, 0-22).

Primary Outcome

Time until fit for discharge. Follow-up for the 2 patients who died in hospital was censored at the time of death. Median times until FFD were 6 days (IQR, 5-7) and 5 days (IQR, 4-6) for the control and treatment groups, respectively, generating a hazard ratio of 1.68 (95% CI, 1.17-2.43; $P = .005$). Figure 2, A shows the cumulative proportion of participants judged FFD with increasing

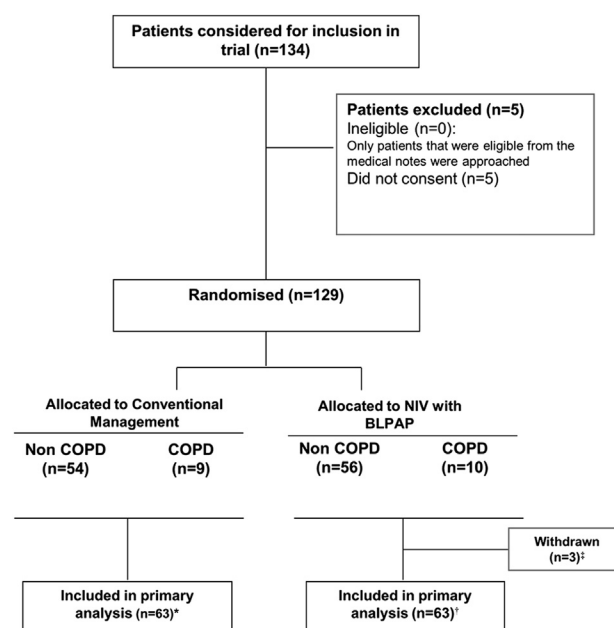


FIGURE 1. Patient flow in the trial. *All received conventional management. For 6 patients requiring noninvasive ventilation support, this included continuous positive air pressure. †All received BLPAP but for varying duration (median time on BLPAP = 16 hours [interquartile range, 11-19]). ‡Three participants asked to be withdrawn because they could not tolerate BLPAP (duration of BLPAP = 0 hours). BLPAP, Bilevel positive airway pressure; NIV, noninvasive ventilation; COPD, chronic obstructive pulmonary disease.

time, with more patients in the BLPAP group than in the usual care group judged FFD from day 3 onward.

Secondary Outcomes

Partial pressure of carbon dioxide. Mean Paco_2 levels at 1, 12, and 24 hours are shown in Table 3. Paco_2 at 1 hour was statistically significantly different (BLPAP vs usual care, -0.41 kPa; 95% CI, -0.73 to -0.09 ; $P = .011$) when adjusted for Paco_2 at the time of extubation, providing evidence that BLPAP was administered efficaciously in the study. The mean difference in Paco_2 over 24 hours (BLPAP vs usual care) was -0.06 kPa (95% CI, -0.25 to 0.13 ; $P = .54$) when adjusted for Paco_2 at the time of extubation.

Forced expiratory volume in 1 second. Mean FEV_1 levels at days 1, 2, and 3 are shown in Table 3. Absolute values were markedly reduced in both groups postoperatively because of wound pain and difficulty performing forced maneuvers. The mean difference in percent predicted FEV_1 at day 3 (BLPAP vs usual care) was 4% (95% CI, -3 to 12 ; $P = .24$) when including preoperative percent predicted FEV_1 as a covariate.

Intensive care unit length of stay. No patient died before discharge from the ICU. Median lengths of ICU stay were 24 hours (IQR, 18-42) and 20 hours (IQR, 17-40) for the usual care and BLPAP groups, respectively. The hazard ratio for BLPAP versus usual care was 1.20 (95% CI, 0.84-1.71; $P = .306$).

