Surfactant phospholipid metabolism and respiratory failure in lung transplantation

A thesis submitted for the degree of Doctor of Philosophy

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Declaration of Originality

This thesis is the result of my own work, and all contributions from others have been acknowledged accordingly. This thesis was written under the supervision of Prof Mark Griffiths, Dr Nandor Marczin, and Dr Anna Reed.

Louit Thakuria

2018 London

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Dedication

It has been the greatest honour and privilege to work with my supervisors. I have learnt so much, you have always looked after me, and I could not have asked for more. I am eternally grateful for all that you have done for me, thank you, Mark, Nandi, and Nonni.

Tony Postle has been a source of great inspiration, and I am especially grateful for all his generous support during this project. His guidance has allowed me to develop laboratory techniques and analytical approaches that I never believed I would be able to comprehend.

Special thanks go to Rosalba Romano, completion of this thesis would not have been possible without her many hours of work and her constant support; it has been a privilege to work with you. I would like to thank the numerous members of the multidisciplinary clinical transplantation team and transplant coordinators who have supported me throughout this study. This study was largely conducted on the Intensive Care Unit at Harefield Hospital, and so I am especially grateful for all the support from the critical care staff.

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The life-saving contributions made by organ donors and their families continues to help so many patients, and their generous contributions to the transplant research programme are equally appreciated. It has been a great privilege to work with the lung transplant recipients who participated in this study and made this project possible, thank you.

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Finally, to my wife for your years of support - thanks HB.

Abstract

Primary graft dysfunction (PGD) after lung transplantation is a multi-factorial syndrome of postoperative lung injury that is similar to the acute respiratory distress syndrome. PGD is a major risk factor for chronic lung allograft dysfunction and perioperative death[1-3]. Pulmonary surfactant dysfunction is frequently seen in PGD and contributes to respiratory failure[4-6]. Surfactant is primarily comprised of lipids, of which phosphatidylcholine (PC) species predominate. Dipalmitoylphosphatidylcholine (DPPC) is the most surface-active species that normally represents over half of all airway PC content[7]. Combining electrospray ionisation tandem mass spectrometry with deuterium labelling of Kennedy's PC synthetic pathway can assess surfactant phospholipid flux, with a molecular specificity that differentiates between lipids with unique functions[8]. This technique was used to quantify perioperative phospholipid metabolic disruption in lung transplantation. The same technique was used in an ex vivo human lung model to isolate the pulmonary contribution to systemic phospholipid metabolism, and evaluate Kennedy pathway integrity during ex vivo lung perfusion (EVLP).

Airway DPPC content was severely reduced after transplantation, and was associated with impaired oxygenation. Variations in Kennedy pathway activity were associated with impaired graft function for months after transplantation. Perioperative flux of phospholipids, like DPPC and polyunsaturated PC species, were strong predictors of poor postoperative lung function. Deuterium labelled ex vivo experiments confirmed continued Kennedy pathway dependent surfactant phospholipid synthesis during EVLP, and could represent a protective mechanism against PGD in clinical EVLP[9]. Ex vivo lungs secreted a different phospholipid profile into the perfusate that is enriched with polyunsaturated PC when compared to the airways, providing compelling evidence of the previously hypothesized basolateral phospholipid secretion by the alveolar epithelium[10,11]. These polyunsaturated PC species are precursors of numerous inflammatory mediators, and could contribute to PGD[12-14]. Continued investigation of phospholipid flux through the Kennedy pathway could identify novel biomarkers and therapeutic targets in lung transplantation.

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Contributions from others

Clinical Study

The Trust statistician Winston Banya reviewed the study design before submission for ethical approval. Patient recruitment, data entry, sample collection and initial processing were performed by the author, Dr Rosalba Romano, and other members of the multidisciplinary transplant research team. Jo Hardy also assisted with patient recruitment. Dr Paras Dalal was our blinded radiologist who reviewed every chest radiograph, to assist with the diagnosis of primary graft dysfunction.

Ex vivo modelling

All organs were obtained by the Harefield retrieval team with the primary intention of clinical transplantation. It would not have been possible to collect the data from these organs without the substantial contributions from the multidisciplinary clinical retrieval teams. Tim Pitt and the Trust Perfusion team provided both equipment and technical expertise throughout this project. Organ support, data entry and initial sample collection was divided between the author and Dr Romano.

Mass Spectrometry

All sample processing, lipid extraction, mass spectrometry and data analyses was performed exclusively by the author. Protocols were devised by Prof Postle and his team at the Southampton Centre for Biomedical Research, with special thanks to Dr Vikki Goss, Paul Townsend, Madhuri Panchal, and David Stockley for their support in the laboratory. Departmental software for the extraction and processing of mass spectra was written by Grielof Koster, who also provided considerable technical expertise throughout this project. Topological data analyses of mass spectra were performed using the Ayasdi Workbench Platform under an academic licence granted by Ayasdi, and supported by Devi Ramanan. All analyses were performed by the author, but Dr Joost Brandsma provided significant support with application of Topological Data Analyses in the analysis of mass spectra.

Other Assays

Tissue staining and image acquisition was performed by Dr P Sarathchandra. The automated image processing macro was designed in collaboration with Steve Rothery. Image processing and analysis was performed exclusively by the author. Assays for NO metabolites and free thiols were performed by Prof Feelisch and Magda Minnion; all subsequent data analysis was performed by the author.

1 Introduction

The first record of a successful lung transplant in animals was performed in 1946 by Vladimir Demikhov[15]. The first 'survivor' of human lung transplantation (single lung) was later recorded in 1963 by James Hardy, and the patient survived for 18 days after surgery[16]. However, it was not until the advent of newer immunosuppressive agents, such as cyclosporine, that meaningful long-term survival was achieved following lung transplantation in the 1980s[17]. The first successful heart-lung transplant was performed by Shumway, Wallwork, and Reitz for a patient with pulmonary hypertension in Stanford in 1981[18]; before the Toronto Lung Transplant Group went on to develop the techniques for single and double lung transplantation in the late 1980's[19,20].

Lung transplantation can prolong life and dramatically improve quality of life in carefully selected patients with advanced respiratory disease[21]. International registry data show a 5 year survival of 54% following double lung transplantation, and the biggest cause of death after lung transplantation is the development of chronic lung allograft dysfunction (CLAD)[22]. Primary graft dysfunction (PGD) is a multi-factorial syndrome of postoperative lung injury that is a major risk factor for the development of CLAD and perioperative death[1].

Ischemia-reperfusion injury (IRI) is a major contributor to the development of PGD, which shares many clinical and pathological similarities with the Acute Respiratory Distress Syndrome (ARDS)[2,3]. As in ARDS, surfactant dysfunction is frequently seen in PGD after lung transplantation[4,5,23-25]. Pulmonary surfactant is primarily comprised of lipids, of which phosphatidylcholine (PC) species predominate. Dipalmitoylphosphatidylcholine (DPPC or PC16:0/16:0) is the most surface-active species that normally represents over half of all airway PC content[7]. This thesis will explore the underlying mechanisms behind the development of perioperative lung injury following lung transplantation, and characterise the perioperative disruption in pulmonary phospholipid biology in both transplant recipients and in a complimentary human ex vivo platform.

1.1 Clinical Problem

1.1.1 Primary Graft Dysfunction (PGD)

The diagnostic criteria for PGD are based on the American-European Consensus Conference (AECC) definition of ARDS - Table 1-1[2,26,27]. PGD by definition occurs in the first 72 hours following lung transplantation affecting 10–25% of lung transplants[28].

Grade	Chest radiograph pulmonary oedema	PaO_2/FiO_2 ratio (mmHg)
PGD Grade 0	No	Any
PGD Grade 1	Yes	>300
PGD Grade 2	Yes	200 to 300
PGD Grade 3	Yes	<200

Table 1-1: The diagnostic criteria for Primary Graft Dysfunction (PGD)
Exclusion criteria for the diagnosis of PGD include; hyper-acute rejection, venous anastomotic obstruction, cardiogenic pulmonary oedema, and pneumonia

PGD is associated with a substantial decrease in both early and late survival, with an all-cause mortality rate of 42.1% at 30 days following surgery[1]. Not only is PGD immediately life-threatening to lung transplant recipients in the early perioperative period[1], PGD also adversely affects medium to long-term lung function and outcomes[29]. Given the clear association between PGD and long-term outcomes[29], every effort must be made to minimise postoperative lung injury in order to maximise the longevity of the graft and the recipient.

IRI is a complex process mediated by oxidative stress, electrolyte disturbance, inflammation and subsequent cell death[30]. Cell injury after transplantation results in the release of damage-associated molecular patterns (DAMPs) that activates the innate immune response in the recipient, further contributing to the development of both acute and chronic graft dysfunction[31-33]. Whilst IRI is a major contributor, there are many other factors which also contribute to the pathogenesis of PGD that include donor brain death, aspiration, ventilator associated lung injury (VALI), inflammation and immune responses[2,27,33-35]. Furthermore, numerous risk factors for PGD have been identified that are seemingly unrelated to IRI, such as blood transfusion requirements, recipient diagnoses, recipient obesity, donor age, donor smoking & alcohol intake, and genetic polymorphisms[36].

1.1.1.1 Similarities with ARDS

PGD after lung transplantation shares many clinical and pathological similarities with ARDS seen in the general ICU population[2,37,38]. Both conditions are characterised by the acute development of poor pulmonary compliance, poor oxygenation, diffuse infiltrates on chest radiography, and a histological appearance termed diffuse alveolar damage (DAD)[2,39,40]. Like PGD, ARDS is also associated with long-term impairment of lung function[41]. ARDS and PGD also share multiple risk factors; which include smoking, obesity, fluid administration, transfusion of blood products and possibly the use of cardiopulmonary bypass[42-49]

ARDS is a common cause of acute respiratory failure that afflicts 20-85 per 100,000 in the general population and carries a mortality of 30-60%[50]. ARDS almost always necessitates mechanical ventilation that inevitably exacerbates lung injury. Despite half a century of research, protective ventilation strategies that mitigate ventilator associated lung injury (VALI) are the only interventions to confer survival benefit[51]. Biochemical, radiographic and epidemiological evidence suggest that even "protective" ventilation will induce further lung injury[52,53]. ARDS causes surfactant depletion which leaves the lung vulnerable to the injurious effects of positive pressure ventilation by exacerbating the development of atelectasis and oedema in the lung[54]. The phospholipid composition of pulmonary surfactant is also altered in patients with both established ARDS and those who are at risk of developing ARDS, with a relative increase in the amount of lysoPC species in relation to DPPC. Whilst previous studies have attempted to quantify the altered phospholipid composition that could contribute to surfactant dysfunction in ARDS[55], the changes in surfactant phospholipid composition in PGD are largely unknown.

Given the many similarities between ARDS and PGD, the recommendations on the management of PGD and protective ventilation after lung transplantation have been extrapolated from studies on

ARDS[2,56]. Therefore, elucidating the pathogenesis of PGD could be extrapolated back into ARDS in the general intensive care population.

Human in vivo models of ARDS have traditionally looked at other patient cohorts that will predictably develop acute lung injury through the effects of intraoperative one-lung ventilation and cardiopulmonary bypass[57]. Given the predictable development of lung injury after transplantation and the many clinical and pathological similarities between PGD and ARDS, lung transplantation could become a valuable human model for research into both ARDS and VALI.

1.1.1.2 Ventilator-associated Lung-Injury (VALI)

VALI is both a risk factor for and a major determinant of outcomes in ARDS, and is the result of volutrauma, barotrauma, atelectrauma, and subsequent biotrauma that occur as a consequence of positive pressure ventilation[51,54,58]. Volutrauma and barotrauma refer to the physical injury sustained by the lungs when exposed to excessive tidal volumes and positive airway pressures; whereas atelectotrauma results from cyclical opening and closing of recruitable lung units. The subsequent inflammatory response that follows is often referred to as biotrauma[51,54].

Mechanical ventilation can add a further insult to the already injured lung and is a key determinant of outcome in patients with ARDS[51,59,60]. Despite the wealth of research on VALI that has been conducted in the general ICU population, data on the topic are extremely limited in the lung transplant cohort[56]. Limited data suggest that injurious ventilation in the donor is a risk factor for the subsequent development of PGD[61-63], but the effects of VALI in the recipient are largely unknown[56].

Lung transplant recipients require invasive ventilation postoperatively, sometimes for prolonged periods. VALI develops in lungs that have already suffered some other form of insult[54]. Hence, after transplantation, IRI and VALI may combine with other risk factors like blood transfusion to cause ARDS[64-66]. Pulmonary surfactant dysfunction will make the lungs particularly susceptible to developing severe VALI, by promoting alveolar collapse and exposing the lung to increasing shear

stress associated with atelectotrauma[67]. Not only does surfactant depletion increase the risk of VALI, injurious ventilation strategies may also cause further damage to the pulmonary surfactant system[68,69]. Experimental models suggest that both endogenous surfactant[70] and the exogenous supplementation of surfactant may protect the lung against the deleterious effects of mechanical ventilation[71-73]. However, the beneficial effects of supplemental surfactant in animal models and neonatal Respiratory Distress Syndrome (RDS) have not translated into the prevention of VALI in adult patients with ARDS; this is discussed in more detail in section 1.2.10.1[74].

1.1.1.3 Management of PGD

There is a paucity of clinical data to support specific treatments or management strategies in patients with PGD. Guidelines on the topic have been mainly extrapolated from studies in ARDS in patients who have not received a lung transplant[2,75]. Protective ventilation (in both the donor and recipient) to prevent VALI is the cornerstone of PGD management[62,76,77]. It is generally accepted that extracorporeal membrane oxygenation (ECMO) may be required as a rescue strategy for those with severe postoperative lung injury (PGD grade 3)[76]. United Network for Organ Sharing (UNOS) registry data and several case series support the use of ECMO within the first few days of developing PGD, although the 6-month survival rate for this subset of patients is reduced to approximately 62%[78]. Prophylactic postoperative ECMO to prevent the development of PGD could be beneficial in selected high-risk patients with severe pulmonary artery hypertension by allowing controlled reperfusion of the lung, and possibly by facilitating protective ventilation; the role of prophylactic ECMO use to prevent PGD through protective ventilation in the wider transplant cohort requires further study[79,80]. Inhaled Nitric Oxide (NO) or prostacyclin may have a role as salvage therapy for severe PGD, refractory hypoxaemia and pulmonary artery hypertension, but data are very limited[75].

Different preservation techniques during organ retrieval can influence the development of PGD in the recipient; combined antegrade and retrograde flush with a solution like Perfadex in organs that have an ischemic time under 8 hours decreased the effects of IRI and protected the lungs from PGD[75].

Ex vivo lung perfusion (EVLP) has recently emerged as clinical tool for the assessment and reconditioning of human lungs for clinical transplantation[81]. EVLP allows for prolonged organ preservation times[82], with successful transplantation after 4-6 hours of ex vivo support in one clinical case series[83], and experimental models demonstrating organ viability after 12 hours of ex vivo support[84]. The Organ Care System (OCS) is a mobile method of perfusing the lungs outside the body, that can also facilitate the transfer and evaluation of organs before clinical transplantation[9]. EVLP has the potential to expand the donor pool of organs by allowing the detailed ex vivo assessment of 'marginal' or 'high-risk organ offers that may have been otherwise rejected, particularly in cases of donation after cardiac death (DCD)[83,85]. The use of EVLP to recondition marginal organs and prevent the incidence of PGD after implantation by reducing cold ischemia (or other undefined mechanisms) is an exciting prospect, but this role is uncertain and remains the subject of ongoing clinical trials[86]. Beyond the potential expansion of the donor organ pool, EVLP presents an opportunity to deliver targeted therapies to prevent the development of PGD, potentially even therapies that are too toxic to be delivered in vivo[87,88]. Experimental models of EVLP with injured lungs have shown that the combination of lung lavage with exogenous surfactant replacement can substantially improve postoperative graft performance[89]. The clinical role of exogenous surfactant replacement therapy remains a promising tool for the treatment of PGD that warrants continued research[75,76,90].

1.2 Pulmonary surfactant biology

1.2.1 The alveolar epithelium

Alveolar type 2 (AT2) cells synthesise pulmonary surfactant which reduces alveolar surface tension. Surfactant is a highly regulated lipid:protein complex, of which phosphatidylcholine (PC) is the most surface-active constituent. By reducing the surface tension of the alveolar lining fluid, surfactant helps to prevent the development of airway and alveolar collapse and pulmonary oedema[91,92].

Alveolar type 1 cells (AT1) are vulnerable to injury; Alveolar type 2 cells (AT2) are more resistant to injury and will dedifferentiate, spread, migrate and proliferate to recover the denuded basement membrane before undergoing apoptosis and differentiation back into type 1 and 2 cell phenotypes[93]. In clinical trials, the recovery of epithelial cell dependent alveolar fluid clearance correlated with patient outcomes[94]. Whilst the AT2 cells can survive prolonged periods of hypoxia, reoxygenation after periods of hypoxia has been associated with significant cell injury and death in vitro[95]. In neonatal RDS AT2 cells fail to differentiate adequately before a gestational age of 34 weeks resulting in post-partum surfactant deficiency, severe respiratory failure and sometimes death[96].

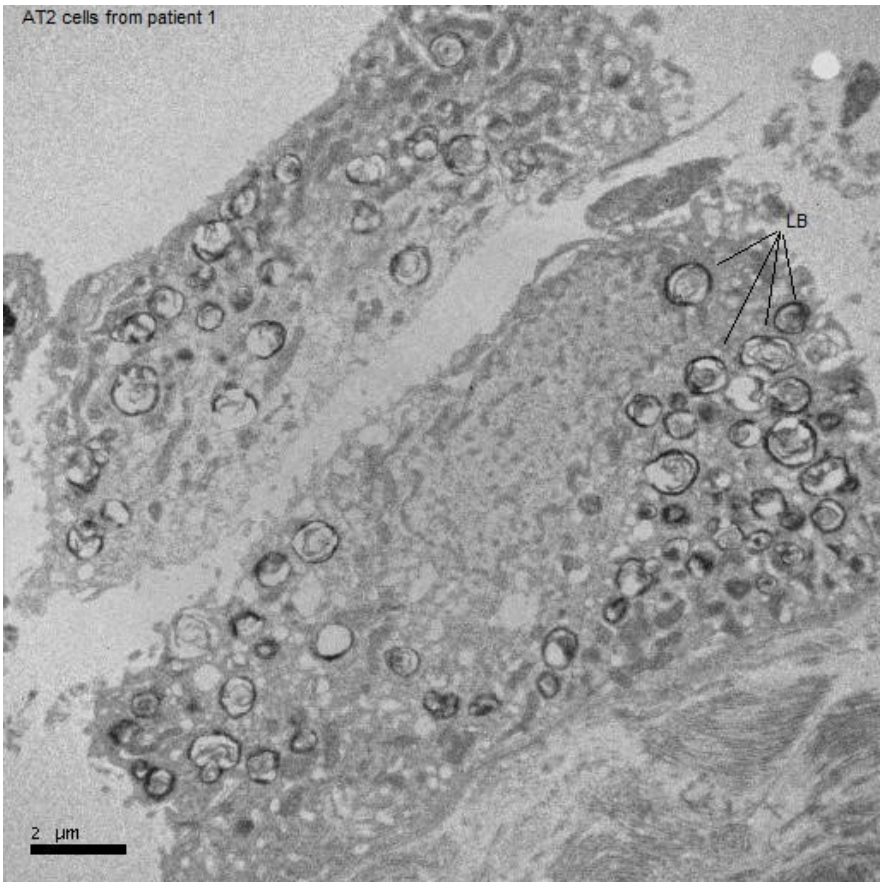


Figure 1-1: Electron microscopy of AT2 cell of a lung transplant recipients
Biopsy taken from patient 1. LB- Lamellar bodies. Electron micrograph produced by Dr Rosalba Romano

Surfactant is initially synthesised in the endoplasmic reticulum, before undergoing post-translational alterations in the Golgi apparatus. Surfactant is then transferred within the cell in multivesicular bodies, prior to storage in the lamellar bodies (Figure 1-2). Fusion of the lamellar bodies with the cellular membrane results in exocytosis of surfactant from the AT2 cells[97]. Cyclical stretch of the alveolar epithelium through ventilation has an important role in up-regulating surfactant secretion, possibly mediated through the mechanosensitive influx of Ca^{2+}[98,99]. Once secreted from AT2 cells, phospholipids arrange into the form of tubular myelin in the alveolar space. Surfactant is then recycled, taken up by alveolar macrophages and by the AT2 cells into multivesicular bodies - Figure 1-2[98,100,101]. Recycling of surfactant from the alveolar space is regulated by the orphan G-protein coupled receptor GPR116 in the AT2[102], and regulated by granulocyte colony stimulating factor (GM-CSF) in alveolar macrophages[103,104].

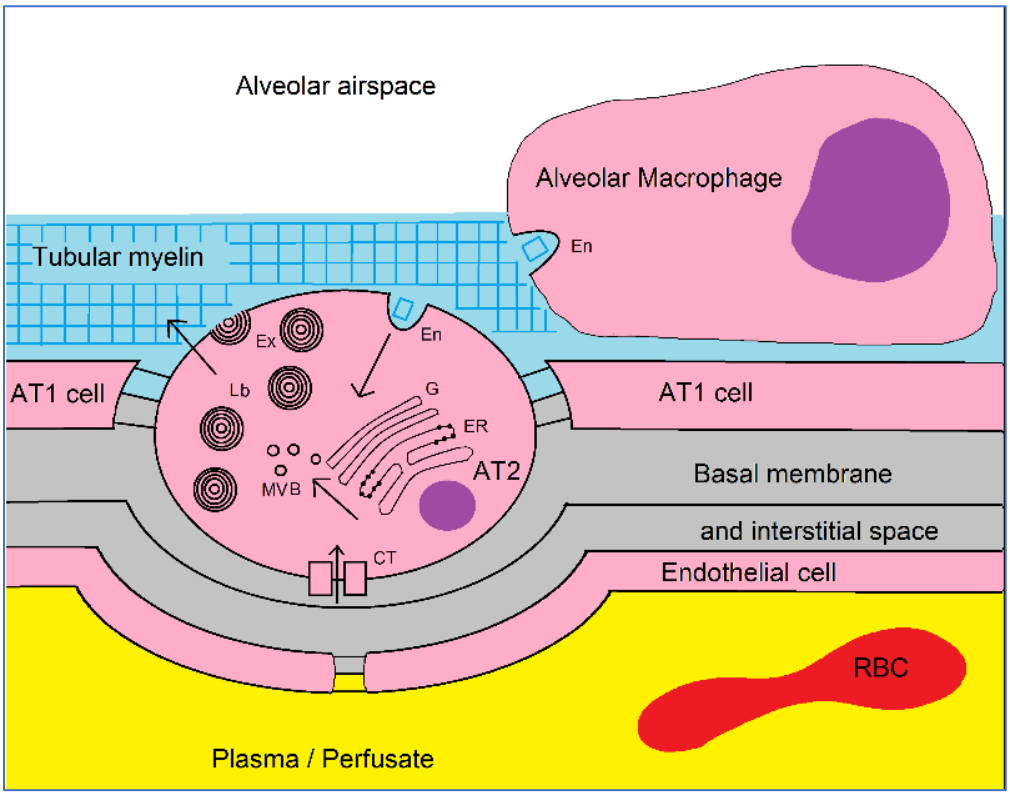


Figure 1-2: Simplified schematic of surfactant metabolism at the alveolar epithelium CT choline transporters. AT2 alveolar type 2 cell. LB lamellar bodies. MVB Microvesicular bodies, ER Endoplasmic reticulum, G Golgi. Ex exocytosis, En Endocytosis. AT1 Alveolar type 1 cell. DPPC Dipalmitoylphosphatidylcholine. RBC Red blood cell

The uptake of choline by the alveolar epithelium is mediated by choline transporter-like protein 1 (CTL1)[105]. It is likely that sodium-coupled transporters are responsible for apical transport of phospholipids from the alveolar epithelium in to the alveolar space, whilst sodium-independent transporters are located in the basolateral surface of the pulmonary epithelium to facilitate the uptake of choline from the plasma by AT2[106]. The ATP-binding cassette (ABC) family of transporters move multiple substrates across the cellular membrane[107]. In vitro models suggest that ABCA3 is an important mediator of ATP dependant uptake of choline phospholipids into the epithelium, the subsequent formation of the lamellar bodies[108], and for surfactant protein processing[109]. ABCA1 is another lipid transporter that also contributes to lipid homeostasis in the lung, and is also expressed on the alveolar epithelium[110].

1.2.2 Surfactant composition

Four surfactant proteins (SP) have been identified, termed SP-A, SP-B, SP-C, and SP-D. Surfactant proteins have multiple roles in surfactant metabolism, as well as antimicrobial and anti-inflammatory properties[111]. Surfactant proteins become incorporated into the network of tubular myelin in the alveolar lining fluid, interacting with the lipid components, contributing to surface tension reduction, facilitating adsorption at the air-liquid interface and spreading of the lipid layer across the air space[112,113]. Surfactant proteins are essential for surfactant function, and deficiency can lead to fatal respiratory failure[114-116]. However, this project will primarily focus on the phospholipid component of surfactant.

1.2.3 Phospholipid nomenclature

Phospholipids classes are defined by their 'head group' (e.g. PC – phosphatidylcholine, Table 1-2), and by any attached fatty acid chains (usually two fatty acid chains are present). Fatty acids are classified by the number of carbon atoms and the number of double bonds that are present[117]. DPPC contains a phosphatidylcholine head attached to two palmitic acid moieties, with each palmitic acid moiety containing 16 carbon bonds without any double bonds[118]. Using this convention, DPPC could also be represented as follows:

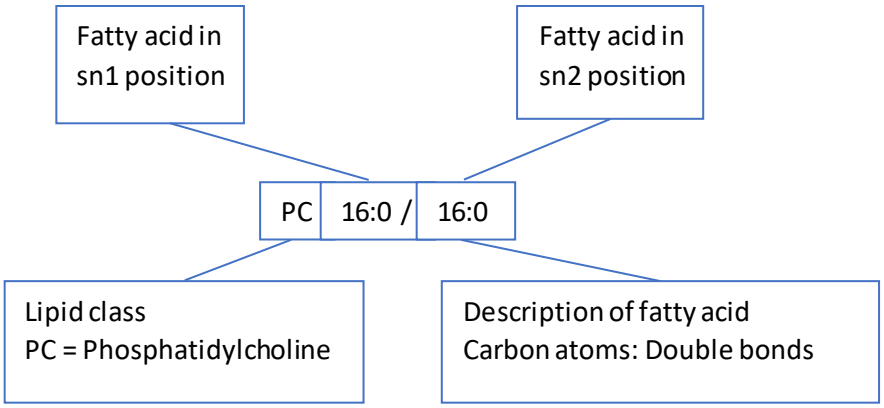


Figure 1-3: Shorthand notation for phospholipids
For example, DPPC is also known as PC16:0/16:0. Sn – stereospecific number, PC - Phosphatidylcholine

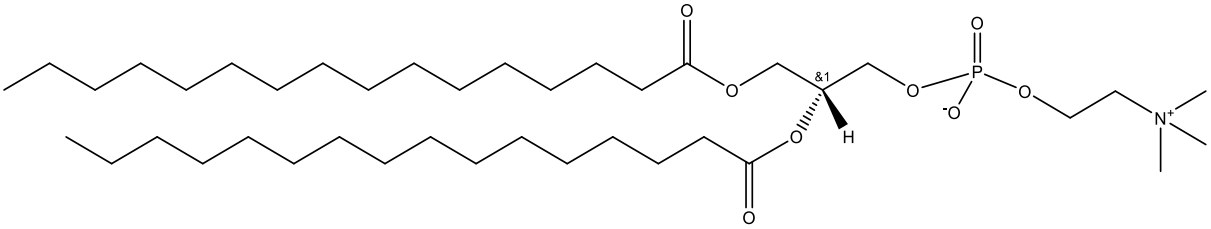


Figure 1-4: Structure of DPPC.
DPPC has a mass of 733.5622 and it consists of two palmitate chains attached to a PC head. Each palmitate chain has 16 carbon atoms with no double bonds – hence the alternative name of PC16:0/16:0

1.2.4 Phospholipid classification

85-90% of surfactant lipids contain a phosphate group (phospholipids), about 5-10% of lipids contain a carbohydrate group (glycolipids), and 5-10% of lipids have no charged/polar group (neutral lipids). Dipalmitoyl phosphatidylcholine (DPPC or PC16:0/16:0[118]) is the most abundant phospholipid in pulmonary surfactant and has the greatest effect on surface tension[101]. The inherent amphiphilic

nature combined with its two non-branching saturated fatty acid chains allows DPPC to form highly compressible monolayers, which probably accounts for its ability to lower surface tension in alveolar surfactant[113,119,120]. Typical surfactant composition is summarised in Table 1-2[74,97,121,122].

	Percentage of total weight
Lipid	85-90%
Protein	10-15%
	Percentage of lipid content
Phospholipids	85-90%
Neutral lipids	5%
Glycolipids	5-10%
	Percentage of phospholipid content
PC	68%
-DPPC or PC16:0/16:0	-Up to 60% of PC content
PG	10%
PE	5%
PI	4%
PS	2%
SM	4%

Table 1-2: Composition of surfactant.
Abbreviations: Dipalmitoyl phosphatidylcholine (DPPC), phosphatidylcholine (PC), phosphatidylgycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SM),

Whilst only present in relatively small quantities in man, other phospholipid classes like PG may indirectly reduce surface tension by facilitating alveolar adsorption of surfactant (i.e. spreading and adherence of the surfactant layer along the alveolar wall)[119]. Neutral lipid species (such as cholesterol) may also increase the fluidity of the surfactant layer in the alveoli, and help re-spreading of the film during normal ventilation[119,123].

1.2.5 PC synthesis

PC is synthesised by three pathways:

1. Kennedy's CDP-choline pathway (de novo PC synthesis)–section 1.2.5.1
2. Land's acyl remodelling pathway (conversion of PC species) –section 1.2.5.2
3. PE Methylation pathway (exclusive to the liver) –section 1.2.5.3

DPPC is the most abundant and surface active of all the PC species, and its deficiency has been demonstrated in other forms of acute lung injury; therefore, DPPC will be the phospholipid of primary interest in this thesis[25]. In the human lungs, most de novo PC synthesis is in the form of PC16:0/18:1 and PC16:0/18:2, with acyl remodelling of these phospholipids into DPPC through the action of phospholipase A_2 (PLA_2) and lyso-PC acyltransferase-1 (LPCAT1)[101,124,125]. Approximately 55% of all DPPC is synthesised through PC remodelling, and the remaining 44% is from de novo synthesis[122]. These pathways will be discussed in greater detail.

1.2.5.1 The Kennedy Pathway

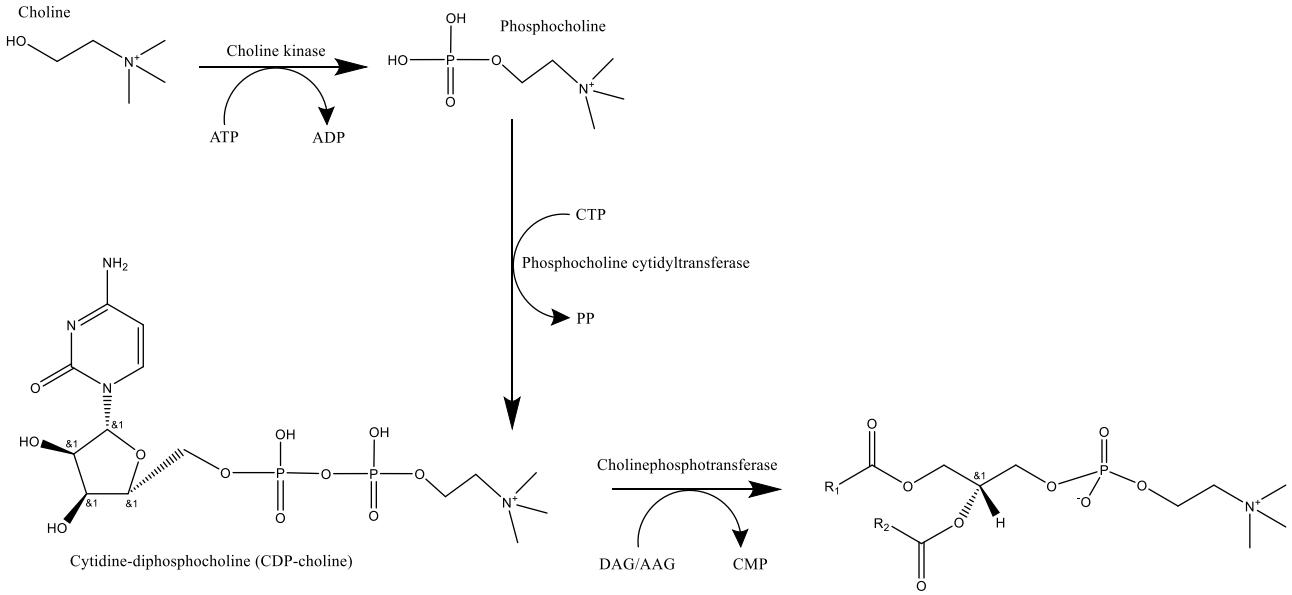


Figure 1-5: The Kennedy Pathway.
ADP – Adenosine diphosphate, ATP – Adenosine triphosphate, CMP – Cytidine monophosphate, DAG – Diacylglycerol, AAG – Alkyl-acyl glycerol, PP Pyrophosphate. R groups typically consist of a fatty acid acyl group of varying chain length and saturation

Eugene Kennedy was an American biochemist who was the first to describe the major pathway of PC synthesis in 1956[126]. The Kennedy pathway takes place in the endoplasmic reticulum and Golgi apparatus of virtually every mammalian cell, and is responsible for the synthesis of most phospholipids[126,127]. Choline is converted into cytidine-diphosphocholine (CDP-choline through the sequential action of choline kinase and phosphocholine cytidyltransferase, before combining with

diacylglycerol (DAG) in a reaction mediated by cholinephosphotransferase to produce a wide range of phosphatidylcholine lipid species[122,126]. Whilst some DPPC is produced de novo by the alveolar epithelium through the Kennedy pathway, most DPPC is remodelled from other phospholipids via Land's cycle[122,128].

1.2.5.2 Land's acyl remodelling cycle

Bill Lands is a nutritional biochemist originally from the University of Michigan, who first described the pathway responsible for the remodelling of phospholipids into different species through a process of deacylation (via phospholipase A_2 (PLA_2) enzymes) and reacylation (via lysophosphatidylcholine acyltransferase) - Figure 1-6[129].

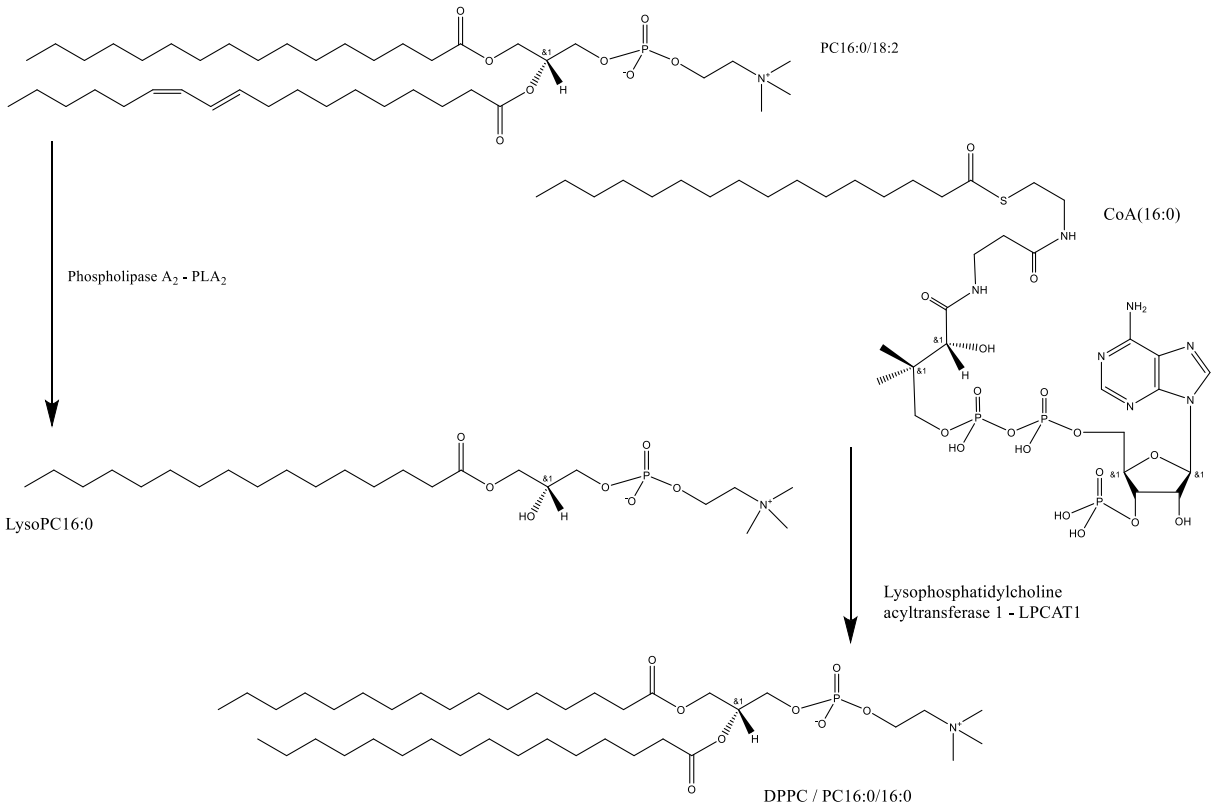


Figure 1-6: Land's Cycle
Also known as the Acyl remodelling pathway. In this example PC16:0/18:2 is remodelled into PC16:0/16:0 after receiving a palmitoyl moiety donated by CoA(16:0) through the action of lysophosphatidylcholine acyltransferase

Palmitate is readily remodelled in the lungs for transfer to phosphatidylcholine[130], and so Land's acyl remodelling acyl remodelling pathway is the source of most DPPC in the airway[122,128].

1.2.5.3 PEMTpathway

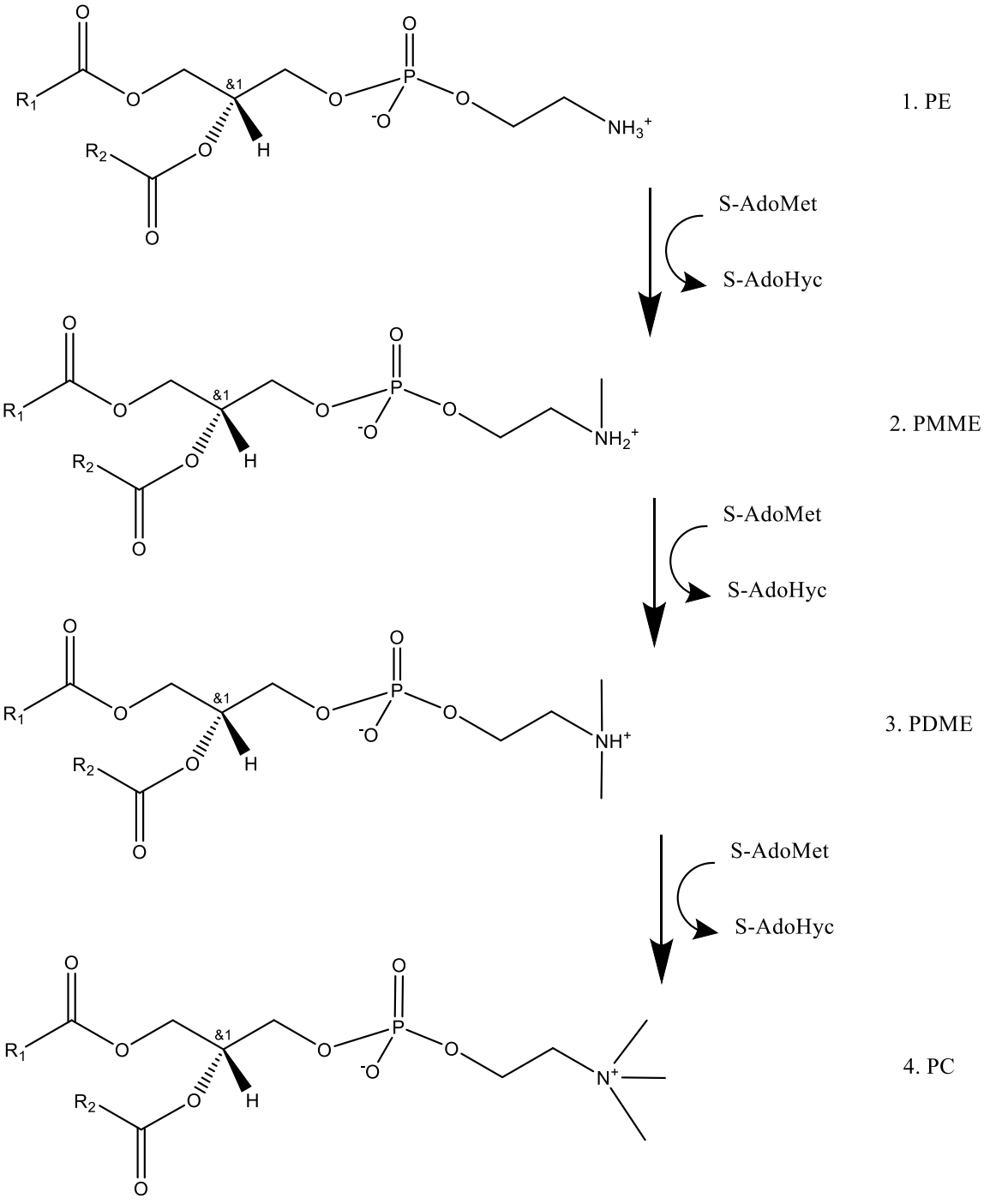


Figure 1-7: PEMT Pathway

Sequential of Phosphatidylethanolamine (1. PE) by Phosphatidylethanolamine N methyltransferase (PEMT) into phosphatidylmonomethylethanolamine (2. PMME), phosphatidyldimethylethanolamine (3. PDME), and then into phosphatidylcholine (4. PC). Methyl groups are donated from the conversion of S-adenosylmethionine (S AdoMet) into S-adenosylhomocysteine(S-AdoHcy)

Thirty percent of PC species are synthesised in the liver through the sequential methylation of phosphatidylethanolamine (PE), mediated by the action of Phosphatidylethanolamine N-methyltransferase (PEMT) as shown in Figure 1-7[131,132]. This PEMT pathway is unique to the hepatocyte, and typically produces a more diverse range of PC species that contain more unsaturated long-chain fatty acids (like arachidonic acid)[133].

1.2.6 Surfactant abnormalities in lung injury

Surfactant dysfunction was quickly established as an important contributor in the pathogenesis of ARDS, soon after the first descriptions of the syndrome were published[23,24,39]. The surfactant abnormality associated with ARDS has several pathological characteristics. The dysfunctional surfactant found in patients with ARDS produces an alveolar film with reduced surface elasticity and increased surface tension[24,134], which increases the tendency for unstable alveolar units to collapse on expiration[135]. This functional impairment is probably the result of changes in both the functional composition of surfactant and in inhibitory factors.

An extensive analysis of pulmonary surfactant sampled from ARDS patients at autopsy demonstrated a relative reduction in the DPPC content and PG class phospholipids with a greater predominance of sphingomyelin and PS species when compared to healthy controls[136]. The total amount of phospholipids retrieved from bronchoalveolar lavage of patients with ARDS was also considerably reduced when compared to healthy controls[137].

1.2.7 Mechanism of surfactant dysfunction in lung injury

All aspects of surfactant metabolism from synthesis to degradation are vulnerable in the acutely injured lung. There is an influx of inflammatory cells into the airspace of injured lungs that release enzymes that could impair surfactant function[68,69]. For example, neutrophil elastases, other proteolytic enzymes and secretory PLA_2 are increased and could degrade surfactant proteins and

phospholipids[138,139]. Complementary in vitro studies suggest that PLA_2 mediated surfactant dysfunction is driven by two mechanisms, the loss of surfactant phospholipids through hydrolysis and the generation of toxic lysophospholipids[140-142]. Lysophospholipids are potent inhibitors of surfactant function, as they are capable of penetrating the phospholipid film and disrupt the normal fluid properties of surfactant[143].

Neutral lipid species (such as cholesterol and triglycerides) have an important physiological role in preserving normal surfactant function (section 1.2.2)[119,123]. Elevated neutral lipids as reported in the airways of patients with ARDS[144], inhibited surfactant function and raised surface tension in experimental in vitro models[145].

Once surfactant dysfunction has been established, increasing surface tension will cause alveolar collapse and result in the formation of a pressure gradient that promotes the continued influx of plasma and the development of pulmonary oedema[146]. The influx of plasma proteins such as albumin, fibrinogen, and haemoglobin[147] can further inhibit surfactant function by preventing adsorption of phospholipids along the alveolar wall[148]. Surfactant proteins, especially SP-A, can reverse the inhibitory effects of plasma proteins in vitro[149]. However, neutrophil elastases released in ARDS patients could degrade these protective surfactant proteins and remove this defence mechanism against surfactant inhibition[138].

Cell membrane phospholipids that have been released through the action of PLA_2 can appear in the alveolar space in ARDS, and could also act as a surfactant inhibitor through a similar concept of competitive adsorption along the alveolar wall, displacing surfactant phospholipids that would otherwise lower surface tension[143].

Surfactant kinetics studies in ARDS suggest that whilst there is a reduction in the amount of surfactant phospholipids in the airways, the turnover of phospholipids is increased in these patients[137,150]; it could be hypothesized that this increased turnover reflects increased surfactant degradation in the injured lung, and possibly a compensatory increase in synthesis[151]. In contrast,

similar in vivo pulse chase experiments have shown a marked reduction in PC metabolism in the first days of life in neonates at risk of RDS[152], and presumably reflects the decreased synthetic ability of the immature AT2 cells. Despite the many clinical and pathological similarities between ARDS and neonatal RDS, complementary studies into surfactant kinetics have demonstrated contrasting patterns of phospholipid metabolism. The impact of lung transplantation on pulmonary surfactant phospholipid kinetics remains to be seen, and the application of these techniques is discussed in greater detail in section 1.3.5 and throughout this thesis.

1.2.8 Mechanisms of surfactant dysfunction following lung transplantation

As with other forms of lung injury, surfactant dysfunction following transplantation is associated with poor pulmonary compliance and oxygenation, and probably contributes to the development of PGD[4,30,153]. The mechanisms described in section 1.2.7 that contribute to surfactant dysfunction in other forms of acute lung injury could also exist in PGD, but there are relatively few studies that have investigated any mechanisms that might be unique to lung transplantation.

Rat models of lung transplantation show that IRI causes the "disintegration" of the tubular myelin that lines the alveolar wall following the influx of pulmonary oedema fluid (Figure 1-2), with clumping of surfactant instead of a smooth and even distribution along the alveolar wall. In this model, IRI induced ultrastructural changes in surfactant that were also associated with impaired pulmonary compliance; pathophysiology that is frequently seen in acute lung injury and PGD[146]. Prolongation of the ischemic time further exacerbated the IRI mediated surfactant dysfunction seen in a canine model of lung transplantation, with decreased surfactant proteins, changes in phospholipids class composition, and an alveolar influx of plasma proteins that may have functionally inhibited surfactant function[153].

Whilst surfactant proteins in the alveoli would normally protect surfactant from inhibition and degradation by serum proteins, these too are deficient after lung transplantation; leaving the pulmonary surfactant system further exposed to disruption after surgery[154,155].

The routine sacrifice of the bronchial circulation during the bilateral sequential single lung transplantation technique may cause further injury to the AT2 cells as the pulmonary epithelium becomes increasingly dependent on the pulmonary circulation[156], and so it could be hypothesised that perioperative changes in blood supply would further compromise surfactant metabolism following lung transplantation.

Oxidative stress represents an imbalance between reactive oxygen and nitrogen species and antioxidant defences, which leads to direct damage to proteins, DNA, and lipids[157]. Lung transplantation is a source of substantial oxidative stress in the early perioperative period[158], which contributes to IRI and PGD[159-161]. Oxidative stress may impair surfactant phospholipid synthesis in the AT2 cells[162]. In vitro AT2 exposure to oxidised lipoproteins will induce a protease-mediated cleavage of phosphocholine cytidylyltransferase, which is the rate-limiting enzyme in the Kennedy pathway of phospholipid synthesis[163,164]. Surfactant producing type 2 alveolar epithelial cells (AT2) in vitro undergo significant injury and cell death on reoxygenation after 72 hours of hypoxia[95], and may contribute to surfactant deficiency after transplantation. AT2 cells that survive IRI appeared swollen and oedematous with decreased numbers of lamellar bodies where surfactant is stored[112]. If the AT2 cells does survive the IRI, surfactant synthesis could be impaired by various preservation fluids and techniques, and the continued exposure to oxidative stress[162,165 166].

Perioperative IRI generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) that react with and damage surfactant phospholipids[30,167,168]. The protein component of pulmonary surfactant is also susceptible to the effects of oxidative stress through protein oxidation, nitration, and carbonylation[169,170]. Whilst not routinely used for all lung transplants, inhaled NO is used to treat pulmonary hypertension and hypoxaemia after transplantation, but can also degrade

pulmonary surfactant in experimental models of lung transplantation[171]. The surfactant toxic effects of inhaled NO are probably the result of nitrosative stress, as NO metabolites like peroxynitrite raised surface tension in an experimental in vitro model[172].

1.2.9 Assessment of surfactant dysfunction in lung transplantation

Several techniques have been used to demonstrate surfactant dysfunction in both animal models and human studies of lung transplantation[4,173]:

Direct measurements of surface tension - The Bubble Surfactometer can assess surface tension through the Laplace equation by measuring the pressure gradient across a bubble of fluid[174]. There is a significant increase in surfactant surface tension within hours of lung transplantation in animal models and patients[4,153].

Small aggregate / large aggregate (SA/LA) ratio - Centrifugation separates surfactant into large and small aggregates[175]. LAs lower surface tension and are comprised of tubular myelin, lamellar bodies, and large vesicles. LAs degrade into SAs, which have little effect of surface tension[176]. The degradation of LAs into SAs is driven by several mechanisms; oxidative inactivation[167], surface area cycling, and enzymatic degradation[177]. Animal models of lung transplantation demonstrated an increase in the SA/LA ratio within hours of transplantation, especially in organs with prolonged ischemic times[153]. The SA/LA ratio and surface tension may remain elevated for years following lung transplantation in humans, implying the prolonged persistence of surfactant dysfunction[173].

Quantitative analysis of alveolar fluid and surfactant composition - Using chromatography to measure the surfactant phospholipid content from bronchoalveolar lavage fluid (BALF) in an animal model demonstrated reduced levels of phosphatidylglycerol following lung transplantation. The amount of surfactant protein A in the BALF was also significantly reduced in the same animal model[153]. Whilst the total concentrations of phospholipids were not changed in one human study of lung transplantation, individual phospholipid species were not assessed[173].

1.2.10 Surfactant replacement therapy in adults

1.2.10.1 Failure in adult lung injury

Neonatal respiratory distress syndrome (RDS) is characterised by surfactant depletion, and exogenous surfactant therapy has become the standard of care for premature neonates with RDS[121]. As with neonatal RDS, surfactant dysfunction has been demonstrated in adults with ARDS[25]. Surfactant dysfunction in ARDS contributes to alveolar collapse and the development of pulmonary oedema and pneumonia[178,179]. Given the many clinical and pathological similarities with neonatal RDS, it has led to the theory that exogenous surfactant therapy may also be beneficial in adult ARDS. However, two meta-analyses have failed to demonstrate any convincing benefit from exogenous surfactant in adult ARDS[180,181].

There are numerous theories as to why surfactant therapy has failed to translate into adult practice. The surface area of adult lungs is much greater than the infant lung, and existing exudative lung injury present stiff challenges for surfactant delivery. Determining the optimal dose, delivery method, and subsequent recruitment manoeuvres to ensure adequate distribution of exogenous surfactant is problematic[182]. Several surfactant inhibitors are seen in ARDS that may not be present neonatal RDS, and increased concentrations of exogenous surfactant could be required to overcome those inhibitory effects[183].

Unlike neonatal RDS, ARDS is a heterogeneous syndrome with many possible causes, so it is possible that there will also be marked heterogeneity in the surfactant biology amongst ARDS patients. Contrasting patterns of phospholipid metabolism between ARDS and neonatal RDS could potentially explain the failure to translate surfactant replacement therapy neonatal RDS into adult ARDS, as discussed in section 1.2.7[74,137,152,184]. In theory, phenotyping surfactant phospholipid metabolism in adults with ARDS could identify patient subsets which would benefit from exogenous surfactant therapy[74].

Authors	Patients receiving surfactant	Setting	Surfactant	Dose	Route	Outcome measures
Struber et al 1995 [185]	1	Case report: Rescue therapy of refractory PGD	Exosurf - synthetic	3g/hr for 1 day	Nebulised	Survival to discharge
Struber et al 1999 [186]	6	Case series: treatment for severe PGD	Exosurf - synthetic	3g/hr	Nebulised	Survival to one year
Della Rocca et al 2002 [187]	1	Case report: Rescue therapy of refractory PGD	Curosurf – porcine	400mg/kg	Bronchoscopic instillation	Survival to one year
Struber et al 2007 [188]	15	Prospective randomized trial: Prophylaxis	Alveofact - bovine	100mg/kg (donor)	Bronchoscopic instillation – pre-implantation	FEV_1 at 1 month
Kermeen et al 2007 [189]	6	Case Series: Treatment of severe PGD	Beractant - bovine	one-vial" /bronchial segment: 20mg/ml	Bronchoscopic instillation	Survival to discharge
Amital et al 2008 [6]	21	Prospective randomised controlled trial: Prophylaxis	Bovine surfactant	20mg/kg	Bronchoscopic instillation	Incidence of PGD, ICU LOS, and PFTs at 1 month
Amital et al 2009 [5]	5	Case series: Rescue therapy for refractory PGD	Curosurf – porcine & Calfactant - bovine	20-90mls	Bronchoscopic instillation	Oxygenation, and survival at 6 months

Table 1-3: Reports of surfactant use in human lung transplantation.

A PubMed search was performed with the keywords "surfactant" and "lung transplant". All reports of surfactant use in human lung transplantation have been included. 7 clinical reports were identified, with a total of 55 transplant recipients. Abbreviations; PGD – Primary Graft Dysfunction, FEV_1 – Forced Expiratory Volume in 1 second, ICU LOS – Intensive Care Unit Length of Stay, PFTs – Pulmonary Function Tests.

1.2.10.2 Surfactant replacement therapy in clinical lung transplantation

Whilst there have been some promising results with exogenous surfactant therapy for both PGD prophylaxis and treatment, they are mostly limited to case reports or small case series (Table 1-3); the role of surfactant replacement in transplantation requires further study[28].

1.2.11 Experimental modelling of phospholipid biology

In vitro cell culture of AT2 has confirmed that they are capable of independent PC synthesis, as would be expected from most mammalian cells that utilise the Kennedy pathway. However, cell culture experiments have thus far failed to replicate the full range of phospholipids species in the proportions found in vivo[101]. Synthesis of some phospholipids requires interaction between multiple different cell types and has been termed 'transcellular biosynthesis'[190]. Leukotrienes (and other eicosanoids – 20 carbon PUFA species containing oxygen) probably depend on transcellular biosynthesis, which could be difficult to replicate within in vitro experiments comprised of a single cell-line[191].

Most experimental models of phospholipid biology in the alveolar epithelium have focused on the airway secretion of phospholipids into the airways that reduce surface tension. Both in vitro cellular modelling and animal experiments have implied that the alveolar epithelium is capable of selectively secreting different phospholipids into the airways and the circulation, but this proposed theory of basolateral phospholipid efflux has never been conclusively proven[10,11]; little is known about any pulmonary contribution to phospholipids in the systemic circulation.

In vitro modelling is further limited by the technical challenges of recreating the mechanically mobile environment created in the lungs during respiration, especially as surfactant release depends on ventilation[192]. The effects of ventilation can be replicated in animal models, ex vivo organ preparations, and even in cell culture plates with deformable silicoelastic membranes exposed to cyclical stretch[57,193,194]. Mechanical stretch of the pulmonary epithelium causes surfactant lamellar body exocytosis of phospholipids in a dose-dependent fashion, by mobilising intracellular calcium

stores[99,195]. Amplitude, timing, and variability of alveolar stretch will affect the release of surfactant phospholipids by the alveolar epithelium, processes that are further attenuated by paracrine effects[196-198]. Cyclical stretch of the alveolar epithelium will induce several enzymes promoting both de novo synthesis and acyl remodelling of DPPC; specifically, enzymes that include LPCAT, PLA_2, CDP-choline:cholinephosphotransferase[199]. These effects of ventilation may not be limited to surfactant phospholipid species like DPPC; it is possible that mechanical stretch associated with breathing could also promote the release of eicosanoid-based mediators from the lungs into the circulation[200].

Given it has been shown that a single deep breath causes a substantial increase in alveolar surfactant phospholipids, the mechanical effects of ventilation must be considered in any model of alveolar phospholipid biology[192,195]. In vivo animal models can address some of these issues and have provided valuable data on pulmonary phospholipid biology, but interspecies variation in pulmonary phospholipid biology must also be considered when establishing animal models to reflect the considerable variation in the phospholipid classes and PG content in surfactant between different mammalian species[201]. Lung size could also be an important consideration in animal models of phospholipid biology when considering trials of exogenous replacement therapy, as differences in organ size (and its associated challenges in surfactant delivery) could have contributed to the failure to translate exogenous surfactant replacement from neonatal RDS into adult critical care[182,202].

The gold standard model for research into human pulmonary phospholipid pathobiology would theoretically have the following attributes:

- Faithfully replicates the physical effects of breathing
    - For its essential role in phospholipid metabolism and the effects of VALI
- Maintains a preserved alveolar & vascular architecture
    - For continued apical and basolateral phospholipid secretion

- Maintains cell-cell interactions that would be expected in the lungs
    - For preserved transcellular biosynthesis and paracrine signalling
- Capable of producing a comparable range of phospholipids expected in man
    - Acknowledging the mammalian interspecies variability in phospholipid content
- Can reliably reproduce injury in a predictable pattern
- Replicates the challenges in drug delivery encountered in adult human lungs
    - i.e. lungs of a comparable size and shape for therapeutic studies of surfactant replacement, and similar therapeutic models that depend on effective delivery

1.2.12 Other biological functions of phospholipids

There are a theoretical 6455 different glycerophospholipids species, without including the many potential isomers[203]. The biological roles for most lipids can be separated into four broad categories: 1) formation of membranes, 2) lipid messengers, 3) creating hydrophobic environments, and 4) energy storage and other applications[204]. A very brief overview of other lipid functions is provided for completeness, but a complete review of lipid biology is beyond the scope of this thesis.

1.2.12.1 Cell membranes

The phospholipid bilayer is the key component of the widely accepted fluid mosaic model of cellular membranes[205]. Membrane phospholipids are of a wide range of different classes and species, which vary with the functional needs of the membrane[206]. Sphingolipids, PE, and PC species represent most of the phospholipids that form cellular membranes[207,208]. Phospholipids also form the membranes of intracellular organelles like the mitochondria[208] and changes in mitochondrial cell membranes have been implicated in apoptosis and cell death[209]. The biology of mitochondrial and cell membrane dysfunction is a vast topic that is beyond the scope of this thesis, and will not be discussed in further detail.

1.2.12.2 Cellular messengers

It is thought that phospholipid PUFA is mobilised from the cellular membranes following the activation of the PLA_2 family of enzymes[210]. Lipid peroxidation of PUFA species released by cells injured by ischemia and reperfusion will create oxidised phospholipids and exposed phosphocholine that will act as DAMPs and trigger the innate immune response [211,212]; presumably through IgM binding with subsequent activation of the lectin-mediated complement cascade and dendritic cells in the transplant recipient[213].

Once released from membrane phospholipids, PUFA species can then become a wide range of inflammatory mediators. Arachidonic acid is one example of a PUFA which enters the cyclooxygenase (COX) pathways where it is converted into a wide range of eicosanoid-based pro-inflammatory mediators[210,214]. In contrast, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are ω-3 PUFA species that are metabolised into a range of specialized pro-resolving mediators like resolvins, protectins, and maresins[215]. The balance between pro-inflammatory and pro-resolving lipid mediators probably plays a crucial role in the development of the systemic inflammatory response syndrome (SIRS), and the subsequent compensatory anti-inflammatory response syndrome (CARS) in all forms of critical illness[216,217]. Breakdown-products of many phospholipids can create a wide range lipid second messengers that are important in the pathogenesis of lung injury and include sphingosylphosphorylcholine, sphingosine-1-phosphate, ceremide, diacylglycerols, and lysoPC species[218-224].

Intact phosphatidylcholine species can themselves act as inflammatory mediators, perhaps one of the best known is platelet activating factor (PAF). PAF (PCo16:0/2:0 - Figure 1-8) was the first PC species discovered to act as an intercellular messenger that triggers coagulation cascades and stimulates a vigorous inflammatory response from leukocytes and endothelial cells (amongst many other actions)[225,226]. As with all other PC species, PAF synthesis is tightly regulated via the Kennedy CDP-choline pathway and Land's Acyl remodelling cycle; however, oxidative degradation of other PC

species will yield phospholipid products that can also bind with the PAF-receptor and induce similar biological effects[227].

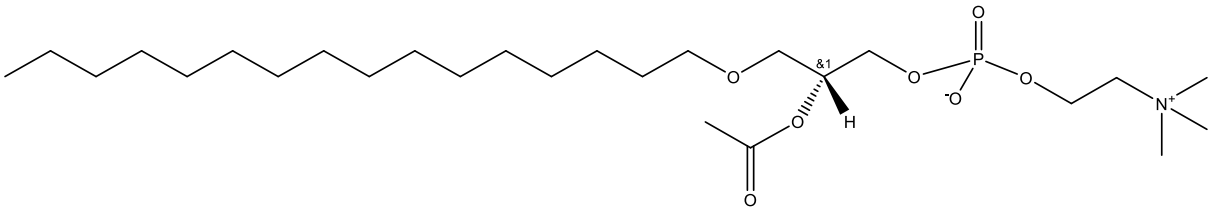


Figure 1-8: Platelet activating factor
PCo16:0/2:0, also known as 1-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine/platelet activating factor (PAF)

1.2.12.3 Hydrophobic environments

Lipids are by definition hydrophobic substances that are soluble in non-polar solvents[228]. Hydrophobicity is the force that stabilises protein structure, extracellular matrices, and membranes; and drives the formation of phospholipid bilayers and monolayers[229,230]. Phospholipids are polar molecules that will naturally form a monolayer at the air-liquid interface with the hydrophobic acyl chains orientated towards the airspace, which is then embedded with the hydrophobic surfactant proteins SP-B and SP-C and further contributes to the stability of the phospholipid layer[230-232].

Elsewhere in the body, bile acids and phospholipids act as a surfactant in the gastrointestinal tract that facilitates the absorption and micelle formation of dietary fat, fat-soluble vitamins, and essential fatty acids[233-235]. Chronic aspiration of bile acids can be particularly problematic following lung transplantation, and is associated with poor postoperative graft function and the development of CLAD[236,237].

1.2.12.4 Energy storage and other functions

Lipids are an important energy source during times of acute physiological stress or fasting[238], and the fat-soluble vitamins (A, D, E and K) are essential micronutrients for health[239]; the nutritional role of lipids is also beyond the scope of this thesis and only mentioned for completeness.

Beyond their role in the reduction of surface tension in pulmonary surfactant, there is a vast range of lipids with essential roles in both health and critical illness[216], and this has understandably prompted the desire to seek a greater understanding of the wider lipidome [204,240,241]. The ability to study the lipidome has been greatly enhanced by technological advancements in mass spectrometry, which are discussed in more detail in section 1.3.

1.3 Mass spectrometry

1.3.1 Application in lipidomic profiling

Previous attempts at analyses of lipid classes and species were both labour-intensive and technically challenging[242]. But with the advent of electrospray ionisation tandem mass spectrometry (ESI-MS/MS) it has become possible to quickly analyse hundreds of lipid species through the direct infusion of a sample into the mass spectrometer, without prior chromatographic separation, allowing the development of so-called 'shotgun lipidomics'[204]. Fragmentation of lipids inside the mass spectrometer through collision-induced dissociation can also provide further information on phospholipids' structure, as found in tandem mass spectrometry[204]. ESI-MS/MS is the primary technique used in this thesis, and so brief overview of the technology is provided with illustrative examples of its application in the field of lung injury.

1.3.2 Basic principles

The Bligh and Dyer method of lipid extraction is frequently used to prepare samples for lipid analysis with mass spectrometry[243]. Extracted lipids can then undergo separation in a chromatography column prior to feeding into the mass spectrometer, or they can be infused directly.

Mass spectrometers analyse samples by charting the relative abundance of various ions against their mass/charge ratio (m/z) and are made of three basic components; an ion source, a mass analyser,

and a detector[244]. Whilst there are many variations on this theme, ESI-MS/MS is primarily used in this project.

1.3.3 Ion source: Electrospray ionisation

Analysis of a sample requires the generation of gas phase ions that can pass through the mass analyser to the detector[244]. The use of electrospray ionisation with mass spectroscopy was described by Yamashita and Fenn both in 1984[245], which earned Fenn the Nobel Prize[246]. An electrical field is applied to a small flow of liquid to produce the so-called electrospray, creating charged droplets of ions that can be sampled by a mass spectrometer - Figure 1-9[204,247].

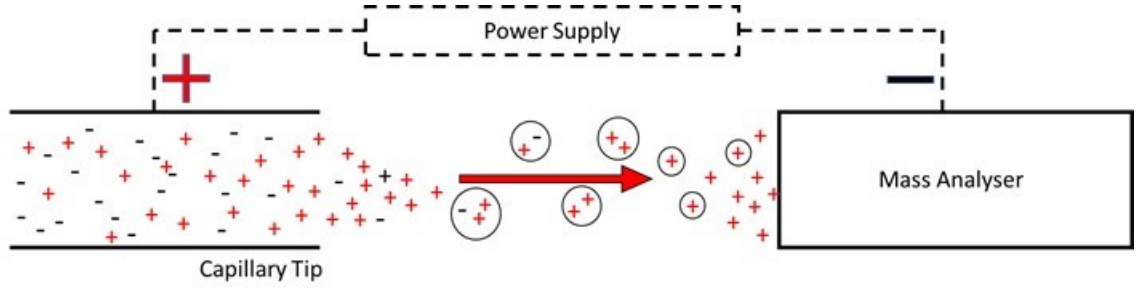


Figure 1-9: Principle of electrospray ionisation in ES-MS/MS (Positive ion mode)
Illustration prepared in collaboration with Dr Hannah Skene, adapted from Han and Gross 2005[204]

1.3.4 Mass analyser: the quadrupole

Once the sample has been ionised, it is delivered to a mass analyser. Whilst several different types are available, this project will use a quadrupole mass analyser. As ions pass through the quadrupole, an electrical field allows ions with a specific m/z (mass/charge ratio)to pass through to the detector, whilst all other ions are filtered out - Figure 1-10[244].

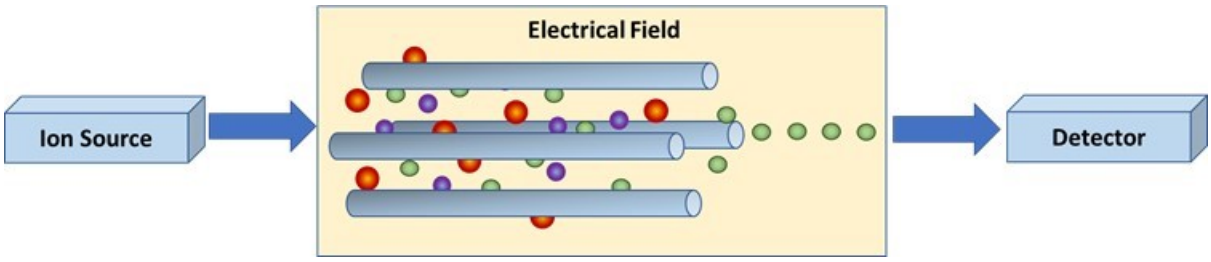


Figure 1-10: Schematic of a quadrupole mass detector.
Four poles are electrically connected. Electrical fields are applied to pairs of poles which will maintain a stable trajectory for ions of a given mass:charge (m/z) ratio, depending on the voltage ratio. Only ions that maintain a stable trajectory from the ion source will reach the detector. Illustration prepared in collaboration with Dr Hannah Skene, adapted from Glish and Vachet 2003[244]

When multiple quadrupoles are used in series to further enhance ion detection it is termed tandem mass spectrometry. By connecting three quadrupoles together in series, the first quadrupole (Q1) can select out a parent ion of interest, which is then fragmented by the second quadrupole (Q2), before the daughter fragments are then sorted by the third quadrupole (Q3) – Figure 1-11[248]

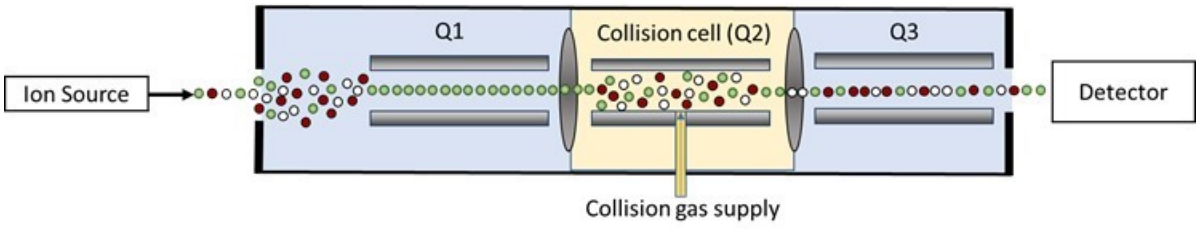


Figure 1-11: Tandem Mass Spectrometry
Three quadrupoles are connected in series. Introduction of gas into Q2 results in collision-induced dissociation (CID) of ions filtered by Q1. Ion fragments leave the collision cell and are then filtered by their m/z in Q3, producing spectra of those fragmented ions. Q1: Quadrupole1, Q2: Quadrupole 2, Q3: Quadrupole 3. Illustration prepared in collaboration with Dr Hannah Skene, adapted from Milne et al 2006[248]

Fragmentation in tandem mass spectrometry can filter out signals that only contain functional groups of interest, for example the polar phosphocholine moiety found in PC and SM lipid species, providing structural data and greatly enhancing the sensitivity and specificity of the technique[249,250].

1.3.5 Isotopic labelling of surfactant phospholipids

Isotopic labelling of choline was the key method that allowed Kennedy to discover his eponymous pathway that is responsible for the majority of phospholipid synthesis in mammalian cells[126]. Experiments based on the labelling of the CDP-choline pathway can continue to provide a dynamic assessment of phospholipid flux that is not possible with static measures of phospholipid content[251,252]. Unlike Kennedy's in vitro radioactive labelling experiments that used unstable isotopes ^{32}P and ^{14}C, stable isotope labelling with deuterium is easy, accurate, and completely safe in man[8,126].

Combining ESI-MS/MS with stable isotope labelling of the Kennedy PC synthetic pathway with D9-choline (deuterated PC precursor) allows the in vivo assessment of surfactant phospholipid flux,

providing molecular specificity that can differentiate between many lipids with unique biological functions[8,253]; a technique that has been utilised in healthy volunteers and the critically ill alike[7,137,254,255].

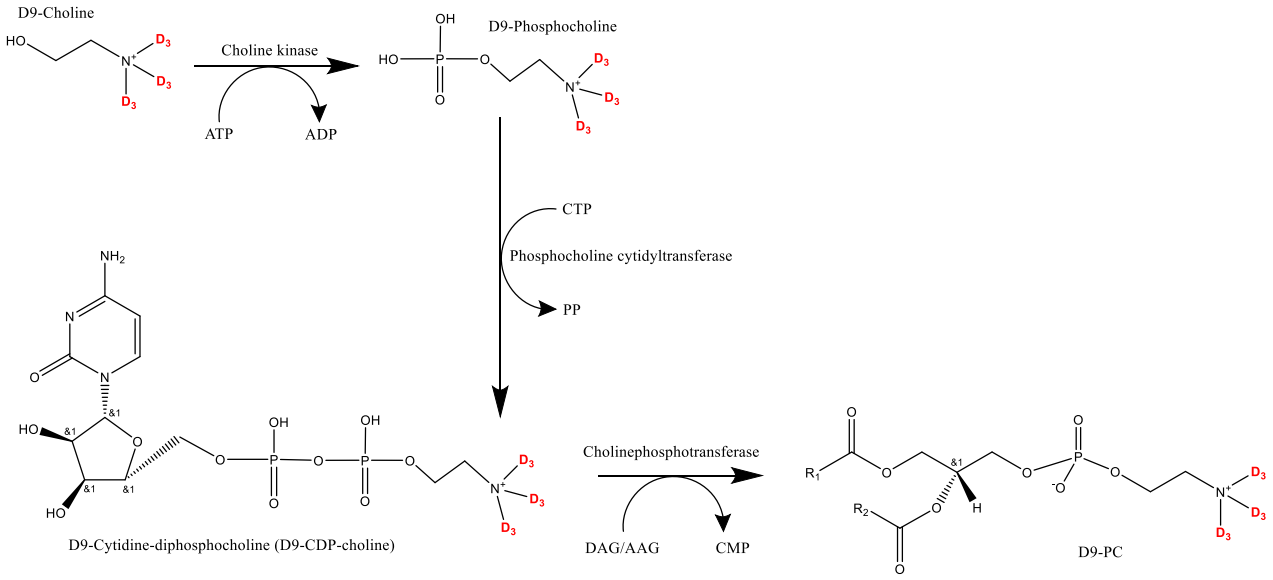


Figure 1-12: Deuterium labelling of the Kennedy CDP-choline pathway.
Nine hydrogen atoms are replaced with deuterium (highlighted in red) resulting in any labelled choline having an m/z ratio that is 9 greater than naturally occurring choline. As the labelled choline moves through the CDP-choline pathway, any newly synthesised PC species will also become enriched with deuterium with similar effects on m/z ratio. Compare with the unlabelled Kennedy CDP-choline pathway described in Figure 1-5

Previous isotope labelling experiments suggest that surfactant phospholipid turnover is increased in ARDS; given the clinical and pathobiological similarities with PGD following lung transplantation it would seem logical to investigate whether this is also the case in this cohort[137,150].

The primary objective of this thesis will be to apply these lipidomic techniques to perioperative lung transplant recipients, to assess the impact of IRI on the Kennedy Pathway, phospholipid biology, and pulmonary surfactant. These techniques have also been applied to a complementary isolated ex vivo perfused and ventilated human lung model to further investigate the pulmonary contribution to systemic phospholipid metabolism, and to explore the protective mechanisms of clinical ex vivo lung perfusion (EVLP) in transplantation.

1.4 Hypotheses

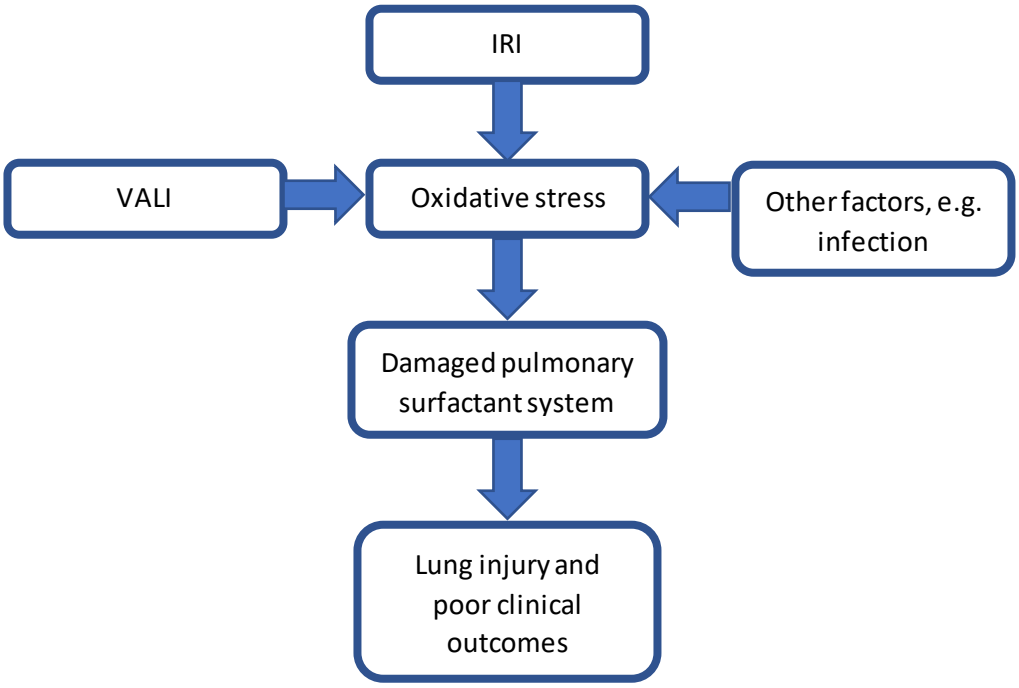


Figure 1-13: Hypothesis.
IRI and VALI (amongst over contributing factors) generate oxidative stress that damage the surfactant system and cause lung injury. IRI – Ischemic reperfusion injury. VALI – Ventilator associated lung injury

Kennedy pathway dependant pulmonary surfactant phospholipid metabolism is disrupted in lung transplantation, which has an adverse effect on clinical outcomes after lung transplantation.

1.5 Objectives

- Establish the viability of lipidomic profiling in perioperative lung transplantation
- Utilise stable isotope labelling of the Kennedy pathway with ESI-MS/MS to explore changes in surfactant phospholipid metabolism following lung transplantation
- Explore associations between lipid flux and clinical outcomes in lung transplantation
- Examine for a link between oxidative stress and disrupted phospholipid metabolism
- Utilise stable isotope labelling to assess the integrity of the Kennedy pathway during ex vivo lung perfusion
- Explore pulmonary surfactant phospholipid metabolism using a human ex vivo platform

2 Methods

2.1 Materials

Material	Source
4-hydroxynoneal (rabbit polyclonal)	Biorbyt. Cambridge, UK
acetonitrile	Thermo Fisher Scientific (Life technologies), Waltham, MA - USA
All solvents – HPLC grade	Thermo Fisher Scientific (Life technologies), Waltham, MA - USA
Ammonium acetate	Thermo Fisher Scientific (Life technologies), Waltham, MA - USA
Ayasdi Workbench Platform	Palo Alto, CA-USA.
Bovine serum albumin (3%)	Sigma-Aldrich. Poole, UK
Butylated Hydroxy Toluene (BHT):	Sigma-Aldrich. Poole, UK
DAPI (4',6-diamidino-2-phenylindole)	Sigma-Aldrich. Poole, UK
Deuterated methyl-D9 choline chloride	CK GAS Products. Hampshire UK
EDTA vacutainer blood tubes	Becton, Dickinson and Company – Franklin Lakes, NJ USA
ENO-20 NOx analyser	Eicom (Amuza Inc.) San Diego, CA-USA
EVLP Disposable circuit tubing	Maquet. Sunderland, UK
Falcon Test Tubes	Becton, Dickinson and Company – Franklin Lakes, NJ USA
FIJI distribution of ImageJ v1.51n	Wayne Rasband, National Institute for Health - USA
Formic acid	Thermo Fisher Scientific (Life technologies), Waltham, MA - USA
Freedom EVO® Platform	Tecan Group. Männedorf, Switzerland
Goat anti mouse Alexa Fluor 594	Thermo Fisher Scientific (Life technologies), Waltham, MA - USA
Goat anti rabbit Alexa Fluor 594	Thermo Fisher Scientific (Life technologies), Waltham, MA - USA
Hemoxygenase 1 (HO-1-mouse monoclonal)	Biorbyt. Cambridge, UK
LSM 880 confocal microscope	Zeiss. Oberkochen, Germany
mass spectrometry glassware	Thermo Fisher Scientific (Life technologies), Waltham, MA - USA
MassLynx™ version 4.0	Waters, Milford, MA-USA
Microsoft Office 365	Microsoft, Redmond, WA-USA
Nitrotyrosine (NT-mouse monoclonal)	Biorbyt. Cambridge, UK
Organ Care System and solutions	OCS – TransMedics, MA. USA
Perfadex	XVIVO Perfusion AB. Goteborg, Sweden
Permafluor	Beckman Coulter. High Wycombe, UK
Phospholipids internal standards	Avanti Polar Lipids. Alabaster, AL - USA
SPSS Statistics V24	IBM North Castle, NY-USA
Steen solution	XVIVO Perfusion AB. Goteborg, Sweden
Ultrapure water, Nanopure Diamond	Thermo Fisher Scientific (Barnstead Thermolyne). MA - USA
Vivoline Ex vivo lung perfusion system	Vivoline Medical AB, Lund. Sweden
Xevo TQ-Mass spectrometer	Waters, Milford, MA-USA

Table 2-1: Materials
Materials used during this project and their source

2.2 Clinical study

This was a single-centre clinical pilot observational study to evaluate perioperative changes in phospholipid metabolism in patients undergoing lung transplantation.

2.2.1 Approval

Ethical approval was granted by the Riverside NRES committee in London (Research Ethics Committee approval reference 13/LO/1052 – See appendix 7.3) and the Royal Brompton & Harefield Research Office provided NHS Management Permission for Research (R&D reference 2013LS001H – See appendix 7.4)

2.2.2 Sample size

As this was a pilot observational study, a formal power calculation was not performed [256]. Whilst a sample size of 12 is acceptable for most pilot studies [257], the larger number of 40 patients was selected to further enhance the statistical power for the study cohort based on the historical annual transplant activity at Harefield Hospital.

2.2.3 Inclusion criteria

2.2.3.1 Inclusion criteria:

- Adult patients undergoing bilateral lung transplantation

2.2.3.2 Exclusion criteria:

- Single lung transplant recipients
- Patients under the age of 18
- Patients extubated in theatre

2.2.4 Entry Procedure

All patients on the active waiting list for bilateral lung transplantation who met the inclusion criteria (section 2.2.3) were provided with a patient information leaflet (see appendix 7.7). Patient information leaflets were provided during hospital attendances, admissions to Harefield Hospital, or they were mailed to their home address. After confirming with the patient that they have had

enough time to study the information sheet and discuss the study with research staff, the patient was invited to confirm their consent to participate in the study by signing the study consent form (see appendix 7.8). Study consent forms were signed prior to surgery for lung transplantation, if it had not already been signed on a previous hospital visit. Consent forms were copied in triplicate; one copy was given to the patient, one copy was filled in the patient notes, and one was stored in the Study Masterfile.

2.2.5 Administration of D9-choline

Sample were collected from the first 10 patients without the administration of D9-choline, to confirm the ability to measure the phospholipids of interest before proceeding to the perioperative labelling of transplant recipients. The subsequent group of 30 patients received an infusion of choline that has been labelled with 9 atoms of deuterium ([methyl-D9] choline chloride – 99% enrichment). D9-Choline was stored in a temperature regulated fridge until required. After the collection of baseline samples, at the end of surgery D9-choline (3.6ml/kg admission body weight) was infused through a central line over 3 hours, as previously described[8].

2.2.6 Patient Sample collection

2.2.6.1 Sample timetable

Samples were taken from transplant recipients according to the following timetable (Table 2-2), immediate sample handling and processing is described in section 2.4.1.

	On waiting list	Start of surgery	Completion of surgery	1-3 hrs	6 hrs	12 hrs	24 hrs	Then daily, until free from ventilation
Screening & invitation	☑							
Informed consent	☑							
Blood		☑	☑		☑	☑	☑	☑
Lung biopsies		☑	☑					
Blind bronchial aspirates		☑	☑		☑	☑	☑	☑
D9-Choline infusion*				☑				

Table 2-2: Time table for sample collection from lung transplant recipients
**Only the final patients were allocated to receive the postoperative infusion of D9-choline*

2.2.6.2 Blood sampling

Blood samples were taken from pre-existing arterial or central venous cannulae into 6ml EDTA (Ethylenediaminetetraacetic acid) Vacutainers®.

2.2.6.3 Blind bronchial aspirates

Regular endotracheal suctioning is routine practice for all ICU nurses looking after intubated patients. This procedure is carried out many times every day as required to clear secretions from the lungs. A closed-suction system is attached to the endotracheal tube of all lung transplant recipients. The suction catheter was advanced to the maximal level, 20mls of normal saline was flushed into the catheter (approximately 5mm diameter and 60cm long), and then all saline/secretions were aspirated back through the catheter into suction traps.

Previous work from our collaborators in Southampton has shown that lipidomic profiles and measures of flux based on deuterium enrichment are identical between blind aspirates from endotracheal tubes and the bronchoalveolar lavage fluid in patients with ARDS. and the results from these two samples types have been put together and treated identically in this study[7].

2.2.6.4 Bronchoscopic sampling

Fibreoptic bronchoscopy is a routine procedure after transplantation, to inspect the anastomoses, to clear secretions from the lung, and to take samples for microbiology. The technique typically uses 150 to 240 ml of 0.9% saline for a bronchoalveolar lavage (BAL), or 20 to 40 ml for bronchial washings. Surplus samples obtained from bronchoscopy were retained for analysis; however, fibreoptic bronchoscopy was only performed at the discretion of the clinical team and the technique was not standardised.

2.2.6.5 Lung biopsies

Lung biopsies were taken at the discretion of the operating consultant surgeon. Small lung biopsies were taken from the donated lungs before and after implantation, aiming for tissue samples of approximately 5-10mm in diameter. If lung biopsies were considered appropriate by the consultant transplant surgeon, the parenchyma was sampled at two perioperative time points; the 1st biopsy was taken shortly after the lungs were removed from cold storage, the 2nd biopsy was sampled from the same lobe immediately prior to closure of the chest. Due to the variable duration of surgery, there was a variable period of in vivo organ reperfusion between the two biopsies.

2.3 Ex vivo study

This project was a pilot single-centre laboratory-based experimental study on surplus human tissue that has been removed from deceased organ donors that cannot be used for clinical transplantation, with the intent of creating a human model of pulmonary phospholipid biology, and demonstrating the ex vivo integrity of the Kennedy pathway.

2.3.1 Approval

Ethical approval was granted by the Riverside NRES committee in London (Research Ethics Committee approval reference 14/LO/0326 – See appendix 7.10) and the Royal Brompton & Harefield Research Office provided NHS Management Permission for research (R&D reference 2013LS002H – See appendix 7.11. Permission for research was also granted by the NHS Blood & Transplant Research office – See appendix 7.12.

2.3.2 Sample size

As previously discussed for the clinical study in section 2.2.2, this ex vivo study was a pilot observational project and a formal power calculation was not performed[256].

2.3.3 Inclusion criteria

2.3.3.1 Inclusion criteria

- Lungs that come with the assent from the next of kin to the use of surplus tissue for research
- Lungs that have been donated into the transplant programme, but subsequently found to be unfit for this purpose following a period of ex vivo assessment: for phospholipid analysis with D9-choline labelling
- Lungs sustained on ex vivo support that are suitable for transplantation: were used for the retention of surplus clinical samples only

2.3.3.2 Exclusion criteria

- Lungs that have been resected without a sufficient length of pulmonary artery to allow cannulation and perfusion
- Lungs that have been resected without a sufficient length of airway that will allow intubation and ventilation
- Lungs that could not be sustained on ex vivo support for at least two hours

2.3.4 Entry procedure

Prior to clinical organ retrieval, the next of kin were specifically asked if they believe that the potential organ donor would have agreed to surplus tissue to be used for research purposes. Transplant coordinators documented if the next of kin had provided their assent for the retention surplus tissue for research. Retrieved lungs were 'link-anonymised' with an NHSBT donor number, and identifiable information on the donor could only be accessed through this number by transplant coordinators via the NHSBT database. NHSBT held all assent forms that were signed by the next of kin (NOK) and were not directly accessible by staff at the transplant centre. Harefield Transplant coordinators confirmed NOK assent for research through the NHSBT donor database and informed researchers whilst maintaining the anonymity of the donor and their family. Only organs that had

been resected from the donor with the primary intention for clinical transplantation were used for this project; no organs were removed from donors with the primary intention of research.

2.3.5 Ex vivo platforms

Two ex vivo platforms were in clinical use during the study period, the Vivoline Ex Vivo Lung Perfusion system, and the TransMedics Organ Care System. Human lungs that have been harvested, but later declined for clinical transplantation were retrieved using established local surgical protocols, as previously described elsewhere[258]. If lungs were sustained on either Vivoline EVLP or the TransMedics OCS systems, they remained on that system using the previously established clinical protocol for ex vivo support, to prevent any further ischaemic insults to the tissue. All ex vivo systems followed the same basic schematic described in Figure 2-1. The PA (pulmonary artery) is

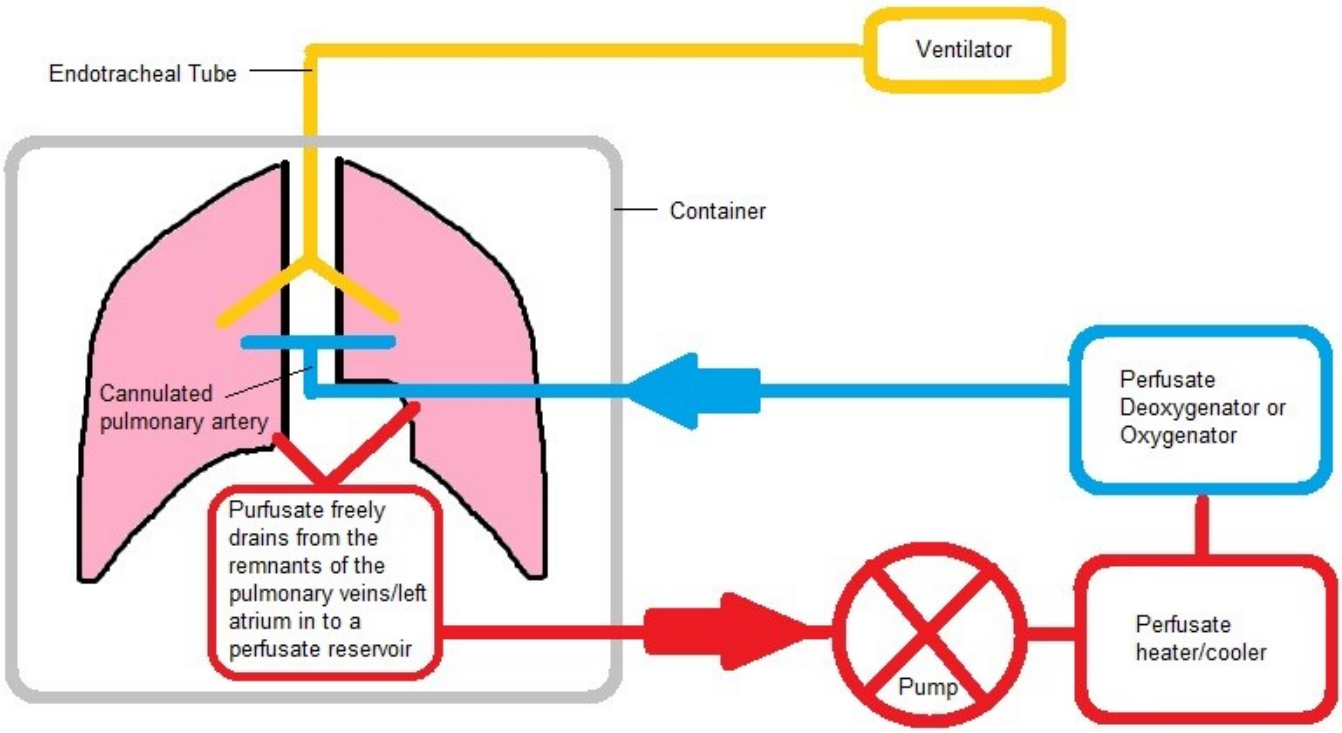


Figure 2-1: Simplified schematic of ex vivo lung perfusion (EVLP)
1) The Reservoir and circuit is primed with perfusate. 2) A cardiopulmonary bypass pump drives the perfusate around the circuit and pulmonary vasculature. 3) After the perfusate leaves the pump, it passes through a counter-current heat-exchanger, providing control over the perfusate temperature. 4) Before entering the lungs, the perfusate will pass through membrane oxygenator, which can also deoxygenate the perfusate. The membrane oxygenator/deoxygenator is not needed in experimental EVLP, if analysis of gas exchange is not required. 5) Perfusate is then pumped into the pulmonary artery, circulates around the pulmonary vasculature, before leaving the lungs via an opened left atrium. Perfusate then flows freely into the perfusate reservoir, before recirculation. 6) Once the lungs are warmed, a ventilator will drive air/oxygen in and out of

cannulated and connected to a warmed perfusion circuit including a pump and membrane oxygenator/deoxygenator, whilst the trachea is intubated and attached to a ventilator.

If the organs were declined for transplantation following ex vivo assessment in cold storage, they were then transferred to the Vivoline EVLP system. Ex vivo support protocols were not standardised across this study, and clinical EVLP techniques were determined by the consultant transplant surgeon. Perfusates typically included either a proprietary OCS solution, Perfadex, or Steen solution, supplemented with heparin, THAM (tromethamine), calcium chloride, sodium bicarbonate, glucose, actrapid insulin as determined by the clinical team. Perfusates on the OCS would typically include cross matched packed red cells, but the Vivoline perfusate was often acellular. Data on ex vivo pulmonary physiology is summarised in section 4.3.1.1.

2.3.6 Sampling

Samples were collected at hourly intervals for organs that had been rejected from transplantation, until the end of the experiment (section 2.3.8). Perfusates and bronchial washings were sampled at the same time. Organs that were still undergoing ex vivo assessment as a potential candidate for clinical transplantation were sampled as clinically indicated, on the direction of the transplant surgical team. Immediate sample handling and processing is described in section 2.4.1. Corresponding data on pulmonary physiology was also recorded, and summarised in section 4.3.1.1.

2.3.6.1 Perfusate sampling

Cellular perfusate from the OCS system was aspirated from the circuit into 6ml EDTA Vacutainers, acellular perfusates were sampled in 5ml syringes and transferred directly into Falcon containers.

2.3.6.2 Bronchoscopic sampling

Bronchial washings were taken via bronchoscopy after instilling 20-40mls of 0.9% saline and transferred into Falcon containers. The middle lobe was typically selected for bronchial washings, unless there were macroscopic signs of injury to that lobe, in which case the least injured lobe was selected for bronchial washings.

2.3.7 Administration of D9-choline

A 50mg bolus of D9-choline was injected into the organ perfusate for lungs that had been rejected for clinical transplantation, after the collection of the baseline samples

2.3.8 Study Endpoints

The experiment is concluded once any of the following criteria are achieved.

- Severe pulmonary oedema
- Depletion of perfusate reservoir

All surplus human tissue was disposed of in full accordance with The Trust's established clinical guidelines.

2.4 Sample Processing

2.4.1 Initial sample processing

2.4.1.1 Fluids

The addition of butylated hydroxytoluene (BHT) to samples has become departmental standard practice for lipidomic profiling[137]. Previous studies into markers of oxidative stress in lung injury have also routinely added BHT to samples without adverse effects[259,260].

All samples were immediately supplemented with BHT at the bedside after collection, prior to transfer on ice to the laboratory. 4mg of BHT was added for approximately every 5ml of sample that was collected from the patients. Prefilled ampules of BHT were created from a stock solution of 2g of BHT in 100ml of methanol; 20µl of stock solution was then dissolved in 500µl of 0.9% saline and loaded into Eppendorf tubes before sample collection (8mg/ml BHT solution). All samples were centrifuged at 1500rpm for 10 minutes at 4°C, before the supernatant was aspirated into 500µl aliquots and frozen to -80°C. Bronchial washings were also filtered through sterile gauze to remove debris and mucus, before centrifugation.

2.4.1.2 Tissue

All lung biopsies were immediately placed on ice and transferred to laboratory for initial processing without delay. Parenchyma was divided into pieces and fixed in formalin, before it was processed through ethanol, xylene, and embedded into paraffin wax. Tissue biopsies were also snap-frozen in liquid nitrogen, preserved in glutaraldehyde, and embedded into Optimal Cutting Temperature (OCT) Compound for future collaborative projects.

2.4.2 Lipid extraction

2.4.2.1 Internal Standards

Stock solutions of internal standards were suspended in a solution of:

- DCM (dichloromethane) 60.2%
- Methanol 32.4%
- Ultrapure Water 7.4%

The addition of internal standards to samples prior to lipid extraction is summarised in Table 2-3.

Lipid	Bronchial washings and ex vivo perfusate	Blood
DMPC	10 nmol	1 nmol
LHPC	1 nmol	0.1 nmol
DMPG	2 nmol	0.2 nmol
DMPE	4 nmol	0.4 nmol
DMPA	1 nmol	0.1 nmol

Table 2-3: Internal standards
Amount of lipid added to each sample prior to lipid extraction. DMPC - dimyristoylglycerophosphocholine, DMPG - dimyristoylglycerophosphoglycerol, LHPC - heptadecanoylglycerophosphocholine, DMPE – dimyristoylglycerophosphoethanolamine, DMPA - dimyristoylglycerophosphate

2.4.2.2 Bligh and Dyer technique

Robot assisted lipid extraction can improve both sample throughput and analytical reproducibility, and this approach was extensively utilised during this thesis (Figure 2-2)[261]. Samples were transferred to borosilicate glass tubes once thawed to room temperature, before the internal standards were added to the samples. Robot-assisted lipid extraction was performed with the Freedom EVO® Platform using a modified version of the Bligh and Dyer method[243]. All samples were transferred into borosilicate glass tubes in 500µl aliquots, except for plasma which was transferred

in 100 µl aliquots with 400 µl of 0.9% saline. The robot added 300ml of 0.9% saline, 2ml of methanol, and 1ml of dichloromethane to each sample. Samples were shaken vigorously before centrifugation at 3,000RPM for 10 minutes at 10°C. Supernatants were decanted into new borosilicate glass tubes after removal of the protein content. A further 1ml of dichloromethane and ultrapure distilled water were added before the samples were centrifuged again at 3,000RPM for 10 minutes at 10°C. Robot-guided separation of the layers took place, before the lipid phase was transferred to mass spectrometry bottles and dried under continuous low flow nitrogen at 37°C. Dried lipid extracts were capped, labelled and frozen at -80°C pending analysis with mass spectrometry. One negative control sample of 0.9% saline was introduced to every batch of 23 samples and subjected to the same lipid extraction and analytical processes, to exclude cross contamination.

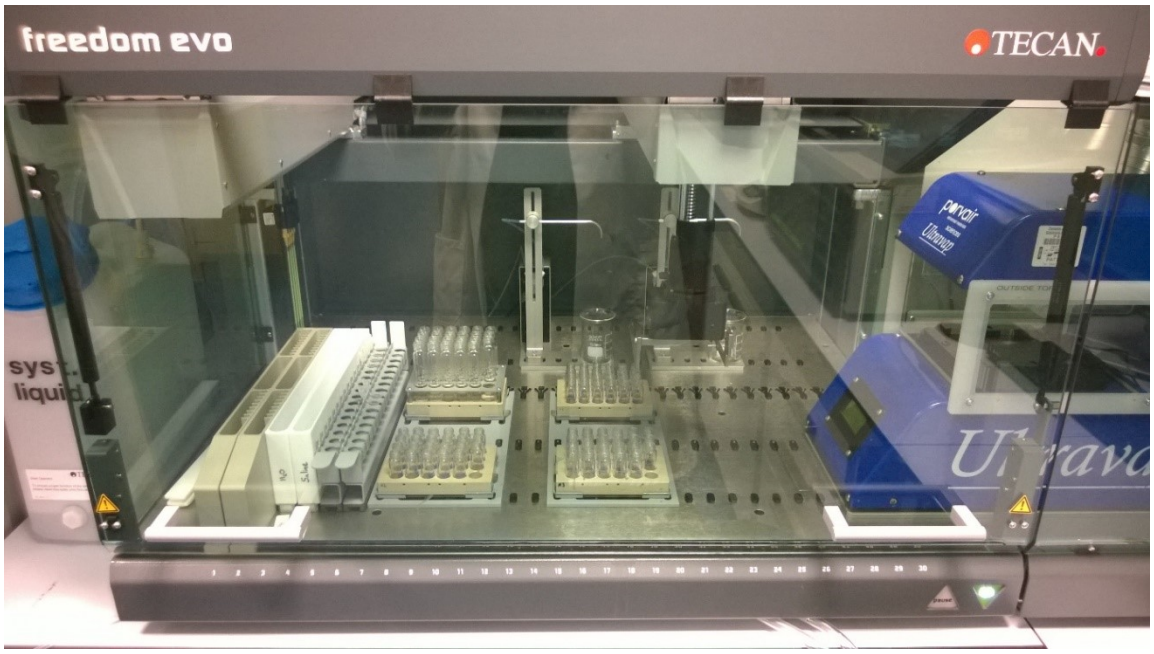


Figure 2-2: The Freedom EVO Platform
Configuration of the Freedom EVO Platform, used for robot assisted lipid extraction

2.5 Assays

2.5.1 Mass Spectrometry

Electrospray Ionisation Tandem mass spectrometry (ESI-MS/MS) was used to measure phospholipid content and deuterium enrichment for all samples. Dried down lipid extracts were reconstituted in

200 µl methanol with 10mM Ammonium Acetate, and stored in the automated sample loader at 4°C prior to automated loop injection into the instrument at a flow rate of 10µl/min. Two mobile phase solvents were used at a ratio of 1:1, and all lines were primed for 4 minutes at a flow of 4ml/minute before the analysis of the first sample:

- Solvent A; UP-H_2O with 10mM ammonium acetate
- Solvent B: methanol with 10mM ammonium acetate

Scans were performed in full positive and full negative ionisation mode, followed by breakdown scans for precursors fragments with a m/z 184 and 193 (phospholipids containing either naturally occurring choline or the deuterium labelled choline respectively). To prevent cross contamination, the whole instrument was flushed prior to injection of the next sample with a cleaning solution that comprised of 60% Methanol, 30% DCM, and 10% Formic acid. Precursor scans for fragments with m/z 184^+ and 193^+ following collision induced dissociation were used to identify phospholipid species containing naturally occurring and deuterium labelled choline respectively. The instrument configuration is described in Table 2-4. Mass spectra were analysed with MassLynx™, and with custom-made departmental software as described in section 2.6

Instrument conditions	Value
Capillary energy	2.8KV
Cone energy	30V
Source temperature	150°C
Desolvation gas flow	300L/Hr
Desolvation temperature	70°C

Table 2-4: Experimental conditions of mass spectrometer

2.5.2 Nitric oxide metabolite profiling

Stable metabolites of NO (nitrate and nitrite) were measured with a combination of High Performance Liquid Chromatography (HPLC) and the Griess reaction. Concentrations of NO metabolites were measured by absorbance at 540nm with spectrophotometry, after generating a standard calibration curve.