TABLE 1. Patients' characteristics and operation details

Patient demographics	BLPAP		Usual care	
	Mean	SD	Mean	SD
Age (y)	65.76	10.77	69.41	8.86
Height (cm)	169.17	7.89	168.03	7.69
Weight (kg)	81.82	18.29	80.39	14.68
BMI (kg/m ²)	28.47	5.28	28.45	4.57
	No. of patients	(%)	No. of patients	(%)
NYHA class				
0	5	7.9	5	8
1	17	27	16	25.8
2	29	46	34	54.8
3	10	15.9	6	9.7
4	2	3.2	1	1.6
Left ventricular function				
Good (>50%)	53	84.1	52	82.5
Moderate (30%-50%)	10	15.9	11	17.5
Comorbidities				
Respiratory				
COPD*	10	15.9	9	14.3
Smoker/ex-smoker	29	46	30	47.6
Other	3	4.8	1	1.6
Cardiovascular				
Hypertension	58	92.1	54	85.7
Hypercholesterolemia	53	84.1	56	88.9
Other	4	6.4	3	4.8
Systemic				
Diabetes mellitus	19	30.2	15	23.8
Other	1	1.6	1	1.6
Other				
Family history	16	15.9	15	14.3
Other	1	1.6	7	11.1
None	0	0	1	1.6
Operation details	Median	IQR	Median	IQR
Bypass (min)	71	63.5-93.5	80	60-92
Crossclamp (min)	38	31-48	46	33-54
Intubation (h)	6	4-10	6	5-8

BLPAP, Bilevel positive airway pressure; SD, standard deviation; BMI, body mass index; NYHA, New York Heart Association; COPD, chronic obstructive pulmonary disease; IQR, interquartile range. *Preoperative COPD was defined as FEV₁% (FEV₁/FVC ratio) <70%.

Time until discharge. By censoring follow-up for the 2 patients who died, median lengths of hospital stay were 6 days (IQR, 5-8) for both groups. The hazard ratio for BLPAP versus usual care was 1.12 (95% CI, 0.78-1.60; *P* = .552).

Postoperative complications. Table 4 describes complications. One or more respiratory complications occurred in 23 patients (37%) and 9 patients (14%) in the usual care and BLPAP groups, respectively. As hypothesized, basal atelectasis, as judged clinically, occurred less often in the BLPAP group (2 patients, 3%) than in the usual care group (15 patients, 24%). Nonrespiratory complications occurred with similar frequency in both groups.

TABLE 2. Baseline respiratory measurements by group

Variable	BLPAP		Usual care	
	Mean	SD	Mean	SD
% predicted FEV ₁ (L)	0.84	0.19	0.88	0.22
FVC	3.26	0.89	3.26	0.93
Paco ₂ (kPA)	5.38	0.70	5.14	0.78
Pao ₂	29.21	13.30	33.17	13.72
Inspiratory mouth pressure*	−84.28	24.08	−80.81	25.82
Expiratory mouth pressure*	136.87	47.65	128.59	35.58

BLPAP, Bilevel positive airway pressure; SD, standard deviation; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity; Paco₂, partial pressure of carbon dioxide; Pao₂, partial pressure of oxygen. *Inspiratory and expiratory mouth pressure measurements were missing for approximately 33% of the patients because they had their lung function tests performed at the referring hospital before their admission to the Hammersmith Hospital. In this case, lung function tests were not repeated when patients were admitted to Hammersmith Hospital.

DISCUSSION

There are 3 main findings of this trial. First, we successfully implemented BLPAP. Among participants allocated to BLPAP, the median duration of BLPAP was 16 hours and 75% managed more than 10 hours. Only 3 participants failed to tolerate BLPAP. Implementation of BLPAP by the CICU staff was accomplished with limited training. This finding is important because continuous NIV for more than a few hours at a time is typically considered unfeasible, and for this reason, it is often implemented intermittently.^{6,13,16,17}

Second, participants allocated to BLPAP were judged, on average, to be FFD 1 day earlier than participants who had usual care alone. Date of FFD relates strictly to postoperative recovery, whereas date of actual discharge is determined by nonmedical factors (eg, social factors). This is important to patients and health service providers because bed availability is a limiting factor on the capacity of cardiac surgery units in the United Kingdom.

Third, overall respiratory complications and, in particular, atelectasis occurred less often in participants allocated to BLPAP. There were no differences between groups in FEV₁ or Paco₂ overall, but Paco₂ did differ between groups 1 hour after extubation, suggesting improved alveolar ventilation due to BLPAP.

Study Strengths and Limitations

We believe that this is the first trial of preventive BLPAP in addition to usual care versus usual care alone (including incentive spirometry) after CABG. There are several strengths of the trial. The primary outcome, time to medically FFD, was chosen a priori as a global measurement of clinical importance. The trial was designed to be able to detect a clinically important difference in the time until FFD. Our findings should be applicable to publicly funded cardiac surgery settings because the trial was carried out in the UK NHS, there were few exclusion criteria, and the intervention was implemented by the CICU staff responsible for day-to-day care, after limited training. We wrote an

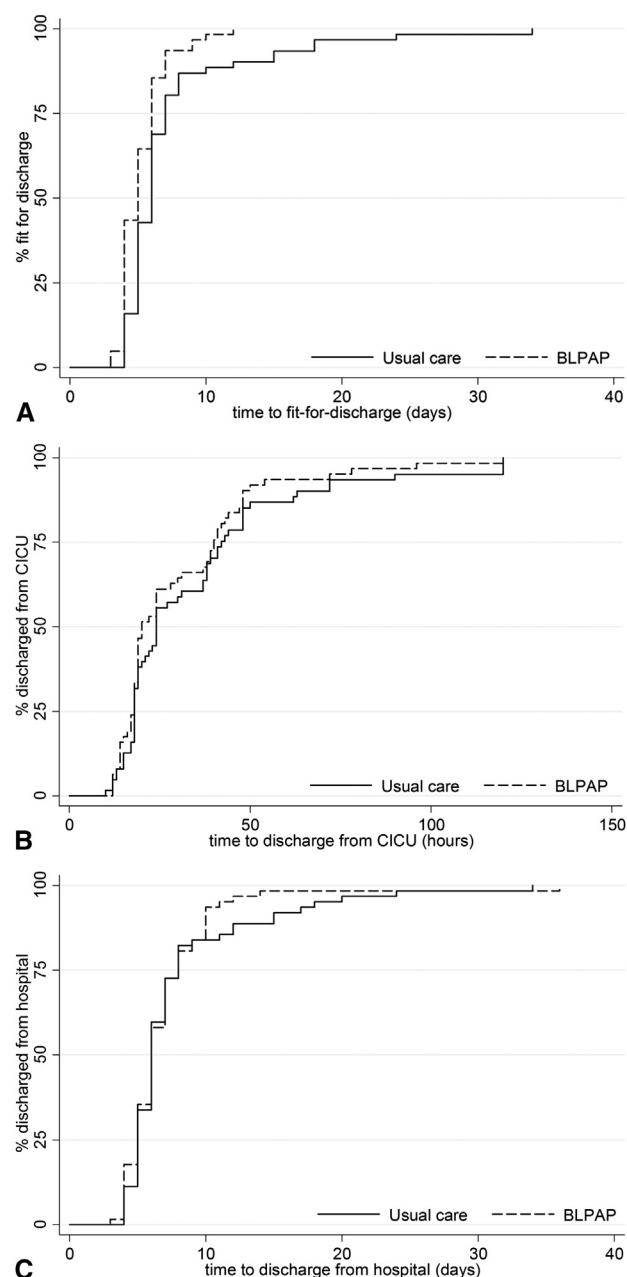


FIGURE 2. A, Proportion of patients fit for discharge. B, Proportion of patients discharged from the CICU. C, Proportion of patients discharged from the hospital. *BLPAP*, Bilevel positive airway pressure; *CICU*, cardiac intensive care unit.

analysis plan in advance of unblinding the data to minimize the risk of within-study selective reporting.^{18,19}

The trial also had limitations. The randomization sequence was not concealed. However, a high percentage of patients were approached and consented, and the analysis of patients' characteristics, as reported in Table 1, shows balance between the 2 groups in patients' demographics and comorbidities. Allocation was not concealed. It was not feasible to blind the participants,

nurses, and research team to allocation; therefore, the primary outcome was at risk of detection bias because the criteria for judging a patient FFD were not completely objective. Ascertainment of complications, including clinical assessment of atelectasis from chest x-rays (reviewed by the cardiothoracic team on ward rounds) and Paco_2 and FEV_1 measurements, was expected to be less at risk of bias. The frequency of atelectasis might have been higher if computed tomography scans had been used to make the diagnosis. Unfortunately, FEV_1 measurement proved extremely difficult. Measurements were low but consistent with postoperative FEV_1 data for patients undergoing CABG reported by other researchers.^{20,21} Ideally, data for the 3 additional patients randomized to the BLPAP group would have been included (more completely reflecting the intention to treat), but data collection was discontinued when the participants asked to be withdrawn.