Sodium nitrate and sodium nitrite were dissolved in ultrapure water to create 59µM nitrate and 58µM nitrite stock solutions, and were used to calibrate a standard curve following sequential dilution; concentrations for the standard curve ranged from 1µM to 58/59µM.

100µL of plasma was dissolved in 100µL of methanol, vortexed for 1 minute before centrifugation, and the supernatant was transferred into vials. Samples were injected into the ENO-20 NOx analyser where nitrates and nitrites were separated with high performance liquid chromatography. Nitrites react with the acidified Griess reagent to generate diazo compounds which are detected by absorbance at 540nm using spectrophotometry. Nitrates were converted to nitrites with a cadmium/copper alloy-based reduction column, before detection with the same Griess reagent spectrophotometry technique.

2.5.3 Measurement of free thiols

Free thiols were detected by absorbance at 412nm with spectrophotometry following the addition of dithionitrobenzoic acid (DTNB) to plasma that had been diluted 1:4 with a 0.1M Tris buffer. Concentrations were calculated against a calibration curve using an L-cysteine standard solution, and corrected for plasma protein concentrations.

A 10mM stock solution of cysteine (10ml of 0.1M Tris 10mM EDTA pH 8.2 buffer) was used to calibrate a standard curve following sequential dilution; concentrations for the standard curve ranged from 0.976µM to 10000µM. 75µL of plasma was mixed with 225µL of the 0.1M Tris pH 8.2 buffer and 90µL of the prepared samples were placed in triplicate into a 96 well plate; the corrected absorbance at 412nm was calculated by subtracting the absorbance at 630nm.

20µL of 1.9nM DTNB in a phosphate buffer (pH 7) was incubated with the samples on a plate shaker in the dark at room temperature for 20 minutes before absorbance was measured again; the corrected absorbance at 412nm was again calculated by subtracting the absorbance at 630nm. Values were averaged across the triplicated readings, and the concentration of free thiols was determined by comparing the difference in pre- and post-DTNB incubation corrected absorbance

against the values generated from the standard curves. Free thiols were corrected for the total protein concentration in the plasma.

2.5.4 Immunohistochemistry imaging

Prior to immunofluorescence staining, 5µm thick paraffin wax sections of Lung tissue were dewaxed and rehydrated in water. Antigen retrieval was carried out by immersing the slides in 0.1M citrate buffer (pH 6), microwaved for 10 minutes, and then left in the same buffer for a further 20 minutes before washing with water. To reduce non-specific binding, the slides were incubated with 3% bovine serum albumin (BSA) for 30 minutes. Specimens were then incubated overnight in a moist chamber with antibodies against Hemoxygenase 1 (HO-1-mouse monoclonal), Nitrotyrosine (NT-mouse monoclonal), and 4-hydroxynoneal (4-HNE-rabbit polyclonal); all antibodies were used at 1:50 dilution, except for HO-1 which was used at 1:100 dilution. Negative controls were incubated with 3% BSA in phosphate buffered saline (PBS). After thorough washing, all the specimens were incubated with secondary antibodies, goat anti rabbit Alexa Fluor 594 for rabbit polyclonals and goat anti-mouse Alexa Fluor 594 for HO1 and NT for 1 hour at 1:1000 dilution. After washing several times with PBS, sections were incubated with DAPI (4',6-diamidino-2-phenylindole - 1:20000) and specimens were mounted using Permafluor. Stained sections were imaged with confocal microscopy. Quality control was ensured by regularly inspecting negative controls for staining with the secondary antibody.

2.6 Data

2.6.1 Clinical data

Routinely collected clinical data were recorded from the medical records for correlation with biological samples. Clinical data were directly transcribed from the Trust IT systems into the study database, with a focus on perioperative oxygenation, length of stay, and postoperative graft function. The diagnosis of primary graft dysfunction was made using The International Society for

Heart and Lung Transplantation 2005 Diagnostic criteria[2], which was based on the P/F ratio, the presence of diffuse interstitial infiltrates on chest radiographs, with the absence of an alternative diagnosis (such as pneumonia or heart failure). Chest radiographs were reviewed for the presence of diffuse infiltrates and alternative diagnoses by a blinded consultant radiologist with experience in thoracic transplantation; a sample data entry form can be found in appendix 7.9.

Clinical data	Notes
	Recipient data
Study number	Link-anonymised identification number
Recipient diagnosis	Primary indication for transplantation
Use of cardiopulmonary bypass	Off pump or with cardiopulmonary bypass
Surgical access	Clam-shell or minimally invasive surgery
Use of ex vivo reconditioning	Either EVLP or OCS
Duration of surgery	Time in minutes
Complications	e.g. need for surgical re-exploration
Use of tracheostomy	To facilitate weaning from ventilation
Blood gases	Taken at the time of lipidomic sampling
Ventilator settings	At the time of blood gases; mode, airway pressures, FiO_2
Duration of ventilation	Time in hours
Length of stay (LOS)	Values for both ICU and Hospital LOS
Spirometry	FEV_1 and FVC, absolute and percentage of predicted values
Date of death (if applicable)	During 1-year of follow-up
	Donor and recipient data
Age	On the date of transplantation
Height/weight	Values for both donor and recipient
Gender	Values for both donor and recipient
	Donor data
Mode of donor death	Cardiac or brain-stem death. Value for donor

Table 2-5: Clinical data collection
A summary of clinical data that was collected, with supporting notes. All data refers to recipient variables, unless specified otherwise. EVLP – Ex vivo lung perfusion. OCS – Organ Care System. LOS – Length of stay. ICU – Intensive Care Unit. FEV1 – Forced Expiratory Volume in 1 second. FVC – Forced Vital Capacity

Study files (such as case report forms and consent forms) were stored in a locked and secure office that were only accessible to NHS Trust research staff. Participants were link-anonymised on

enrolment, and were allocated a unique study number which could be traced to their hospital number. The document of this linkage was only accessible to named research staff members. The research was conducted to ensure the confidentiality of personal data, in compliance with the Data Protection Act 1998[262] and the NHS Code of Confidentiality[263]. All identifiable data were treated in accordance with the Caldicott Principles.

2.6.2 Processing of mass spectra

Mass spectra were initially generated with MassLynx™ version 4.0 (Waters, Milford, MA-USA). Mass spectra were processed and analysed with software developed in the Southampton Centre for Biomedical Research as previously described[137], which included corrections for adduct formation and natural isotope distribution before lipid identification and quantification against internal standards. This software was written by Dr Grielof Koster (Southampton Biomedical Research Unit) in Visual Basic and run in Excel. In summary the first step of the software extracted the raw data from the summation of peak areas, before correcting for background noise for all mass spectra. The second step of the analysis assigned a phospholipid species identity based on the m/z (mass/charge ratio) for every peak. Phospholipid identification included automatic corrections for the distribution of naturally occurring isotopes (like ^{13}C) and adduct formation following ionisation (i.e. the combination of sodium with the ion of interest). Internal standards were subtracted from all spectra, and used to quantify phospholipid concentrations. Phospholipids are then presented as their concentration, their relative abundance, and their deuterium label incorporation.

2.6.3 Immunohistochemistry image analyses

Five images at 20x magnification were assessed as a set for each biopsy, and all images were reviewed for quality control by a blinded assessor. Specifically, images were reviewed for adequate exposure, technical failures, and artefact; the author was blinded to the origins of the images during this process. Technically inadequate images were removed from the study. Biopsy image sets were excluded from analysis if there were fewer than 3 images available for any one tissue sample.

The area of tissue staining with the primary antibody was calculated automatically using a bespoke algorithm that was written in ImageJ macro script. Images were analysed using the FIJI distribution of ImageJ v1.51n (Wayne Rasband, National Institute for Health - USA). Immunofluorescence images were automatically split into images masks that either represented signals generated by the primary antibody or the tissue as a whole, using an approach that has been adapted from the principles described by Kaczmarek[264]. The key steps processed by our bespoke macro included: 1) calculation of the percentage area of the biopsy area that stained with the primary antibody, 2) correction for auto fluorescence using digital image subtraction, 3) adjustment for the total area in the image field containing any stainable tissue as opposed to air in the alveolar space. Automatically generated values were averaged across the set of 5 images for each biopsy (median), before un-blinding of the data and subsequent analysis.

2.6.4 Statistical analyses

All data were stored in Excel spreadsheets and Access databases. Basic statistical analyses were performed with IBM SPSS Statistics, and topological data analyses were performed with the Ayasdi Workbench Platform.

2.6.4.1 Data presentation and analysis

Unless otherwise stated, data were presented as the median and interquartile range, and analysed using the appropriate non-parametric test. When a normal distribution has been confirmed, the use of the relevant parametric tests is highlighted accordingly. Two-tailed p-values are reported throughout, with values of <0.05 considered as statistically significant.

Methods designed to control the family wise error rate (such as the Bonferroni method) would result in an unacceptably high rate of type 2 errors, and are inappropriate for this kind of dataset [265,266]. Calculation of the false discovery rate is also inappropriate for this form of pilot dataset, as additional candidate phospholipid species will not incur additional time, labour, or other costs with subsequent studies based on an ESI-MS/MS platform[267]; on that basis, the unadjusted raw data of all candidate species is presented[265,266].

3 Results: Phospholipid profiling in clinical lung transplantation

3.1 Introduction

3.1.1 Background

90% of pulmonary surfactant is comprised of a diverse mixture of phospholipids[101]. Clinical lung transplantation is associated with postoperative surfactant dysfunction that could contribute to primary graft dysfunction (PGD) after surgery[5], yet the underlying phospholipid biology has not been studied in this cohort[173]. Kennedy's CDP-choline pathway is the source of most de novo synthesis of phosphatidylcholine (PC), which is the predominant class of phospholipid species in surfactant[101,126]. Dipalmitoyl phosphatidylcholine (DPPC, also referred to as PC16:0/16:0) is the most abundant and surface active of all the PC species in pulmonary surfactant, and DPPC disruption has been associated with acute lung injury in the general (non-transplant) ICU population[25,137]. Changes in PC kinetics have been associated with acute lung injury in the general population[137], and therefore investigation of DPPC flux in perioperative lung transplantation is a logical step for a cohort at risk of developing acute lung injury following ischemia and reperfusion[2].

Perioperative changes in DPPC after lung transplantation were investigated using electrospray ionisation tandem mass spectrometry (ESI-MS/MS) combined with stable isotope labelling of Kennedy's CDP-choline pathway to assess PC flux in this unique cohort[7,251]. ESI-MS/MS has the ability measure to large numbers of unique lipid species, offering the opportunity for a comprehensive characterisation of the perioperative lipidome following lung transplantation. In addition to the focussed analyses of airway DPPC, other phospholipid species have also been screened for potential future investigation. Large datasets provide unique challenges, and so a novel topological data analysis platform was applied to explore the temporal changes in the lipidome of transplant recipients[268].

Study of phospholipid biology immediately after lung transplantation could provide insights into the pathogenesis of Primary Graft Dysfunction (PGD) after lung transplantation, identity novel therapeutic targets, and could be extrapolated into the wider population with acute lung injury[184].

3.1.2 Hypothesis

Lung transplantation is associated with changes in pulmonary surfactant phospholipid metabolism, which in turn affects clinical outcomes.

3.1.3 Chapter objectives

- Describe the study cohort
- Report the immediate perioperative impact on the lipidome of transplant recipients
- Present a case report of extended serial measurements in airway DPPC for a lung transplant recipient with a prolonged ICU stay
- Explore perioperative phospholipid kinetics in transplant recipients through stable isotope labelling of the Kennedy pathway
- Explore the association between perioperative changes in phospholipids and subsequent clinical outcomes after lung transplantation

3.2 Methods

Patients were recruited from the lung transplant waiting list. Blind bronchial aspirates and plasma, samples were collected, processed stored as described in Table 3-1, and in sections 2.2.4 & 2.2.6.

	On waiting list	Start of surgery	Completion of surgery	1-3 hrs	6 hrs	12 hrs	24 hrs	Then daily, until free from ventilation
Screening & invitation	☑							
Informed consent	☑							
Blood		☑	☑		☑	☑	☑	☑
Lung biopsies		☑	☑					
Blind bronchial aspirates		☑	☑		☑	☑	☑	☑
D9-Choline infusion*				☑				

Table 3-1: Study timetable
Timetable of events for all patients enrolled onto the study. Note that the first ten patients did not receive Methyl-D9 choline chloride. ivi- Intravenous infusion.

Anonymised clinical data were stored in a study database. Lipids were extracted using the modified Bligh & Dyer method, prior to analysis with ESI-MS/MS (see section 2.5.1). Samples were scanned for precursors of m/z 184 and m/z 193 (P184 and P193 respectively), and this chapter will deal exclusively with phospholipids that are detectable on those precursor scans. Mass spectra were processed and analysed with our departmental software, which included corrections for adduct formation, natural isotope distribution and subsequent identification and quantification of lipid species from their m/z ratio. Following the identification of lipid species, analyses were performed with Microsoft Excel and IBM SPSS. Topological analyses were performed using the Ayasdi Machine Intelligence Platform – see section 2.6.

3.3 Results

3.3.1 Clinical characteristics of the study cohort

3.3.1.1 Patient recruitment

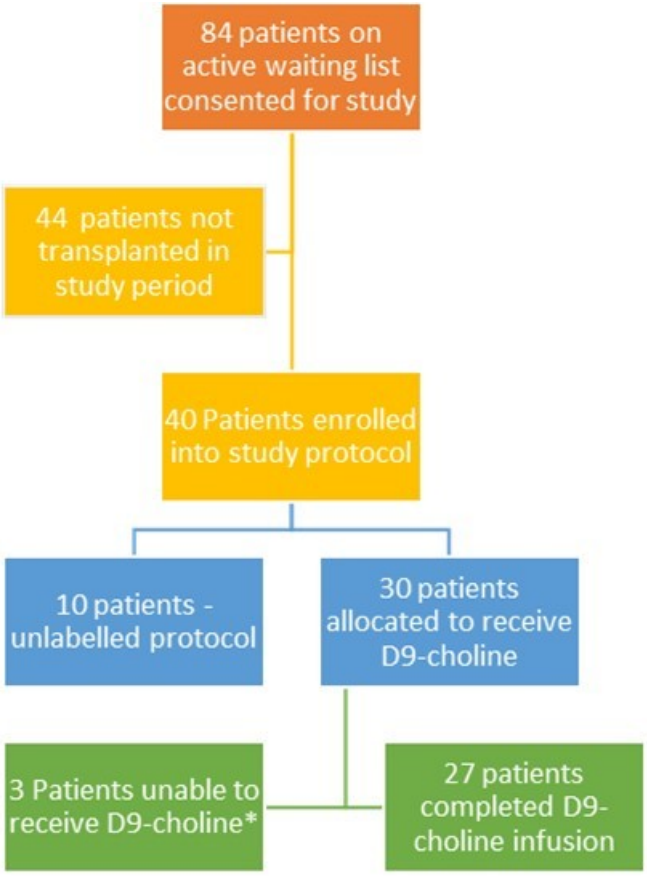


Figure 3-1: Patient recruitment summary
*Flow chart of study patients. *1 patient suffered catastrophic postoperative bleeding, D9-choline infusion was stopped before completion to allow ongoing therapy as patient was returned to theatre. D9-choline was not available for 2 patients who would have otherwise been allocated for deuterium labelling.*

There were no significant adverse events that were related to any of the study procedures. The first 10 patients of the study were a pilot group that were studied without the deuterium label, to ensure the adequacy of sampling techniques to obtain the necessary lipidomic data, prior to the start of the deuterium labelling experiment. After lipidomic profiling was confirmed in the first 10 patients to be feasible, administration of the deuterated choline label commenced for the remainder of the cohort.

3.3.1.2 Patient characteristics

A basic summary of the study cohort's key clinical characteristics is presented in Table 3-2.

Recipient indication for transplantation	
Alpha 1 antitrypsin deficiency (n)	5
COPD (n)	13
CF (n)	10
Non-CF Bronchiectasis (n)	3
Diffuse interstitial lung disease (n)	7
Failure of previous lung transplant (n)	2
Operative details	
Ex vivo organ support (n)	5
Need for cardiopulmonary bypass	
On-pump (n)	16
Off-pump (n)	24
Surgical approach	
Clam-shell (n)	25
Minimally invasive (n)	15
Duration of surgery (minutes)	320 (272.5 - 427.5)
Surgical re-exploration (n)	4
Postoperative recovery	
Duration of postoperative mechanical ventilation (hours)	44.5 (18.25 - 510)
Recipients receiving a tracheostomy (n)	13
Intensive care unit LOS (days)	5.5 (3 - 22)
Hospital LOS (days)	31.5 (20 - 54.5)
Deaths within the first year after surgery (n)	5

Table 3-2: Summary of the clinical characteristics of the study cohort
Median values are reported (interquartile range). COPD – Chronic obstructive pulmonary disease. CF – Cystic fibrosis. LOS – Length of stay

Our previously published data reported an average duration of mechanical ventilation after transplantation of approximately 3 days[56]. The average duration of mechanical ventilation in the study cohort was under 2 days, which reduces the number of subsequent samples collected from each patient and affects the lipidomic data presented in section 3.3.2.. A shorter duration of mechanical ventilation curtails serial sample collection from the lower respiratory tract following extubation. This limits the assessment of deuterium incorporation and the ability to comment on

temporal changes in phospholipid profiles after lung transplantation; these issues are illustrated and later discussed in sections 3.3.2.4, 3.3.2.5, and 3.4.3. Study cohort mortality was in keeping with figures available from international registry data (section 3.4.2)[22].

3.3.1.3 Postoperative graft function – P/F ratio

In the early perioperative period (the first 3 days), primary graft dysfunction has been reported in accordance with the ISHLT guidelines[2]. The severity of PGD, is largely based on arterial blood gas criteria, using the ratio of the arterial oxygen tension and fraction of inspired oxygen (P/F ratio – summarised in section 1.1.1). The presence of PGD grades 1-3 is described for each of the first three days after surgery in Figure 3-2.

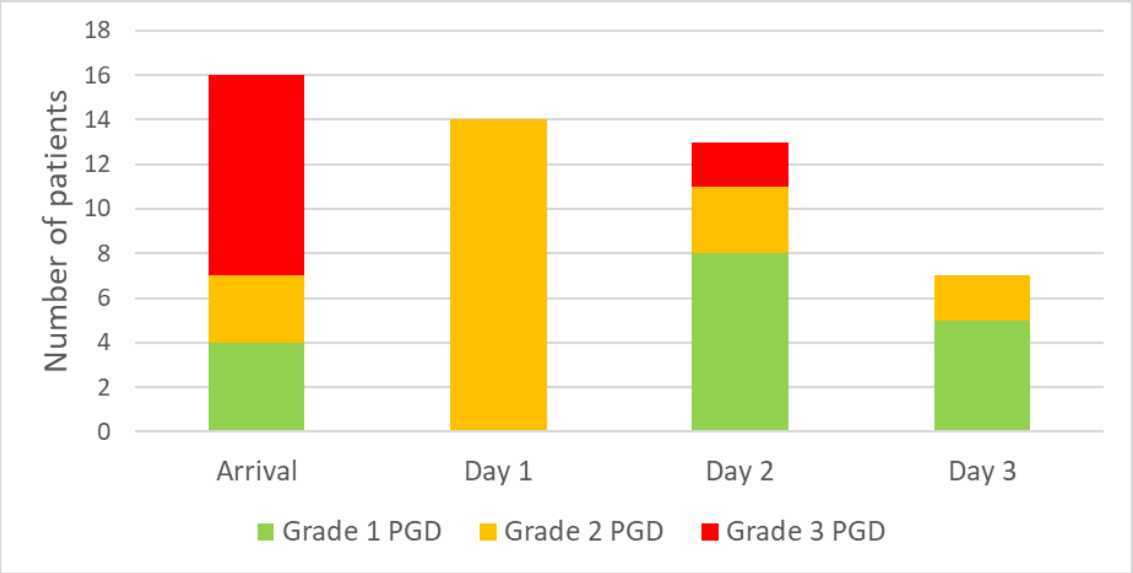


Figure 3-2: Postoperative primary graft dysfunction
The number of study patients with Primary Graft Dysfunction in the first 3 days after surgery. The criteria for diagnosis are based on the chest radiograph, combined with blood gas and clinical parameters, as described elsewhere. The first bloods gas was sampled on arrival at the Intensive Care unit immediately after the conclusion of surgery (Arrival, day 0). The blood gas with the lowest p/f ratio

Prevalence of PGD decreased over time, with the most severe PGD primarily occurring in the first 24 hours of arrival on the ICU. 23% of patients (9 patients) developed severe Grade 3 PGD during the first three days after surgery, compared to an incidence of 35% in our previously published reports (appendix 7.2)[56].

3.3.1.4 *Postoperative graft function - Spirometry*

Pulmonary function was measured over first year after surgery, at routine postoperative follow-up appointments (summarised in Figure 3-3).

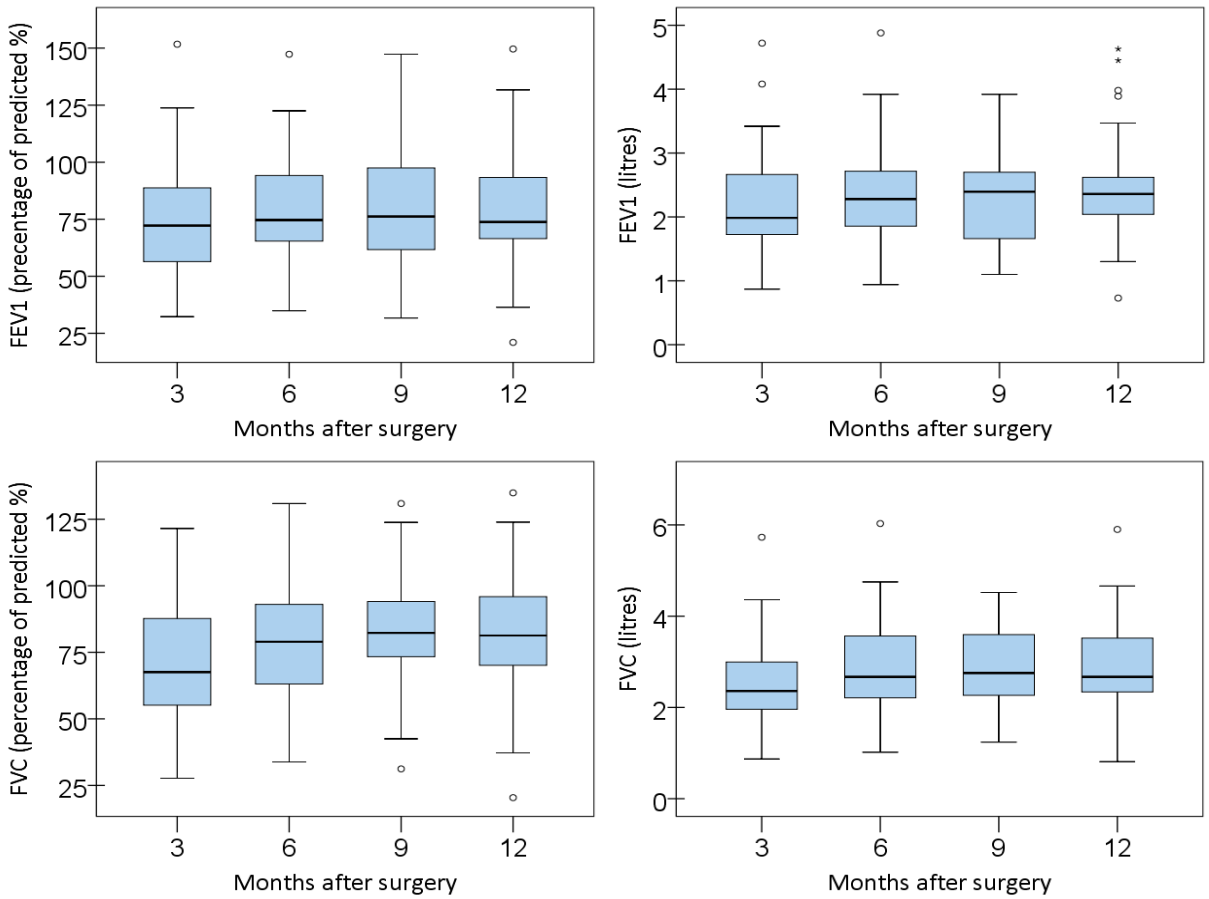


Figure 3-3: Pulmonary function for the whole study cohort after lung transplantation
Box and whisker plot, demonstrating median, interquartile range, and range. FEV_1 after surgery expressed as a percentage of the recipients predicted values (panel A), and in litres (panel B). FVC after surgery expressed as a percentage of the recipients predicted values (panel C), and in litres (panel D)

On average, patients achieved 72% of their predicted FEV_1 at 3 months following surgery. FEV_1 remained relatively stable at 6, 9 and 12 months after surgery, with an average predicted FEV_1 of 75%, 76%, and 74% respectively, with similar trends observed for the FVC -Figure 3-3.

3.3.1.5 *Key findings - patient characteristics*

- Mortality for the study cohort was similar to established registry data.
- The incidence of PGD and duration of mechanical ventilation were lower when compared to a historical cohort.

3.3.2 Mass spectrometry data

3.3.2.1 Typical examples of spectra generated from precursor scans from samples taken from lung transplant recipients after surgery.

Typical spectra that have been generated from the plasma and airways are shown below. Peaks at 678 correspond to the internal standard that was added to each sample prior to lipid extraction.

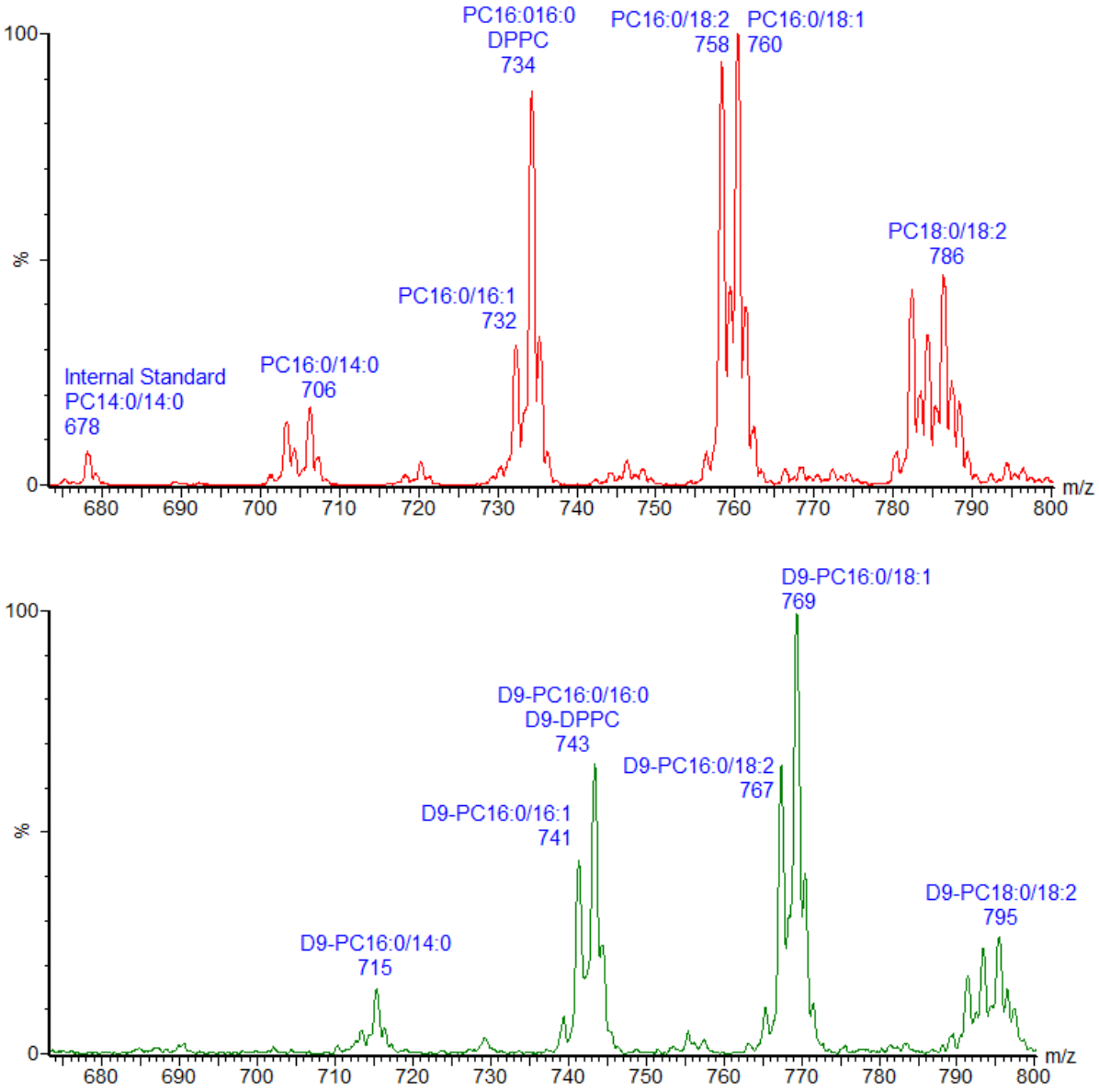


Figure 3-4: Typical spectra from airway samples
Typical spectra generated from bronchial washings of a lung transplant recipient who has received the D9-choline label. Top panel - Precursor 184 scan, bottom panel - Precursor 193 scan. Y-axis: Signal intensity (%), normalized at 100% to the most abundant ion. X-axis: mass charge ratio (m/z)

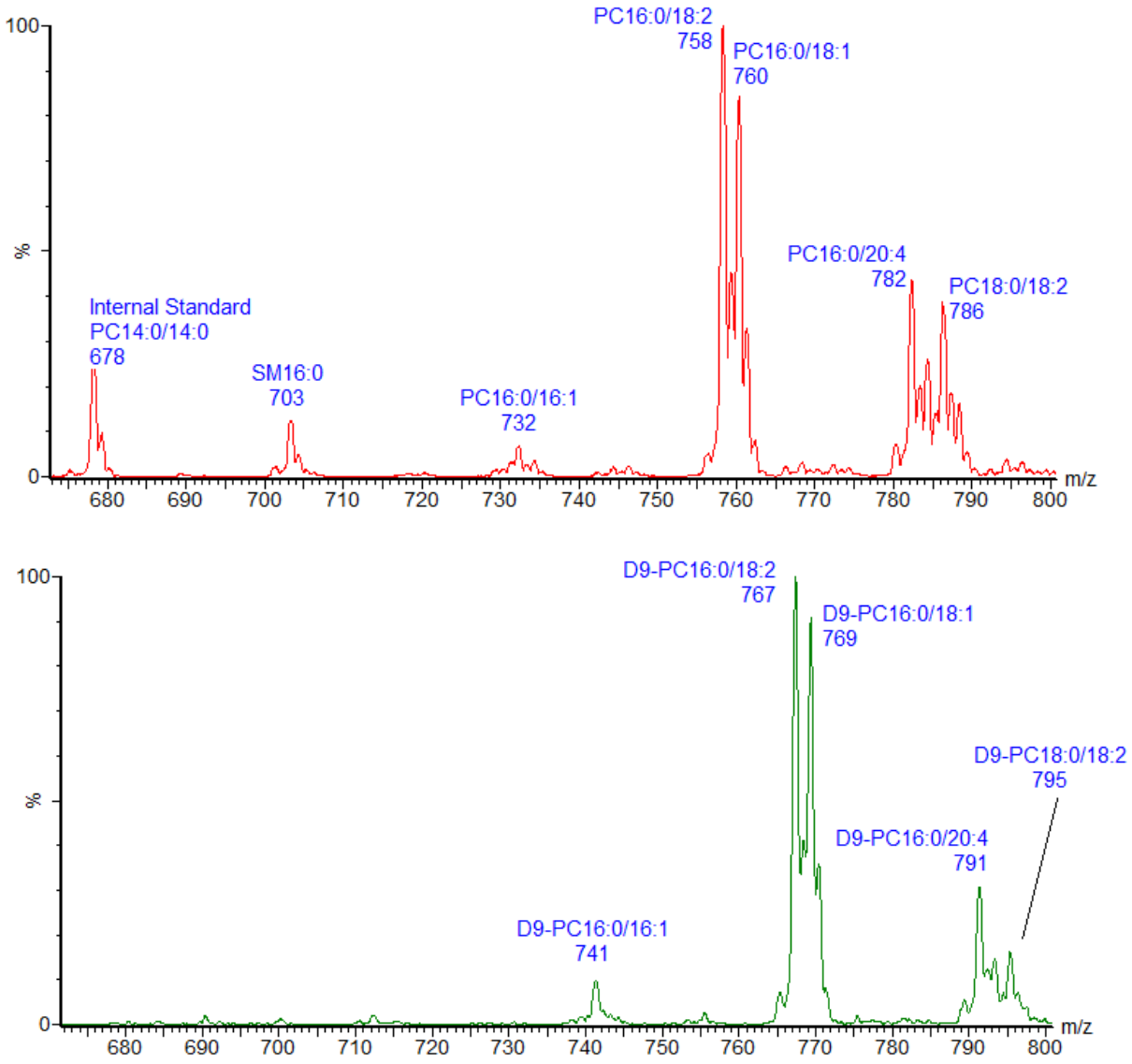


Figure 3-5: Typical spectra from plasma samples
Typical spectra generated from the plasma of a lung transplant recipient who has received the D9-choline label. Top panel - Precursor 184 scan, bottom panel - Precursor 193 scan. Y-axis: Signal intensity (%), normalized at 100% to the most abundant ion. X-axis: mass charge ratio (m/z)

Note the lack of saturated PC in the mass spectra from the plasma sample, when compared to the spectra generated from airway samples.

These spectra illustrate the detection of phospholipids that have been synthesised from the labelled D9-choline. Phospholipids synthesised from D9-choline have an m/z that is 9 greater than phospholipids synthesised from naturally occurring choline.

To put the data into context and aid in the interpretation, typical spectra from the BAL of healthy volunteers are illustrated in the following figure (Figure 3-6 - reproduced from earlier work conducted by Prof Postle's group)[137].

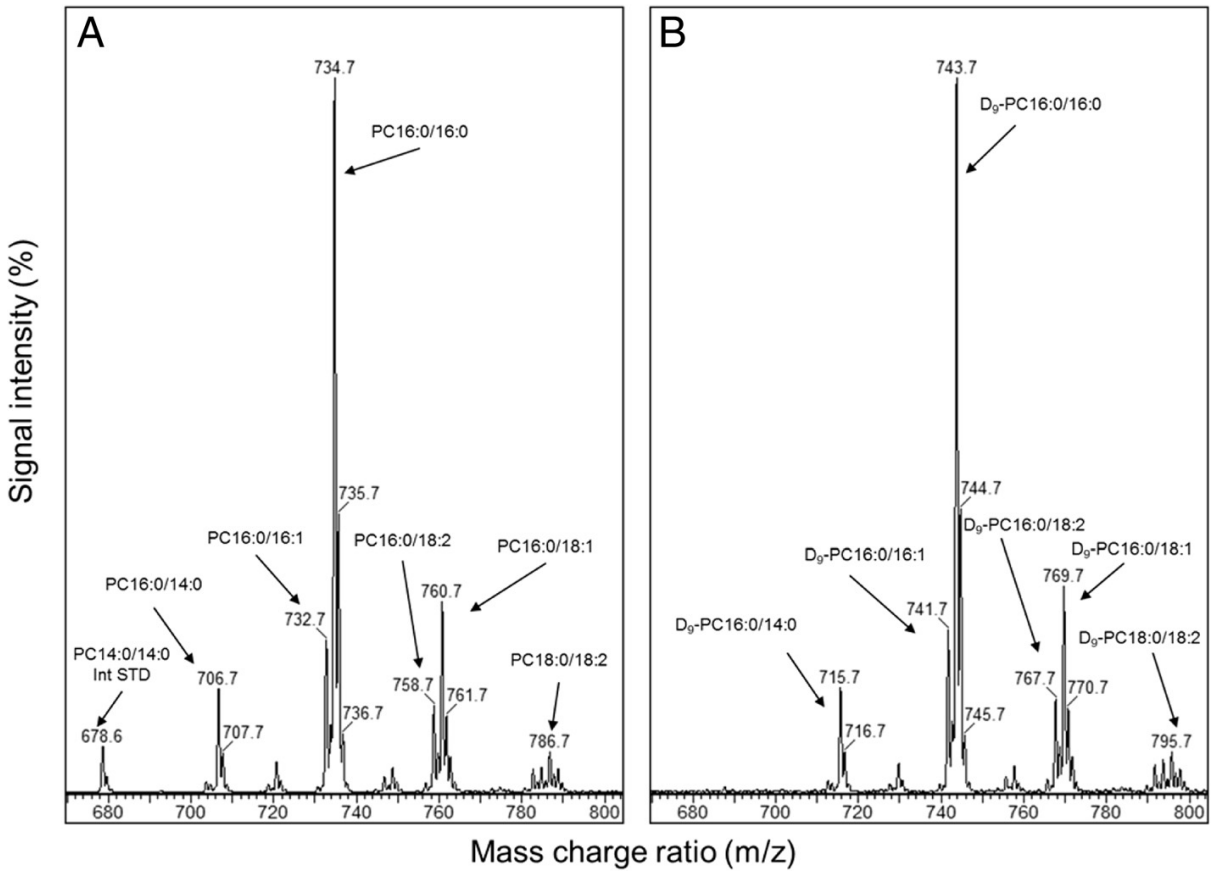


Figure 3-6: Mass spectra of the bronchoalveolar lavage from a healthy volunteer Panel A: precursor scan P184, Panel B: precursor scan P193. Y-axis: Signal intensity (%), normalized at 100% to the most abundant ion. X-axis: mass charge ratio (m/z). Reproduced with permission, from earlier work conducted by Prof. Postle's group, originally published by BioMed Central under Open Access[137].

Examining the spectra generated from the airways of a transplant recipient in comparison with the typical spectra from the airways of a healthy volunteer reveals obvious differences. There is a clear deficiency in the peaks at 734 (corresponding to DPPC) in the transplant recipient, with a similar deficiency seen in the peak at 743 on the matching P193 scan (corresponding to D9-labelled DPPC). This reduction in the relative abundance of DDPC is highlighted again across the whole study cohort in the following section (3.3.2.2).

3.3.2.1.1 Key findings – typical mass spectra

- Examples are provided of the mass spectra generated from the airways and the plasma
- Newly synthesised PC were identified by deuterium labelling

3.3.2.2 Perioperative phospholipid composition in lung transplant recipients –Airways

The relative abundance of the major species is shown from washings taken immediately after surgery Figure 3-7. As expected, PC16:0:16:0 was the most abundant species in the bronchial washings. However, there were two striking abnormalities:

1) There was a marked reduction in the relative abundance in airway DPPC after transplantation when compared to values from healthy volunteers (DPPC typically accounts for 40% of all PC content)[137]

2) The abundance of DPPC was highly variable.

This reduction in airway DPPC could also be clinically significant, as it was associated with initial difficulties oxygenating patients after transplantation (see section 3.3.4: Table 3-8).

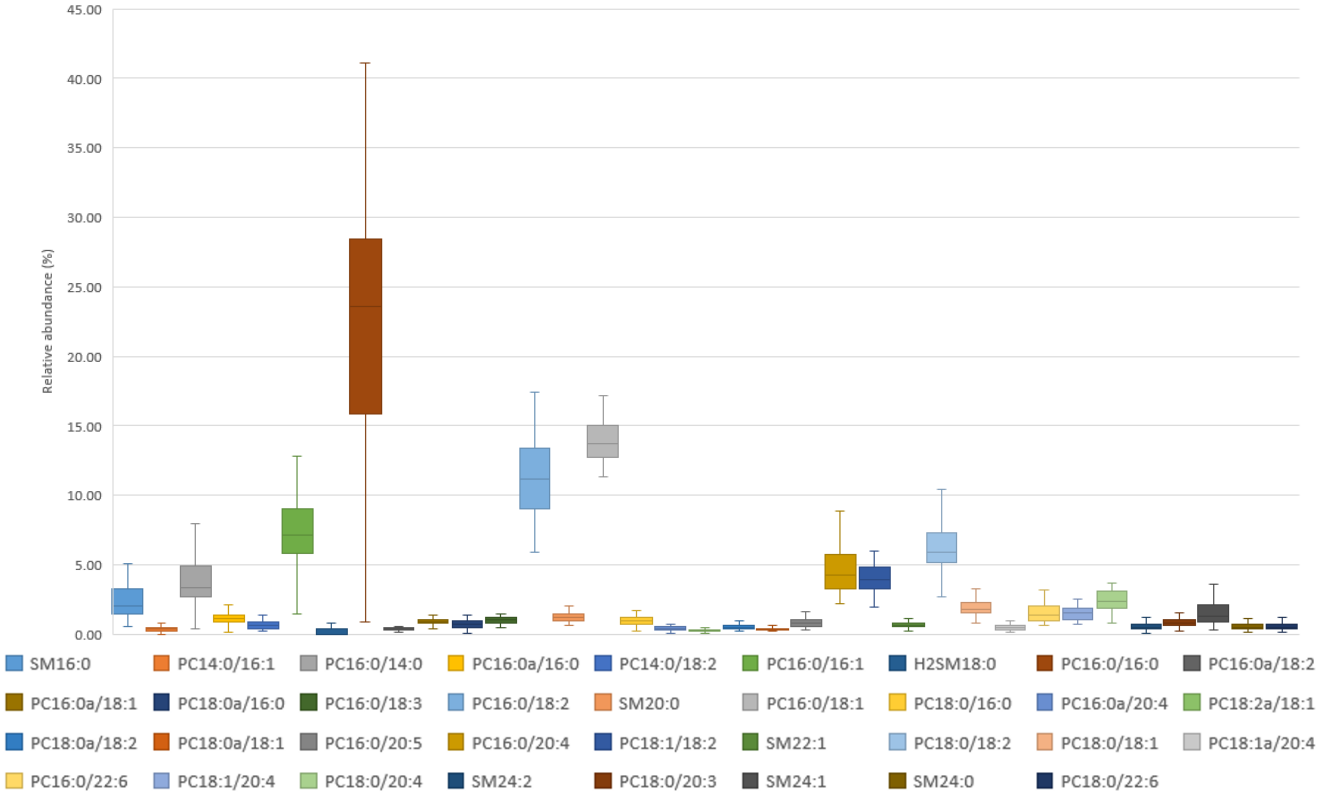


Figure 3-7: Postoperative airway lipid profile – relative abundance
Box and whisker plot. Relative abundance of the phospholipid species detected on a precursor 184 scan in the airways of patients immediately after lung transplantation

Focussing on those deficiencies in airway DPPC, the relative abundance is charted for the whole cohort over time following surgery. There is a continued fall in airway DPPC down to a nadir at day 5, after which levels start to recover slowly. The median abundance of airway DPPC at baseline for all patients was 23.26%, in contrast to a median abundance of 10.76% at 5 days (Mann-Whitney 2-tailed p=0.000).

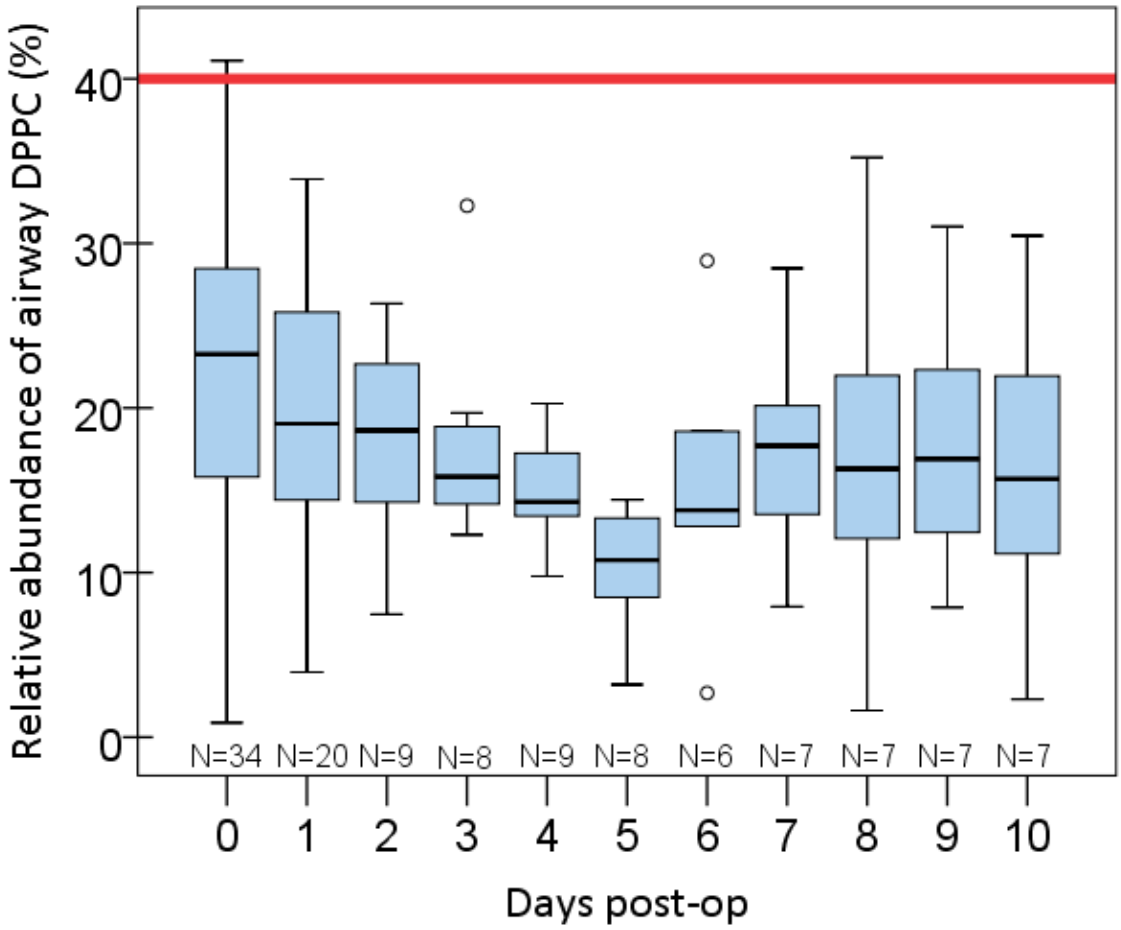


Figure 3-8: Trends in airway DPPC after surgery - whole cohort
Box and whisker plot. Y axis - the median relative abundance of DPPC (PC16:0/16:0), with the range and interquartile ranges. X axis – time after surgery in days (non-linear scale). The relative abundance of airway DPPC is then charted against time after surgery for the whole cohort. The red line illustrates the relative abundance of airway DPPC that is typically seen in healthy volunteers. The number of samples available for analysis at each timepoint is listed along the top of the x-axis. DPPC – Dipalmitoylphosphatidylcholine

It is important to highlight that not all patients who provided samples at baseline remained intubated at 5 days. Given only 8 patients remained ventilated and therefore able to provide samples at 5 days, those patients have been investigated in closer detail. Evaluating the changes over time within these selected patients, we still see that there is a substantial and statistically significant fall in the relative abundance of DPPC from an average of 19.65% at 12 hours following surgery to 10.76% (Figure 3-9: Wilcoxon signed ranks, 2-tailed p=0.017).

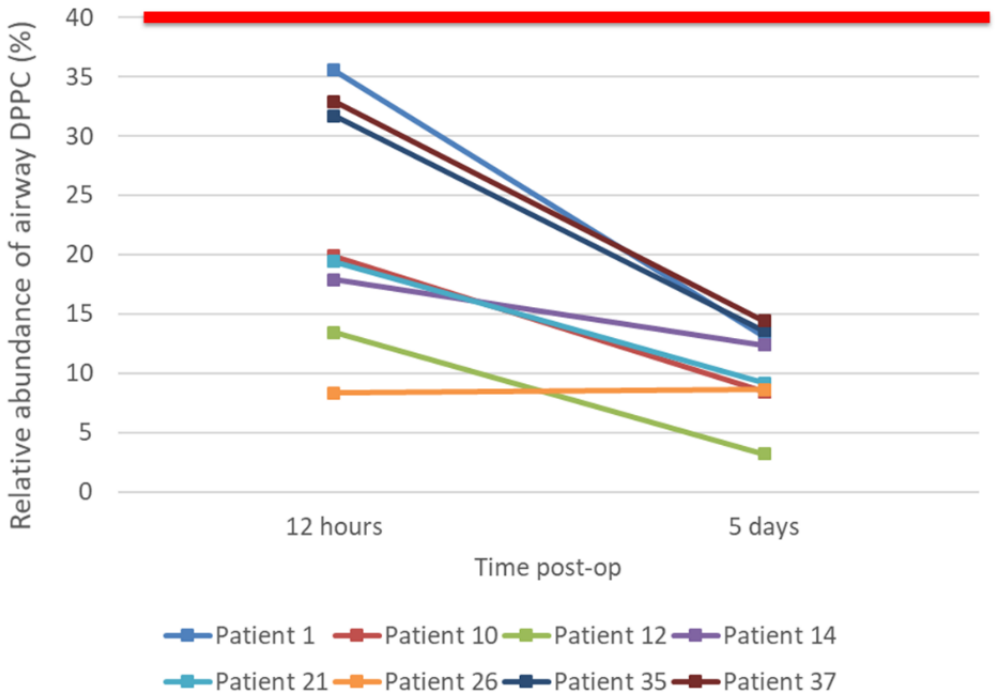


Figure 3-9: Postoperative trends in airway DPPC – patients needing prolonged respiratory support
The relative abundance of DPPC in the sub-group of patients who remained intubated at 5 days (n=8). DPPC levels from 12 hours and 5 days are compared for this sub-group. The red line illustrates the relative abundance of airway DPPC that is typically seen in healthy volunteers.

3.3.2.3 Perioperative phospholipid composition in lung transplant recipients – Plasma

Whilst the measurement of phospholipid concentrations in bronchial washings is limited by the unknown dilution factor associated with the technique, measurements of concentrations are possible for plasma. As it was possible to collect samples before and after transplantation, a comparison of those measurements is also presented.

The preoperative concentrations of the most abundant phospholipids are described in Figure 3-10. The most notable difference between plasma and the bronchial washings is that the plasma was relatively devoid of saturated PC species that were found in the airways, and the plasma has a higher content of PC species containing unsaturated and polyunsaturated fatty acids (PUFA) (as expected).

The species with the highest concentration in the plasma is PC16:0/18:2, followed by PC16:0/18:1, PC18:0/18:2, and then PC16:0/20:4.