RESULTS IN THE CONTEXT OF OTHER LITERATURE

The previous Cochrane review of incentive spirometry (date of last search December 2004, content assessed as up to date in April 2007) identified 4 relevant trials.¹⁴ Two trials compared postoperative physical therapy (PPT) with PPT plus incentive spirometry.^{16,17} One trial randomized patients into 3 groups, PPT plus incentive spirometry versus PPT plus NIV using CPAP versus PPT plus NIV with 2 levels of pressure⁶; this latter intervention is similar to BLPAP but not as implemented in our trial. The fourth trial compared PPT plus incentive spirometry versus PPT plus CPAP NIV versus PPT plus NIV with intermittent positive pressure breathing.²² Thus, although all of these trials included 1 group with incentive spirometry, the comparator groups varied substantially. We used the same search strategy as the Cochrane review to identify more recent relevant literature.

In one trial, BLPAP was not started until 4 hours after extubation and was applied for only 1 in every 3 hours.⁶ The authors reported the physiologic and lung function outcomes, ICU stay, and proportion developing atelectasis on day 2 but reported changes between preoperative and day 1 measurements, and between day 1 and day 2 measurements, instead of referring both day 1 and day 2 measurements to the baseline. The authors also combined CPAP and BLPAP groups to test the effect of NIV, reporting a statistically significant difference in the mean change in vital capacity and FEV_1 from day 1 to day 2 between the combined groups and usual care. However, this finding may arise from selective outcome reporting.^{18,19} The difference in the frequency of postoperative atelectasis (30% with usual care vs 15% with CPAP or BLPAP combined, with blinded assessment) was consistent with our own findings.

TABLE 3. Partial pressure of carbon dioxide and percentage of predicted forced expiratory volume in 1 second

Paco ₂ (kPa)*					Percentage of predicted FEV ₁ (L)†				
Time	BLPAP		Usual care		Time	BLPAP		Usual care	
	Mean	SD	Mean	SD		Mean	SD	Mean	SD
Post-extubation	5.19	0.69	5.35	0.84					
1 h	5.10	0.85	5.58	1.09	Day 1	0.30	0.19	0.37	0.23
12 h	4.95	0.72	5.13	0.60	Day 2	0.33	0.18	0.38	0.20
24 h	4.91	0.60	4.95	0.53	Day 3	0.43	0.22	0.40	0.20

Paco₂, Partial pressure of carbon dioxide; FEV₁, forced expiratory volume in 1 second; BLPAP, bilevel positive airway pressure; SD, standard deviation. *Differences in mean Paco₂ between groups were tested at 1 hour (BLPAP vs usual care, −0.41 kPa; 95% CI, −0.73 to −0.09; *P* = .011) and across the first 24 hours (BLPAP vs usual care, 0.06 kPa; 95% CI, −0.25 to 0.13; *P* = .54). †The mean difference in percent predicted FEV₁ between groups at day 3 (BLPAP vs usual care) was 4% (95% CI, −3 to 12; *P* = .24), adjusted for preoperative percent predicted FEV₁.

Another trial compared intermittent noninvasive pressure support ventilation (NIPSV, a form of BLPAP, applied 4 times per day) with CPAP in 150 patients with evidence of postoperative radiologic atelectasis.¹¹ The patients received CPAP or NIPSV (the maximal pressure delivered was 30 cm H₂O, positive end-expiratory pressure was 5 cm H₂O, and the minimal trigger flow was selected) 4 times per day for 30 minutes. Compared with the CPAP group, the NIPSV group had reduced atelectasis according to a radiologic score. However, there were no statistically significant differences in other clinical outcomes (eg, lung function).

TABLE 4. Adverse events

Adverse event	BLPAP		Usual care	
	n	%	n	%
Respiratory				
CPAP	4	6.4	6	9.5
Reintubation	1	1.6	2	3.2
Basal atelectasis	2	3.2	15	23.8
Bibasilar collapse	0	0	1	1.6
Pneumonia	0	0	2	3.2
Pleural effusion	2	3.2	0	0
Cardiovascular				
Atrial fibrillation	10	15.9	7	11.1
Sinus tachycardia	0	0	1	1.6
Ventricular tachycardia	0	0	1	1.6
Cardiac arrest	1	1.6	0	0
Re-sternotomy	1	1.6	3	4.8
Ischemia	0	0	1	1.6
Neurologic				
Confusion	1	1.6	2	3.17
Transient ischemic attack	0	0	1	1.6
Renal				
Renal failure	4	6.4	7	11.1
Other				
Liver infarct	0	0	2	3.2
Diarrhea	1	1.6	0	0
Sternal wound infection	1	1.6	1	1.6
Death	1	1.6	1	1.6
Other	0	0	1	1.6
None	44	69.8	26	41.3

BLPAP, Bilevel positive airway pressure; CPAP, continuous positive airway pressure.

A third trial, with 4 groups (25 patients per group), evaluated the pulmonary effects of intermittent NIV with BLPAP (1 hour in every 6 hours), or recruitment maneuver, compared with usual care, after open surgery.¹² Although this trial was factorial in its design, results were reported for the 4 groups separately. All 3 intervention groups had significantly reduced atelectasis scores (assessed blind to allocation), with the lowest scores in the 2 BLPAP groups. However, no differences were observed in the duration of ICU stay or the length of hospitalization.

INTERPRETATION

Our finding that BLPAP reduces atelectasis is consistent with 3 previous trials in which assessment of atelectasis was carried out blind to treatment allocation. This finding alone is important, because pulmonary complications and atelectasis are associated with longer duration of hospital stay after other types of thoracic surgery and cardiac surgery.²³⁻²⁵ Patients who stay in the hospital longer than expected reduce the availability of beds for patients awaiting elective surgery and decrease the capacity of cardiac surgery centers. The reduction in atelectasis with BLPAP probably explains our finding of a reduction in time until FFD.

Previous trials also found no significant effect of NIV interventions similar to BLPAP for other outcome measures, such as Paco₂ and FEV₁. Our failure to observe differences is unlikely to be explained by a lack of power because these outcomes are measured on continuous scales, and, in the case of Paco₂, repeated measurements were analyzed. We also considered that these measures would be less at risk of detection bias and, inasmuch as they are intermediary measures of improving respiratory function, more likely to show a benefit. However, practical difficulties in this pragmatic setting, with lack of absolute standardization, for example, with respect to the fraction of inspired oxygen during BLPAP, make them less robust.

In reconciling the findings of the primary and secondary outcomes, one faces a choice between an explanation in terms of bias (which could explain the difference in FFD but not in the frequency of atelectasis) and an explanation

in terms of the intrinsic variability of postoperative measures of Paco_2 and FEV_1 . The latter were not robust in the trial setting and are subject to large individual differences arising from variation in patients' pain thresholds, respiratory effort in the early postoperative period, and adherence to instructions for lung function tests. These variations would be expected to contribute noise to measurements of Paco_2 and FEV_1 .

The consistency of the findings regarding atelectasis across 4 trials strengthens our results and provides a mechanism for the clinical benefit we observed with BLPAP. However, the case for implementation of routine BLPAP after extubation in patients who have undergone CABG cannot be considered proven, and we are exploring the feasibility of performing a larger multicenter trial with features to reduce the risk of bias. Given the potential resource implications of BLPAP reducing length of stay, it will be important to include an economic evaluation alongside the trial. It may also be possible to further optimize the application of BLPAP.

CONCLUSIONS

Participants who received NIV with BLPAP in addition to usual care were, on average, judged to be medically FFD (study primary outcome) 1 day earlier than participants who had usual care only. If this reduction in hospital stay could be confirmed in a larger multicenter trial, it would represent an important benefit to patients and health services provision, reducing the cost of an admission for CABG and increasing the capacity of cardiac surgery units. We conclude that BLPAP could be an important part of an enhanced recovery program for patients undergoing CABG.

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