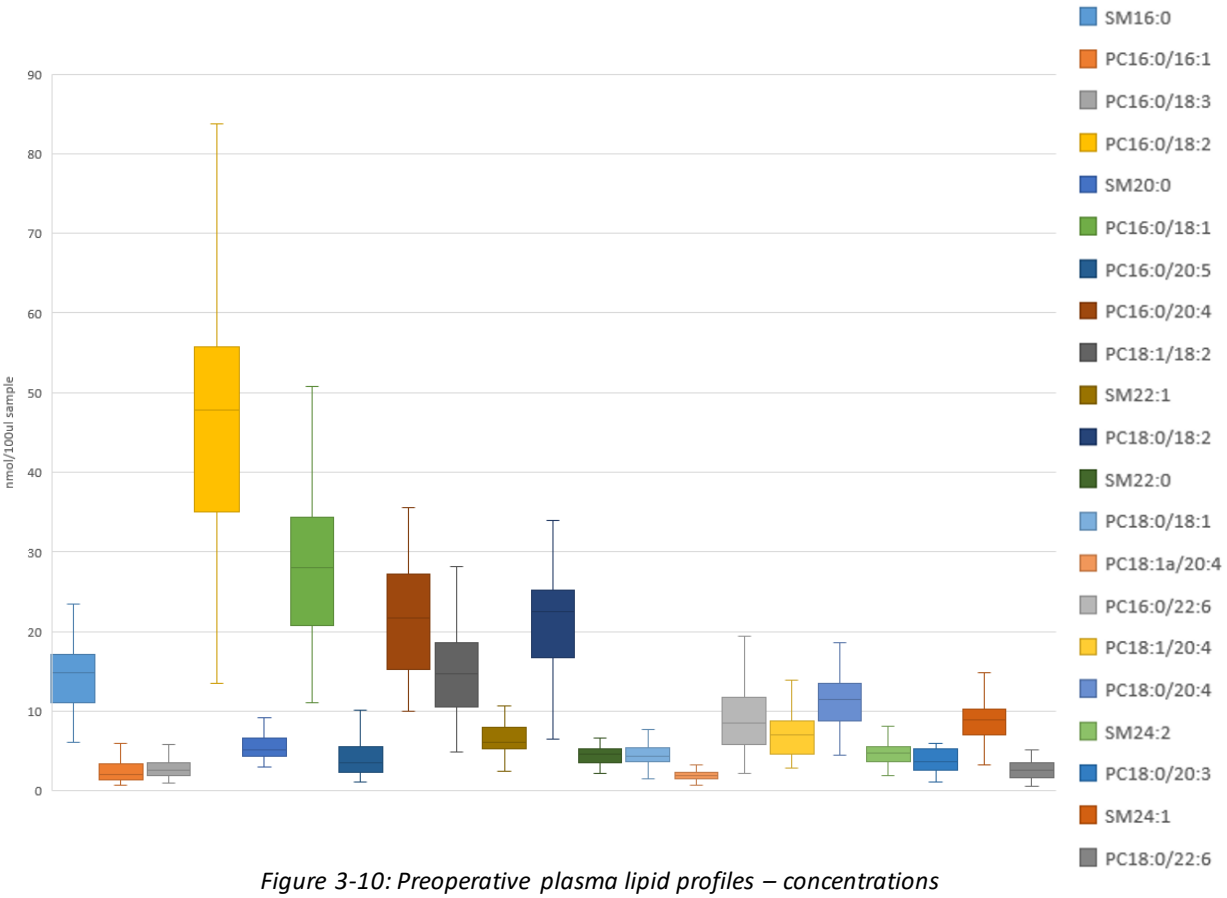


Figure 3-10: Preoperative plasma lipid profiles – concentrations
Box and whisker plot. Concentration of the most abundant phospholipid species detected on a precursor 184 scan on the plasma of patients prior to lung transplantation

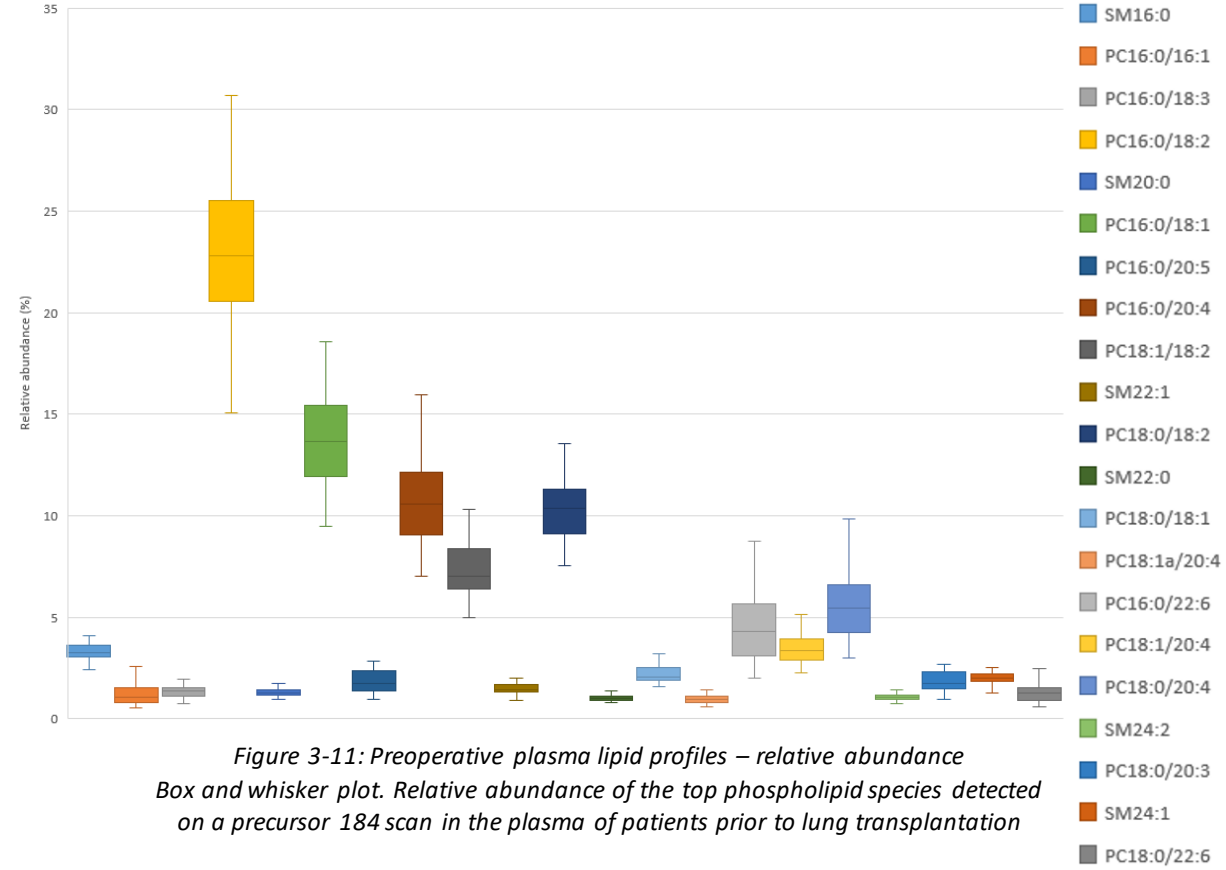


Figure 3-11: Preoperative plasma lipid profiles – relative abundance
Box and whisker plot. Relative abundance of the top phospholipid species detected on a precursor 184 scan in the plasma of patients prior to lung transplantation

Presenting plasma phospholipids as their concentration and their relative abundance shows a comparable distribution of the major species. Again, the species with the greatest relative abundance in the plasma is PC16:0/18:2, followed by PC16:0/18:1, PC18:0/18:2, and then PC16:0/20:4; this pattern was seen both pre- and post-transplant.

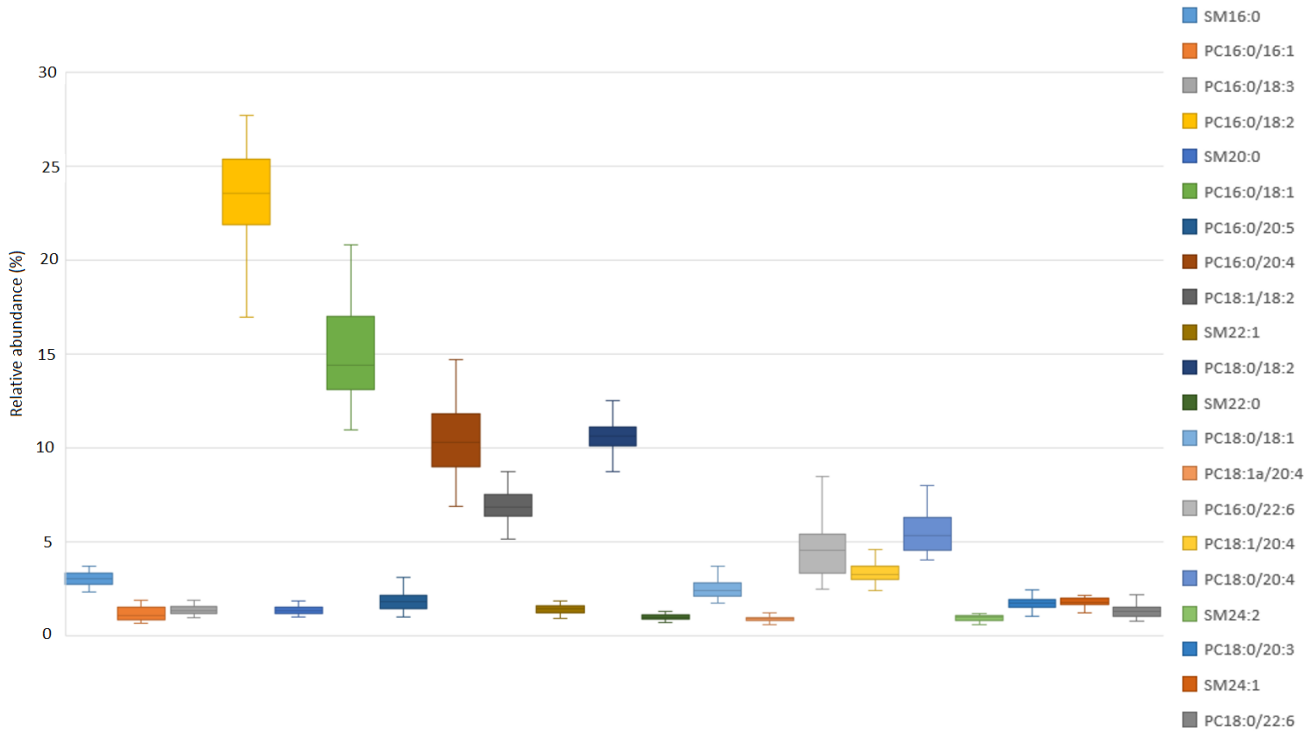


Figure 3-12: Postoperative plasma lipid profile – relative abundance
Box and whisker plot. Relative abundance of the top phospholipid species detected on a precursor 184 scan in the plasma of patients immediately after lung transplantation

A general decrease is seen in the concentration of all the major phospholipids in the plasma during lung transplantation. Figure 3-13 shows the change in concentration in each patient in plasma samples taken immediately after chest closure. PC16:0/18:2 demonstrated the largest intraoperative fall in concentration, followed by PC16:0/18:1, PC18:0/18:2, and then PC16:0/20:4 (i.e. the most abundant species illustrated in Figure 3-10). Whilst there was a generalised reduction in the concentration of all the major plasma phospholipid species, there were only subtle changes in the relative abundances of some phospholipid species that are observed during lung transplantation (Figure 3-14 & Table 3-3).

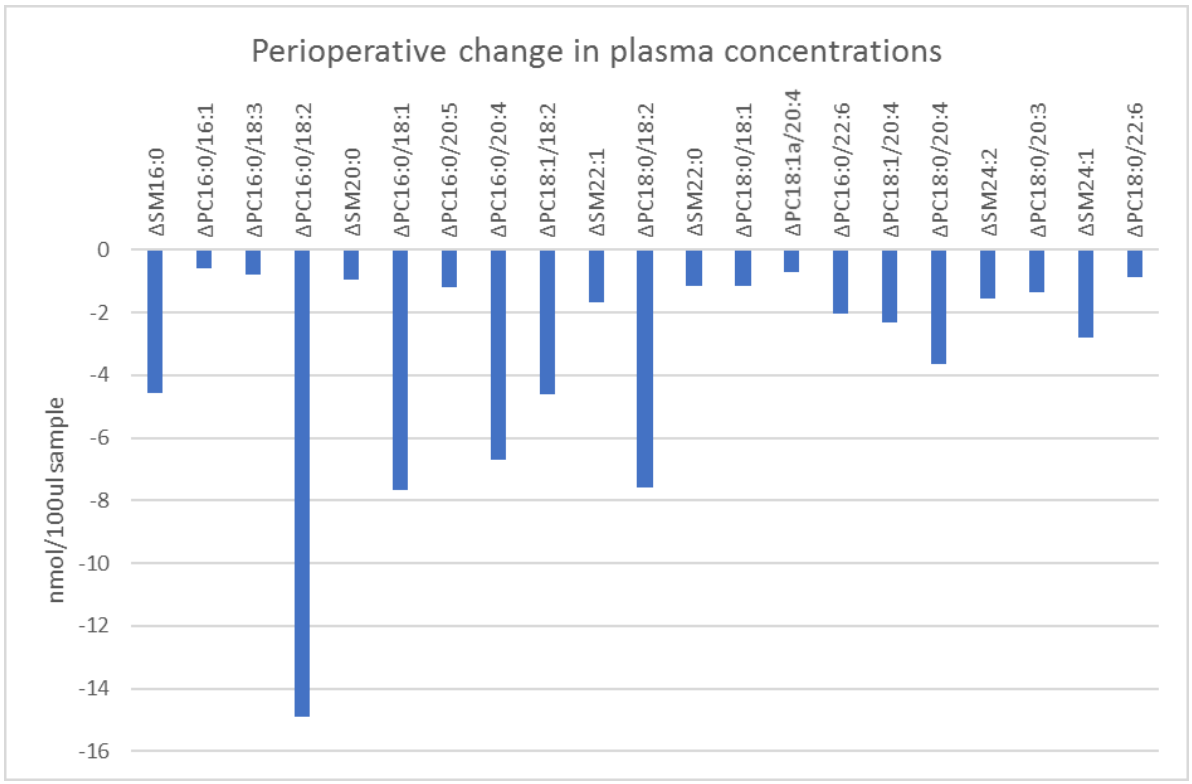


Figure 3-13: Intraoperative changes in plasma lipid concentration
Median change in amounts of each PC species measured before and after transplantation in the plasma

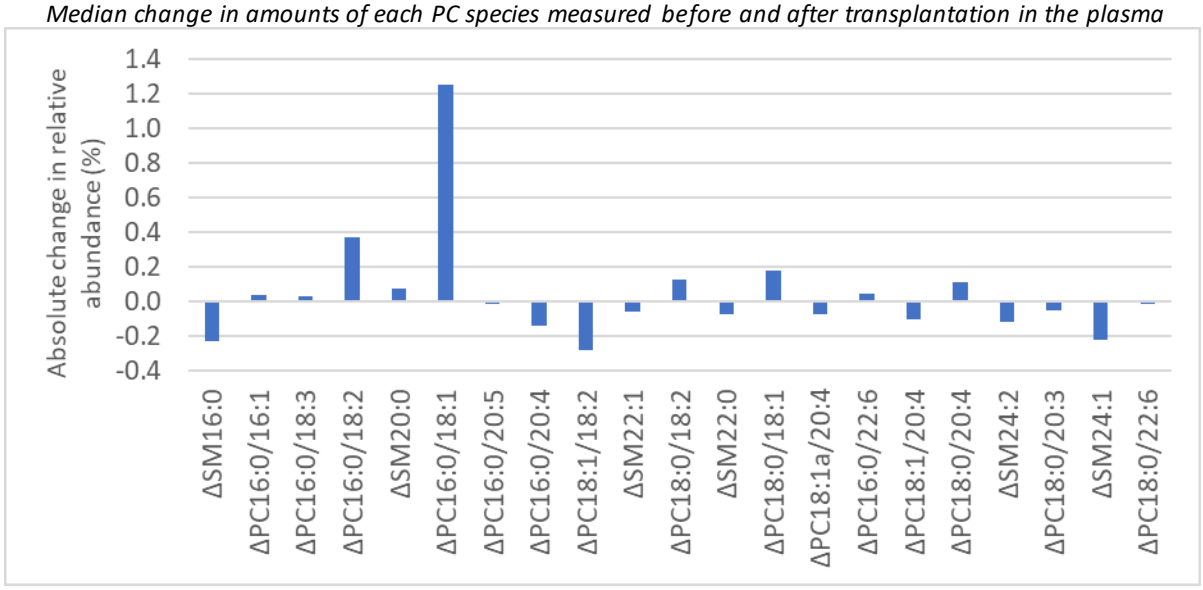


Figure 3-14: Intraoperative change in plasma lipid abundance
Immediate perioperative changes in the relative abundance of the top phospholipid species found in the plasma of lung transplant recipients

Species	Median perioperative change in relative abundance (% abundance)	p-value
ΔSM16:0	-0.230	0.000
ΔPC16:0/16:1	0.035	0.834
ΔPC16:0/18:3	0.033	0.232
ΔPC16:0/18:2	0.373	0.115
ΔSM20:0	0.074	0.013
ΔPC16:0/18:1	1.258	0.000
ΔPC16:0/20:5	-0.013	0.293
ΔPC16:0/20:4	-0.136	0.152
ΔPC18:1/18:2	-0.278	0.004
ΔSM22:1	-0.058	0.023
ΔPC18:0/18:2	0.130	0.786
ΔSM22:0	-0.070	0.025
ΔPC18:0/18:1	0.181	0.000
ΔPC18:1a/20:4	-0.072	0.000
ΔPC16:0/22:6	0.044	0.346
ΔPC18:1/20:4	-0.101	0.025
ΔPC18:0/20:4	0.111	0.550
ΔSM24:2	-0.115	0.000
ΔPC18:0/20:3	-0.054	0.044
ΔSM24:1	-0.220	0.000
ΔPC18:0/22:6	-0.014	0.647

Table 3-3: Intraoperative change in plasma lipid abundance
Median intraoperative changes in relative abundance of plasma lipid species. Wilcoxon signed ranks test with 2-tailed p-values

3.3.2.3.1 Key findings – lipid profiles during lung transplantation

- DPPC was markedly reduced in the airways immediately after lung transplantation
- Levels of airway DPPC continued to fall in those patients who remained intubated for the first 5 days
- The relative abundance of airway DPPC was highly variable
- There was an immediate fall in the plasma concentration of all PC species during lung transplantation
- Plasma contains more unsaturated and polyunsaturated PC species compared to the airways (as expected)
- A description of the lipidome is provided for the plasma and airways of perioperative lung transplant recipients

3.3.2.4 A case report of extended serial lipidomic measurements after transplantation during a prolonged ICU stay

As the average duration of ventilation after transplantation was under two days, most study participants provided fewer than 6 bronchial washings that sampled DPPC content in their airways.

A case study of airway DPPC data is presented from one lung transplant recipient who had a prolonged stay on the ICU. Without corresponding samples at similar time-points from other transplant recipients, comparisons and most statistical analyses are not possible. However, this case report has been presented to highlight the temporal changes in DPPC, to aid subsequent data interpretation and to highlight the challenges of measuring DPPC with bronchial washings.

Patient 12 was a 34-year-old female who underwent bilateral sequential lung transplantation from a brain-dead donor for non-CF bronchiectasis, her postoperative recovery was hindered by the development of Grade 2 PGD, recurrent episodes of chest infections and presumed acute cellular rejection. She died from multi-organ failure on Day 85 after transplantation. Sequential measurements of DPPC are presented for her admission.

3.3.2.4.1 Airway DPPC

When airway DPPC data are presented as an absolute amount (nmol in 500ul samples), there is no discernible pattern - Figure 3-15.

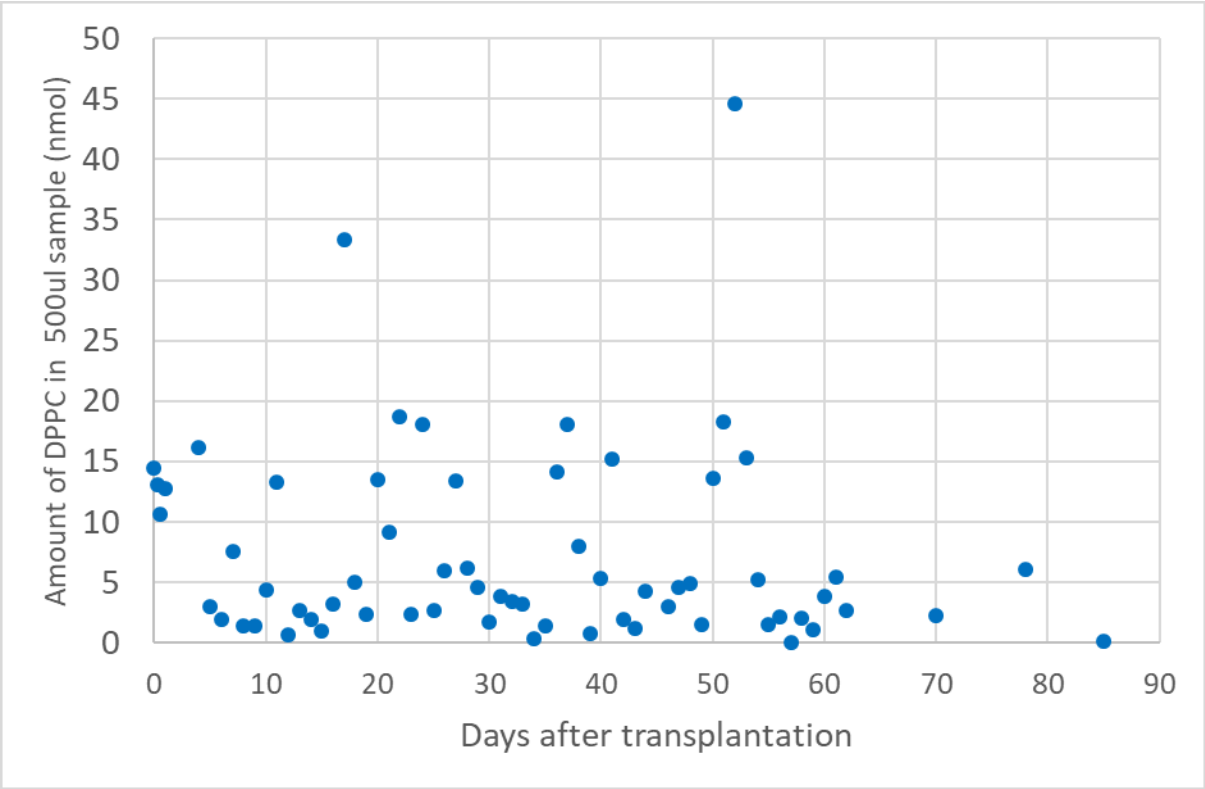


Figure 3-15: Case study – amount of DPPC in bronchial washings
Amount of DPPC in bronchial washings over the duration of one patient's prolonged ICU stay following lung transplantation

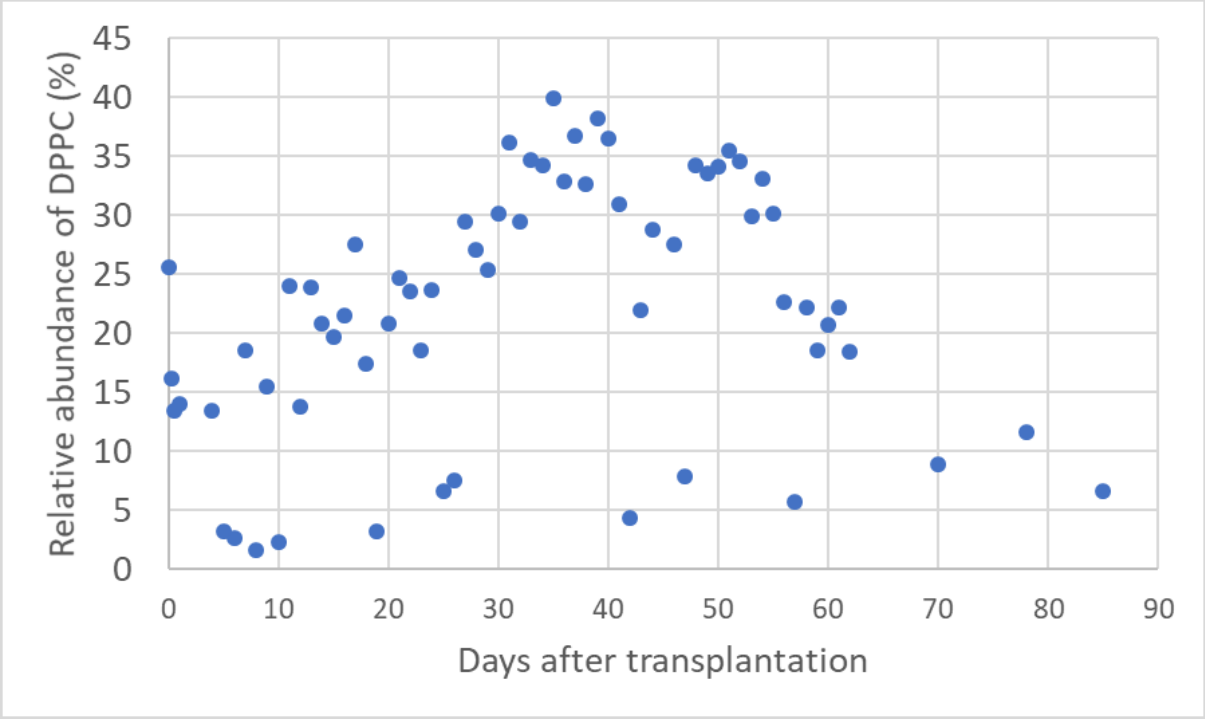


Figure 3-16: Case study – relative abundance of DPPC in bronchial washings
Relative abundance of DPPC in bronchial washings over the duration of one patient's prolonged ICU stay following lung transplantation

If the relative abundance of airway DPPC is charted for this patient, there appears to be a trend towards recovery of a near normal proportion of DPPC after one month, which then decreased again to very low levels before the patient's death (Figure 3-16).

Following the introduction of the D9-choline label, it is possible to assess DPPC by the percentage of lipid that has incorporated the choline label. In contrast to the previous figures of abundance and concentration, charting the incorporation of the deuterium label into DPPC produces a very clear line; with peak incorporation taking place between 1 and 4 days after transplantation, before undergoing an exponential decay - Figure 3-17. This very clear trend suggests that measurements of airway D9-DPPC enrichment are not subject to the same variability seen in measurements of airway abundance or concentration. It is important to note that it was not possible to collect samples from this patient between days 2 and 3, making it difficult to comment on peak deuterium enrichment for this patient.

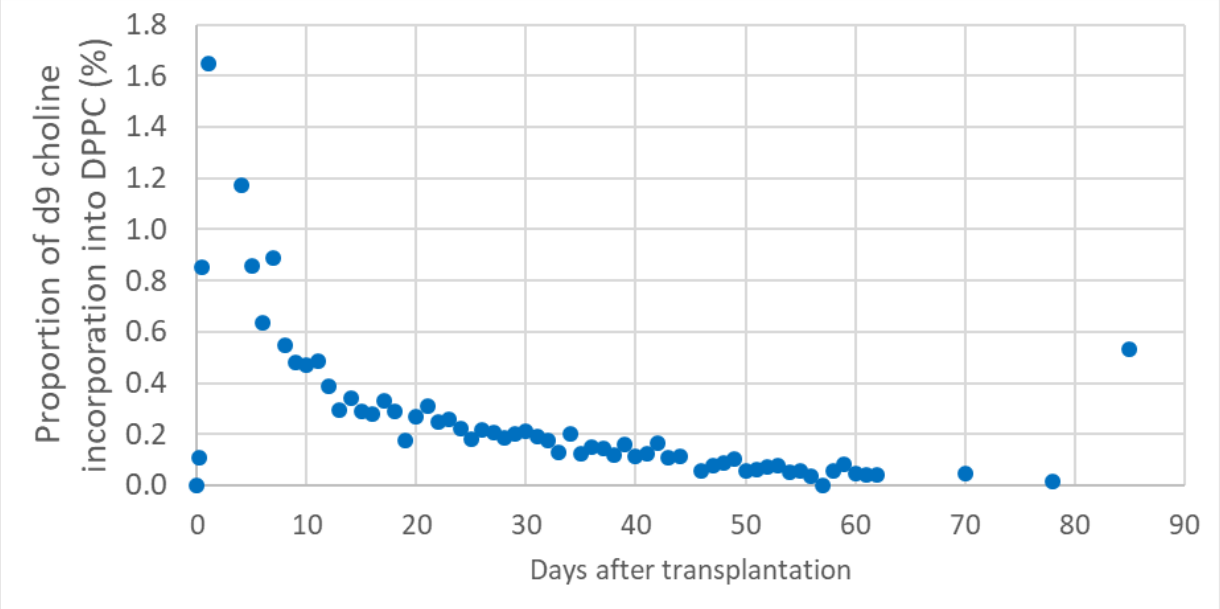


Figure 3-17: Case study – D9-DPPC incorporation in bronchial washings
Proportion of D9-choline incorporation into DPPC in bronchial washings over the duration of one patient's prolonged ICU stay following lung transplantation

3.3.2.4.2 Plasma DPPC

Concentration, relative abundance, and percentage deuterium incorporation in PC16:0/16:0 has also been charted for the plasma in the same patient in the following three figures (Figure 3-18 to Figure 3-20). Whilst PC16:0/16:0 was not particularly abundant in the plasma (never above 1% in this patient), plasma levels for this patient have been shown for comparison against the corresponding airway measurements.

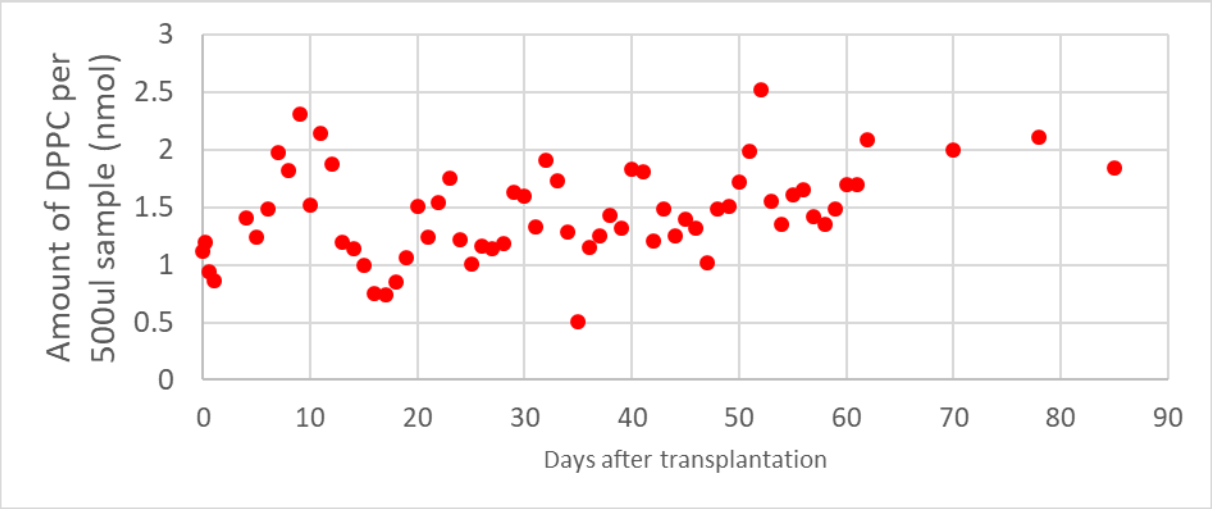


Figure 3-18: Case study – amount of DPPC in plasma
Amount of DPPC in the plasma over the duration of one patient's prolonged ICU stay following lung transplantation

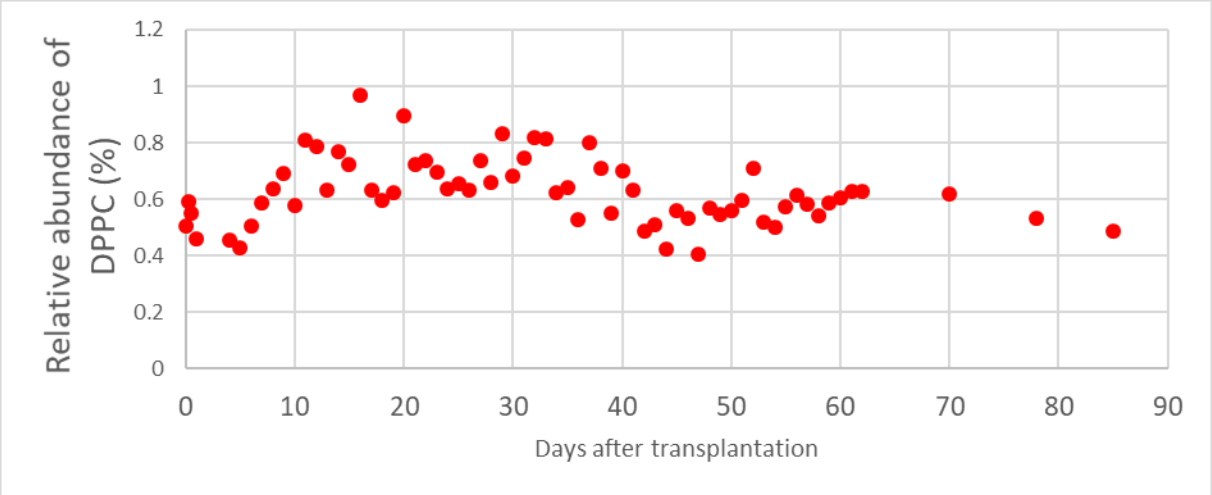


Figure 3-19: Case study – relative abundance of DPPC in plasma
Relative abundance of DPPC in the plasma over the duration of one patient's prolonged ICU stay following lung transplantation

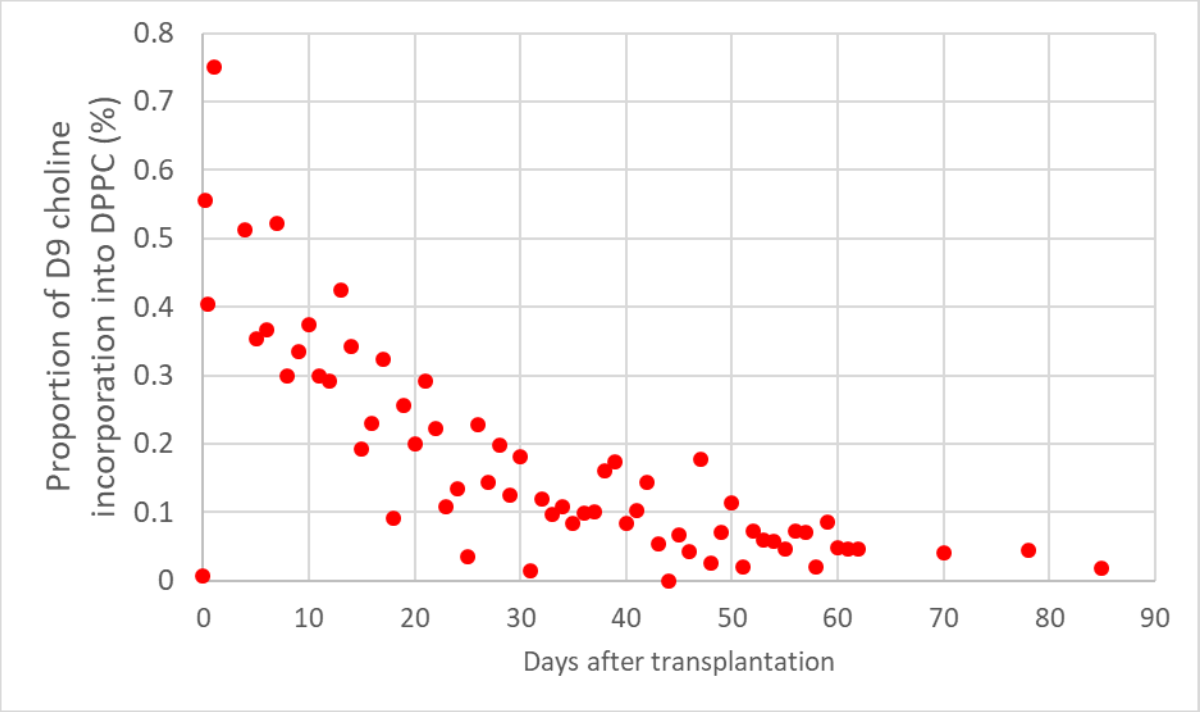


Figure 3-20: Case study – D9-DPPC incorporation in plasma
Proportion of D9-choline incorporation into DPPC in the plasma over the duration of one patient's prolonged ICU stay following lung transplantation

3.3.2.4.3 Mapping of deuterium labelled species

To assess deuterium labelling of the other measured phospholipids over the course of her admission, the data have been presented as a heat-map to highlight the most abundant species containing the deuterium label.

All phospholipid species identified within the detection threshold of the instrument on precursor 193 scans are displayed, with lipids arranged along the x-axis in order of ascending m/z ratio; therefore, each vertical line represents one phospholipid species measured in the many samples collected from this patient. The full list of lipids that are detected by the instrument are summarised in appendix 7.13. All samples collected for this patient are displayed, with samples arranged along the y-axis in order of time after surgery (non-linear y-axis); therefore, each horizontal line represents one sample collected from the patient, with the many phospholipid species measured by ESI-MS/MS. The colour of each pixel reflects the concentration of deuterated phospholipid species, expressed as a centile of all deuterium phospholipid concentrations for the study cohort. Thus, each pixel of the heat map represents the amount of deuterium labelled, phospholipid species at one

sample time-point, expressed as a centile for the study cohort. The heat-map was interrogated for phospholipids with the most intense localisation of the deuterium label, and highlighted accordingly. The lower threshold of the 55th centile provided an acceptable lower limit for noise, based on data from unlabelled samples.

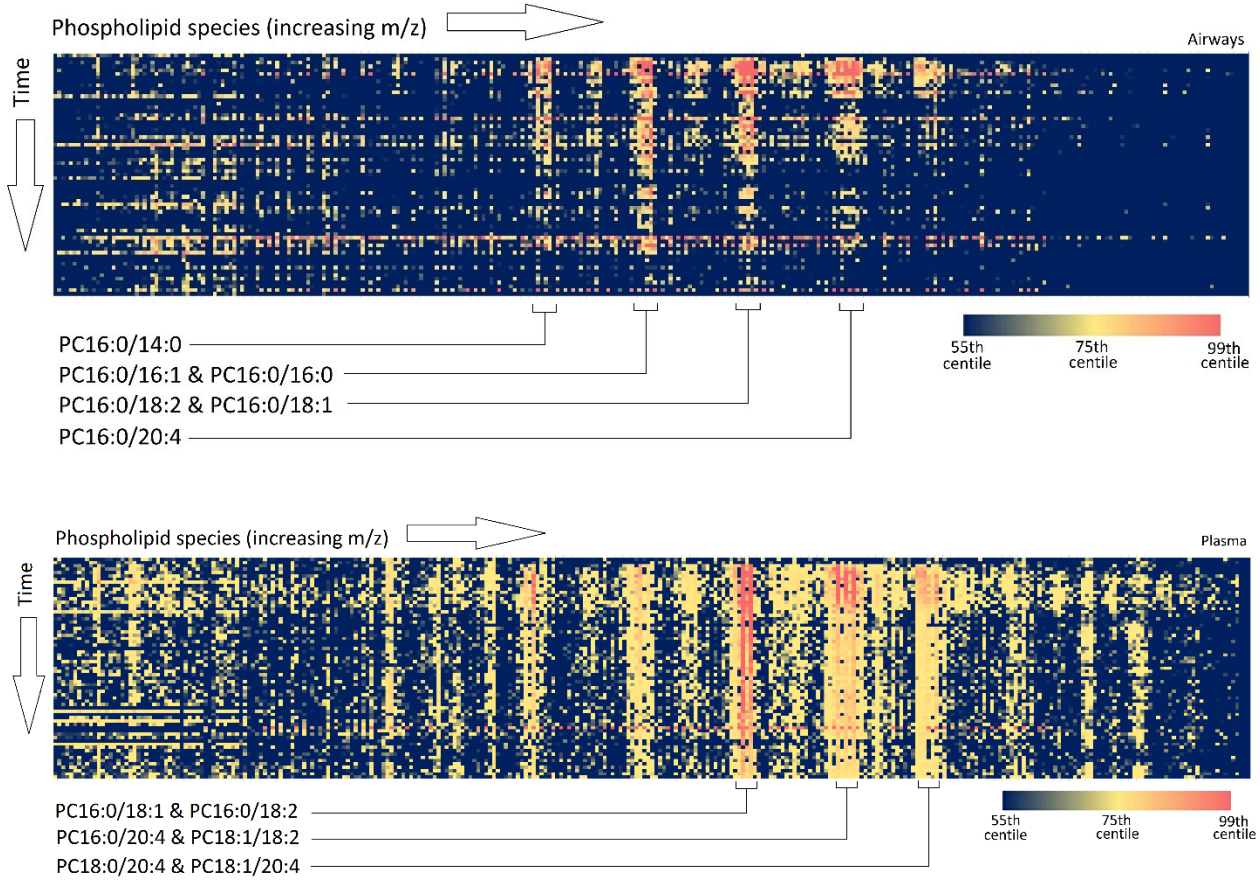


Figure 3-21: Deuterium label heat map – Plasma and bronchial washings from one patient
Amount of deuterium label within each lipid species detected across all bronchial washing samples taken from one patient who had a prolonged stay on the ICU. Each horizontal line represents one sample. Time after surgery increases vertically down the y-axis. Phospholipid species detected by the instrument are listed in order of increasing m/z ratio from left to right along the x axis, and the complete list of species is detailed in appendix 7.13. Each pixel represents the deuterium label content of one phospholipid species. Colour represents amount of lipid containing Deuterium label, expressed as a study centile, species are highlighted with the greatest perioperative deuterium enrichment. Top panel – bronchial washings. Bottom panel – plasma samples

Looking at these heat maps, the lipid species that tend to contain the greatest amount of the deuterium label contain fatty acids with chain lengths of either 16 or 18 carbon atoms and varying degrees of saturation. Looking at the plasma, deuterium incorporation is also seen in the

arachidonic acid containing species (such as PC16:0/20:4), and faint deuterium incorporation into several lyso-PC species is also noted, seen at the far left of the heat-map. Halfway through the patient's stay on the ICU, there is a slight shift in the localisation of the deuterium signal from PC species containing 16 & 18 length fatty acid towards lyso-PC species in the bronchial washings. Deuterium labelled lyso species were generally more apparent in the plasma heat map.

3.3.2.4.4 Key observations – case study

- Unlabelled DPPC concentrations measured in blind bronchial washings are highly variable
- There is less variability in the measured values of the relative abundance of airway DPPC
- Measurement of deuterium incorporation in airway DPPC is not subject to the variability seen in measurements of concentration and relative abundance, and provides consistent readings over time
- Illustrating deuterium labelled PC species as a heat map is trialled for one patient, and will be applied for the rest of the cohort in the next section

3.3.2.5 Deuterium labelled species across the study cohort

3.3.2.5.1 Mapping of deuterium labelled species

As study patients were ventilated for an average of 2 days, the in-depth analyses presented in section 3.3.2.4 are not possible for all the study patients. Instead, the same heat map approach of displaying deuterated phospholipid has been used for the whole study cohort to illustrate the localisation of the deuterium label across the whole study (as described in section 3.3.2.4.3).

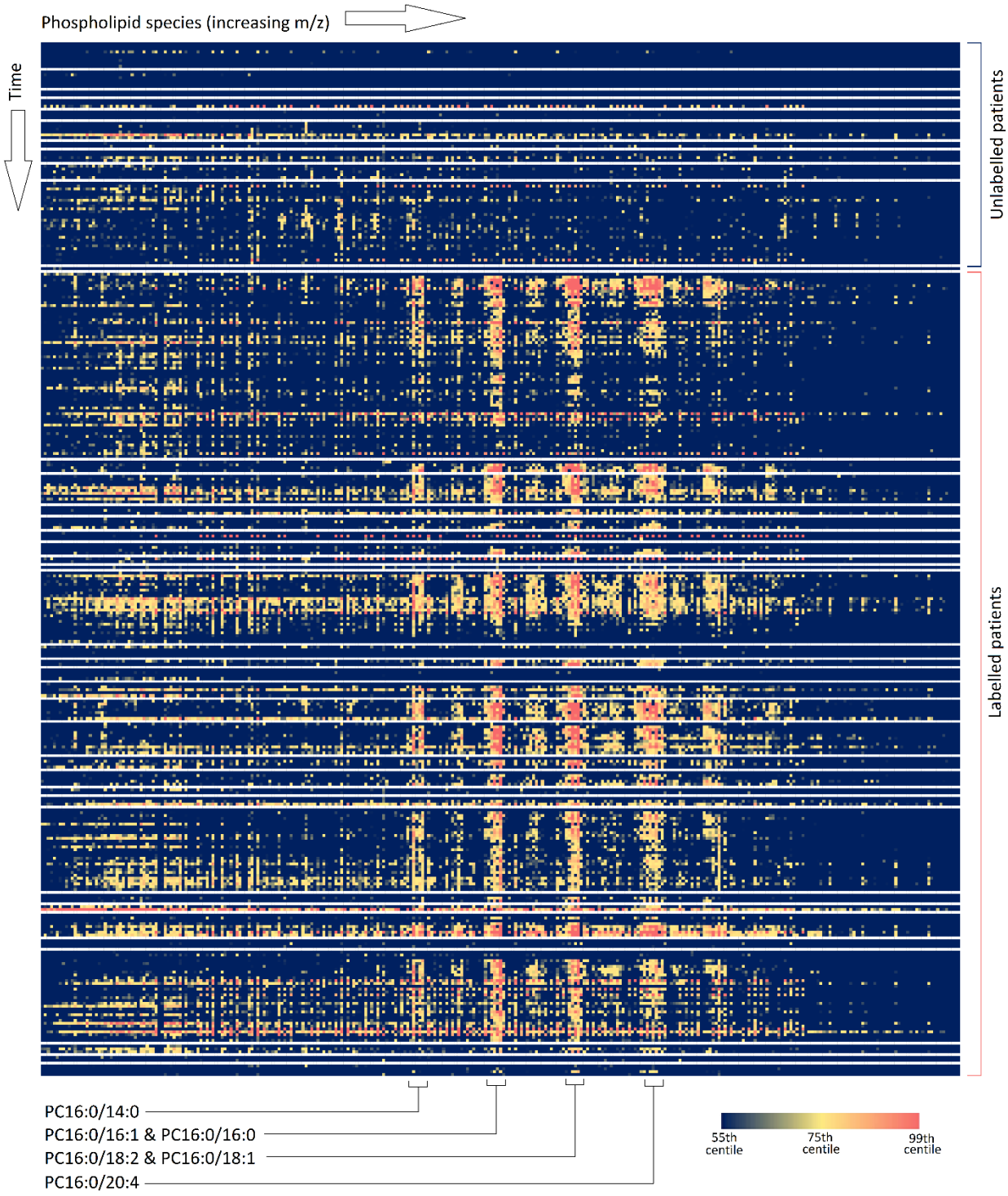


Figure 3-22: Deuterium label heat map – Bronchial washings from the whole study cohort
Amount of deuterium labelled lipid species detected across all bronchial washing samples taken from the entire study cohort. Each horizontal line represents one sample. Time after surgery increases vertically down the y-axis for each patient. Phospholipid species detected by the instrument are listed in order of increasing m/z ratio along the right axis, and the complete list of species is detailed in appendix 7.13. Each pixel represents the deuterium label content of one phospholipid species. Each horizontal white line represents a new patient. Colour represents amount of lipid containing Deuterium label, expressed as a study centile, species are highlighted with the greatest perioperative deuterium enrichment.

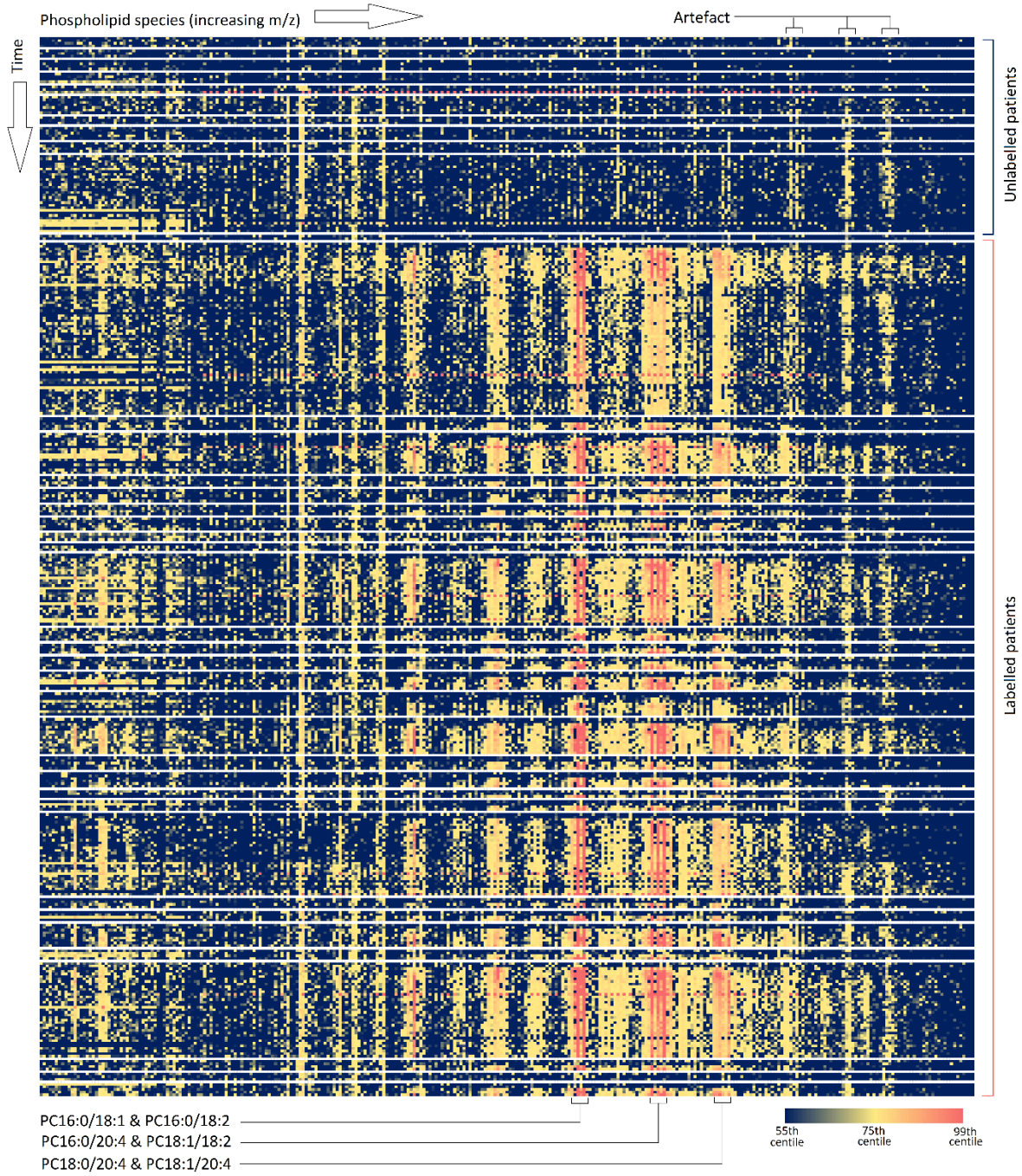


Figure 3-23: Deuterium label heat map – Plasma samples from the whole study cohort
Amount of deuterium labelled lipid species detected across all plasma samples taken from the entire study cohort. Each horizontal line represents one sample. Time after surgery increases vertically down the y-axis for each patient. Phospholipid species detected by the instrument are listed in order of increasing m/z ratio along the right axis, and the complete list of species is detailed in appendix 7.13. Each pixel represents the deuterium label content of one phospholipid species. Colour represents amount of lipid containing Deuterium label, expressed as a study centile, species are highlighted with the greatest perioperative deuterium enrichment. Species with consistent deuterium enrichment are highlighted. Species with the appearance of deuterium enrichment in unlabelled patients are highlighted as artefacts.

The following observations can be made by examining the localisation of the deuterium label in the plasma and the bronchial washings across the whole cohort. Most deuterium label is again seen in phospholipid species containing 16 and 18 carbon fatty acids.

In the plasma, the deuterium label is localised in multiple PC species containing unsaturated and polyunsaturated PC species. Deuterated labelling is more apparent in saturated PC species (like DPPC) in the airways when compared to the plasma. The plasma shows a faint signal for deuterium labelled lysoPC species in the plasma, which is less apparent in the airways. Examining the deuterium heat-map also helps to exclude artefacts from subsequent analysis, i.e. highlighting species that give the appearance of deuterium enrichment in patients who did not receive the D9-choline label.

3.3.2.5.1.1 Key findings – deuterium mapping

- Many phospholipid species are consistently labelled with deuterium across the study cohort following D9-choline administration
    - Especially in PC species with 16 and 18 chain length fatty acids
- A novel technique for representing dynamic lipidomic datasets has been presented

3.3.2.5.2 Deuterium enrichment

As described in section 1.3.5, deuterated choline labelling reveals the synthetic activity of the Kennedy CDP choline pathway. Previous data have established that deuterium enrichment reaches a peak at 24-48 hours[8,137].

Due to a relative lack of samples beyond 2-3 days after choline administration, it is difficult to demonstrate that the peak enrichment has been observed for most patients. The median duration of ventilation was 44.5 hours (interquartile range 18.25-510), and so the 12-hour time-point has been used for a comparison of deuterium enrichment, as samples were available for 75% of patients at this time-point and this enabled meaningful statistical analyses. Deuterium enrichment is

standardised at the 12-hour time-point across the whole study cohort, and presented for all phospholipid species identified in Figure 3-7 and Figure 3-12; species are presented in Table 3-5, ranked in order of their deuterium enrichment.

The most striking observations from Table 3-5 are that deuterium enrichment is highly variable amongst the PC species, and enrichment is not related to content. If Table 3-5 is compared to the lipid profiles presented in Figure 3-7 and Figure 3-12, high levels of deuterium enrichment can be seen in both very abundant species and in minor phospholipid species that appear in low quantities. To illustrate this point further with DPPC, it is the most abundant phospholipid in the bronchial washings but ranks as 26th for deuterium enrichment out of the 34 phospholipid species measured in the airways.

Plasma species	Median incorporation at 12 hours
D9PC16:0/16:1	1.116279573
D9PC16:0/18:3	0.847273749
D9PC16:0/18:2	0.635079397
D9PC16:0/18:1	0.628207633
D9SM20:0	0.52449622
D9PC16:0/20:5	0.465662836
D9PC16:0/20:4	0.464705204
D9PC18:1/20:4	0.406845363
D9PC18:1/18:2	0.36979647
D9SM22:1	0.35308052
D9PC16:0/22:6	0.349478977
D9PC18:0/18:2	0.306990848
D9SM24:2	0.272044875
D9PC18:0/20:4	0.214958153
D9SM24:1	0.176849874
D9PC18:0/18:1	0.165536261
D9SM22:0	0.1476941
D9PC18:0/20:3	0.141773881
D9PC18:0/22:6	0.137696549
D9PC18:1a/20:4	0.133921534
D9SM16:0	0.113203088

Airway species	Median incorporation at 12 hours
D9PC14:0/16:1	1.013673103
D9PC16:0/18:3	0.954849809
D9PC14:0/18:2	0.890518264
D9PC18:0/22:6	0.652058576
D9PC16:0/20:5	0.594241616
D9PC18:0a/18:1	0.552099824
D9SM20:0	0.54932636
D9PC18:1/18:2	0.531123504
D9PC16:0/18:1	0.497966679
D9SM22:1	0.496339793
D9PC16:0/18:2	0.493698865
D9PC16:0a/18:2	0.467659852
D9PC16:0/16:1	0.428895971
D9PC16:0/22:6	0.413528292
D9PC18:1a/20:4	0.41334709
D9PC18:0/16:0	0.405615891
D9PC18:0/18:2	0.401835421
D9PC18:0a/16:0	0.397098918
D9PC18:1/20:4	0.394441848
D9PC18:2a/18:1	0.37172755
D9PC16:0/20:4	0.366834373
D9PC16:0/14:0	0.351589039
D9PC18:0/18:1	0.327374338
D9PC16:0a/18:1	0.31317567
D9PC18:0a/18:2	0.282875076
D9PC16:0/16:0	0.275436764
D9PC16:0a/20:4	0.268355594
D9SM24:0	0.25662806
D9PC18:0/20:4	0.243222473
D9PC16:0a/16:0	0.230396283
D9PC18:0/20:3	0.219567903
D9SM24:2	0.195698756
D9SM24:1	0.148802592
D9SM16:0	0.135778228

Table 3-4: Ranking of species by deuterium incorporation
The median incorporation of deuterium after 12 hours into the top species within the plasma (table on the left) and airways (table on the right). Species are now ranked in descending order of deuterium incorporation

The determinants of deuterium enrichment are evidently different in the airways and the circulation. Using DPPC as an example shows that deuterium enrichment is significantly different in the circulation when compared with that in the airways. - Figure 3-24.

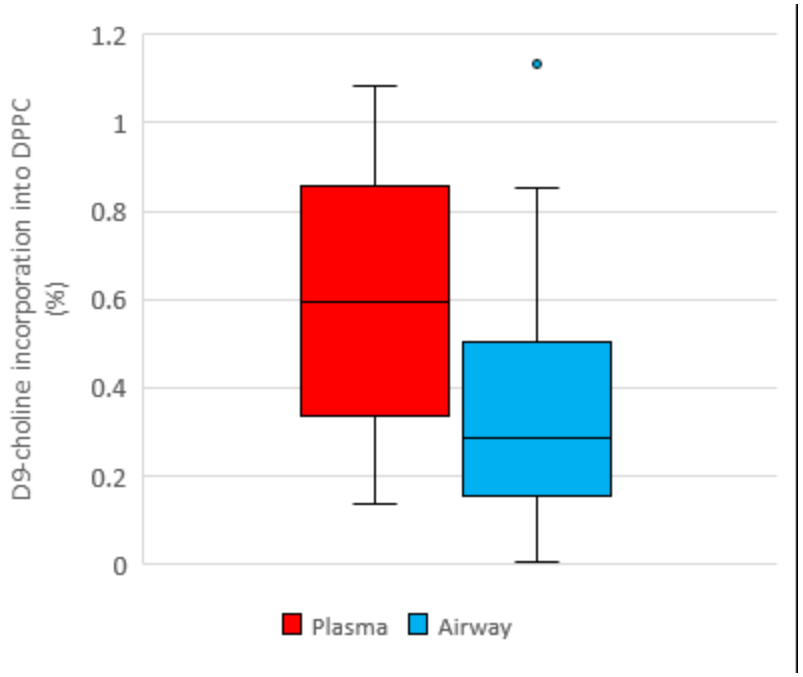


Figure 3-24: D9-choline incorporation into DPPC in the plasma and airways
Box and whisker plot. Comparison of D9 enrichment of DPPC in the plasma and the bronchial washings, 12 hours after the administration of the D9-choline label. p=0.006, 2-tailed Wilcoxon Signed Ranks Test

3.3.2.5.2.1 Key findings – deuterium enrichment

- Deuterium enrichment is highly variable amongst different phospholipid species
- Phospholipids that are highly enriched with deuterium are not necessarily the most abundant species
- Factors that determine deuterium enrichment differ between the airways and the circulation
    - This concept is explored in more detail in chapter 4

3.3.3 Topological data analyses

Large lipidomic datasets can be difficult to interpret; especially if data is available from many patients, for many species, over many time-points. Topological data analyses have been employed to illustrate the changing trends in the lipid profile over time after lung transplantation. The relative abundance of plasma species (unlabelled data) were entered with clinical metadata (as described in Table 2-5) into the Ayasdi Topological data analysis (TDA) platform to further explore lipidomic changes over time. A topological model of the data was created using variance normalized Euclidean metrics with L-infinity centrality lens (resolution 55, gain 3). The blue flare represents samples taken prior to transplantation, whereas the red flare was comprised of samples that were taken an average of 22 days after surgery.

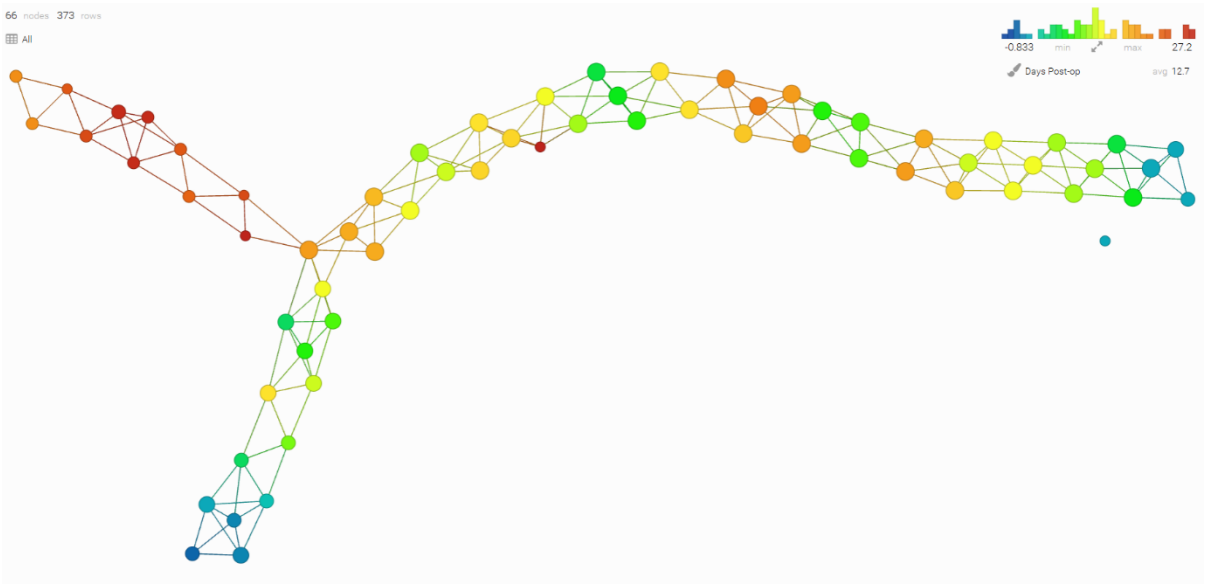


Figure 3-25: Topological data analysis of all plasma samples
TDA Network of plasma phospholipids from all patients at all time-points.
Generated by performing Topological Data Analysis (TDA) on plasma phospholipids expressed as a relative abundance with the Ayasdi Iris software platform (ayasdi.com). Nodes in the network represent clusters of plasma samples and edges connect nodes that contain phospholipid species in common. Nodes are coloured by the average value of their samples for the time-point of sampling (days after surgery). TDA network was created with variance normalized Euclidean metrics with L-infinity centrality lens (resolution 55, gain 3) The red flare of samples is largely comprised of samples that were taken a long time after surgery, whilst the blue flare on the network is largely comprised of samples from early time points obtained prior to transplantation or soon after surgery - Figure 3-25. The model was interrogated to determine the lipidomic differences between these early and late sample clusters, and some of those differences are summarised in Table 3-5.

Species	*Relative abundance % early sample cluster*	*Relative abundance % late sample cluster*	*p-value*	*KS Score*
PC16:0/18:1	12.7	22.0	2.56e-13	1
PC16:0/16:1	0.802	3.86	5.24e-13	0.971
PC16:0/18:3	1.14	2.4	5.24e-13	0.971
PC18:1/20:4	3.64	1.69	5.24e-13	0.971
SM24:1	2.09	1.07	5.24e-13	0.971
PC18:0/20:4	6.48	2.52	7.96e-13	0.964
PC18:0/22:6	1.48	0.415	7.96e-13	0.964
SM16:0	3.32	2.26	2.98e-12	0.941
SM22:0	1.05	2.26	2.98e-12	0.941
SM22:1	1.51	1.14	4.00e-12	0.935
PC18:1a/20:4	1.01	0.467	4.00e-12	0.935
PC16:0/20:4	10.9	6.35	1.48e-11	0.912
SM24:2	1.11	0.699	1.48e-11	0.912
PC16:0/22:6	4.87	1.52	2.26e-11	0.905
PC16:0/18:2	22.7	30.7	1.59e-10	0.870

Table 3-5: Temporal changes in plasma phospholipids
Differences in the abundance of selected phospholipids between the early and late sample clusters from the topological model in Figure 3-25. KS = Kolmogorov–Smirnov. Numbers highlighted in blue signify a group with a greater abundance of a lipid species, red signifies a group with a lower abundance of the lipid species.

A similar approach has been used with the phospholipid data obtained from the bronchial washings, and TDA modelling has been used to explore the changes in lipid profiles over time across the study cohort. The top phospholipid species found within every bronchial washing have been expressed as their relative abundance, and entered into the topology platform using variance normalized Euclidean metrics with L-infinity centrality lens (resolution 48, gain 3).

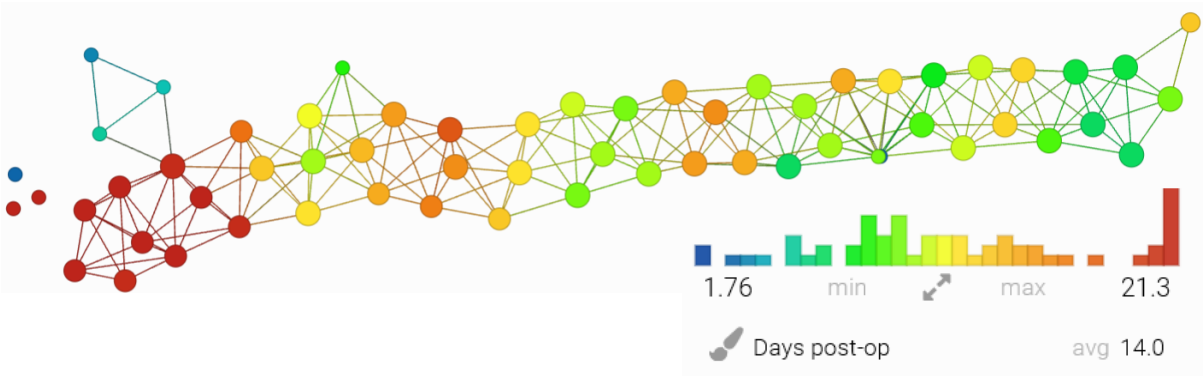


Figure 3-26: Topological data analysis of all airway samples
TDA Network of airway phospholipids from all patients at all time points.
Generated by performing Topological Data Analysis (TDA) on airway phospholipids expressed as a relative abundance with the Ayasdi Iris software platform (ayasdi.com). Nodes in the network represent clusters of airway samples and edges connect nodes that contain phospholipid species in common. Nodes are coloured by the average value of their samples for the time-point of sampling (days after surgery). TDA network was created with variance normalized Euclidean metrics with L-infinity centrality lens (resolution 55, gain 4)

As with the plasma samples, the topological model has identified a cluster of samples with similar lipid profiles that are associated with later time-points. The model was interrogated for lipidomic features that distinguished this late sample cluster against the remainder of the network. Statistically significant differences in species with a KS score above 0.5 are reported in Table 3-6. The red cluster of samples in Figure 3-26 was taken at an average of 21 days after surgery, in contrast to the remainder of the network which contained samples taken at an average of 5 days after surgery. This cluster of late samples tended to have a greater abundance of saturated PC species (PC16:0/16:0 and PC16:0/14:0) and PC16:0/16:1, whilst all other airway species decreased in their relative abundance (Table 3-6).

Species	Relative abundance % late sample cluster	Relative abundance % remainder of network	p-value	KS Score
PC18:0/18:2	4.12	6.66	5.37e-13	0.785
PC18:0/20:3	0.380	0.830	5.37e-13	0.770
PC18:1/20:4	0.600	1.34	5.37e-13	0.706
PC18:0/20:4	0.720	1.90	5.37e-13	0.686
PC18:0/22:6	0.140	0.370	5.37e-13	0.668
PC16:0/16:0	32.9	17.3	5.37e-13	0.653
SM22:1	0.400	0.760	5.37e-13	0.653
PC16:0/18:2	7.26	10.7	5.37e-13	0.637
PC16:0/16:1	11.8	6.97	5.37e-13	0.636
PC16:0/14:0	4.33	3.41	5.37e-13	0.635
PC16:0/22:6	0.390	1.00	5.37e-13	0.620
PC16:0/20:4	1.62	3.59	5.37e-13	0.616
SM24:2	0.330	0.660	5.37e-13	0.607
PC18:0/18:1	1.18	2.07	5.37e-13	0.601
PC18:1/18:2	2.79	4.17	5.37e-13	0.599
PC18:1a20:4	0.370	0.720	2.87e-13	0.556
SM24:0	0.280	0.480	1.43e-12	0.528

Table 3-6: Temporal changes in airway phospholipids
Differences in the abundance of selected phospholipids between the late sample cluster and the remainder of the network from the topological model in Figure 3-26. KS = Kolmogorov–Smirnov. Numbers highlighted in blue signifies a group with a greater abundance of a lipid species, red signifies a group with a lower abundance of the lipid species.

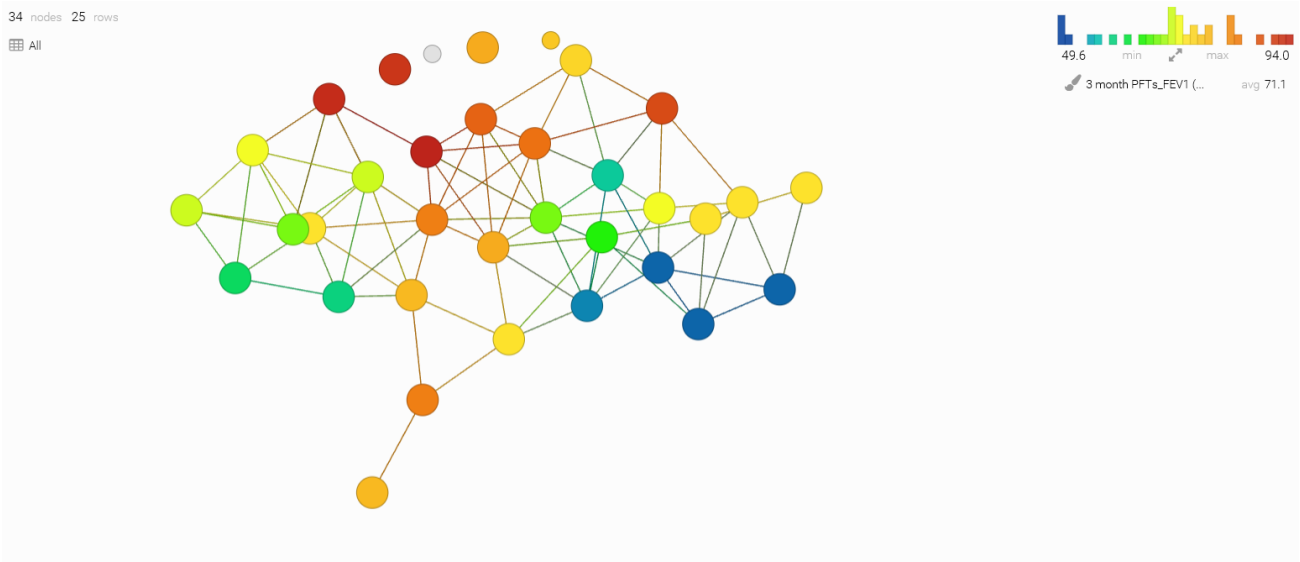


Figure 3-27: Topological data analysis of deuterium incorporation in 12-hour airway samples
TDA network of D9-choline incorporation into airway species from all labelled patients at 12 hours. Neighbourhood lens with cosine metrics, resolution 25 and gain 5. Coloured by predicted FEV1 at 3 months, blue for the lowest and red for the highest

Focusing on deuterium incorporation at the 12-hour postoperative time-point, another TDA network has been created in Figure 3-27, and subsequently interrogated for changes associated with poor postoperative graft function. A small cluster of samples (blue) was identified as having particularly poor graft function at 3 months, and the network was interrogated for distinguishing lipidomic features.

Species	*D9 incorporation % Low FEV_1 cluster*	*D9 incorporation % remainder of network*	*p-value*	*KS Score*
PC16:0/16:1	0.917	0.353	0.0209	0.750
PC16:0/14:0	0.599	0.292	0.0397	0.700
PC16:0a/16:0	0.576	0.195	0.0397	0.700
PC16:0/16:0	0.557	0.262	0.0397	0.700
PC16:0/18:1	0.941	0.362	0.0397	0.700
PC18:0a/18:2	0.348	0.215	0.0397	0.700
PC18:1/20:4	0.774	0.382	0.0397	0.700

Table 3-7: Candidate phospholipid species for investigation into markers of postoperative spirometry Differences in the abundance of selected phospholipids between the poor graft function cluster and the remainder of the network from the topological model in Figure 3-27. KS = Kolmogorov–Smirnov. Numbers highlighted in blue signifies a group with a greater abundance of a lipid species, red signifies a group with a lower abundance of the lipid species.

Lipid species with statistically significant differences in D9-incorporation between the poor graft function cluster and the remainder of the network are highlighted in Table 3-7. Whilst the initial hypothesis was focused on perioperative changes in airway PC16:0/16:0 (DPPC), Table 3-7 has highlighted 6 additional species within the airways as candidates for further investigation.

A similar TDA network was created for D9 labelled plasma samples obtained 12 hours after surgery, and interrogated for lipids associated with poor graft function at 3 months.

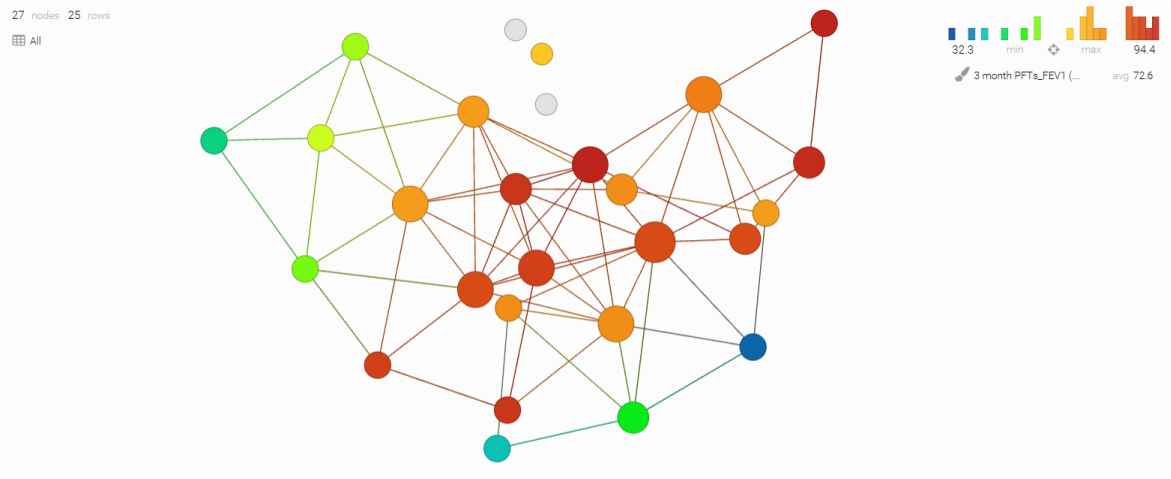


Figure 3-28: Topological data analysis of deuterium in corporation in 12-hour plasma samples TDA network of D9-choline incorporation into plasma species from all labelled patients at 12 hours. Neighbourhood lens with cosine metrics, resolution 25 and gain 5. Coloured by predicted FEV1 at 3 months, blue for the lowest and red for the highest

A small 3-node cluster of samples associated with poor lung function was selected from the bottom of the TDA network in Figure 3-28. In this instance, D9-incorporation was highlighted as a distinguishing feature for only two plasma phospholipid species. Within the poor FEV_1 cluster, D9-incorporation was reduced in SM24:2 (KS0.8, p=0.0120), and increased for PC18:1/18:2 (KS0.75, p=0.0209).

3.3.3.1 Key findings - TDA

- TDA could be used to present the temporal changes in large lipidomic datasets
- TDA could be used to identify phospholipids of interest for further study

3.3.4 Associations between lipid profiles and postoperative clinical outcomes

As airway DPPC is the primary target for investigation, levels have been compared against perioperative P/F ratios and postoperative pulmonary function tests for the study cohort. Again, focussing on the 12-hour time point, Table 3-8 reports the DPPC as both its relative abundance and its proportion of D9-choline incorporation.

12 Hour airway samples	PC16:0/16:0		D9PC16:0/16:0	
	Correlation coefficient	p-value	Correlation coefficient	p-value
Minimum P/F T24	.438*	0.029	-0.020	0.924
Minimum P/F T48	0.272	0.188	0.082	0.696
Minimum P/F T72	.430*	0.032	-0.083	0.693

Table 3-8: Association between DPPC and perioperative P/F ratio
Correlation between DPPC early perioperative P/F ratio. Columns on the left refer to the relative abundance of DPPC within the airways 12 hours after surgery, Columns on the right refer to the proportion of D-choline incorporation into airway DPPC at 12 hours. The lowest P/F ratio is recorded for each patient over the first three postoperative days, before correlation with DPPC data. Correlation coefficients refer to Spearman's Rank tests. P values are two tailed, and p<0.05 are highlighted in red.

12 Hour airway samples	PC16:0/16:0		D9PC16:0/16:0	
	Correlation coefficient	p-value	Correlation coefficient	p-value
3 month FVC (litres)	-0.216	0.348	-0.312	0.169
3 month FVC (% predicted)	-0.240	0.294	-.497*	0.022
3 month FEV1 (litres)	-0.108	0.642	-0.257	0.260
3 month FEV1 (% predicted)	-0.178	0.440	-.522*	0.015
6 month FVC (litres)	-0.255	0.265	-0.197	0.391
6 month FVC (% predicted)	-0.332	0.141	-.456*	0.038
6 month FEV1 (litres)	-0.103	0.656	-0.166	0.473
6 month FEV1 (% predicted)	-0.166	0.471	-0.376	0.093
9 month FVC (litres)	-0.043	0.870	-0.169	0.516
9 month FVC (% predicted)	-0.189	0.468	-.511*	0.036
9 month FEV1 (litres)	0.156	0.551	-0.199	0.445
9 month FEV1 (% predicted)	0.059	0.823	-0.434	0.082
12 month FVC (litres)	-0.056	0.816	-0.186	0.431
12 month FVC (% predicted)	-0.180	0.446	-0.337	0.146
12 month FEV1 (litres)	-0.159	0.504	-0.148	0.533
12 month FEV1 (% predicted)	-0.236	0.316	-0.254	0.280

Table 3-9: Association between DPPC and postoperative spirometry
Correlation between DPPC and spirometry for the first year. Columns on the left refer to the relative abundance of DPPC within the airways 12 hours after surgery, Columns on the right refer to the proportion of D-choline incorporation into airway DPPC at 12 hours. Correlation coefficients refer to Spearman's Rank tests. Two tailed p values are reported, and p<0.05 are highlighted in red.

Having observed the trend between D9-choline incorporation into DPPC and postoperative graft function, this concept has been explored in further detail.

The study cohort was divided into low and high lung function groups, using the median FEV_1 at 3 months (72.5%) as the cut-off. The low graft function group had a statically higher proportion of D9-choline incorporation into DPPC at 12 hours, when compared to the high graft function group (0.457% vs 0.161%, p=0.003* - two-tailed Man Whitney test).

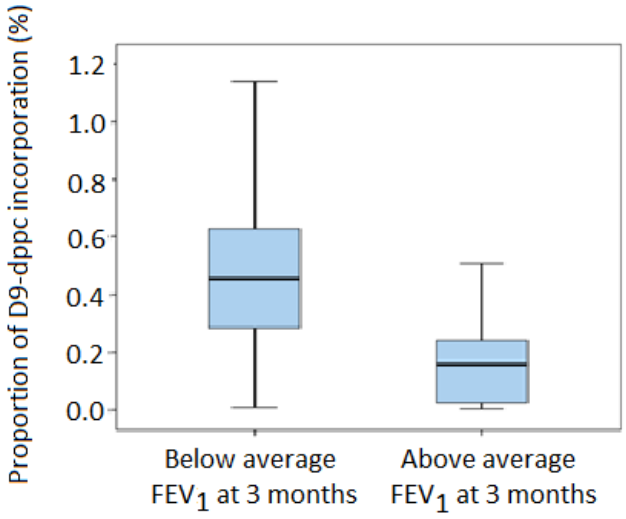


Figure 3-29: Differences in D9-DPPC incorporation by postoperative spirometry
Box and whisker plot of the proportion of D9 incorporation into DPPC at 12 hrs post-transplantation. Patients with above average FEV (right) vs below average FEV (left) at 3 months

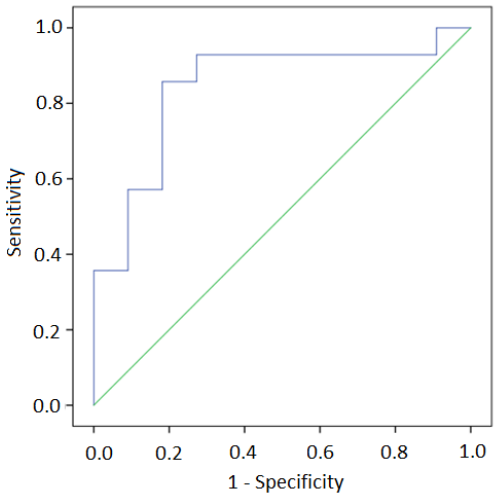


Figure 3-30: D9-DPPC incorporation as a predictor of below-average graft function
Receiver operator characteristics of D9 incorporation into DPPC as a predictor of below average graft function at 3 months

Receiver operator characteristics for D9-DPPC incorporation as a predictor of poorer graft function at 3 months reveal an area under the curve of 0.844 (95% confidence interval 0.678-1.000, p=0.004).

TDA networks have identified several additional candidate lipid species that might be associated with poor outcomes, and so they have also been evaluated for their ability to predict below average postoperative graft function at 3 months as above. Receiver operator characteristics are displayed for the additional lipid species identified in Table 3-7.

Deuterium incorporation at 12 hours	*Area under curve*	*p-value*	*95% Confidence Interval*	
			Lower Bound	*Upper Bound*
D9PC16:0/16:1	*.756*	*.031*	*.562*	*.951*
D9PC16:0/14:0	*.773*	*.021*	*.566*	*.979*
D9PC16:0a/16:0	*.773*	*.021*	*.584*	*.961*
D9PC16:0/18:1	*.818*	*.007*	*.651*	*.985*
D9PC18:0a/18:2	*.828*	*.006*	*.654*	*1.000*
D9PC18:1/20:4	*.870*	*.002*	*.718*	*1.000*

Table 3-10: D9-PC incorporation as a predictor of below-average graft function – Multiple species Receiver operator characteristics for 12-hour deuterium incorporation into multiple airways species as a predictor for below average graft function. Area under the curve, p-value, and 95% confidence interval for species described in Figure 3-31

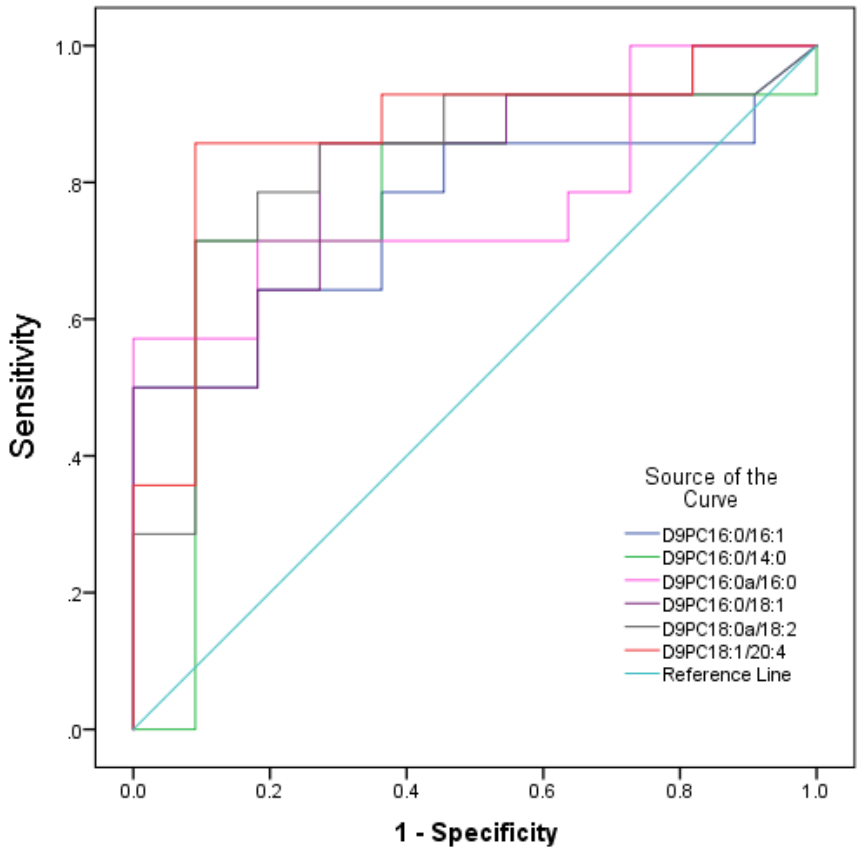


Figure 3-31: D9-PC incorporation as a predictor of below-average graft function – Multiple species Receiver operator characteristics for 12-hour deuterium incorporation into airways species identified in Table 3-7 as a predictor for below average graft function

3.4 Discussion

3.4.1 Summary of key findings

- Airway DPPC is reduced after lung transplantation, and continues to fall for 5 days in the patients who remain intubated.
- The relative abundance of DPPC is highly variable, and is associated with impaired oxygenation after lung transplantation.
- Deuterium enrichment is highly variable amongst phospholipid species, and differs between the airways and circulation.
- Deuterium enrichment of DPPC and PC18:1/20:4 are predictors of below average postoperative graft function.
- Deuterium heat-maps and topological data analyses are novel techniques for interpreting lipidomic datasets.

This study provides the first detailed description of the perioperative changes in phospholipids in clinical lung transplantation; specifically including unique data on a wide range of distinct phosphatidylcholine lipid species and phospholipid kinetics. Lung transplantation immediately changes the lipidomic profile in the airways of lung transplant recipients, and further changes can be seen during postoperative recovery. The most striking finding is the marked reduction of DPPC in the airways immediately after lung transplantation. This decline in airway DPPC continued over the first 5 days after lung transplantation for some of the sickest patients, and serial measurements of airway DPPC could be of value as the reduction in airway DPPC was associated with P/F ratios. Stable isotope labelling experiments have demonstrated increased perioperative DPPC flux through the Kennedy pathway in patients who develop impaired graft function after surgery, and could provide prognostic information that is unavailable from static measurements of important phospholipids. Novel techniques of interpreting lipidomic data have been presented, and could be of value in identifying lipids of interest in subsequent studies (like airway PC species containing

arachidonic acid). Lung transplantation causes variability in Kennedy CDP-choline pathway dependent metabolism that is associated with poor graft function, and is worthy of continued investigation.

3.4.2 Study cohort characteristics

The study cohort's one-year survival rate of 88% is comparable to the international registry one-year survival rate of 80%[22]. The incidence of severe PGD for the study cohort was 23%, which is lower than the 35% incidence of Grade 3 PGD that has been previously reported for our institution[56]. The 3-month predicted FEV_1 of 72% was similar to the 69% that has been previously reported for our institution[56].

Our previous work has recorded a median duration of postoperative mechanical ventilation of just under three days[56], yet the duration of postoperative ventilation in the study cohort was just under two days (Table 3-2). As patients were extubated sooner than expected, the number of postoperative samples obtained from each patient was lower than initially expected. Because peak deuterium incorporation is typically seen at about 2-3 days after in vivo administration of the D9-choline label, it has not been possible to calculate peak D9-DPPC incorporation for most lung transplant recipients enrolled on this study[7,137].

3.4.3 Perioperative changes in phospholipids

3.4.3.1 Airway phospholipids

On inspection of spectra from the airways of postoperative lung transplant recipients, there is a marked reduction in airway DPPC abundance, when compared to historical spectra from healthy volunteers - Figure 3-4 and Figure 3-6. Previous studies have shown that DPPC typically accounts for 50% of all PC species in healthy volunteers [7], which is considerably higher than the average of 23.26% that was seen for our postoperative lung transplant recipients. Not one patient included

within this study had a relative DPPC content that reached 45% on completion of surgery (Figure 3-7). Furthermore, those few patients who required prolonged invasive mechanical ventilation experienced further falls in their airway DPPC content up to day 5 after lung transplantation (Figure 3-8 & Figure 3-9). It is not possible to say from this data if this continued fall in DPPC content would be seen in those healthier transplant recipients who were extubated after two days, or if this is a finding that would be confined to a sicker sub-set of patients who require prolonged invasive ventilation. Without examining airway phospholipids of healthier recipients after extubation at comparable time-points, it is not possible to generalise the continued falls in DPPC content to the wider perioperative lung transplant population. Lower levels of airway DPPC were associated with poor P/F ratio after lung transplantation (Table 3-8), and so the mechanisms behind this fall in DPPC should be explored.

The case report in section 3.3.2.4 presented serial measurements of DPPC in one patient over an extended period of time, and suggested airway DPPC levels started to recover after reaching a nadir at one week after transplantation. Sadly, after airway DPPC levels recovered to 40% of PC content, there was another steady decline in airway DPPC until the patient's death. Limited conclusions can be drawn from a single case report, but it would further support the hypothesis that airway phospholipids will vary over the course of an ICU admission – as highlighted in Figure 3-8 and Figure 3-9.

Describing the temporal changes for many lipid species across many patients is challenging. Topological data analyses offer a novel approach for dealing with large and complex datasets, and TDA networks have been used to provide an alternative method illustrating the changes in the lipidome over time in this very heterogeneous dataset, as illustrated in Figure 3-25 to Figure 3-26. The TDA networks are also limited by the small number of sick patients, and so conclusions must be drawn with caution. Acknowledging the limitations of observing changes in a small heterogenous

group, TDA networks showed that surfactant phospholipids (PC16:0/16:0, PC16:0/16:1, and PC16:0/14:0) returned to predominance over the course of 3 weeks for those patients who remained intubated, with values that were nearing those seen in healthy volunteers[7]. The advantage of the TDA network is that it will also highlight temporal changes of relatively minor phospholipid species that have not previously been of interest; however, the potential lipidomic applications of the TDA platform will require continued investigation.

Chapter 4 provides a description of DPPC in the airways of lungs sustained ex vivo, prior to transplantation, and DPPC levels were consistently better than the levels seen in airways of postoperative lung transplant recipients - see section 4.3.2.1. These data suggest that there is a pathological derangement of DPPC metabolism in the transplanted lung, after it is reperfused in vivo; possibly either decreased synthesis or increased degradation. As the Kennedy's CDP choline pathway is a major source of DPPC synthesis, measuring PC flux through this pathway with stable isotope labelling could provide valuable insights and this is discussed in greater detail in section 3.4.4.

3.4.3.2 Plasma phospholipids

Lung transplantation is associated with an immediate fall in the concentration of all the major circulating phospholipids. Perioperative haemodilution could explain the reduced concentration of circulating phospholipids following intravenous fluid resuscitation, but the subtle changes in the relative abundance of several species imply that other factors may have also contributed to the perioperative changes in phospholipid composition (Table 3-3). Similarly, ARDS is associated with a prompt fall in circulating plasma PC species when compared to healthy controls[269]. That study did not demonstrate any recovery in plasma phospholipids over the 96 hour study period, but our data suggest that follow-up for 3 weeks might be necessary to see plasma phospholipid recovery. As previously stated in section 3.4.3.1, the study design limits the conclusions that can drawn from this

dataset; it is difficult to say if the observed temporal changes in plasma phospholipids would be seen in those healthier transplant recipients who were extubated after two days, or if this is a finding that would be confined to a sicker sub-set of patients who require prolonged invasive ventilation. The plasma does show a trend for increasing levels of PC16:1/18:1 and decreasing PC18:1/20:4 over time (section 3.3.3). Unlike observations from ARDS patients, perioperative lung transplantation was not associated with an increased plasma concentration of PC16:0/18:1[269] (Figure 3-14).

There are differences in the phospholipid composition of plasma compared with airway phospholipids; the airways have a greater proportion of saturated PC species, whilst the plasma has a greater amount of unsaturated PC. DPPC is not abundant in the plasma, representing less than 1% of circulating PC species with concentrations that range between 1-5nmol/ml (Figure 3-18 and Figure 3-19).

3.4.4 Label incorporation

Phosphatidylcholine and sphingomyelin species are synthesised from choline via the Kennedy pathway. Phosphatidylcholine and sphingomyelin phospholipids will generate choline fragments with a m/z 184 following collision induced dissociation (CID) in the Q2 quadrupole in ESI-MS/MS, and thus appear on scans for precursors of m/z 184 fragments (P184 scan). D9-choline (choline labelled with 9 atoms of deuterium) is 9Da heavier than naturally occurring choline, and so phospholipids synthesised from labelled choline are 9Da heavier, generate 193m/z fragments following CID, and appear on P193 scans (184 + 9 = 193). The detection of the D9-choline label on the P184 and P193 scans is illustrated in Figure 3-4 & Figure 3-5.

The proportion of deuterium incorporation for each species is determined by 3 variables: synthetic rate, catabolic rate, and the baseline amount of the species at the time of label introduction. Species with high synthetic and catabolic rates will have a greater proportion of deuterium

incorporation, and thus one could think of deuterium incorporation as a reflection of phospholipid turnover.

If surfactant dysfunction were exclusively the result of DPPC depletion because of diminished synthesis through the Kennedy pathway, deuterium enrichment would be diminished or delayed; as is the case in neonatal RDS[152]. Instead, patients with poor postoperative graft function had increased deuterium enrichment of their airway DPPC, implying increased flux through the Kennedy pathway. This is in keeping with previous ARDS studies that also demonstrated increased deuterium enrichment of DPPC when compared to healthy volunteers[150,269]. This is also in keeping with a mouse model of acid aspiration, which used an alternative deuterium labelling technique to illustrate increased DPPC metabolism in response to acute lung injury[151].

3.4.4.1 Relationship between incorporation and content

The four most abundant phospholipids in the plasma were (in descending order) PC16:0/18:2, PC16:0/18:1, PC18:0/18:2, and then PC18:0/20:4 (section 1.3.3). However, the four plasma species with the greatest deuterium incorporation were PC16:0/16:1, PC16:0/18:3, PC16:0/18:2, and PC16:0/18:1 (section 3.3.2.5.2, again in descending order). Therefore, high levels of PC flux through the Kennedy pathway is not limited to the most abundant phospholipid species.

Similarly in the airways, lipid flux does not always directly correlate with the abundance of phospholipids. DPPC is the most abundant airway phospholipid, yet relatively low levels of deuterium enrichment were seen compared to other species -section 3.3.2.5.2. These discrepancies imply that there are additional mechanisms beyond the Kennedy pathway that influence pulmonary phospholipid homeostasis, and PC metabolism through Land's acyl remodelling cycle could account for some of these discrepancies[7,8,101,137]. It is known that over half of DPPC is synthesised through PC remodelling[122], perhaps these minor species with high proportions of deuterium enrichment are rapidly converted into other species like DPPC soon after their initial synthesis via the Kennedy

pathway. An alternative explanation is that these minor PC species undergo rapid metabolism because they are synthesised for some other biological role, like the synthesis of lipid based inflammatory mediators[210]. Increased deuterium enrichment of these less abundant phospholipids is associated with poor graft function, so further study of these phospholipids would be justified – section 3.3.4.

3.4.4.2 Airways and circulation

PC flux is different in the airways and the circulation, as evidenced by the varying proportions of deuterium enrichment in lipid species measured in samples from different compartments. DPPC is not abundant in the plasma, yet deuterium enrichment is greater in plasma DPPC than in airway DPPC - Figure 3-24. Table 3-5 also illustrates this discrepancy for other phospholipids; 12 hour deuterium enrichment of PC16:0/16:1 is 1.16% in the plasma, but only 0.43% in the airways. 12 hour deuterium enrichment of PC18:0/22:6 is 0.138% in the plasma, but only 0.652% in the airways.

Phospholipid content is very different in the airways and the circulation, suggesting that there are different homeostatic mechanisms regulating phospholipid content in those two compartments. The liver is a major source of circulating polyunsaturated PC via the Phosphatidylethanolamine N-methyltransferase pathway (PEMT), which could account for some of these differences in PC flux [133]. In vitro and animal experiments have led to the hypothesis that basolateral phospholipid efflux from the pulmonary epithelium could transport unsaturated PC from the lungs in to the circulation, and this concept is explored in greater detail in the next chapter[10,11].

3.4.5 Associations between lipid profiles and clinical outcomes

Perioperative lung injury in the form of PGD has a lasting association with graft function and survival[1], and these data suggest that disrupted surfactant metabolism could represent one contributing mechanism. DPPC is the most abundant and surface active of all the pulmonary

surfactant phospholipids, and so it was hypothesised that perioperative changes in DPPC would be associated with clinical outcomes.

Decreased airway DPPC abundance is associated with impaired postoperative oxygenation, as measured by the P/F ratio – the major determinant of PGD. In contrast, increased DPPC flux is associated with poor postoperative graft function, an association that is present for 9 months after surgery - section 3.3.4. Measures of phospholipid flux and abundance are each associated with different clinical outcomes, and so both parameters could hold prognostic value.

Changes in phospholipid flux through the Kennedy pathway are associated with important clinical outcomes following lung transplantation. Further study of this pathway could shed new insights into surfactant biology in the injured lung, offer new clinical biomarkers of lung injury, and identify new therapeutic targets for investigation. The perioperative changes in DPPC metabolism after transplantation, and their subsequent association with adverse clinical outcome measures are compelling evidence in support of the original hypothesis: "Lung transplantation is associated with changes in pulmonary surfactant phospholipid metabolism, which in turn affects clinical outcomes."

However, ESI-MS/MS presents the opportunity to measure a vast range of phospholipid species beyond DPPC; providing insights into the wider lipidome and generating new hypotheses for further investigation. The TDA platform identified several unsaturated and polyunsaturated PC species in the airways as potential markers of poor postoperative graft function. PC18:1/20:4 is one of the species identified by the TDA platform that contains arachidonic acid, and could be a better predictor of postoperative graft function than DPPC. PC species are a source of arachidonic acid, which is synthesised during acute inflammation and has been previously indicted in the development oxidative stress mediated acute lung injury[270]. As arachidonic acid is believed to have a role in pulmonary inflammation, oxidative stress, and neutrophil and monocyte activation[12,270], the

identification of this species as a potential biomarker of poor graft function after lung transplantation is of great interest.

In summary, measures of both phospholipid abundance and flux through the Kennedy pathway could provide valuable prognostic information, and TDA could help identify other phospholipids as candidates for further study.

3.4.6 Limitations of the experimental methods

3.4.6.1 Impact of study design

Logistical constraints prevented sampling from recipients after endotracheal extubation. The resulting variable number of time-points for each patient limits the ability to draw comparisons or conclusions, this is particularly the case for samples collected from patients who required prolonged periods of respiratory support because of the lack of samples from healthier recipients for comparison. This issue has been partially addressed by focussing analyses on a standardised time-point that provides sufficient time for deuterium enrichment of newly synthesised phospholipids, before too many study participants have left the study because they have been extubated and can no longer provide airway samples.

3.4.6.2 Surrogate clinical endpoints

The small size of this pilot study cohort precludes using either CLAD or mortality as a study endpoint. However, as the median survival after lung transplantation is 5.8 years, further analyses in the association between lipid profiles and mortality could become feasible in a few years [22].

Postoperative spirometry and P/F ratio have been used as surrogate clinical end points as they are the major determinants of CLAD and PGD respectively. Having identified a correlation between

phospholipid metabolism and graft dysfunction, it will now be possible to perform the relevant power calculation for the subsequent studies using these data.

3.4.6.3 Lung function

Absolute values for FEV_1 and FVC in litres are greatly influenced by the size of the donor and the recipient. Postoperative lung volumes (specifically the total lung capacity) will soon approach the recipients' predicted values after surgery, irrespective of the donor size[271,272]. Referring to spirometry as a percentage of the recipients' predicted value offers a convenient and readily available method to adjust values of lung function for the size of the recipient (and donated lungs).

Chronic lung allograft dysfunction is routinely assessed by measuring the patients' spirometry after surgery[273]. It is has been previously observed that nearly all of the postoperative improvement in lung function is seen within the first 3 months after surgery[272]. One can only speculate as to why the association between D9-DPPC incorporation and lung function is lost after 9 months. It is possible that subsequent postoperative events that can affect postoperative graft function will start to exert a greater influence beyond 9 months, such as episodes of infection & acute rejection.

3.4.6.4 Bronchial washings

The addition of an internal standard into samples prior to ESI-MS/MS analysis allows the calculation of phospholipid concentrations. However, limitations of the sampling technique make the measurement of airway concentrations very problematic with bronchial washings because of the variable and unknown dilution factor. When airway DPPC data was presented as a concentration in the bronchial washings for one patient over time, the data points are highly erratic without any discernible pattern (Figure 3-15). Measuring DPPC concentrations in blind bronchial washings provided little usable information, with a seemingly random distribution of values over time. Previous studies have used paired measurements of urea in the plasma and bronchial washings as an

internal standard to adjust for the variable dilution factor[274]; this approach could be used for phospholipid measurements in the airways.

The problems of variable dilution can be partially overcome by presenting lipid data from bronchial washings as their relative abundance. Because the relative abundance only considers the lipid component of the samples, the variable addition of saline (or any other aqueous solvent removed by the Bligh and Dyer technique) is immediately disregarded from the analyses.

Airway contamination with blood could give the appearance of reduced airway DPPC by flooding mass spectra with plasma PC that are also detectable on P184 scans. Contaminating blood is rich in polyunsaturated PC species but has a relatively poor DPPC content, and this could result in artificially reduced measurement of airway DPPC when expressed as a relative abundance. However, it is unlikely that the low levels of airway DPPC after surgery are an artefact that is the result of intraoperative bleeding, as a continued fall in DPPC was demonstrated for 5 days after surgery – long after any intraoperative bleeding would have stopped. Airway bleeding could be assessed by measuring the haemoglobin in the bronchial washings, but this is complicated by the fact that cell-free haemoglobin in the alveoli could also act as a marker of lung injury[275].

The problem of airway lipid contaminants can be addressed with the introduction of the D9-choline label. Airway DPPC is predominantly synthesised by the type 2 alveolar epithelial cells[122], and this is true for both naturally occurring and deuterium enriched DPPC. In this regard, deuterium enrichment could be considered as a marker of AT2 DPPC metabolism that is relatively spared from the variability introduced by blood contaminants or dilution; the clear trends observed in Figure 3-17 would support this hypothesis.

Blind bronchial aspirates via the endotracheal tube are a convenient method of sampling the airways without a bronchoscope, and previous work has shown that the phospholipid profile of bind tracheal aspirates is comparable to bronchoalveolar lavage fluid[7,276].

3.4.6.5 Statistical analyses

Associations have been sought between multiple phospholipid species and many clinical outcomes, and so many comparisons come with an inescapable risk of type 1 errors. However, methods designed to control the family-wise error rate (such as the Bonferroni method) would also result in an unacceptably high rate of type 2 errors with this type of study, and are arguably inappropriate for this kind of dataset [265,266]. Whilst the calculation of the false discovery rate has been used in other 'omics' studies to curtail the costs of subsequent studies by reducing the number of candidate biomarkers for further investigation, such concerns would not be applicable with this type of study[267]. Indeed, identifying phospholipid candidates other than DPPC for further investigation would not be enhanced by the removal of species, as ESI-MS/MS easily allows for the simultaneous assessment of these species without the investment of additional time, labour, or costs. Therefore, unadjusted p-values are reported for all species and clinical observations, whilst acknowledging the inherent risk of type 1 errors within this secondary hypothesis generating exercise.

This chapter has presented several novel approaches for the interpretation of lipidomic data. Whilst the deuterium heat-maps in section 3.3.2.5.1 provide a graphical illustration that highlights phospholipids that incorporate the deuterium label, it does not permit meaningful statistical analyses. Instead, deuterium enrichment has been tabulated as a percentage at a standardised 12 hour time-point (section 3.3.2.5.2), and this value of 12 hour deuterium incorporation has been used for analyses against clinical outcomes (section 3.3.4).

Similarly, this novel application of TDA to lipidomic datasets illustrates that it can be used to describe temporal changes in lipid profiles, but the significance of any such identified changes would require continued investigation. Lipid species of interest that were identified by TDA were subjected to testing with more conventional statistical analyses, in the form of Receiver Operator Characteristics and Man Whitney tests - section 3.3.4.

3.4.7 Conclusions

This study provides the most detailed analysis of the lipidome in perioperative patients recovering from lung transplantation to date. Deuterium labelling of the Kennedy pathway combined with ESI-MS/MS allows a detailed and dynamic assessment of patients' lipidomic profile during recovery from lung transplantation, it is a clinically viable technique, and may hold prognostic value in predicting outcomes. Lung transplantation is associated with multiple changes in the patients' lipidome in the immediate perioperative period. Assessment of phospholipid flux using the stable isotope technique can reveal changes that are associated with clinically important outcomes that are not easily detected by looking at static amounts of phospholipid species. Changes in airway phospholipids that lower pulmonary surface tension and PUFA containing species are associated with impaired postoperative graft function, and the metabolism of these species warrants further investigation.

4 Results: Phospholipid metabolism in human ex vivo lung perfusion

4.1 Introduction

4.1.1 Background

The previous chapter demonstrated that phospholipid metabolism is associated with important clinical outcomes following lung transplantation. Ex vivo lung perfusion (EVLP) may have a role in the assessment, transport, and reconditioning of lungs for use in clinical lung transplantation[9,81], but the effects of EVLP on pulmonary surfactant phospholipid biology are largely unknown. In studying the effects of EVLP on surfactant, we are also offered a unique opportunity to study human pulmonary phospholipid biology in isolation from the rest of the body.

Whilst much research has been conducted into the pulmonary synthesis and secretion of surfactant phospholipids into the airway, little is known about any pulmonary contribution to systemic phospholipids in the circulation. Phosphatidylcholine (PC) species are synthesised in the Golgi and endoplasmic reticulum of nearly every mammalian cell, typically through the Kennedy CDP-choline pathway[127]. These surfactant phospholipids are stored in lamellar bodies before their contents are released into the alveolar space through exocytosis, and this process of apical secretion of phospholipids in to the alveolar space has been well described[97]. However, the nature of pulmonary phospholipid transport back into the systemic circulation is largely unknown, and difficult to ascertain in vivo as the circulation contains polyunsaturated PC that are also synthesised by the liver[131,132,277]. Polarised cell culture models and animal experiments have both suggested that the alveolar epithelium is capable of selectively secreting different phospholipids into the airways and the circulation, but this proposed theory of basolateral phospholipid efflux from the lung into the circulation has never been conclusively proven[10,11,278]; neither in experimental models nor in man. To address this issue, this chapter provides the first description of intact human lungs secreting a range of newly synthesised phospholipid species into two different compartments; the airways and the circulation.

As with most mammalian cells, isolation and culture of primary alveolar type II cells (AT2) has confirmed that they are capable of independent PC synthesis. However, cell culture experiments have thus far failed to replicate the full range of phospholipids species in the proportions found in vivo[101,279]. Breathing-related cyclical mechanical stretch of the alveolar epithelium plays an important role in the lamellar body exocytosis of surfactant[99], which can be technically challenging to recreate in vitro. Human EVLP has the potential to address the limitations of other experimental models of pulmonary phospholipid biology, and this novel application of EVLP could provide unique insights into pulmonary phospholipid biology. Combining a human ex vivo model with electrospray ionisation tandem mass spectrometry and stable isotope labelling of the Kennedy pathway can provide novel data on pulmonary phospholipid flux with a molecular specificity that has not been previously achieved[137,251].

Several platforms of ex vivo perfusion have recently entered clinical use in human lung transplantation, in the hope of minimising ischemia reperfusion injury (IRI) to the lung[9,258] – described in section 1.1.1.3. Further investigation into EVLP phospholipid metabolism might also provide mechanistic insights that could allow further optimisation of this evolving technology to protect the graft during transplantation.

4.1.2 Hypotheses

- The Kennedy pathway continues to produce and release surfactant phospholipids during normothermic ex vivo lung perfusion.
- The lungs are capable of surfactant phospholipid synthesis in isolation from hepatic PEMT metabolism.
- Lungs release phospholipids back into the circulation, which are different from the surfactant phospholipids that are secreted into the airways.

4.1.3 Chapter objectives

- Explore the viability of human ex vivo lung perfusion as a platform for research into pulmonary phospholipid biology
- Demonstrate continued pulmonary surfactant phospholipid secretion into the airways during normothermic ex vivo lung perfusion.
- Measure the pulmonary secretion of newly synthesised phospholipid into the circulation, and contrast those with the species measured in the airways.

4.2 Methods

4.2.1 Ex vivo organ support

Human organs that had been brought to Harefield hospital with the primary intent of clinical transplantation were sampled in accordance with the protocol described in section 2.3.

Organs deemed fit for transplantation had surplus perfusate and bronchial washings sampled as deemed clinically appropriate by the organ retrieval team. The exact method for ex vivo support was initiated at the discretion of the transplant surgeons whilst the organs were under assessment for potential transplantation, and so it was not logistically possible to standardise the protocol or platform for the organs included in this chapter.

The decision to accept organs for clinical transplantation ultimately rests with the transplant surgeon, with indicative inclusion criteria for transplantation summarised in Table 4-1[280]. Extension of those criteria is possible at the discretion of the transplant surgeon, with acceptable results for those so-called marginal organs[281,282].

Ideal lung transplant donor criteria
Age <55 years
ABO compatibility
Clear chest x-ray
$PaO_2 \geq 300$ mmHg on $FiO_2 = 1.0$ and PEEP = 5cm H_2O
≤ 20-pack-year smoking history
No previous cardiopulmonary surgery
No evidence of aspiration or sepsis
Absence of purulent secretions at bronchoscopy
Negative microbiology

Table 4-1: Ideal lung transplant donor criteria. Description of the ideal donor organ criteria, adapted from Frost 1997[280]

In organs that were rejected for transplantation, D9-choline was added to the perfusate reservoir. Bronchial washings were sampled hourly via bronchoscopy with matching perfusate samples, until the organs were no longer viable.

The onset of pulmonary oedema after the lungs have been established on EVLP is believed to represent development of lung injury, with the failure of the alveolar-capillary barrier[283]. Organs were no longer considered viable, if either of the following conditions occurred:

1. Critical depletion or the perfusate reservoir
2. Pulmonary oedema visible at the endotracheal tube



Figure 4-1: The TransMedics Organ Care System
Human lungs sustained on TransMedics Organ Care System, after they were rejected for lung transplantation. The lungs are perfused with cellular perfusate. (i.e. contains erythrocytes).

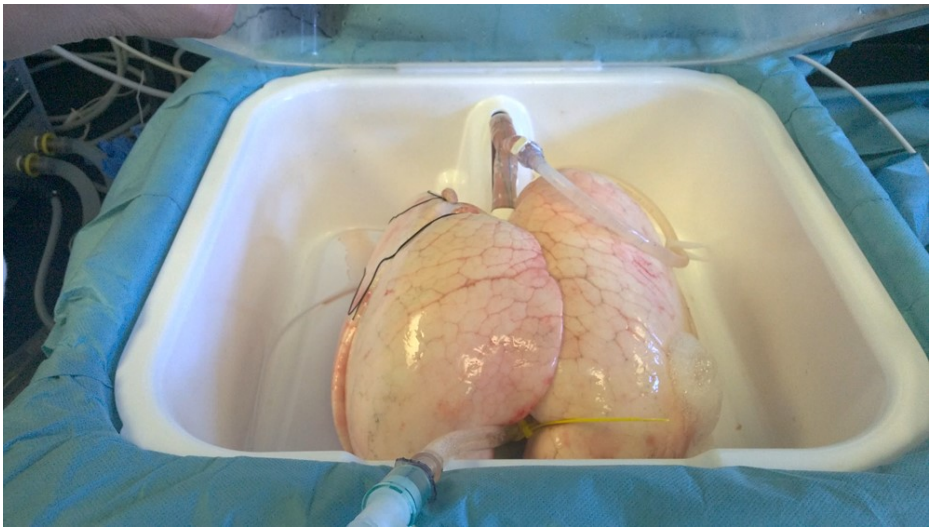


Figure 4-2: Vivoline organ chamber
Human lungs sustained on the Vivoline Ex Vivo Lung Perfusion platform with an acellular perfusate (i.e. does not contains erythrocytes).

Observations on pulmonary physiology were recorded for every organ enrolled into the deuterium labelling experiment – section 4.3.1.1. Pulmonary vascular resistance and pulmonary compliance were calculated with the following formulae:

$$Compliance = \frac{V_t}{P_{insp} - PEEP}$$

$$PVR = \frac{80\,(PAP - 0)}{Perfusate\ flow}$$

Equation 1: Formulae for pulmonary physiology
PAP = Pulmonary Artery Pressure, PEEP = Positive End Expiratory Pressure, P_{insp} = Peak inspiratory pressure, PVR = Pulmonary Vascular Resistance, V_t = Tidal volume

Note that the pressure gradient across the pulmonary vasculature was equal to the pulmonary artery pressure, as the left atrial cuff was open to atmospheric pressure.

4.2.2 Sample processing and analyses

The anti-oxidant butylated hydroxytoluene (BHT) was added to each sample on collection, and immediately placed in ice before subsequent sample processing. All samples were centrifuged and aliquoted before freezing at -80°c, pending ESI-MS/MS analysis at a later date. Bronchial washings were also filtered for debris prior to centrifugation. The initial sample processing protocol is described in greater detail in section 2.4.

Lipids were extracted using the modified Bligh & Dyer method, prior to analysis with ESI-MS/MS. Samples were scanned for precursors of m/z 184 and m/z 193 (P184 and P193 respectively), and this chapter will deal exclusively with phospholipids that are detectable on those precursor scans 2.5.1. Mass spectra were processed and analysed with our departmental macros, which included corrections for adduct formation, natural isotope distribution and subsequent identification and quantification of lipid species from their m/z ratio. Following the identification of lipid species, analyses were performed with Excel and SPSS – section 2.6.

4.3 Results

4.3.1 Source of ex vivo organs

The destination of all organs brought to Harefield for potential transplantation during the study period is summarised in the following flow diagram - Figure 4-3.

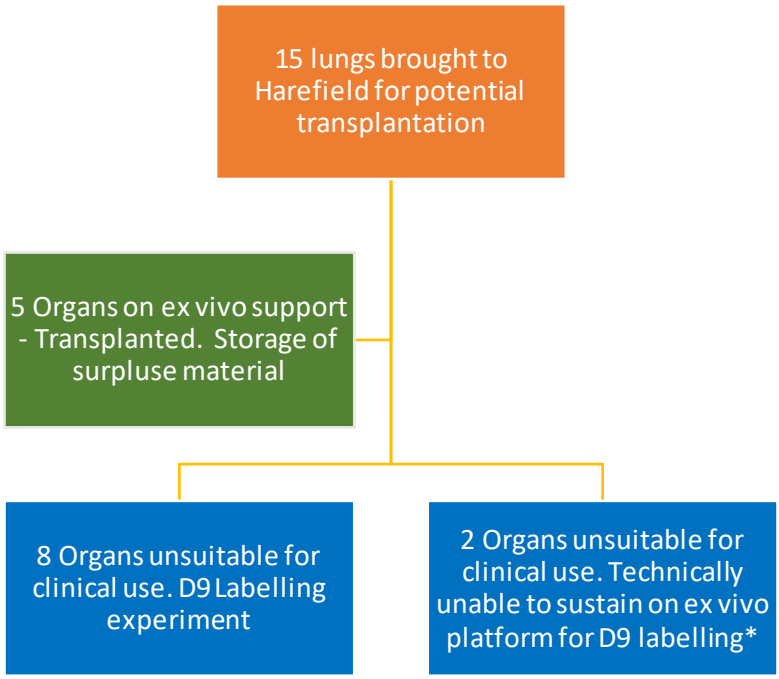
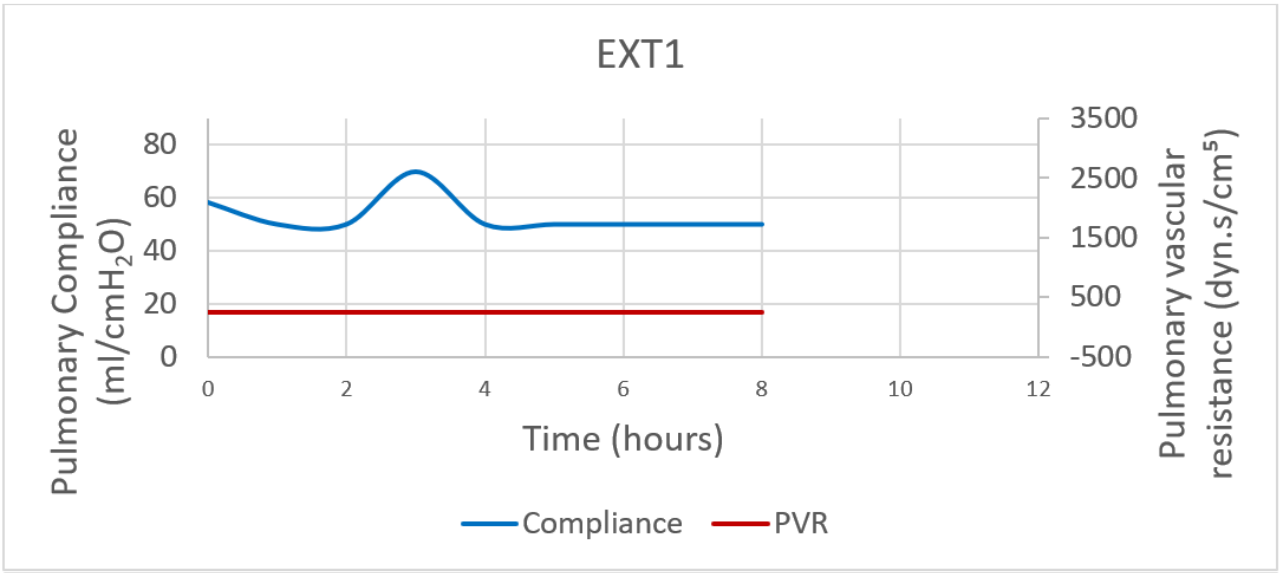
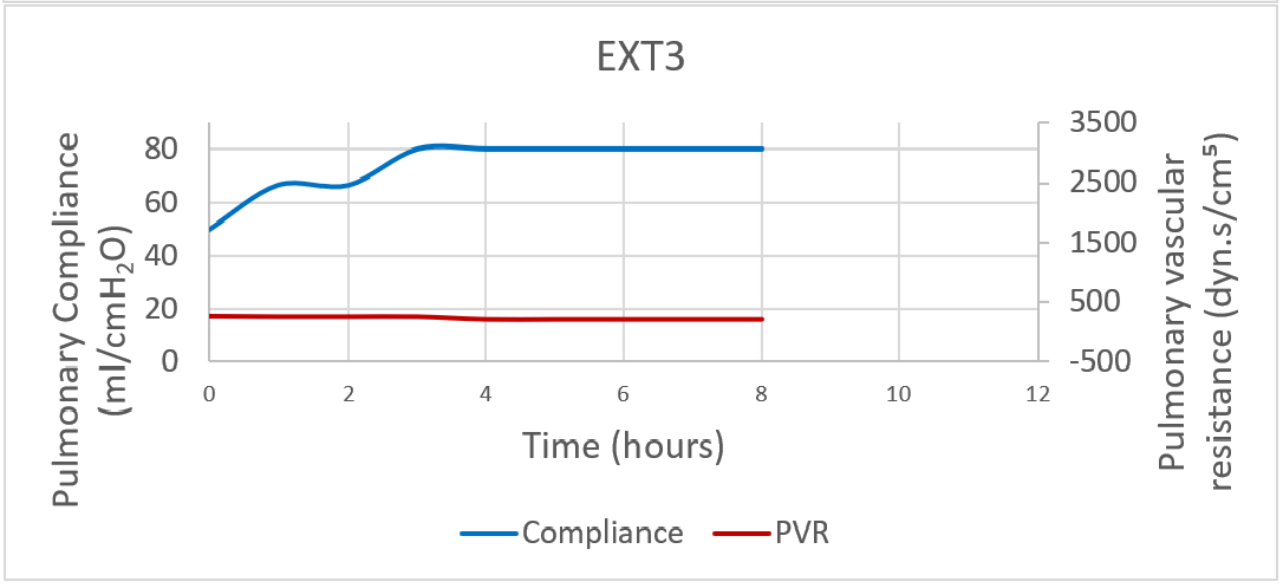


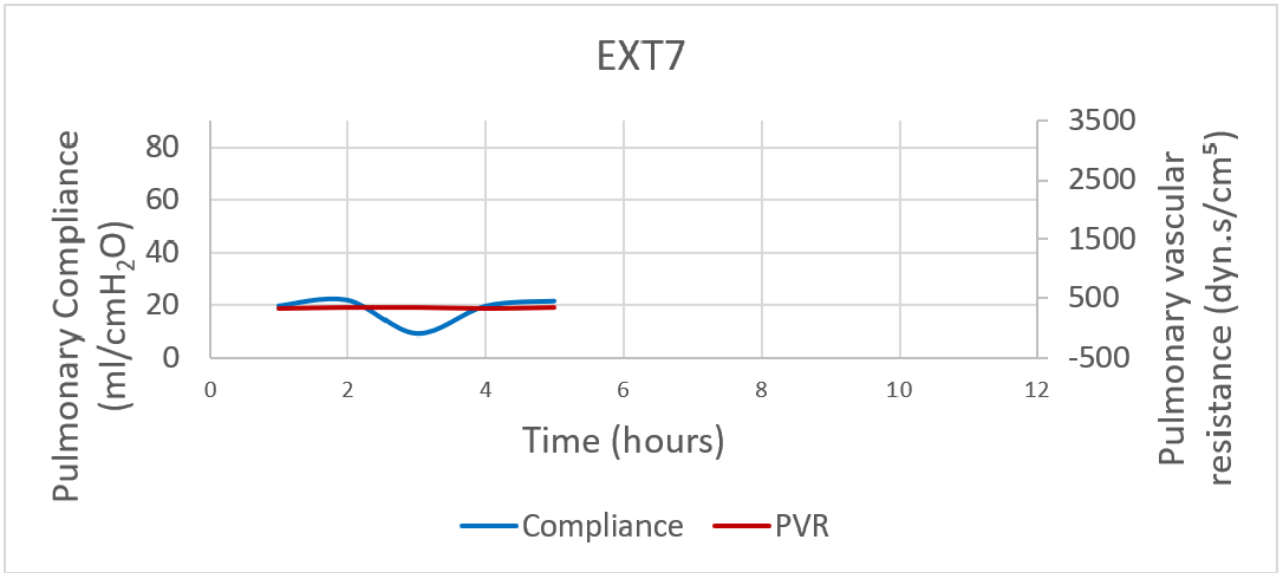
Figure 4-3: Ex vivo organ outcomes
*Flow chart illustrating the outcomes of ex vivo organs brought to Harefield that met study criteria. *On first inspection at Harefield, these organs had severe macroscopic injury that precluded attempts at ex vivo support.*

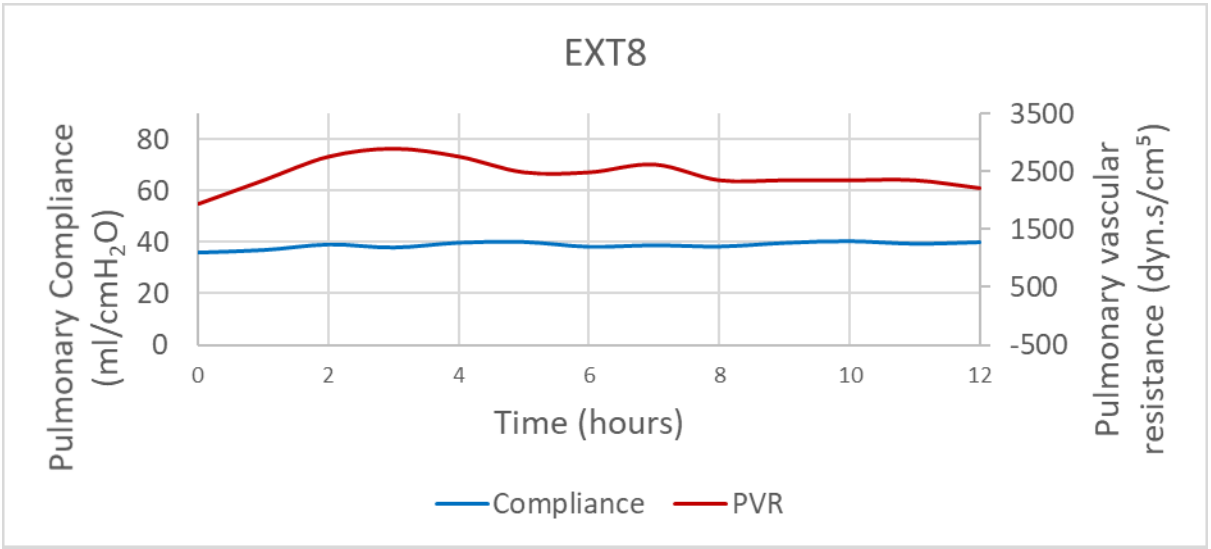
4.3.1.1 Ex vivo pulmonary physiology

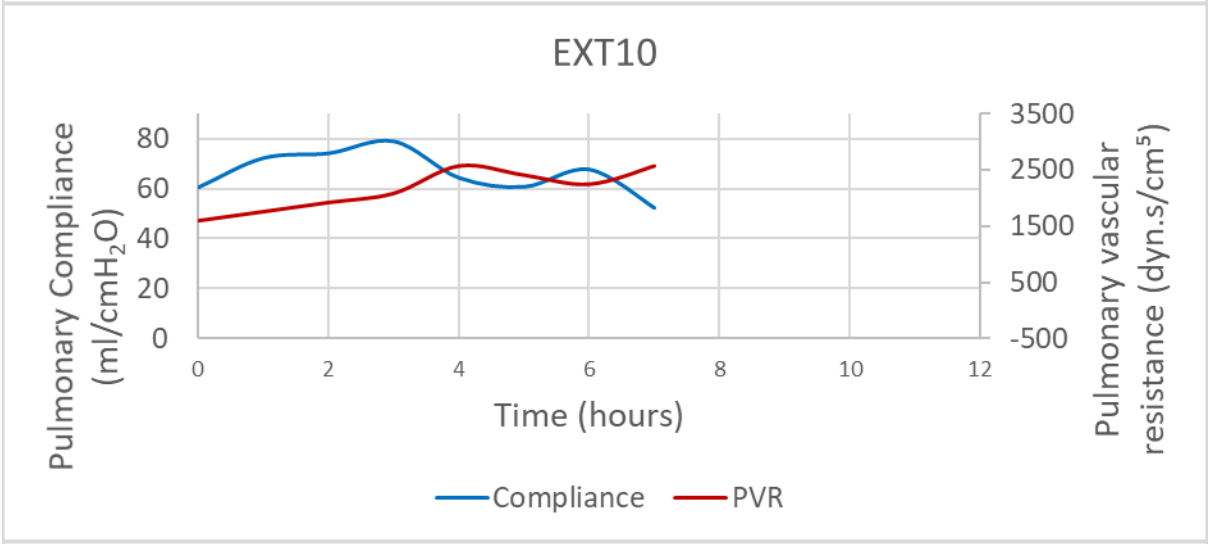
Physiological parameters of every organ enrolled into the D9 labelling experimented were recorded, and included measures of pulmonary perfusate flow, perfusate pressure, tidal volumes, and airway pressures. These values have been used to calculate pulmonary vascular resistance and compliance for each organ, and these parameters are summarised for each organ.

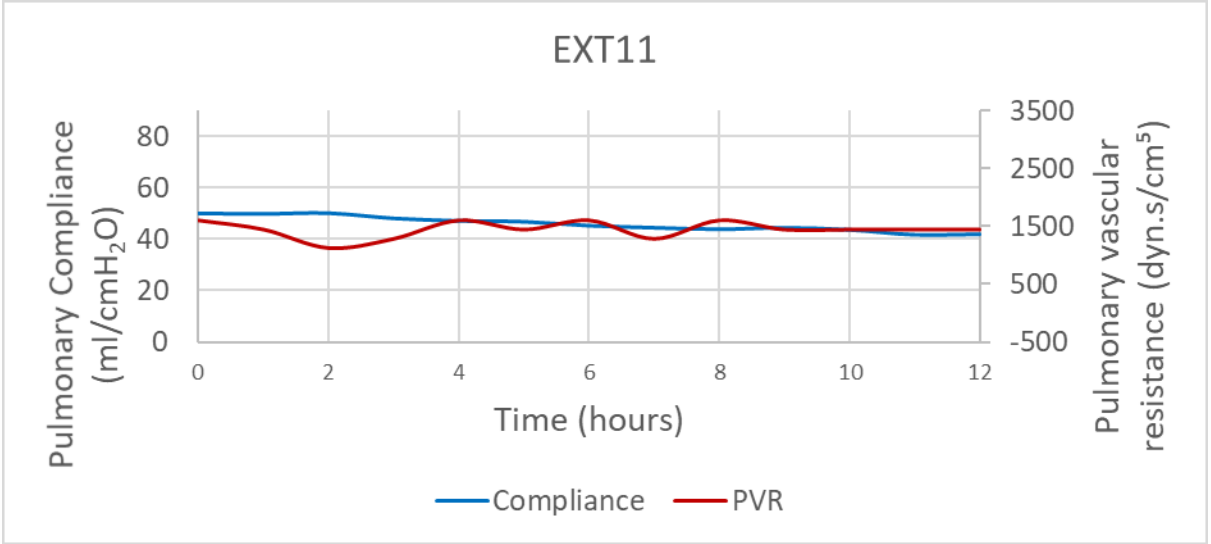

EXT1
Pulmonary Compliance (ml/cmH2O)
80
60
40
20
0
0
2
4
6
8
10
12
3500
2500
1500
500
-500
Pulmonary vascular resistance (dyn.s/cm5)
Time (hours)
Compliance
PVR


EXT3
Pulmonary Compliance (ml/cmH2O)
80
60
40
20
0
0
2
4
6
8
10
12
3500
2500
1500
500
-500
Pulmonary vascular resistance (dyn.s/cm5)
Time (hours)
Compliance
PVR


EXT7
Pulmonary Compliance (ml/cmH2O)
80
60
40
20
0
0
2
4
6
8
10
12
3500
2500
1500
500
-500
Pulmonary vascular resistance (dyn.s/cm5)
Time (hours)
Compliance
PVR


EXT8
Pulmonary Compliance (ml/cmH2O)
80
60
40
20
0
3500
2500
1500
500
-500
0
2
4
6
8
10
12
Time (hours)
Pulmonary vascular resistance (dyn.s/cm5)
Compliance
PVR


EXT10
Pulmonary Compliance (ml/cmH2O)
80
60
40
20
0
3500
2500
1500
500
-500
0
2
4
6
8
10
12
Time (hours)
Pulmonary vascular resistance (dyn.s/cm5)
Compliance
PVR


EXT11
Pulmonary Compliance (ml/cmH2O)
80
60
40
20
0
3500
2500
1500
500
-500
0
2
4
6
8
10
12
Time (hours)
Pulmonary vascular resistance (dyn.s/cm5)
Compliance
PVR

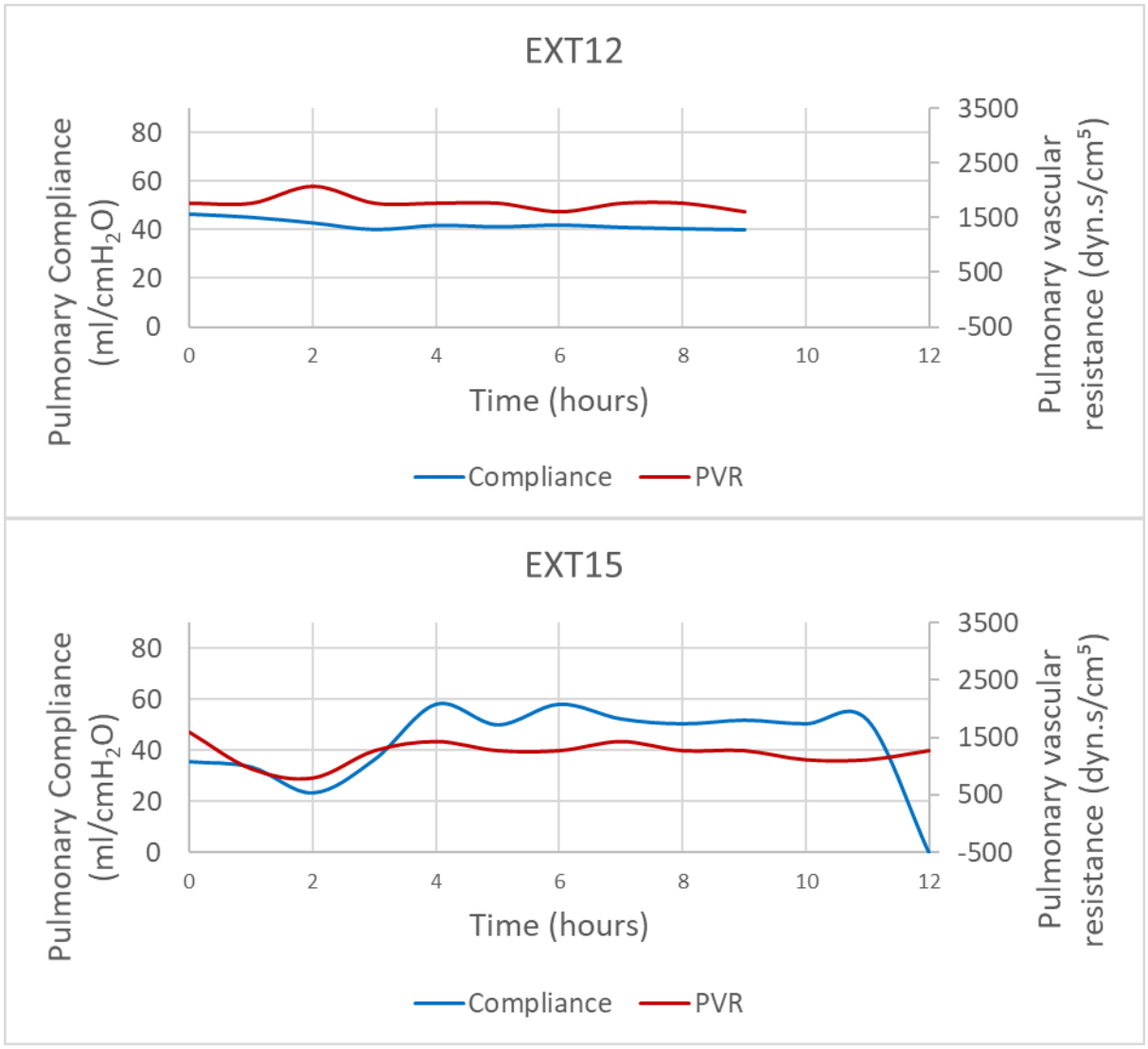


Figure 4-4: Ex vivo pulmonary physiology.
Physiological observation charts of every organ sustained on ex vivo support for deuterium labelling experiments. Each organ studied is displayed with a unique study identifier at the top of its panel. Dynamic pulmonary compliance is displayed in blue on the left vertical axis; pulmonary vascular resistance is displayed in red on the right vertical axes. Hours after the administration of the D9-choline label is displayed in hours along the horizontal axes. Physiology is demonstrated for 12 hours of ex vivo support, and lines terminate before then if the organs failed on ex vivo support - see study end-points in section 4.2. There is a missing data point for EXT7 at T=0.

4.3.1.2 Key Findings – Study organs

- The ex vivo study was comprised of a small heterogeneous cohort of organs, with widely varying physiology
- The ex vivo life span of these organs was variable

4.3.2 Baseline phospholipid profiles

The phospholipid profile has been described for the bronchial washings and perfusate of all ex vivo organs, by summarising the relative abundance of all species from the baseline samples that were taken as soon as the organs became available.

4.3.2.1 Airway phospholipid profile

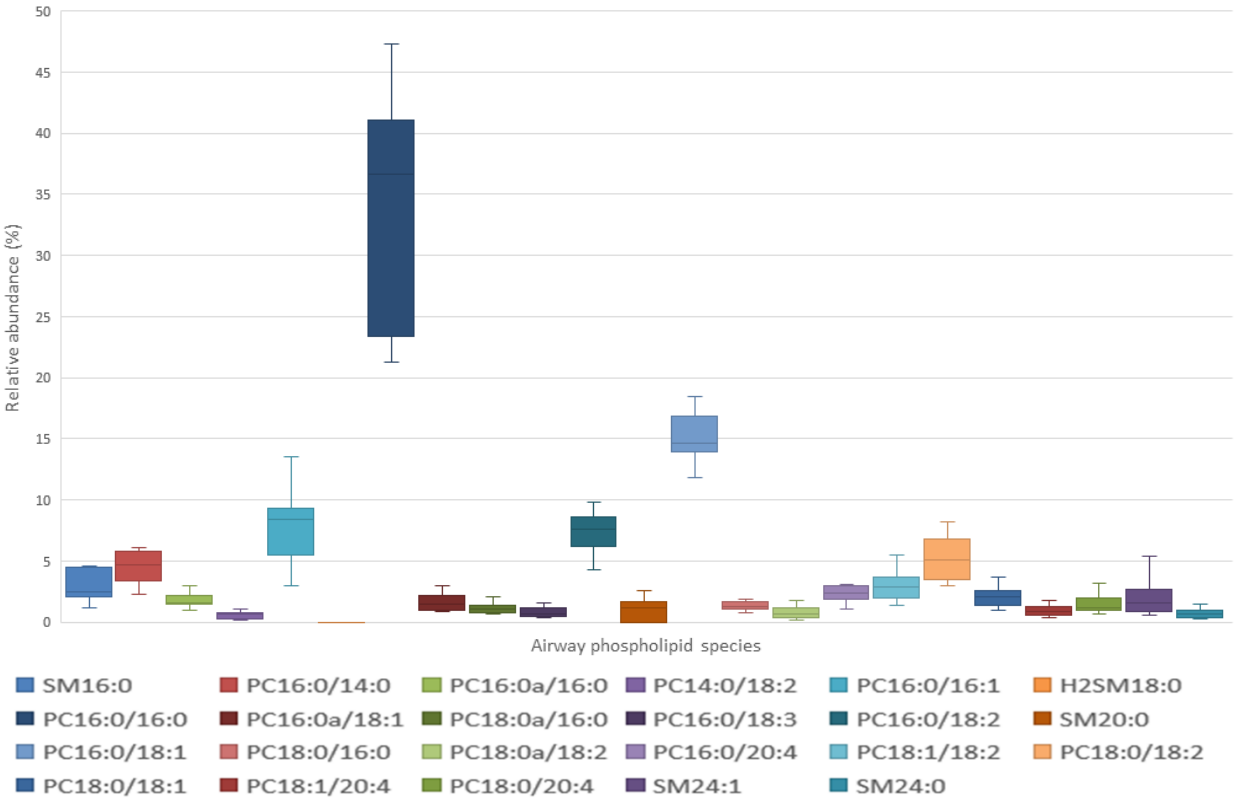


Figure 4-5: Airway phospholipid composition of lungs on ex vivo support
Box and whisker plot of the relative abundance of the baseline (first available sample) phospholipid species in the bronchoalveolar lavage from lungs sustained ex vivo, illustrating the median, range, and interquartile range of values for each species. n=12

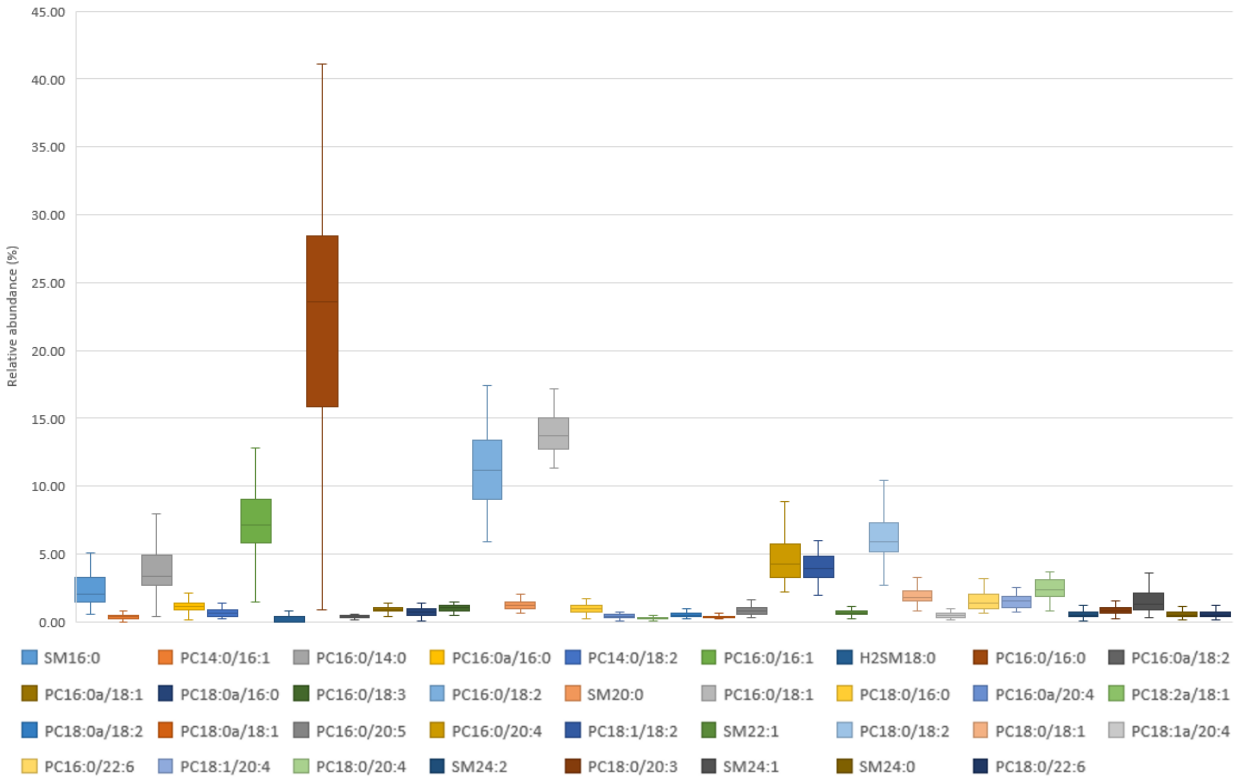


Figure 4-6: Postoperative airway lipid profile – relative abundance
Reproduced from Figure 3-7 in section 3.3.2.2; to illustrate the similarities in phospholipid profiles. Box and whisker plot. Relative abundance of the top phospholipid species detected on a precursor 184 scan in the airways of patients immediately after lung transplantation. n=34

Examining the first available BAL samples from all EVLP preparations, a range of phospholipid species that are comparable to those seen in vivo in postoperative transplant recipients were observed. Figure 4-6 has been reproduced from the previous chapter, to illustrate the comparable phospholipid profiles seen in vivo (Figure 3-7 in section 3.3.2.2). A detailed comparison of the differences between the ex vivo data and transplant recipient data is presented in Table 4-2. Whilst most of the differences in the relative abundance of many species appeared to be small, there was a marked difference in the abundance of DPPC. DPPC accounted for 36.7% of the airway PC content in the ex vivo lungs, but only accounted for 23.3% of the airway PC content immediately after transplantation (p=0.003, two-tailed Mann Whitney test, described in Table 4-2).

	Ex vivo relative abundance (%)		*In vivo relative abundance (%)*		*p value*
SM16:0	2.493	(2.102 - 4.493)	1.996	(1.466 - 3.391)	0.1189
PC16:0/14:0	4.706	(3.443 - 5.771)	3.362	(2.265 - 4.919)	0.0867
PC16:0a/16:0	1.589	(1.436 - 2.164)	1.131	(0.876 - 1.403)	**0.0004**
PC14:0/18:2	0.679	(0.322 - 0.762)	0.637	(0.426 - 0.87)	0.7204
PC16:0/16:1	8.421	(5.545 - 9.356)	6.909	(5.241 - 9.246)	0.3931
H2SM18:0	0	(0 - 0)	0	(0 - 0.473)	0.4430
PC16:0/16:0	36.656	(23.414 - 41.067)	23.264	(15.7 - 28.638)	**0.0030**
PC16:0a/18:1	1.47	(0.978 - 2.227)	0.907	(0.8 - 1.088)	**0.0012**
PC18:0a/16:0	1.039	(0.814 - 1.415)	0.662	(0.507 - 0.929)	**0.0007**
PC16:0/18:3	0.731	(0.444 - 1.143)	1.018	(0.826 - 1.19)	0.0867
PC16:0/18:2	7.631	(6.179 - 8.601)	11.603	(9.251 - 13.48)	**0.0035**
SM20:0	1.141	(0 - 1.677)	1.166	(0.975 - 1.369)	0.6609
PC16:0/18:1	14.619	(13.972 - 16.875)	13.71	(12.577 - 14.721)	**0.0311**
PC18:0/16:0	1.331	(1.105 - 1.678)	1.007	(0.74 - 1.192)	**0.0002**
PC18:0a/18:2	0.732	(0.343 - 1.2)	0.459	(0.378 - 0.63)	0.1918
PC16:0/20:4	2.341	(1.869 - 2.985)	4.444	(3.331 - 5.723)	**0.0002**
PC18:1/18:2	2.913	(2.014 - 3.702)	4.071	(3.252 - 5.199)	**0.0071**
PC18:0/18:2	5.149	(3.538 - 6.806)	6.025	(5.161 - 7.645)	0.1251
PC18:0/18:1	2.069	(1.352 - 2.581)	1.743	(1.437 - 2.142)	0.3277
PC18:1/20:4	0.838	(0.619 - 1.317)	1.539	(1.207 - 2.017)	**0.0004**
PC18:0/20:4	1.15	(0.946 - 1.989)	2.374	(1.803 - 3.121)	**0.0042**
SM24:1	1.58	(0.904 - 2.724)	1.373	(0.915 - 2.131)	0.5953
SM24:0	0.684	(0.363 - 0.969)	0.485	(0.347 - 0.705)	0.2696

Table 4-2: Comparison of airway phospholipids between ex vivo lungs and lung transplant recipients Baseline blind bronchial aspirates are compared from 34 transplant recipients and compared against the first available bronchial washing from 12 ex vivo organs. The relative abundance is reported as the median value for each species, with the interquartile range in brackets. Two-tailed p-values were calculated with the Mann Whitney test, and values below 0.05 have been highlighted in red.

4.3.2.1.1 Key findings – ex vivo airway lipids profiles

- A description of airway phospholipids on EVLP is provided
- There were some differences between the airways phospholipids of EVLP lungs compared to lungs that were transplanted
    - Notably, there was more airway DPPC in EVLP lungs than there was in the recipient's lungs described in the previous chapter

4.3.2.2 Perfusate phospholipid profiles

The relative abundance of phospholipid species in the first available perfusate sample taken from all ex vivo organs is illustrated in Figure 4-7. As seen in the plasma of postoperative lung transplant recipients, PC16:0/18:2, PC16:0/18:1, PC16:0/20:4, PC18:1/18:2, and PC18:0/18:2 are the five most abundant species.

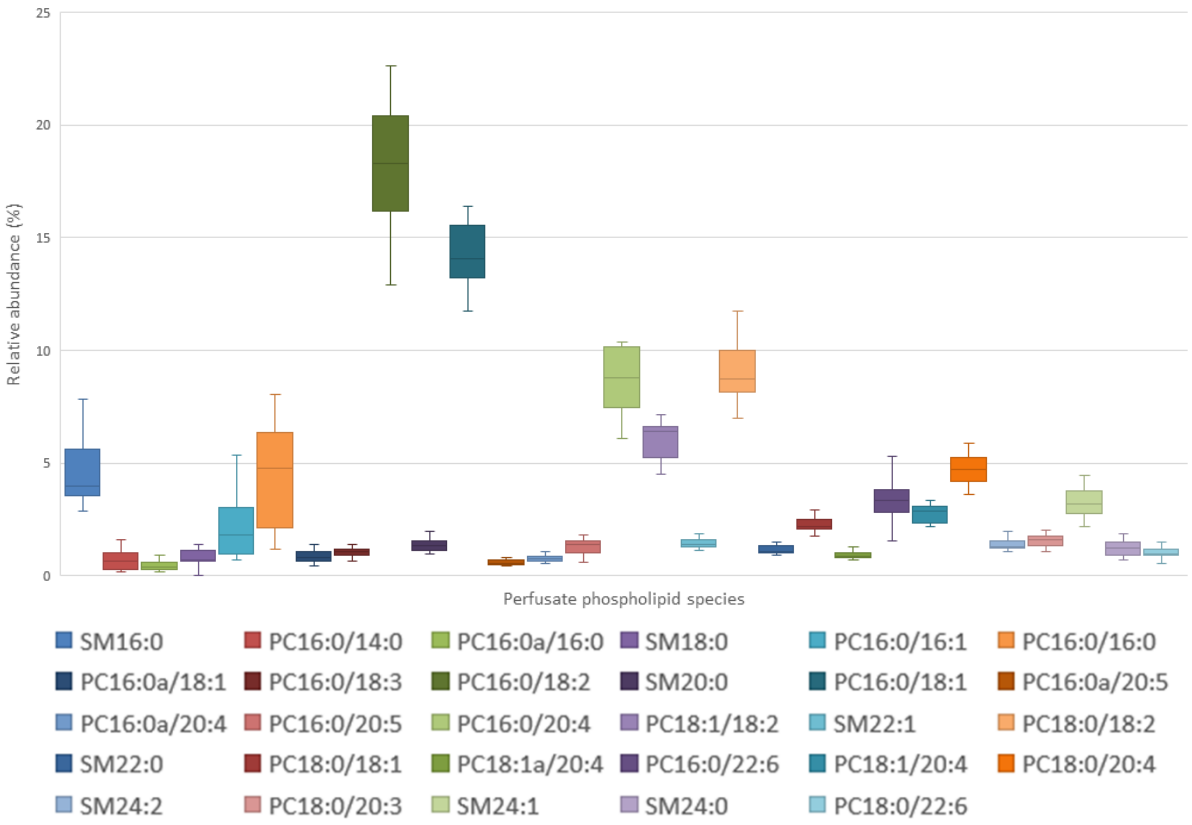


Figure 4-7: Baseline profiles in perfusate phospholipids. Box and whisker plot of the relative abundance of the baseline (first available sample) phospholipid species in the perfusate from lungs sustained ex vivo, illustrating the median, range, and interquartile range of values for each species, n=13.

4.3.2.2.1 Differences in lipidomic profile between transplanted and non-transplanted organs

Comparison of the perfusate lipidomic profiles revealed significant differences between the organs that were transplanted and those that were rejected by the transplant team. Standard acceptance criteria are summarised in Table 4-1, and illustrate some of the reasons for rejection. The perfusate of rejected organs had a greater relative abundance of PC16:0/14:0 (p=0.08), PC16:0/16:1 (p=0.019), and PC 16:0/16:0 (p=0.013), three species typically associated with pulmonary surfactant in the airways[284]; with a corresponding reduction in the relative abundance of PC18:1/18:2 (p=0.013).

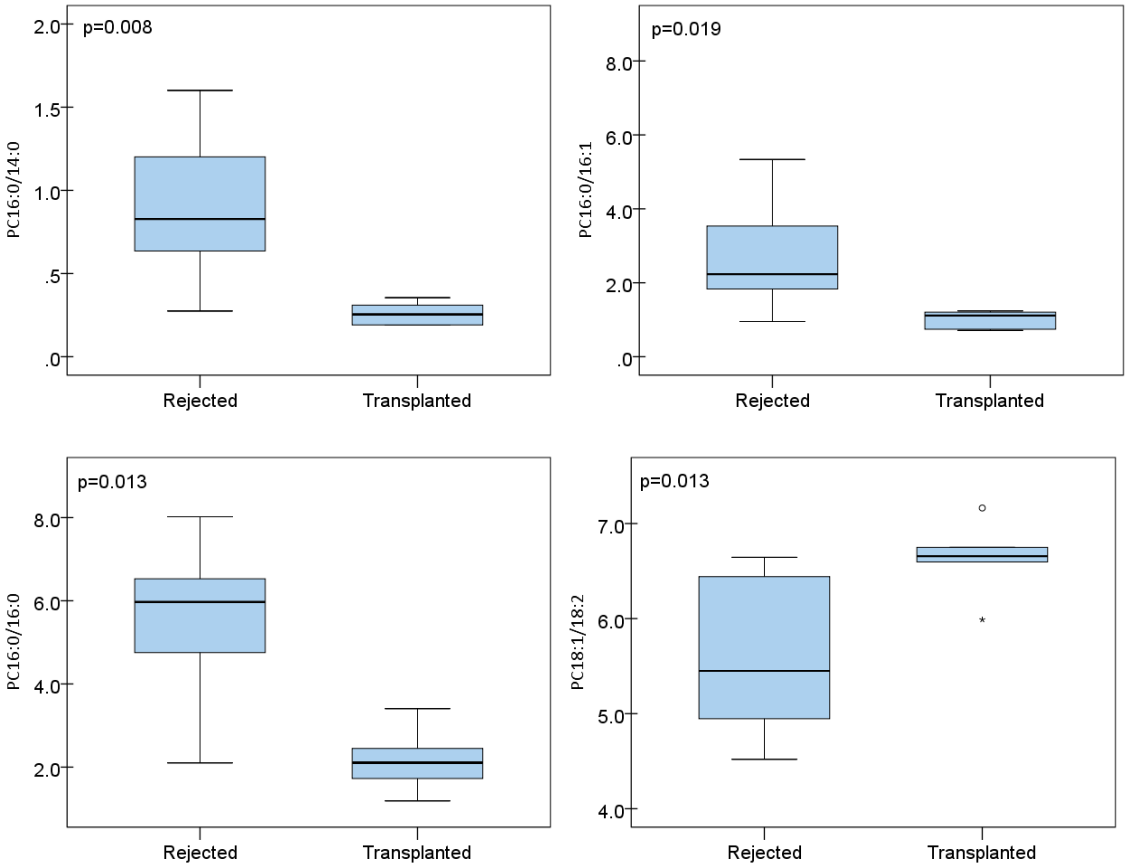


Figure 4-8: Differences between transplanted and rejected lungs in perfusate lipid profiles. Box plot of the relative abundance (%) of perfusate phospholipid species, demonstrating the range, interquartile range and median values. Rejected ex vivo lungs (n=10) on the left box are compared against transplanted ex vivo lungs (n=5) on the right box. Two-tailed Mann-Whitney p-values are reported.

4.3.2.2.2 Key findings

- There is an increase of surfactant phospholipid species in the perfusate of lungs that are considered unsuitable for clinical transplantation
- A description of the phospholipids found in EVLP perfusate is provided

4.3.3 Deuterium labelling of the Kennedy CDP-choline pathway

4.3.3.1 The range of deuterium labelled phospholipids in the perfusate

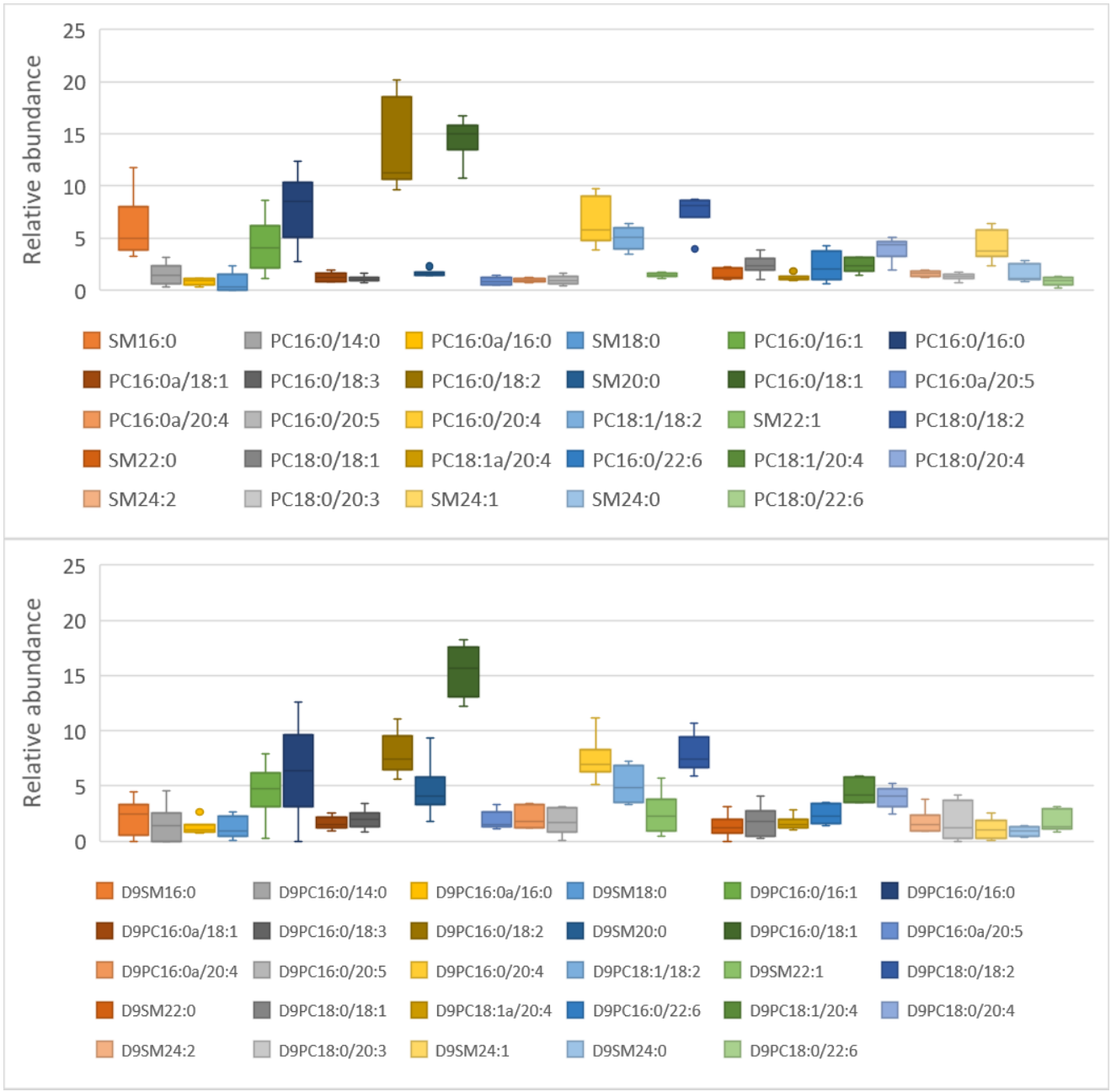


Figure 4-9: Perfusate lipid profile at 9 hours in labelled ex vivo lungs. Box plot showing range, interquartile range, and median values for the relative abundance of phospholipid species detected in the perfusate samples taken 9 hours after label administration, N=6. Top Panel – unlabelled species. Bottom Panel – labelled species.

The 9-hour time point has been selected for in-depth analysis of labelled phospholipids, as this provided sufficient time for incorporation of the deuterium label in a sufficient number of surviving ex vivo organs. The unlabelled lipid profile is first illustrated as the relative abundance of PC species

for all perfusate samples taken 9 hours after the administration of the deuterium label; deuterium-labelled species are then displayed as their relative abundance in the same samples.

4.3.3.2 Time-course of ex vivo deuterium incorporation into phospholipids

Deuterium incorporation into DPPC can be seen in the airways within a matter of hours; however, the rate of label incorporation varied between organs. Figure 4-10 highlights the substantial variability in D9-DPPC incorporation between organs, with organ EXT7 showing rapid incorporation of the deuterium label, whereas organs EXT11 and EXT15 showing minimal to no D9-DPPC incorporation.

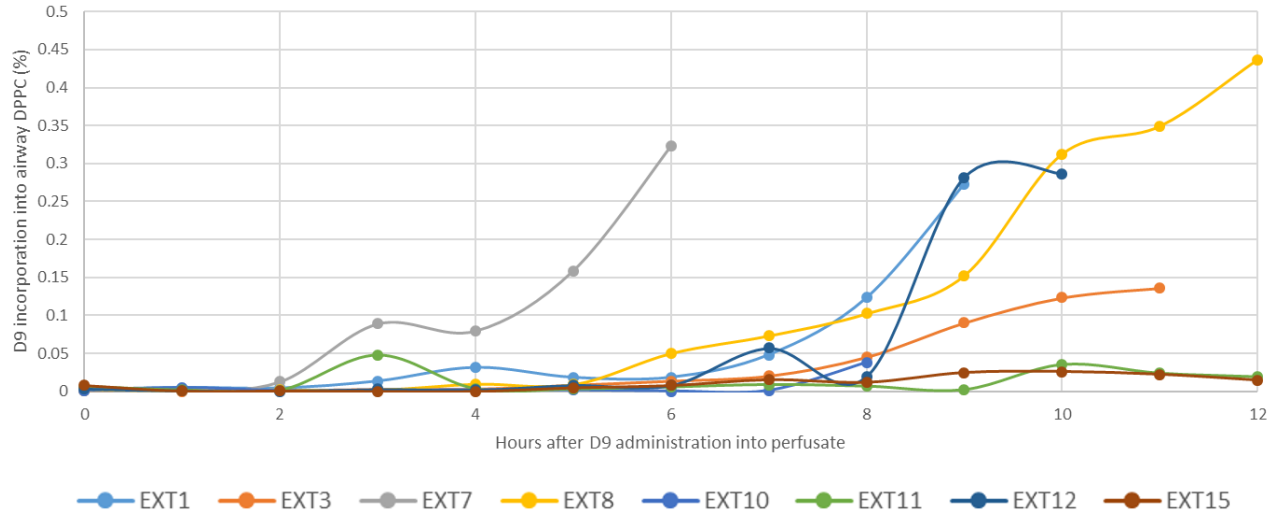


Figure 4-10: D9-choline incorporation into airway DPPC into all labelled ex vivo organs. The proportion of D9 label incorporation airway PC16:0/16:0 (DPPC) is shown as a percentage on the y-axis, against the time (in hours) after the label was introduced to the organs (x-axis), for every labelled organ (n=8).

For comparison, D9-incorporation has also been plotted against time for two selected perfusate species; DPPC (as it is the key phospholipid species of interest in the airways), and PC16:0/18:2 (as it is the most abundant species in the perfusate). Whilst the time-course graphs appear to show different trends when comparing deuterium incorporation in the BAL and perfusate for each organ, there was no statistical difference when comparing deuterium incorporation between the BAL and perfusate at 9 hours - Figure 4-12.

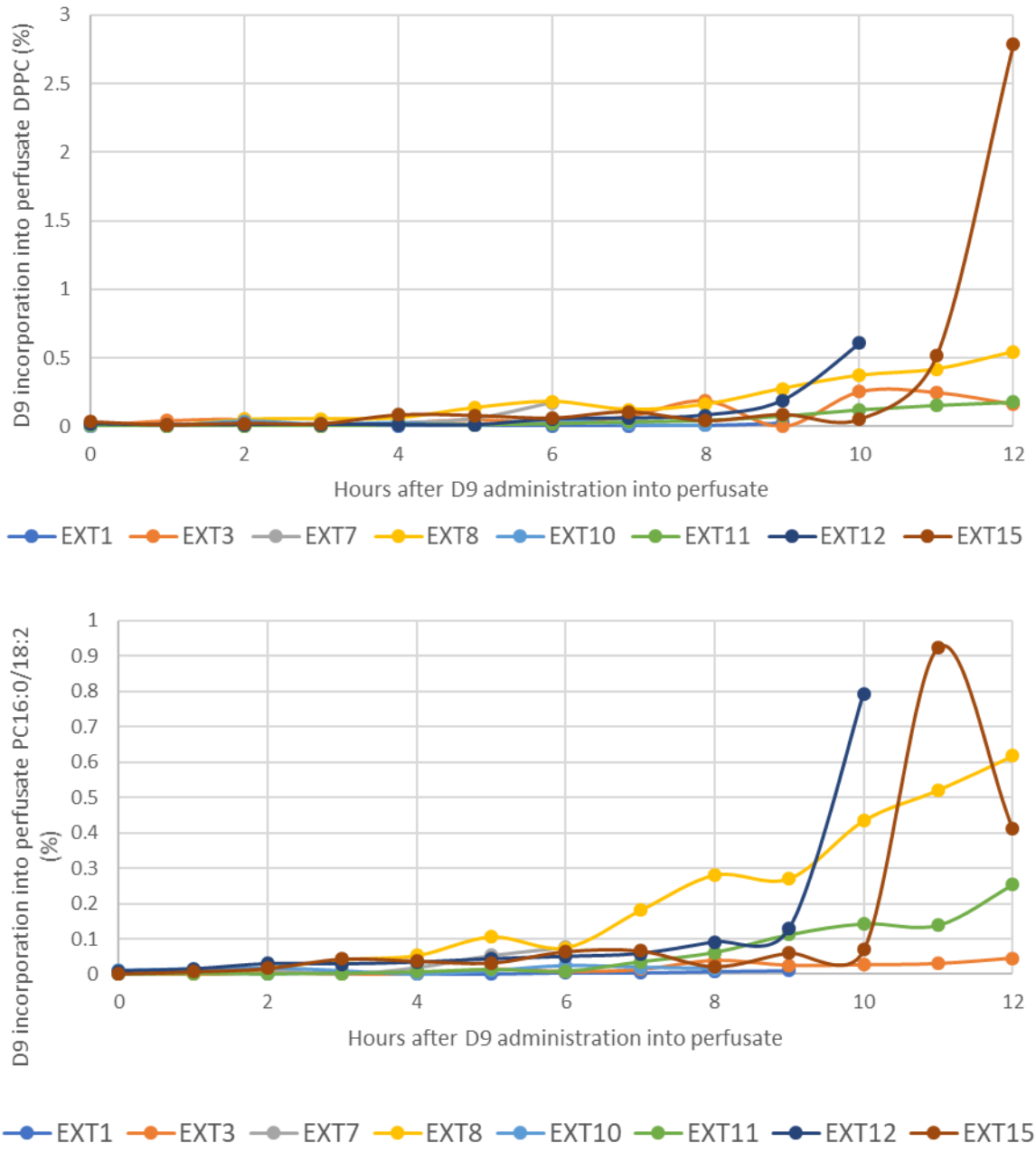


Figure 4-11; Time course of ex vivo deuterium incorporation into perfusate phospholipids. The proportion of D9 label incorporation into the phospholipid species is shown as a percentage on the y-axis, against the time (in hours) after the label was introduced to the organs (x-axis), for every labelled organ (n=8). Top panel-PC16:0/16:0 (aka DPPC), bottom panel-PC16:0/18:2.

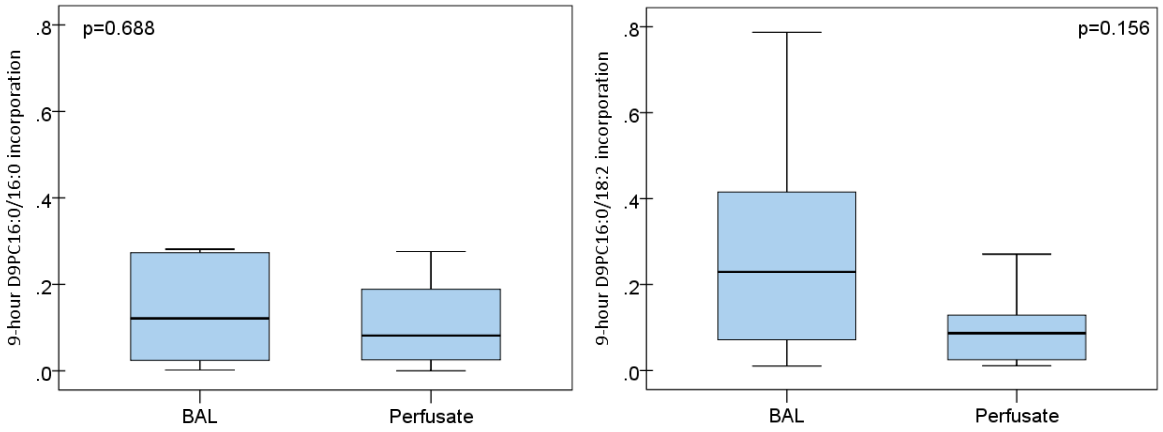


Figure 4-12: D9 incorporation compared between the perfusate and the BAL. Box plot of deuterium incorporation demonstrating the range, interquartile range and median values in the BAL and perfusate of ex vivo lungs at 9 hours (n=6). Left panel, PC16:0/16:0 (DPPC). Right panel, PC16:0/18:2. Wilcoxon signed rank test with 2-tailed p-values reported.

Whilst deuterium enrichment of DPPC after 12 hours was significantly different when measured in the plasma and airways of lung transplant recipients (Figure 3-24), similar differences were not detected in the airways and perfusate of this ex vivo cohort of lungs.

4.3.3.2.1 Key findings – Ex vivo deuterium labelling

- There is Kennedy pathway activity during normothermic EVLP
- Kennedy pathway activity is highly variable amongst EVLP organs that have been rejected for transplantation

4.3.3.3 Differences between airway and vascular secretion of pulmonary phospholipids.

The relative abundance of deuterated phospholipids was compared between the perfusate and the BAL of all labelled ex vivo organs that survived for 9 hours. There were significant differences in several species (Figure 4-13).

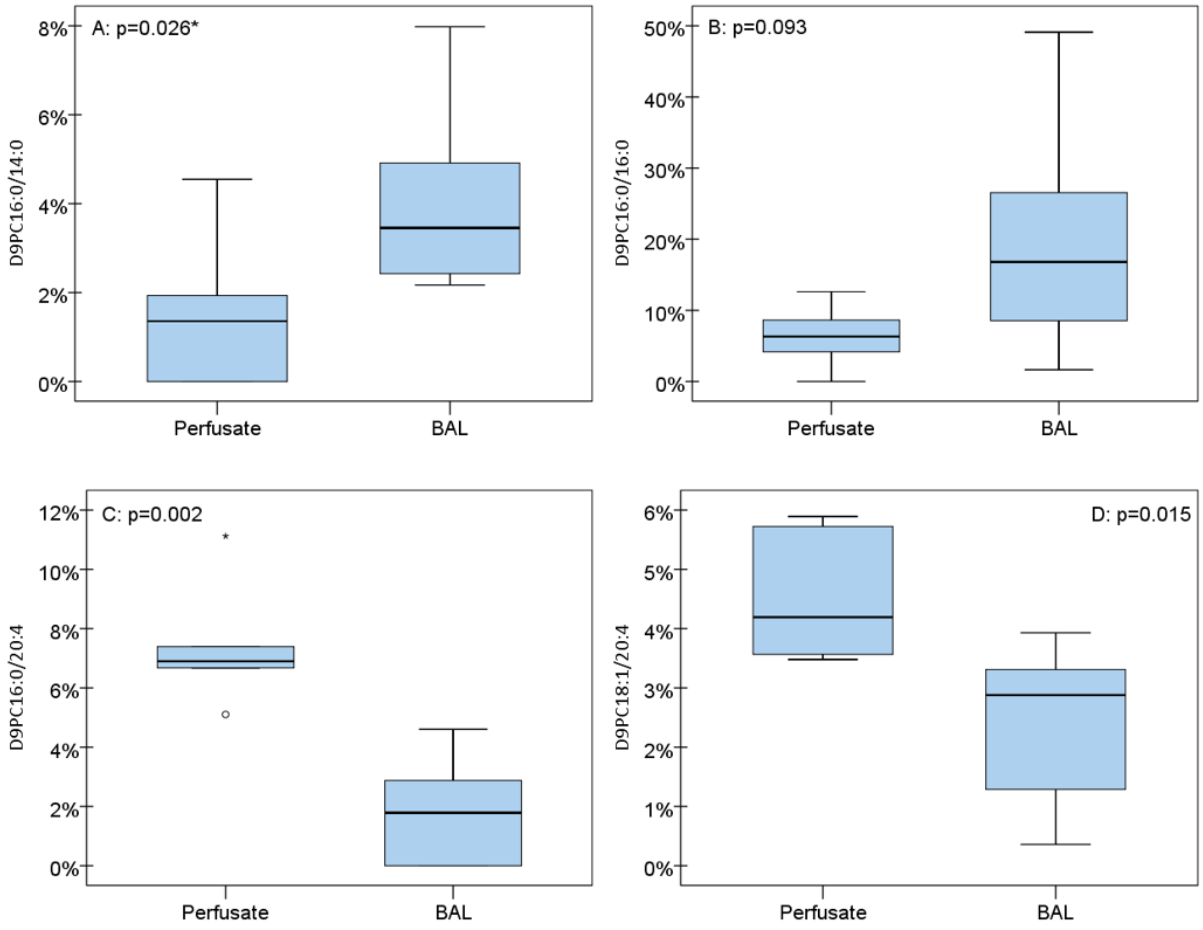


Figure 4-13: Differences in newly synthesised phospholipids in the airways and the perfusate. Relative abundance of selected deuterated PC species in the lavage and perfusate in lungs that have been sustained ex vivo for 9 hours. PC16:0/14:0 (A), PC16:0/16:0 (B), PC16:0/20:4 (C), PC 18:1/20:4 (D). n=6. Two-tailed Mann-Whitney p-values are reported

As expected, there is a tendency for the lungs to release more surface active saturated phospholipid species into the airways when compared to the circulation. In contrast, the phospholipids released into the circulation had relatively more newly synthesised arachidonic acid containing phosphatidylcholine species - Figure 4-13.

4.3.3.3.1 Key findings – comparison of deuterium labelling in the perfusate and airways

- <u>The composition of phospholipids that are synthesised and released by the lungs is different in the airways and the circulation</u>

4.4 Discussion

4.4.1 Summary of key findings

- Surfactant phospholipid synthesis continued via the Kennedy CDP-choline pathway during normothermic ex vivo lung perfusion and ventilation
- Kennedy pathway activity was highly variable in EVLP using rejected organs
- Surfactant phospholipids were synthesised by the lung and secreted into the airspace in isolation from the liver and other organs
- Lungs synthesised and actively secreted a range of phospholipids into the circulation that are relatively rich in PUFA. In contrast, lungs secreted phospholipids that are relatively rich in saturated species into the airways
- Human ex vivo lung perfusion proved to be a viable platform for research that can provide unique insights into pulmonary phospholipid biology

This study provides the first description of human lungs actively secreting a distinct phospholipid profile into the circulation, in addition to the secretion of surfactant phospholipids into the airways. These data provide compelling evidence in support of the previously hypothesised basolateral phospholipid secretion by the alveolar epithelium, and these valuable insights will further our understanding of phospholipid trafficking in the lung.

We have confirmed that the presence of Kennedy pathway activity in EVLP, and preservation of this pathway could represent one way in which the EVLP platform protects the lung. However, Kennedy pathway activity was highly variable in this heterogeneous cohort of lungs, and the origins of this variability will require further study.

4.4.2 Deuterium label incorporation

4.4.2.1 Timing of phospholipid synthesis

As the deuterated choline was introduced after the organs were sustained on ex vivo support, it can be said with certainty that all deuterium labelled phospholipids had been synthesised by the lungs via the Kennedy pathway after they were established on EVLP. The incorporation of the D9-choline label into phospholipids during EVLP offers definitive proof that pulmonary surfactant phospholipid metabolism can continue during normothermic ex vivo support, and could represent one of the protective mechanisms behind clinical EVLP in lung transplantation.

It is likely that some of the unlabelled phospholipids in the airways of these EVLP lungs may have been synthesised prior to organ retrieval, as the half-life of airway DPPC is probably no less than 24 hours[285]. Similarly, some of the unlabelled perfusate phospholipids may also have originated from residual donor plasma that was incompletely flushed through during organ retrieval. However, deuterium labelled phospholipids can only have been synthesised by the lungs after they were established on EVLP and infused with the D9-choline label. Therefore, any deuterium labelled phospholipids were actively synthesised by the lung during ex vivo support through the Kennedy CDP-choline pathway, and were not residual phospholipids that were synthesised by other organs in the donor prior to retrieval.

4.4.2.2 Airway D9-DPPC incorporation

The ex vivo incorporation of the D9 label varied between the organs studied – section 4.3.3.2. Organ EXT7 demonstrated rapid airway D9-DPPC incorporation within six hours, yet two organs (EXT11 & EXT 15) showed minimal airway D9-DPPC incorporation after 12 hours (Figure 4-10). This lack of deuterium enrichment of airway DPPC cannot be explained by assuming that those organs were dead or that there was a problem with the labelling technique, as EXT15 had high levels of deuterium enrichment in perfusate DPPC and PC16:0/18:2. Therefore, it can be concluded that

EXT15 was alive with some Kennedy pathway activity during the experiment, even if the pathway was functioning abnormally with minimal airway DPPC synthesis.

It is possible that the abnormal PC metabolism seen in EXT15 reflects a greater degree of lung injury, but there were many other variables that could have accounted for altered PC metabolism; like differences in the EVLP platform or perfusate. Addressing this question in future studies would require a larger number of ex vivo organs with standardised EVLP operating conditions combined with additional measures of lung injury. The determinants of abnormal airway DPPC metabolism warrants further study as this could provide insights into the surfactant dysfunction seen in acute lung injury, and potentially identify novel biomarkers and therapeutic targets. Enhanced protection of ex vivo DPPC synthesis could also theoretically improve the ability of EVLP to protect the transplanted lung, but addressing this hypothesis would require continued research.

4.4.2.3 Pulmonary phospholipid secretion into the circulation

It is possible that some airway phospholipids could passively 'leak' into the perfusate, especially if the alveolar barrier function is compromised in rejected organs with more severe lung injury. DPPC was not typically seen in the circulation of postoperative recipients (section 3.3.2.3), but it was repeatedly detected at low levels in the perfusate in EVLP; particularly in organs that were rejected (Figure 4-8). It is possible that rejected lungs had more DPPC in their perfusate because their alveolar barrier function was impaired, and surfactant phospholipids could leak from the alveolar space into the perfusate. However, the differences in labelled phospholipids in the bronchial washings and the perfusate are highly suggestive of an active and selective process that determines which phospholipids are released into the alveolar space or the circulation. If the only source of labelled phospholipids in the perfusate was through the passive leak from the alveolar space, there would be an identical phospholipid profile in both compartments. Instead, we see differing profiles,

with the airways containing more surface-active saturated phospholipids and the circulation containing more polyunsaturated PC species – section 4.3.3.3.

The appearance of deuterium-labelled PUFA-containing PC species in the EVLP perfusate is of particular interest. Arachidonic acid has been described as the "mother substance of pro-inflammatory eicosanoids", and it is thought to be released by membrane phospholipids[210]. Arachidonic acid containing PC species are also markers of monocyte activation, which is another process that has been implicated in the development of primary graft dysfunction[12,13]. Arachidonic acid is actually much more than the "mother substance of pro-inflammatory eicosanoids"[210]. Arachidonic acid in converted in a diverse range of lipid mediators, initially via cyclooxygenase (COX) and lipoxygenase (LO) pathways[14]. Lipoxins are an example of pro-resolving mediators that are derived from arachidonic acid via LO pathway interactions[286]. Lipoxin A4 has previously been found in the lavage fluid of human lung transplant recipients and was associated with severe allograft dysfunction[287].

Apart from arachidonic acid, there are other PUFA species that are also converted into biologically important mediators. Several newly synthesised PC species containing eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) were detected in the perfusate (but not in the airways), and they are precursors for several specialised anti-inflammatory mediators that include resolvins, protectins and maresins[288]. These data confirm that the lung is a source of several PUFA precursors of both proinflammatory and proresolving lipid mediators that are released into the pulmonary circulation.

Beyond their role in the generation of lipid mediators of inflammation, long-chain PUFA may exert an antioxidant effect that protects the lungs through free radical-scavenging[289]. It is also possible that circulating PUFAs may also exert systemic cardiovascular effects, as administering lipid emulsions rich in PUFAs in animal models produces a positive inotropic effect and promotes cardiac diastolic relaxation[290]; which would be a desirable haemodynamic response during periods of pathophysiological stress.

As stated earlier, it is believed that arachidonic acid is mobilised from membrane phospholipids[210]. Data from this chapter shows that pulmonary secretion of PC species containing arachidonic acid occurred within hours of de novo synthesis on EVLP. However, it is difficult to determine whether these PC species containing arachidonic acid had spent any time in the membrane pool of phospholipids.

Polarised murine cell culture experiments have previously suggested that pulmonary epithelium is capable of secreting phospholipids in both apical and basolateral directions, and that basolateral phospholipid secretion is mediated by the ATP-binding cassette transporter A1 (ABCA1)[10,278]. However, it had not been confirmed that these vitro observations equate to pulmonary phospholipid secretion into the circulation, and any PC species that may have been secreted by the lung into the circulation were previously unknown. The data presented in this chapter confirms that the lungs can selectively secrete polyunsaturated PC into the circulation.

In summary, pulmonary phospholipid metabolism probably extends beyond the synthesis of surface active phospholipids that prevent alveolar collapse. The lungs also release phospholipids into the circulation that probably have an important role in orchestrating the inflammatory response; continued research is indicated.

4.4.3 Baseline airway and perfusate phospholipid composition

Previous studies using nearly identical techniques in healthy volunteers have shown that DPPC typically accounts for 50% of all PC species[7]. Interestingly, DPPC levels in the airways of the ex vivo lungs were closer to the levels seen in health at 36.7%, when compared to the proportion of DPPC of 23.3% seen in perioperative lung transplant recipients immediately after surgery (Figure 4-5 & Figure 4-6). Depleted surfactant phospholipids might be expected in a study that included some organs that were considered to be too injured for clinical transplantation as well as healthy organs that

were subsequently used for transplantation, yet the relative abundance of airway DPPC in these ex vivo organs was better than those seen in postoperative transplant recipients. These data suggest that some pulmonary DPPC is lost during the process of organ retrieval and ex vivo support, but more is rapidly lost in the hours after reperfusion associated with transplantation. Certainly, continued deterioration in airway DPPC abundance was seen in recipients for days after lung transplantation (see section 3.3.2.2). Taking these two observations together suggests that some airway DPPC is lost during both retrieval and ischemia, but more is lost during the subsequent in vivo reperfusion.

4.4.4 Cellular origins of newly synthesised pulmonary phospholipids

Nearly every eukaryotic cell is capable of PC synthesis via Kennedy's CDP-choline pathway[127]. Type I alveolar cells are the most abundant cell type and represent nearly 95% of the alveolar surface, yet they are relatively devoid of the cellular organelles that are typically associated with phospholipid synthesis and secretion as they form the thin air-blood barrier for gas exchange. By contrast, AT2 contain lamellar bodies storing surfactant phospholipids that have been synthesised in the endoplasmic reticulum and Golgi apparatus, and release multiple phospholipids in isolated cell culture experiments [291,292]. Therefore, it would be reasonable to presume that AT2 are the primary source of deuterated phospholipids seen in the airways of the ex vivo lungs presented in this chapter. However, AT2 cell culture models in isolation from other cell types found in the lung synthesised different phospholipids when compared to isolated perfused rat lung models [122,279,293].

Pulmonary lipofibroblasts (sub-type of fibroblasts) are mesenchymal cells that are laden with lipid droplets and reside in the alveolar interstitium, and probably contribute to surfactant metabolism; although, their role in human lungs remains controversial[294]. Lipofibroblasts support the growth and differentiation of AT2, and provide ongoing support through the storage and supply of triglycerides for the use in surfactant phospholipid synthesis[295]. Lipofibroblasts are also independently capable of

phospholipid synthesis in animal models, and it is also conceivable that they may have contributed to phospholipid synthesis in this human ex vivo model[296].

Donor alveolar macrophages travel with the graft during lung transplantation, and are responsible for the removal and catabolism of surfactant phospholipids from the airways prior to recycling by AT2[101,297]. Alveolar macrophages also secrete PLA_2 enzymes that are an integral part of Land's acyl remodelling pathways [101,297,298]. Even if these passenger alveolar macrophages do not directly contribute to phospholipid synthesis, it is likely that they have indirectly contributed to the molecular diversity of phospholipid species that have been identified in these ex vivo lungs through their role in acyl remodelling. PLA_2 are a super-family of esterases that catalyse the hydrolysis of acyl groups from phospholipids, and have a diverse range of biological functions amongst the numerous isotypes that have been identified. Beyond their role in surfactant homeostasis, PLA_2 enzymes have been implicated in the pathogenesis of ARDS through the generation of eicosanoids and other lipid based inflammatory mediators[299]. PLA_2 could also have also have a more direct role in the development of ARDS though the local release of lyso PC species, which have been associated leukocyte recruitment, impaired surfactant tensioactivity, and the injury of type I alveolar epithelial cell membranes[300]. PLA_2 enzymes are also expressed in: monocytes, mast cells, smooth muscle cells, and fibroblasts[301]; it is conceivable that all of these cell types have contributed to the molecular diversity of phospholipid species. This hypothesis could be tested by co-culture of the various cell types or by determining how lipid profiles in AT2 conditioned media were affected by transfer to other cell types, like alveolar macrophages.

Pulmonary phospholipid secretion into the circulation has not been previously studied, and the results from this chapter suggest that the lungs actively secrete PUFA containing phospholipids into the blood. Human vascular endothelial cells actively remodel phospholipids via the Land's cycle, but the de novo synthesis of phospholipids occurs slowly via the Kennedy pathway in endothelial cells

when compared to other tissues[302]. One could hypothesise that the differing phospholipid profile secreted by the lungs into the circulation could result from endothelial phospholipid synthesis (as opposed to epithelial synthesis), or possibly through the interactions between pulmonary epithelial and endothelial cells. Eicosanoid synthesis depends on the interaction of many different cell types, and this has been referred to as 'transcellular biosynthesis'[190]. This concept of transcellular biosynthesis has already been illustrated in leukotriene metabolism using a rabbit EVLP model, and further highlights the advantage of using whole-organ preparations when investigating lipid biology[191]. Spatial localisation of these lipids to specific cell types in the lung is difficult, but MALDI-IMS (matrix-assisted laser desorption ionization imaging mass spectrometry) is one of several novel techniques that could be used to visualise pulmonary phospholipids[303]. Combining novel MS imaging with the dynamic lipidomic techniques presented in this chapter is an exciting future direction, that could allow the cellular/extracellular localisation of the numerous metabolic steps that are required in pulmonary phospholipid homeostasis[251].

4.4.5 Synthetic independence from the liver

Phosphatidylcholine (PC) species are synthesised in the Golgi and endoplasmic reticulum of nearly every mammalian cell, typically through the Kennedy CDP-choline pathway[126]. A notable exception is the hepatocyte, which can also synthesise phospholipids via a secondary phosphatidylethanolamine methyltransferase (PEMT) pathway[304]. Hepatic PEMT phospholipid synthesis creates a wider range of PC species containing greater amounts of long-chain polyunsaturated fatty acids (PUFA, such as arachidonic acid) when compared to the species that are synthesised via Kennedy's CDP-choline pathway found in the lung[133]. Animal knock-down models of these hepatic methylation pathways can result in severe disruption of circulating phosphatidylcholine (PC) species, suggesting that the liver is a major source of systemic phospholipids[277]. The true extent to which the lungs depend on the liver for phospholipid

metabolism was previously unknown. However, this study has proven that the lungs can synthesise a diverse range of phospholipid species in isolation from the hepatic PEMT pathways, including those PUFA species, which have little impact on alveolar surface tension.

Apolipoproteins are vital for lipid transport in both the circulation and the lymphatics; many of these apolipoproteins are synthesised in the liver and so were not present in the perfusate of these experiments[305]. Beyond its role as a lipid transporter, ApoA-I also interacts with ABCA1, stabilising this transmembrane protein by protecting it from degradation by proteases[306]. ABCA1 is responsible for cellular efflux of cholesterols and phospholipids into apolipoproteins[307], and has been proposed as the mediator of basolateral phospholipid secretion by the alveolar epithelium[10]. Therefore, it is possible that the lack of these apolipoproteins in the perfusate could impair or alter pulmonary phospholipid secretion into the circulation in an EVLP model.

4.4.6 Validity of the ex vivo platform for research into pulmonary surfactant biology

This study has demonstrated activity of the Kennedy pathway in EVLP, which is a prerequisite for establishing human EVLP as a model of pulmonary phospholipid biology.

Lipidomic profiling has demonstrated a wide range of phospholipids in the airways of these EVLP lungs. DPPC remains the predominant phospholipid species in ex vivo airways, although levels were slightly lower than those seen in healthy volunteers[7]. However, the abundance of DPPC in the airways was generally better in these rejected EVLP lungs than those levels seen in lung transplant recipients immediately after surgery – section 4.3.2.1.

Most of the airway phospholipid species seen in postoperative lung transplant recipients were seen at comparable proportions in ex vivo airways. Importantly, this chapter has also shown that human EVLP can reproduce a wide range of phospholipids in isolation from hepatocyte based PEMT

pathways. Continued ex vivo synthesis of a diverse range of airway phospholipid species that are rich in DPPC would support the use of this isolated perfused and ventilated lung model as a platform for research into human pulmonary surfactant biology.

Ongoing work is required to fully establish EVLP as a platform for surfactant research. Statistically significant differences were detected in the airway phospholipid profile of ex vivo lungs, when compared to the airway phospholipids measured in postoperative lung transplant recipients, but the biological significance of these differences is uncertain. Notably, ex vivo airways had a greater abundance of DPPC than the samples obtained from transplant recipients had a greater proportion of PC species containing PUFA (Table 4-2 and discussed in section 4.4.3).

The reason for these differences is not clear, but there are several possible explanations. Acute lung injury is associated with reduced airway DPPC[137], and so continued lung injury sustained following reperfusion in the postoperative period would be one possible explanation. The most likely explanation is that ischemia reperfusion injury in transplant recipients has caused greater disruption to surfactant phospholipids. Airway DPPC in EVLP was closer to normal healthy values than those seen in transplant recipients[7], and so a more detailed comparison of the lipidomic profiles from EVLP and healthy volunteers could also help to characterise EVLP as a platform for surfactant research.

The reasons behind the variation in ex vivo Kennedy pathway activity also require continued investigation, in order the define EVLP as a model for pulmonary phospholipid biology. Furthermore, understanding the mechanisms behind impaired Kennedy pathway metabolism could lead to new therapeutic strategies to protect the lungs on EVLP. The Toronto group have demonstrated that surfactant replacement therapy can improve graft performance in an animal model of EVLP and transplantation, and so identifying and targeting abnormal surfactant biology could theoretically lead to treatments that could recondition lungs for transplantation[89].

Human EVLP could provide a comprehensive experimental platform for the study of pulmonary surfactant biology by preserving the relationships between multiple cells types within the alveolar architecture at the air-blood interface; thus overcoming many of the inherent limitations that are associated with other in vitro models[57]. The wide range of phospholipid species that have been measured in this chapter probably reflects this preservation of diverse cellular interactions in the lungs, like transcellular biosynthesis.

Surfactant replacement therapy has already been attempted in animal models of EVLP[9], and this human EVLP model further evaluate this experimental strategy to recondition lungs for transplantation. The development and application of human EVLP as a model of pulmonary phospholipid biology is discussed in more detail in section 6.4.3.

4.4.7 Limitations of the methods

4.4.7.1 Small heterogeneous cohort

Human lungs that are suitable for ex vivo modelling are in extremely limited supply, even in a centre with high levels of transplant activity, such as Harefield Hospital. This chapter includes data from a very limited number of organs, and so the conclusions that can be drawn from this data set are limited. The study cohort included a very heterogeneous set of organs, with varying degrees of injury that is difficult to quantify objectively. Donor lungs were sustained ex vivo at the discretion of the transplant surgeon, and varying techniques of ex vivo support were employed in accordance with the wishes of the clinical transplant team. Ex vivo support protocols were not standardised across the cohort, and this is likely to have introduced a source of further variability into the phospholipid dataset. Multiple confounders arising from differences in ex vivo support protocols between organs could introduce systematic bias and limits the conclusions that can be drawn from the dataset. Instead, it would be better to consider this study as a pilot dataset that provides "proof of concept". Now that this study has confirmed the technical viability of the platform for research into pulmonary phospholipid biology, collected preliminary/pilot data, and identified phospholipids

of potential interest; these limitations could be addressed with a standardised protocol in subsequent studies.

4.4.7.2 Identification of minor species

Fewer phospholipid species were identified in ex vivo airways than in post-transplant airways. Our departmental software identified 35 phospholipid species in the airways of transplant recipients, yet only 23 species were identified in airways of the ex vivo platform (Figure 4-5 & Figure 4-6). Our departmental software will identify species that are repeatedly detected at a relative abundance of greater than 1%. A greater number of samples in the clinical study will increase the probability of identifying species that have an average airway abundance that is close to 1%

The seemingly reduced number of airway phospholipid species detected ex vivo probably reflects the smaller dataset, and subsequently reduced ability to reliably detect minor phospholipid species that occur in very low quantities. A larger set of samples from more organs could make it easier to detect these minor airway phospholipid species. Alternatively, high performance liquid chromatography combined with tandem mass spectrometry (HPLC-MS/MS) has been used to detect lipid based mediators that occur in very small quantities, and could also theoretically assist in the detection of minor phospholipid species[308]. It is possible that separating the lungs in EVLP from the liver, and possibly the supply of other essential phospholipid precursors (such as fatty acids and glycerol) could have had some effect on the diversity of synthesised phospholipids, but further research would be required to determine whether this is the case.

4.4.7.3 Lack of a healthy ex vivo control group

Keeping healthy lungs on EVLP for 9-12 hours for deuterium labelling experiments would be unethical, as prolonged support could compromise their potential for transplantation. Therefore, it is impossible to compare these data with deuterium labelling experiments in corresponding cohort

of healthy human lungs on EVLP. Deuterium incorporation data are only available for organs that have been deemed unsuitable for clinical transplantation, and observations made on diseased organs may not be generalisable to the healthy human lungs. Nonetheless, the data from these limited experiments offer can still offer valuable insights into pulmonary phospholipid biology.

4.4.8 Clinical significance

As novel platforms evolve for the ex vivo support of organs during lung transplantation, a greater understanding of their impact on pulmonary biology will also be required. Surfactant dysfunction is a contributing factor in the pathogenesis of Primary Graft Dysfunction[5], and these data prove that normothermic ex vivo lung perfusion can preserve the function of the pulmonary surfactant system by facilitating continued phospholipid synthesis via the Kennedy Pathway. These data have also demonstrated substantial variability in ex vivo phospholipid metabolism, which raises important questions for clinical transplantation.

1) Can ex vivo measurement of surfactant biology assist in the selection of lungs for transplantation?
2) Could further optimisation of ex vivo support strategies offer additional protection of the Kennedy Pathway and pulmonary surfactant system to prevent the development of Primary Graft Dysfunction?

Based on these data, it could be hypothesised that normothermic ex vivo lung perfusion protects the graft during lung transplantation by preserving Kennedy pathway dependent surfactant metabolism. Animal models of transplantation suggest that ex vivo reconditioning of lungs through exogenous surfactant replacement has the potential to reverse lung injury and increase the donor pool of organs that could be suitable for transplantation[89]; continued investigation into the pulmonary surfactant system in EVLP is justified.

Beyond gaining insights into the protective mechanisms of clinical normothermic EVLP in clinical lung transplantation, these data provide valuable information that increases our understanding of pulmonary phospholipid biology. This study has provided first in-human proof that the lungs secrete arachidonic acid PC species into the circulation; species that have been associated with monocyte activation and the development of ARDS[270]. This human ex vivo model provides a valuable platform for the research of pulmonary phospholipid biology, and presents a unique opportunity to study lipid mediators originating from the lung, and could lead to novel targets for the treatment and prevention of lung injury.

4.4.9 Conclusions

Experimental human EVLP is a viable method for the study of pulmonary lipid biology. This chapter provides a detailed account of the range of PC species that are synthesised by the lungs in isolation from the rest of the body, and provides insights into the pulmonary contribution to phospholipid metabolism. The human lung actively secretes a distinct profile of phospholipids into the circulation that is rich in polyunsaturated fatty acids.

Ventilation-induced surfactant release is an energy dependent process that is lost when the lungs are cooled[99,309]. Demonstrating continued surfactant metabolism and secretion in this chapter offers mechanistic insights into graft preservation during clinical EVLP in lung transplantation.

5 Results: Oxido-nitrosative stress and phospholipid metabolism in lung transplantation

5.1 Introduction

5.1.1 Background

Having established that lung transplantation is associated with changes in phospholipid biology, this chapter will explore the hypothesis that oxidative stress is a mechanism that contributes to the perioperative disruption in phospholipid metabolism. Lung transplantation is a source of substantial oxidative stress in the early perioperative period[158], that contributes to Primary Graft Dysfunction (PGD)[159-161]. Ischemia and reperfusion during lung transplantation generates unstable reactive oxygen species (ROS) and reactive nitrogen species (RNS) that readily react with nearby lipids and damage surfactant[30,168]. Oxidative and nitrosative stress are toxic to the alveolar epithelium[166], and have been implicated in the development of pulmonary ischemia-reperfusion injury[310]. Oxidative stress may impair surfactant phospholipid synthesis by type 2 alveolar epithelial cells (AT2)[162]. AT2 exposure to oxidised lipoproteins will induce protease-mediated cleavage of phosphocholine cytidylyltransferase, which is the rate-limiting enzyme in the Kennedy pathway of phospholipid synthesis[163,164]. Oxidative stress can also damage existing surfactant through lipid peroxidation[167], and has been implicated in the development of PGD[46]. Beyond the potential effects on pulmonary surfactant tensioactivity, oxidative/nitrosative stress induced peroxidation of polyunsaturated fatty acids (PUFA) can start a 'chain reaction' that generates many toxic peroxides and aldehydes that will also precipitate further peroxidation of nearby PUFA species[311]. Malondialdehyde and 4-hydroxynoneal (4-HNE) are examples of the reactive products of lipid peroxidation that are generated when surfactant phospholipids are exposed to ROS[312], and will also degrade nearby DNA and proteins[313]. Protein thiol groups are particularly susceptible to oxidative and nitrosative stress, often resulting in changes in both protein structure and function[313,314]. Oxidised thiols are associated with increasing lipid peroxidation and reflect overall redox status[315]. The two-major thiol-dependant antioxidant systems are the thioredoxin system and the glutathione system[316], and disruption of

both antioxidant systems have been associated with adverse outcomes in human lung transplantation[317,318].

Changes in nitrosative stress markers are associated with surfactant dysfunction in lung transplant recipients, and RNS driven alveolar inflammation may further contribute to surfactant degradation through lipid peroxidation[158,171,319]. Nitric Oxide (NO) is a signalling molecule with many roles that paradoxically has both antioxidant properties, and can be converted into the potent RNS peroxynitrite through the reaction with superoxide ions ($O_2^{\bullet-}$)[320]. Nitrotyrosine (NT) is a marker of protein nitration found in the airways in the acute respiratory distress syndrome (ARDS)[321], and has been associated with surfactant dysfunction in lung transplantation[319].

Lung transplantation is associated with changes in NO metabolism and oxidative stress, and further investigation into the impact on pulmonary surfactant and phospholipid biology is warranted. Having demonstrated perioperative changes in phospholipid metabolism, this chapter will attempt to integrate the analyses of NO metabolism, oxido-nitrosative stress, with perioperative lipidomic profiles and measures of phospholipid flux.

5.1.2 Hypotheses

1. Disrupted NO metabolism and oxido-nitrosative stress are associated with changes in the pulmonary surfactant phospholipid Dipalmitoyl Phosphatidylcholine (DPPC) after lung transplantation.
2. Disrupted NO metabolism and oxido-nitrosative stress are associated with changes in wider phospholipid metabolism in clinical lung transplantation.

5.1.3 Objectives

- Assess NO metabolites in perioperative lung transplantation: Nitrates, nitrites, nitrotyrosine
- Measure markers of oxidative stress in perioperative lung transplantation: HO1, 4HNE, and free thiols
- Correlate markers of redox status with changes in perioperative phospholipid metabolism

5.2 Methods

5.2.1 Clinical sample and data collection

Patients were recruited from the lung transplant waiting list. Blind bronchial aspirates and plasma, samples were collected, processed and stored as described in Table 3-1. Biopsies were taken at the discretion of the consultant transplant surgeon. Where possible, paired biopsies were taken: First sample collected immediately prior to donor implantation, second sample collected immediately prior to chest closure.

Anonymised clinical data were stored in a study database (section 2.6). Measures of clinical outcome focused on the P/F ratio (ratio between arterial oxygen tension and inspired oxygen fraction) and medium-term graft function (spirometry recorded every three months for the first year after transplantation). Forced Expiratory Volume in 1 second (FEV_1) and the Forced Vital Capacity (FVC) were reported as a percentage of their predicted values.

	Induction of anaesthesia	Prior to implantation	Prior to chest closure	End of surgery	1-3 hrs	12 hrs
Blood	☑			☑		☑
Lung biopsies		☑	☑			
Bronchial washings				☑		☑
D9-Choline infusion*					☑	

Table 5-1: Study timetable
Timetable of events that describe the collection of samples used to generate the data in this chapter. Modified from the table shown in section 2.2.6.1, to illustrate the precise timing of biopsies

5.2.2 Lipidomic data

Lipids were extracted using the modified Bligh & Dyer method, prior to ESI-MS/MS – sections 2.4.2 & 2.5.1. Samples were scanned for precursors of m/z184 and m/z 193 (P184 and P193 respectively), and this chapter will deal exclusively with phospholipids that are detectable on those precursor scans. Mass spectra were processed and analysed as described in section 2.6.2.

As in chapter 3, analyses have focused on the sample collected 12 hours after the D9-Choline infusion was started, to allow sufficient time for incorporation of the deuterium label whilst still providing enough patients to perform meaningful analyses. Peak deuterium incorporation is typically seen after approximately two days[8], yet the median time on postoperative respiratory support was 44.5 hours (Interquartile range 18.25 – 510 hours); focused analyses on the 12 hour time point offered a compromise between allowing sufficient time to allow incorporation of the D9-Choline label, whilst still analysing samples from 75% of the study cohort. Furthermore, the previous chapter has already demonstrated that changes in 12-hour phospholipids are associated important clinical outcomes, and so further investigation into the mechanisms behind those changes at that time point would appear to be justified – section 3.3.4.

5.2.3 NO metabolites

Stable metabolites of NO (nitrate and nitrite) were measured with a combination of High Performance Liquid Chromatography (HPLC) and the Griess reaction. Concentrations of NO metabolites were measured by absorbance at 540nm with spectrophotometry, after generating a calibration curve using standard solutions – section 2.5.2.

5.2.4 Free thiols

Free thiols were detected by absorbance at 412nm with spectrophotometry following the addition of dithionitrobenzoic acid (DTNB) to plasma that had been diluted 1:4 with a 0.1M Tris buffer. Concentrations were calculated against a calibration curve using an L-cysteine standard solution, and corrected for plasma protein concentrations – section 2.5.3.

5.2.5 Immunohistochemistry and image acquisition

Paraffin wax embedded sections of lung tissue (5µm thick) were incubated with the primary antibodies against Haem oxygenase 1 (HO-1, mouse monoclonal), Nitrotyrosine (NT, mouse monoclonal), 4-hydroxynoneal (4-HNE, rabbit polyclonal); prior to incubation with the fluorescent labelled secondary antibody and subsequent staining for DAPI (4',6-diamidino-2-phenylindole).

Images were then acquired with confocal microscopy (section 2.5.4). After screening for technical adequacy by a blinded reviewer, all images were digitally analysed for the area of tissue stained with the primary antibodies and values were averaged across 5 images from each biopsy – section 2.6.3.

5.3 Results

5.3.1 Perioperative changes in oxido-nitrosative stress and NO metabolites

5.3.1.1 NO Metabolites

The total concentration of NO metabolites (NO_x = sum of NO_3 and NO_2) increased intra-operatively during transplantation from 30.95 µM to 41.48 µM (two-tailed Mann Whitney p=0.007). Nitrate was the predominant NO metabolite in plasma before implantation at 98.4% of all NO_x, and there was no statistically significant difference after lung transplantation with nitrate remaining at 98.6% of all plasma NO_x (two-tailed Mann Whitney p=0.609). Small (but statistically significant) rises were also seen in the plasma concentration of nitrites and nitrates, but the ratio of Nitrate:Nitrite plasma concentration was unchanged by lung transplantation -Table 5-2.

	Pre-transplantation		Post-transplantation		P value
Nitrite (µM)	0.51	(0.40-0.58)	0.59	(0.40-0.75)	**0.006**
Nitrate (µM)	30.44	(25.18-38.66)	40.89	(32.22-53.24)	**<0.001**
Nitrate:Nitrite ratio	60.48	(41.95-94.19)	68.91	(43.87-102.42)	0.225

Table 5-2:Intraoperative change in NO metabolites

NO metabolites measured in the plasma sampled after induction of general anaesthesia and immediately following the completion of surgery. Distributions for all variables were statistically significant on testing for normality with Shapiro-Wilk (p<0.05). Median values are reported with the interquartile range in brackets. Statistical significance tested with Wilcoxon signed ranks test, 2-tailed p-values are reported and highlighted in red when below 0.05. n=39, pre-transplant plasma was not available for one patient.

5.3.1.2 Free thiols

Lung transplantation was associated with an immediate decrease in protein and free thiol concentrations. Free thiols were adjusted for protein concentration, because proteins are the major

source of free thiols in human plasma[322]. Once adjusted for the plasma concentration of protein, there was a statistically significant rise in free thiol concentration during lung transplantation.

	Pre-transplantation		Post-transplantation		P value
Protein (g/L)	44.31	(38.91-51.95)	24.15	(21.51-29.44)	**<0.001**
Total Free Thiols (µM)	214.93	(204.96-224.91)*	163.60	(149.58-177.61)*	**<0.001**
Total Free Thiols (µM/g protein)	4.89	(4.66-5.12)*	6.23	(5.85-6.61)*	**<0.001**

Table 5-3: Intraoperative change in free thiols
*Protein and thiols were measured in the plasma sampled after induction of general anaesthesia and immediately following the completion of surgery. Median values are reported with the interquartile range in brackets for plasma protein, and statistical significance assessed with Wilcoxon signed ranks test. *Thiol data was normally distributed on testing with Shapiro-Wilks test and so mean values are reported with the 95% confidence interval, and assessed with paired t-tests. 2-tailed p-values are reported and highlighted in red when below 0.05. n=39 paired samples, pre-transplant plasma was not available for one patient.*

5.3.1.3 Immunohistochemistry – Tissue markers of oxidative and nitrosative stress

Images for lung biopsies are shown for the three primary antibodies that were used markers of oxidative and nitrosative stress. Sample images with corresponding masks are shown below, to illustrate their distribution of antibody staining in the lung parenchyma. All three primary antibodies (HO1, NT, and 4-HNE) resulted in diffuse staining across the whole of the alveolus. The proportion of lung tissue stained with the primary antibody is summarised for all available biopsies in section 5.3.1.3.4.

5.3.1.3.1 Haem oxygenase 1

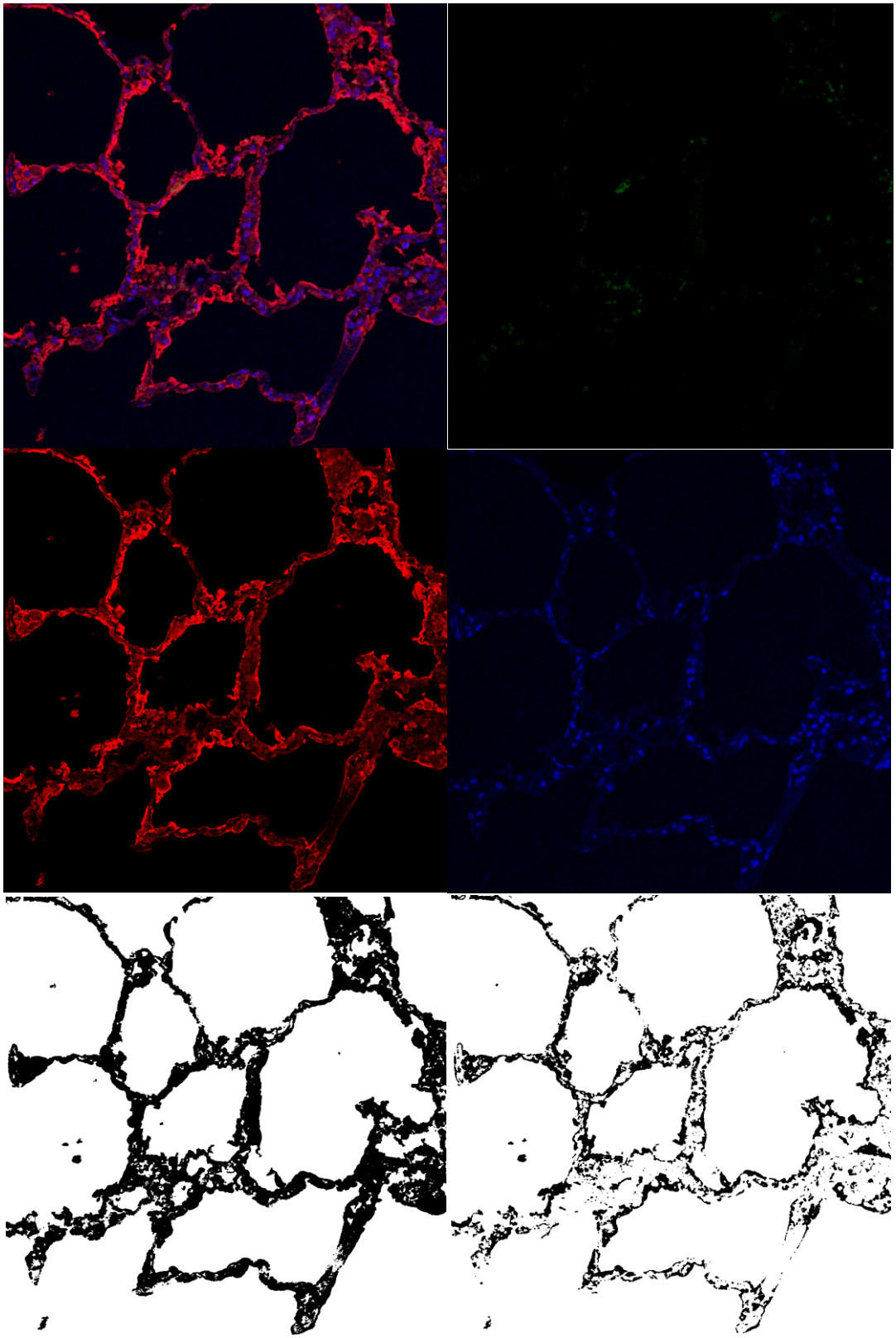


Figure 5-1: HO-1 stained biopsy taken during lung transplantation
Top-left, composite image. Top-right, green channel auto-fluorescence. Middle-left, red channel HO-1 antibody stain. Middle-right channel DAPI stain. Bottom-left, tissue mask. Bottom-right, HO-1 signal mask. All images at 20x magnification. DAPI: 4',6-diamidino-2-phenylindole. HO-1: Haem oxygenase-1

5.3.1.3.2 Nitrotyrosine

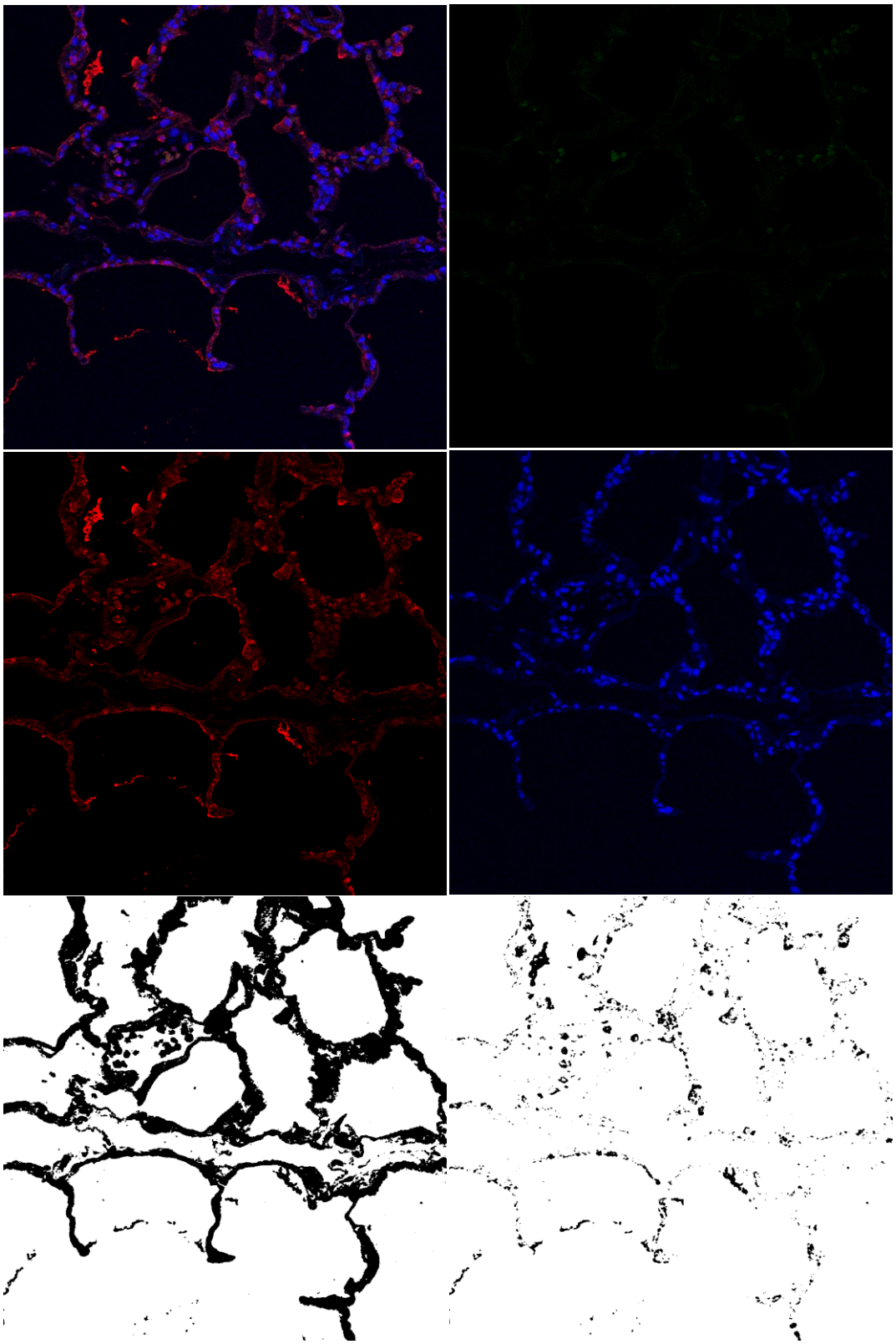


Figure 5-2: NT stained biopsy taken during lung transplantation
Top-left, composite image. Top-right, green channel auto-fluorescence. Middle-left, red channel HO-1 antibody stain. Middle-right channel DAPI stain. Bottom-left, tissue mask. Bottom-right, NT signal mask. All images at 20x magnification. DAPI: 4',6-diamidino-2-phenylindole. NT: Nitrotyrosine

5.3.1.3.3 4-hydroxynoneal

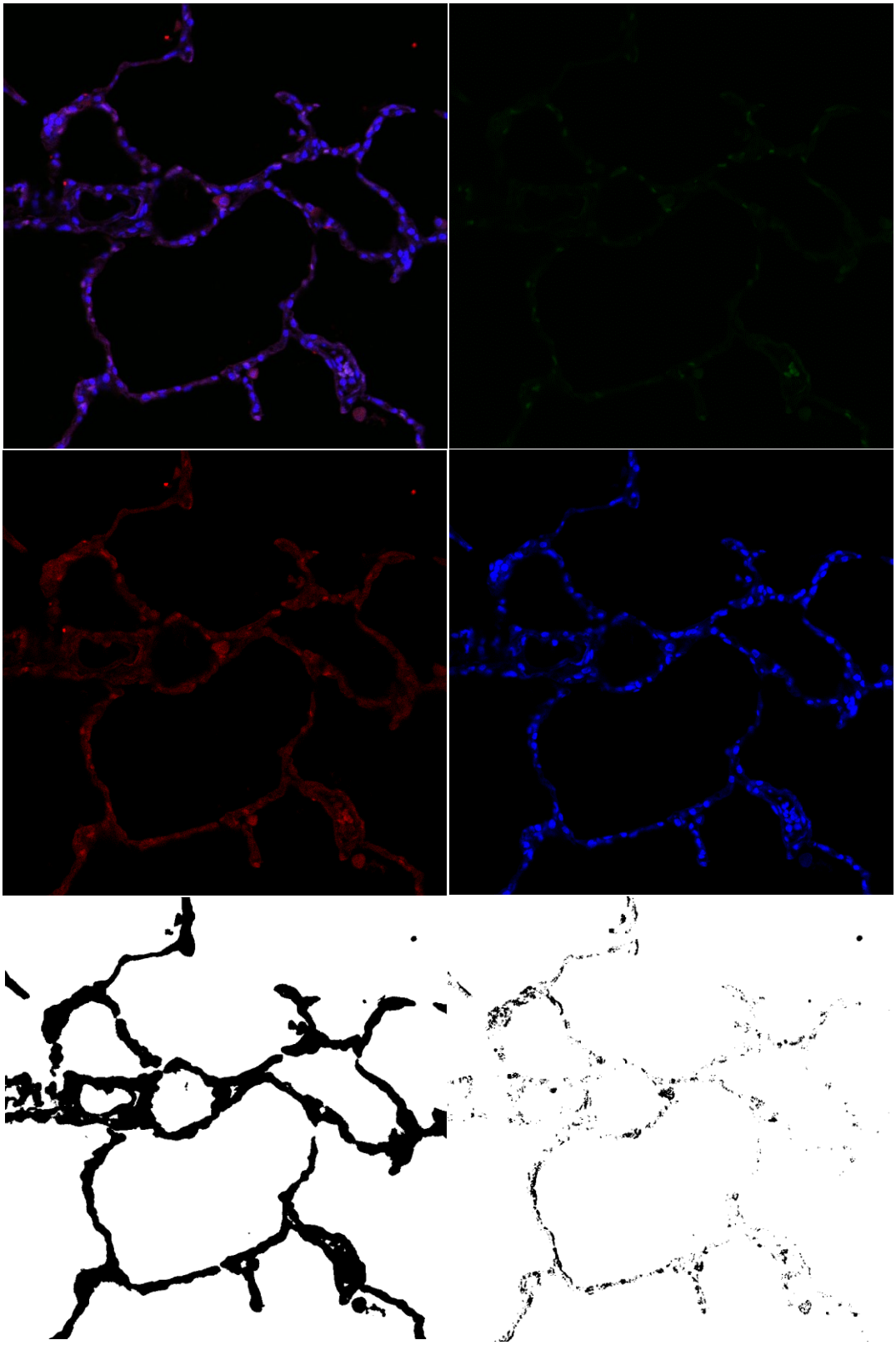


Figure 5-3: 4-HNE stained biopsy taken during lung transplantation
Top-left, composite image. Top-right, green channel auto-fluorescence. Middle-left, red channel 4HNE antibody stain. Middle-right channel DAPI stain. Bottom-left, tissue mask. Bottom-right, 4-HNE signal mask. All images at 20x magnification. DAPI: 4',6-diamidino-2-phenylindole. 4-HNE: 4-hydroxynoneal

5.3.1.3.4 Summary of tissue staining across study cohort

The area of lung tissue stained with the primary antibody has been summarised in Table 5-4. There was no statistically significant difference in the proportion of tissue stained with HO-1, NT, or 4-HNE when comparing tissue that had been biopsied before or after surgical implantation.

	Pre-implantation	Post-implantation	Sample pairs	P value
HO1	26.0 (19.0-36.2) n=17	25.3 (11.3-42.6) n=15	5	0.686
NT	23.4 (14.3-29.6) n=20	27.4 (16.7-37.6) n=15	6	0.345
4HNE	14.9 (8.3-22.9) n=29	11.6 (4.7-16.5) n=27	19	0.070

Table 5-4: Intraoperative change in tissue markers.
Values represent the percentage of the lung biopsy that has been stained with the primary antibody, averaged from multiple images. Median values are reported, with the interquartile range presented in brackets. Statistical significance tested with Wilcoxon signed ranks test, two-tailed p-values are reported. The number of available data points are specified for each marker, as numerous image sets were removed during quality control.

Fewer lung biopsy samples were available for analysis following implantation compared to pre-implantation biopsies. As there was no statistically significant difference between pre-implantation and post-implantation samples, subsequent analyses have focused on pre-implantation biopsies, as a greater number of tissue samples were available for analyses.

5.3.1.4 Association between NO metabolites and oxidative stress markers

No statistically significant associations were seen between tissue markers of oxidative and nitrosative stress derived by immunohistochemistry and perioperative plasma NO metabolites and free thiols. Multiple associations were assessed with Spearman's rank using two-tailed p-values tested at the p=0.05 significance level.

5.3.1.5 Perioperative NO metabolism, redox status, and subsequent clinical outcomes

Postoperative P/F ratio, and spirometry out to one year was screened for associations with NO metabolites, free thiols, and tissue markers using Spearman's rank; none were found (Spearman's rank with two-tailed p-values at p=0.05, but data not shown). Similarly, there was no significant difference in NO metabolites, free thiols nor tissue markers of oxidative and nitrosative stress when compared between the various diagnostic grades of primary graft dysfunction (Kruskal Wallis tested with two-tailed p=values, no associations seen at the p=0.05 level – data not shown).

5.3.1.6 Key findings – Intraoperative changes in NO metabolites and oxido-nitrosative stress

- Lung transplantation was associated with a prompt increase in plasma nitrates and nitrites
- There was an increase in free thiols during lung transplantation
- No intraoperative changes were detected in tissue markers oxido-nitrosative stress

5.3.2 Association with airway phospholipids

5.3.2.1 NO metabolites and free thiols

No association was seen between NO metabolites and airway DPPC, either expressed as its relative abundance or its D9-incorporation - Figure 5-4.

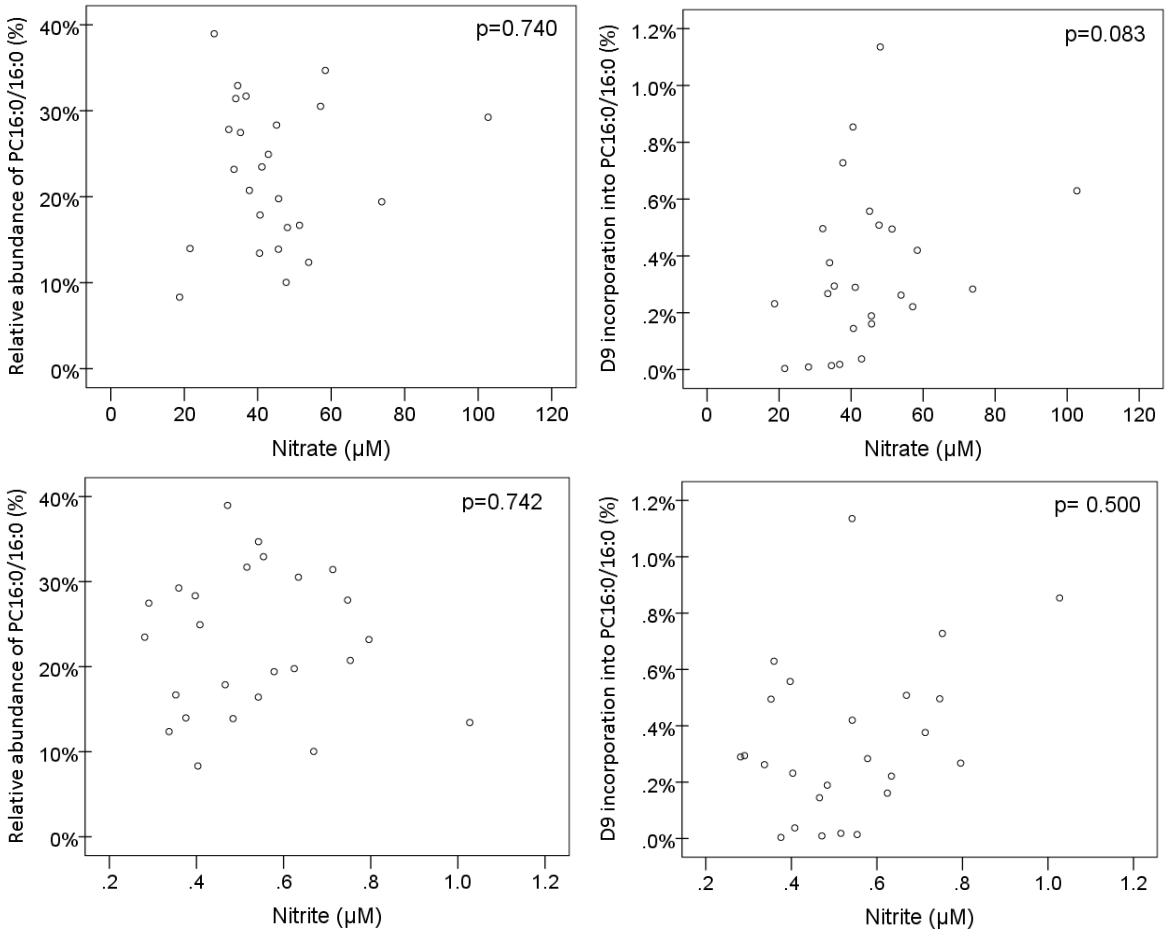


Figure 5-4: Association between NO metabolites and airway DPPPC after lung transplantation. Scatter plot of lung transplant recipients who received the D9-choline label after transplantation and remained intubated at 12 hours (n=25). X-axes: bottom panels – nitrite plasma concentration immediately after lung transplantation, top panels – nitrate plasma concentration immediately after lung transplantation. Y-axes: left panels – relative abundance of DPPC in the airways of transplant recipients at 12 hours (%), right panels – Proportion of D9-choline incorporation into airway DPPC after 12 hours (%). Two-tailed p-values are reported, and calculated with Spearman's Rank test.

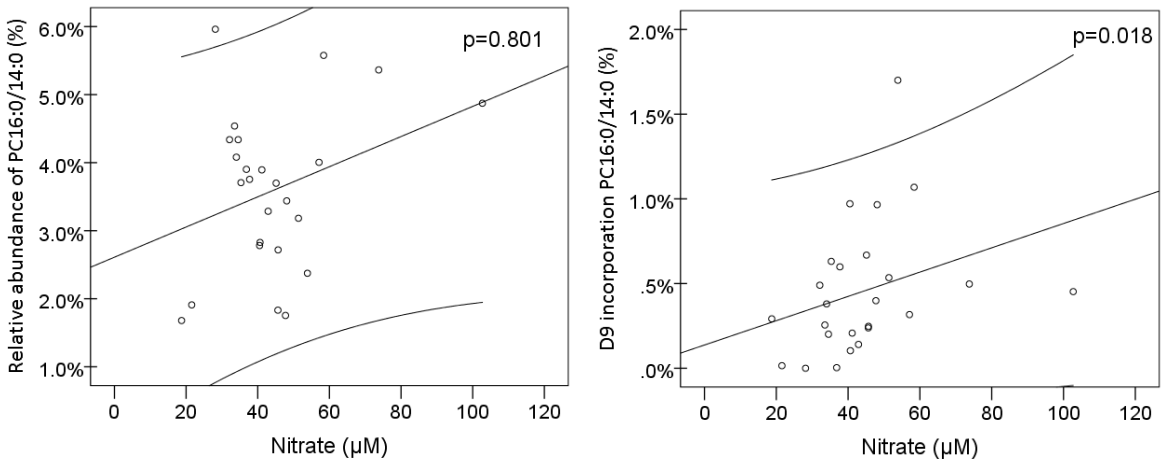


Figure 5-5: Post-transplant plasma nitrate and airway PC16:0/14:0
Scatter plot of lung transplant recipients who received the D9-choline label after transplantation and remained intubated at 12 hours (n=25). X-axis: plasma nitrate concentration in samples taken immediately after lung transplantation. Left panel. Y-axis: 12-hour airway samples showing the relative abundance of PC16:0/14:0; linear regression line R^2=0.101, with 95% confidence interval, Spearman rank's correlation coefficient = 0.053, p=0.801. Right panel. Y-axis: 12-hour airway samples with the proportion of PC16:0/14:0 that has incorporated the D9-choline label, expressed as a percentage; linear regression line R^2=0.096, with 95% confidence interval, Spearman's correlation coefficient= 0.468, p=0.018.*

A weak association was demonstrated between postoperative nitrate concentration and 12 hour D9-incorporation into PC16:0/14:0 (another phospholipid that is typically found in pulmonary surfactant[284]) - Figure 5-5. Another modest association was seen between post-transplantation plasma nitrite concentration and 12 hour D9-incorporation into PC16:0/18:3 (Spearman's rank correlation coefficient 0.445, two-tailed p=0.026, n=25). Postoperative nitrates, nitrites, and free thiols failed to demonstrate any significant association with either the 12-hour relative abundance nor the 12-hour D9-incorporation into any other phospholipid species (multiple bivariate correlations assessed with Spearman Rank, multiple two-tailed p-values that failed to reach significance at the level of p=0.05 - species as listed in Table 3-4.

5.3.2.2 Nitrotyrosine

5.3.2.2.1 Relative abundance of phospholipid species

Increased staining for NT in pre-implantation biopsies was associated with an increasing abundance of sphingomyelin species SM16:0 (Spearman's rank coefficient 0.622, p=0.018, R^2=0.157), SM24:1 (Spearman's rank coefficient 0.556, p=0.039, R^2=0.287), SM22:1 (Spearman's rank coefficient 0.591, p=0.026, R2=0.164), and SM24:2 (Spearman's rank coefficient 0.534, p=0.049, R^2=0.141).

It should be noted that these sphingomyelins that demonstrated an association with NT staining were only present in the bronchial washings at very low levels, none were detected at a relative abundance greater than 6%. SM24:2 was not detected at a level above 1% in those labelled patients who remained intubated at 12 hours, and barely reached the 5% threshold for statistical significance.

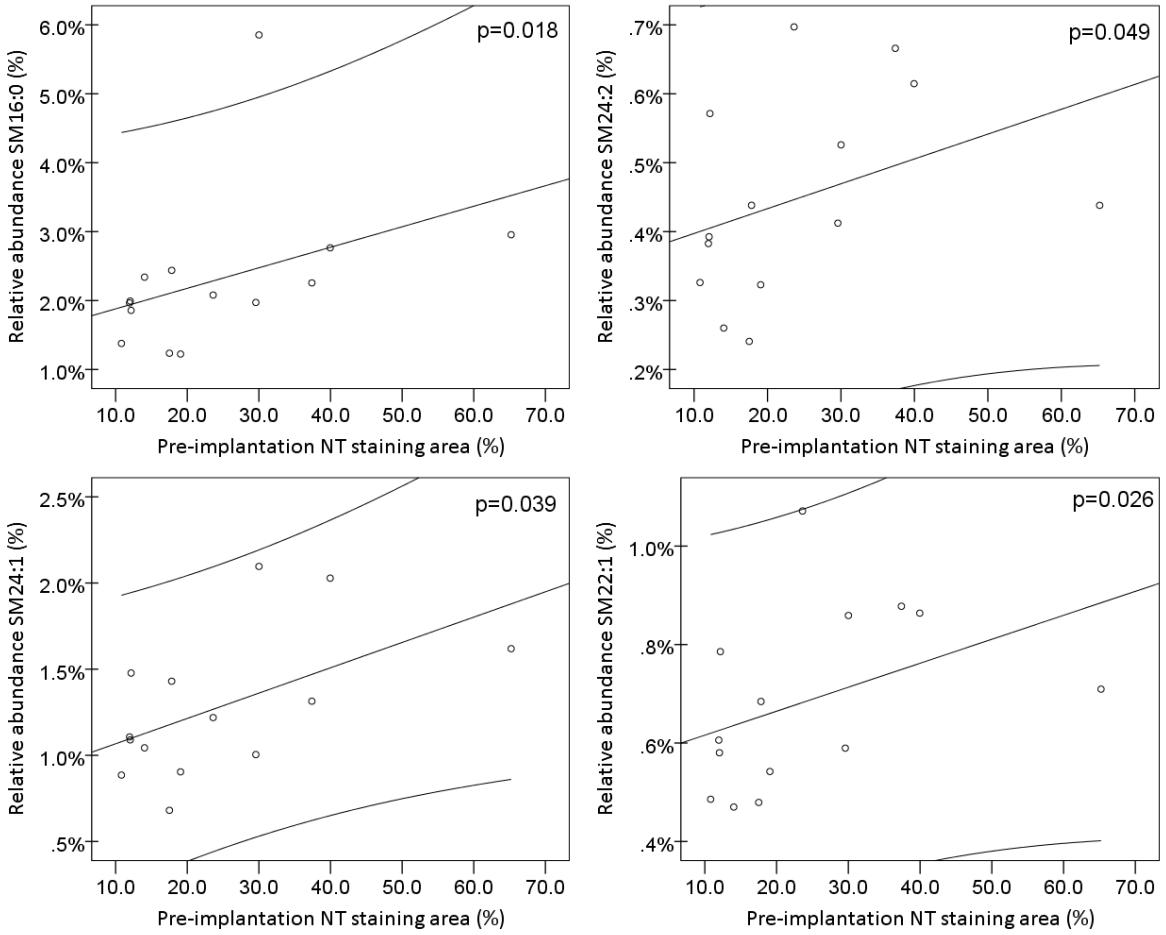


Figure 5-6: Nitrotyrosine staining and airway sphingomyelin species. The association between NT staining in pre-implantation lung biopsies and airway phospholipid species sampled by bronchial washings at 12 hours after transplantation. X-axes all panels – Area of lung tissue stained for NT in pre-implantation biopsies (%). Y-axis (top left) – relative abundance of SM16:0 (%). Y-axis (top right) relative abundance of SM24:2 (%). Y-axis (bottom left) – relative abundance of SM24:1 (%). Y-axis (bottom right) – relative abundance of SM22:1 (%). Linear regression lines with 95% confidence intervals are illustrated for each panel. Two tailed p-values for Spearman's rank are reported at the p=0.05 level. N=14.

Pre-implantation NT staining was only associated with two phosphatidylcholine species that contain arachidonic acid; PC16:0a/20:4 (Spearman's rank coefficient 0.692, p=0.006, R^2=0.581), and PC18:1a/20:4 (Spearman's rank coefficient 0.582, p=0.029, R^2=0.367). Whilst the correlation

coefficient and p=value for these PC species are compelling, it must be highlighted that both species are minor airway phospholipids that were typically detected in the airways at a very low level, with a typical relative abundance below 1% (Figure 5-7).

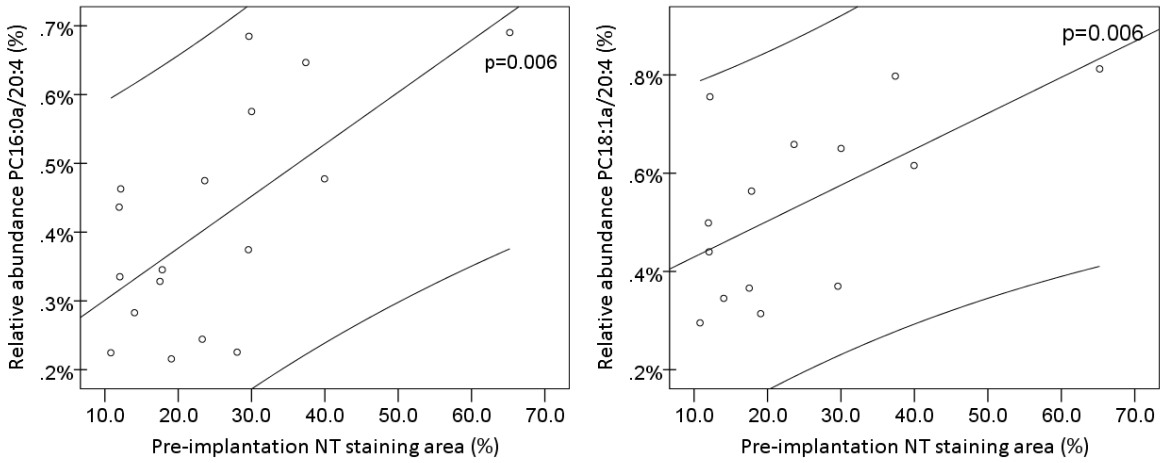


Figure 5-7: Pre-implantation nitrotyrosine staining and abundance of airway phospholipid species. The association between NT staining in pre-implantation lung biopsies and airway phospholipid species sampled by bronchial washings at 12 hours after transplantation. X-axes– Area of lung tissue stained for NT in pre-implantation biopsies (%). Y-axis (left panel) - Relative abundance of PC16:0a/20:4 (%). Y-axis (right panel) - Relative abundance of PC18:1a/20:4 (%). Linear regression lines with 95% confidence intervals are illustrated for each panel. Two tailed p-values for Spearman's rank are reported. N=14

5.3.2.2.2 Label incorporation into phospholipid species

Pre-implantation NT biopsy staining was associated with increased D9-incorporation for SM16:0 (Spearman's rank coefficient 0.569 p=0.034, R^2=0.445), SM20:0 (Spearman's rank coefficient 0.582, p=0.029, R^2=0.577), PC18:0/16:0 (Spearman's rank coefficient 0.622, p=0.018, R^2=0.324), PC18:0/18:1 (Spearman's rank coefficient 0.609, p=0.021, R^2=0.564), PC16:0a/16:0 (Spearman's rank coefficient 0.662, p=0.010, R^2=0.401). PC18:0a/16:0 also show a statistically significant association with pre-implantation staining for NT (Spearman's rank coefficient 0.578, p=0.030, R^2=0.711). However, the scatter plot of the data shows one data-point with deuterium enrichment of 5.13%. This level of deuterium enrichment is considerably greater than any value seen for other lipids in other samples; this outlier could represent a spurious reading or other artefact (Figure 5-8).

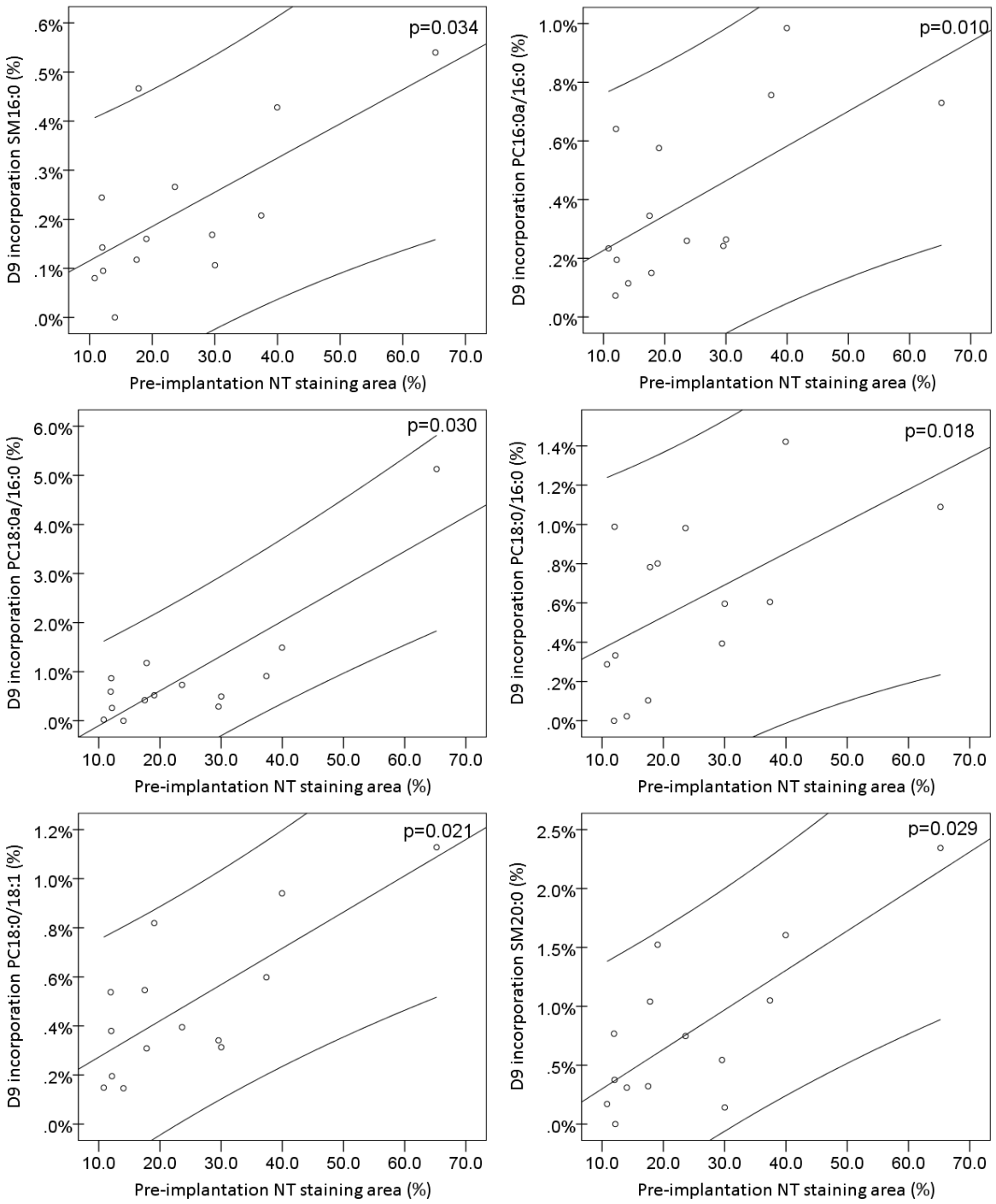


Figure 5-8: Pre-implantation nitrotyrosine staining and deuterium incorporation into airway phospholipids. The association between NT staining in pre-implantation lung biopsies and D9-incorporation into airway phospholipid species sampled by bronchial washings at 12 hours after transplantation. X-axes all panels – Area of lung tissue stained for NT in preimplantation biopsies (%). Y-axis (top left) – Deuterium incorporation into airway SM16:0 at 12 hours (%). Y-axis (top right – Deuterium incorporation into airway PC16:0a/16:0 at 12 hours (%). Y-axis (middle left) – Deuterium incorporation into airway PC18:0a/16:0 at 12 hours (%). Y-axis (middle right) – Deuterium incorporation into airway PC18:0/16:0 at 12 hours (%). Y-axis (bottom left) – Deuterium incorporation into airway PC18:0/18:1 at 12 hours (%). Y-axis (bottom right) – Deuterium incorporation into airway SM20:0 at 12 hours (%). Linear regression lines with 95% confidence intervals are illustrated for each panel. Two tailed p-values for Spearman's rank are reported. n=14.

5.3.2.3 Haem oxygenase 1

HO-1 staining was associated with changes in the relative abundance of two minor airway species that occur in very low proportions. Pre-implant tissue staining for HO-1 was only associated with changes in the abundance of PC14:0/18:2 when examining airway samples taken at 12 hours after surgery (Spearman's rank coefficient 0.629, p=0.028, R^2=0.417). Post-implant tissue staining for HO-1 was associated with changes in the abundance of PC18:0/16:0 when examining airway samples taken at 12 hours after surgery (Spearman's rank coefficient 0.721, p=0.019, R^2=0.539).

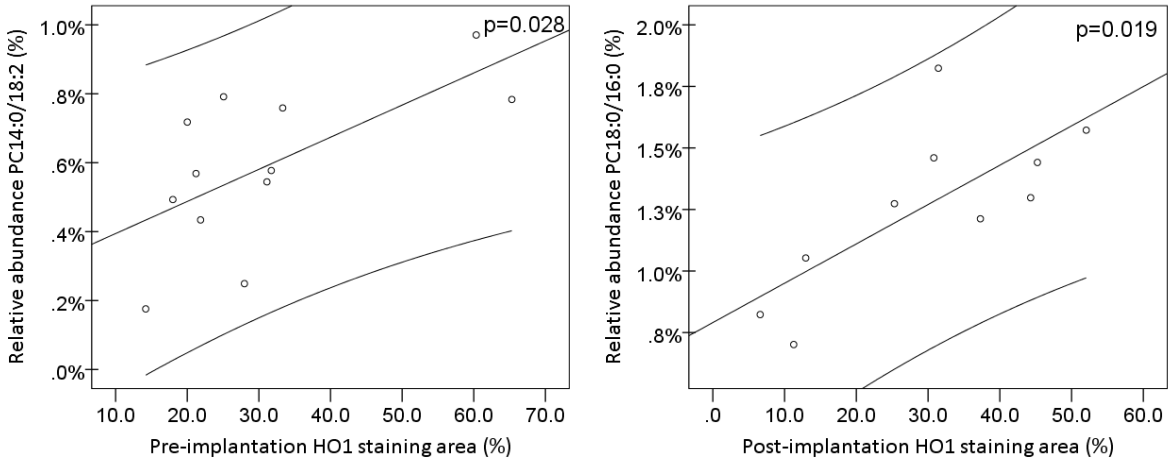


Figure 5-9: HO-1 staining and relative abundance of airway phospholipid species. The association between HO-1 staining in lung biopsies and airway phospholipids sampled by bronchial washings at 12 hours after transplantation. X-axes– Area of lung tissue stained for HO-1 in lung biopsies (left panel –pre-implantation, right panel – post-implantation) (%). Y-axis (left panel)– Relative abundance of airway PC14:0/18:2 at 12 hours (%). Y-axis (right panel) – Relative abundance of airway PC18:0/16:0 at 12-hours (%). Linear regression lines with 95% confidence intervals are illustrated for each panel. Two tailed p-values for Spearman's rank are reported at the p=0.05 level. N=12

Changes in pre-implantation staining for HO-1 was associated with D9-choline incorporation for PC18:0a:/18:1 (Spearman's rank coefficient 0.678, p=0.015, R^2=0.592) and PC18:0/22:6 (Spearman's rank coefficient 0.587, p=0.045, R^2=0.499) – n=12.

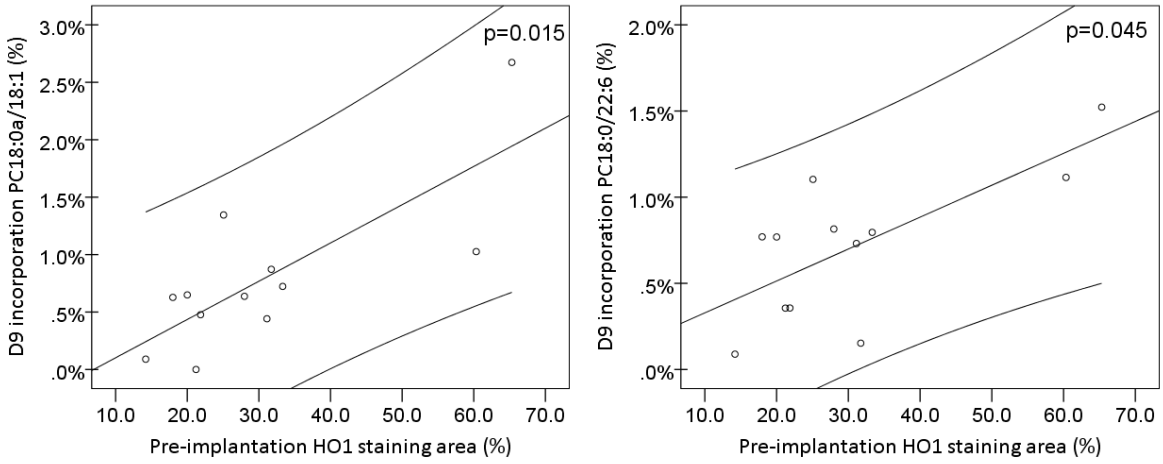


Figure 5-10: HO-1 and deuterium incorporation into airway phospholipids. The association between HO1 staining in pre-implantation lung biopsies and airway phospholipid species sampled by bronchial washings at 12 hours after transplantation. X-axes– Area of lung tissue stained for HO-1 in pre-implantation biopsies (%). Y-axis (left panel) – Deuterium incorporation into airway PC18:0a/18:1 at 12 hours (%). Y-axis (right panel) – Deuterium incorporation into airway PC18:0/22:6 at 12 hours (%). Linear regression lines with 95% confidence intervals are illustrated for each panel. Two tailed p-values for Spearman's rank are reported at the p=0.05 level. N=12

5.3.2.4 4-hydroxynoneal

Lung biopsies stained for 4-HNE did not show any association with 12-hour airway phospholipids (data not shown, multiple associations tested with Spearman's rank and selected at the two-tailed significance level of p=0.05, n=17).

5.3.2.5 Key findings – association with airway phospholipids

- Perioperative changes in NO metabolites were not associated with the amount of airway DPPC, nor DPPC flux through the Kennedy pathway
- A weak association between postoperative nitrates and deuterium labelling of PC16:0/14:0 was observed (another surfactant phospholipid that is typically less abundant than DPPC)
- Pre-implantation tissue staining for nitrotyrosine was weakly associated with changes in the abundance and deuterium enrichment of several sphingomyelin species (phospholipids that are typically found in the cell membrane)
- Pre-implantation tissue staining for nitrotyrosine was weakly associated with changes in airway PC 16:0a/20:4 and PC18:1a/20:4 (phospholipids containing arachidonic acid)
- Perioperative free thiols and tissue staining for 4-HNE was not associated with perioperative changes in airway phospholipids

5.3.3 Association with plasma phospholipids

Bivariate correlation analyses were performed as a screening exercise to look for associations between NO metabolites, free thiols, and plasma phospholipids measured at 12 hours after lung transplantation. The relative abundance and D9-choline incorporation was assessed for the major phospholipid species in the plasma, as listed in Table 3-4

5.3.3.1 Free thiols

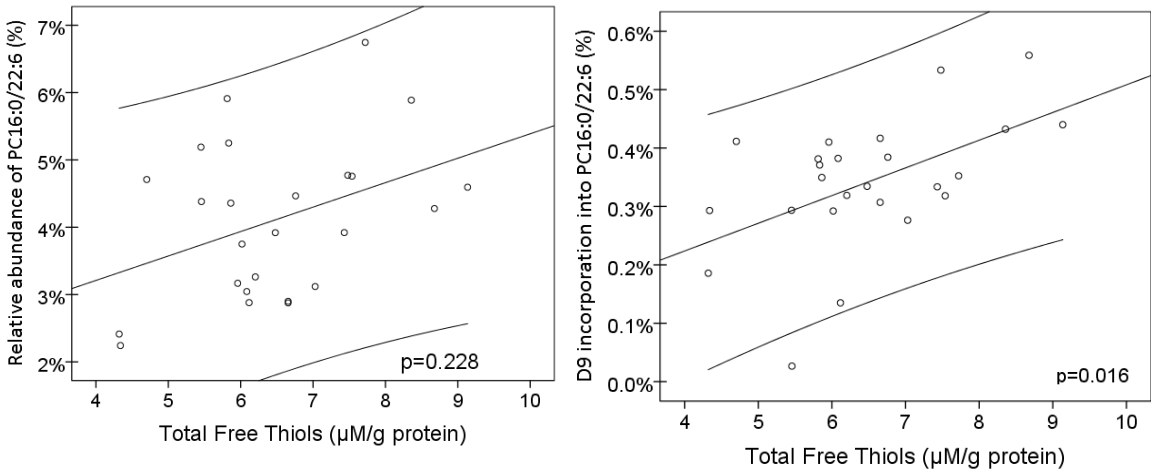


Figure 5-11: Comparison of post-transplant free thiols and PC16:0/22:6
Scatter plot of lung transplant recipients who received the D9-choline label after transplantation and remained intubated at 12-hours (n=25). X-axis: plasma concentration of free thiols, adjusted for protein concentration from samples taken immediately after lung transplantation. Left panel. Y-axis: 12-hour plasma samples showing the relative abundance of PC16:0/22:6; linear regression line R^2=0.151, with 95% confidence interval, Spearman rank's correlation coefficient = 0.250, p=0.228. Right panel. Y-axis: 12-hour plasma samples with the proportion of PC16:0/22:6 that has incorporated the D9-choline label, expressed as a percentage; linear regression line R^2=0.275, with 95% confidence interval, Spearman's correlation coefficient= 0.476, p=0.016.*

Postoperative free thiols were positively associated with D9-choline incorporation into PC16:0/22:6, which is a PUFA PC species containing docosahexaenoic acid (DHA). However, no association was seen when free thiols were measured against the static measure of relative abundance for PC16:0/22:6 - Figure 5-11

5.3.3.2 NO metabolites

Postoperative nitrite concentrations were positively associated with the relative abundance of two phospholipid species SM20:0 and PC16:0/18:1, but there was no association with D9-choline incorporation for those species.

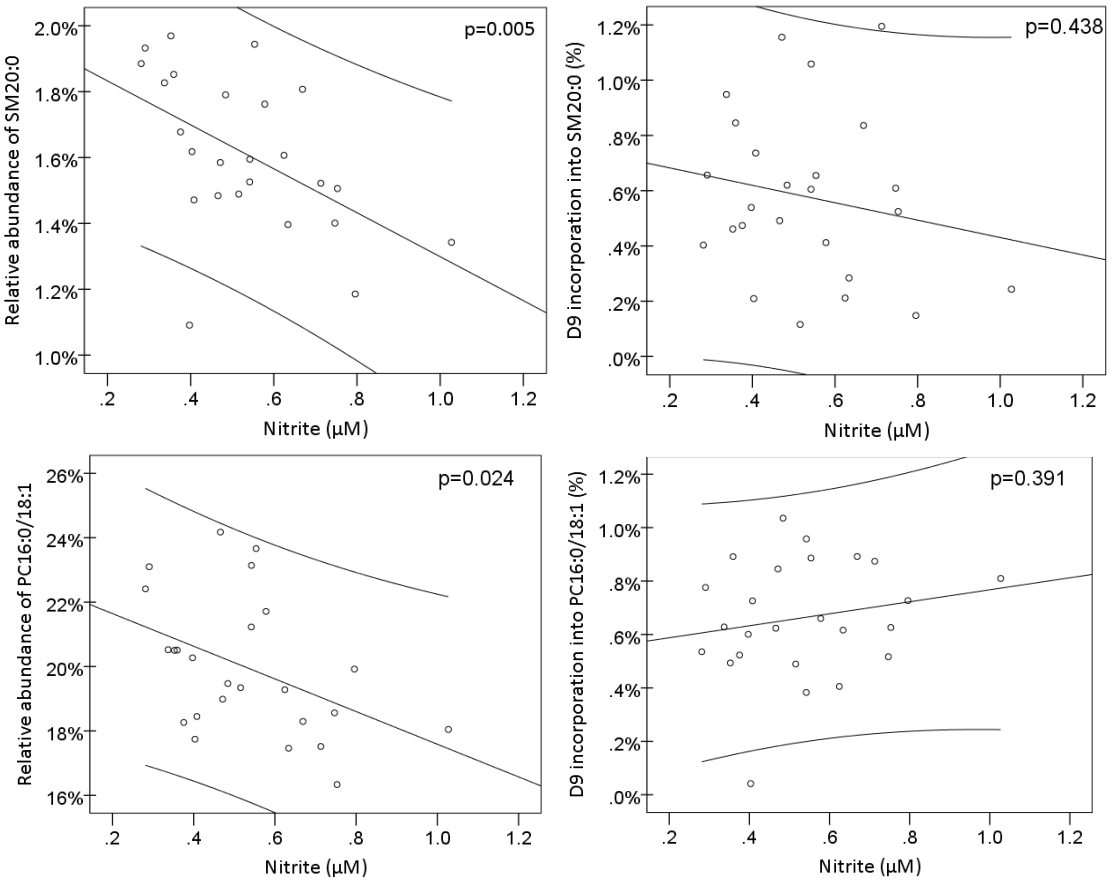


Figure 5-12: Postoperative nitrite concentration association with 12 hours plasma phospholipids. Scatter plot comparing the postoperative plasma nitrite concentration (x-axes) with plasma phospholipids sampled 12 hours later. Left panels: Y-axes -relative abundance of SM20:0 (top-left) and the relative abundance of PC16:0/18:1 (bottom-left). Right panels: Y axes -D9 incorporation in to SM20:0 (top-right) and PC16:0/18:1 (bottom-right). Linear regression lines with 95% confidence intervals are illustrated for each panel. Two-tailed p-values are reported from Spearman's rank test. N=25.

Further screening failed to identify any other phospholipids with a statistically significant association between 12 D9-incorporation and postoperative nitrite concentration (Spearman's rank examining for associations at the two-tailed significance level of p=0.05, n=25 - multiple associations with lipid species as specified above).

5.3.3.3 Tissue markers of oxidative stress

Pre-implantation staining for NT was associated with the relative abundance of SM16:0 in the plasma (Spearman's rank coefficient 0.587 p=0.027, R^2=0.523), but no other phospholipids. Note that NT staining was also associated with numerous sphingomyelin species in the airways – section

5.3.2.2.1. Weak associations were noted between NT staining and deuterium incorporation for two plasma species: SM16:0 (Spearman's rank coefficient -0.560 p=0.037, R^2=0.186), and PC18:1a/20:4 (Spearman's rank coefficient -0.622, p=0.018, R^2=0.268, n=14).

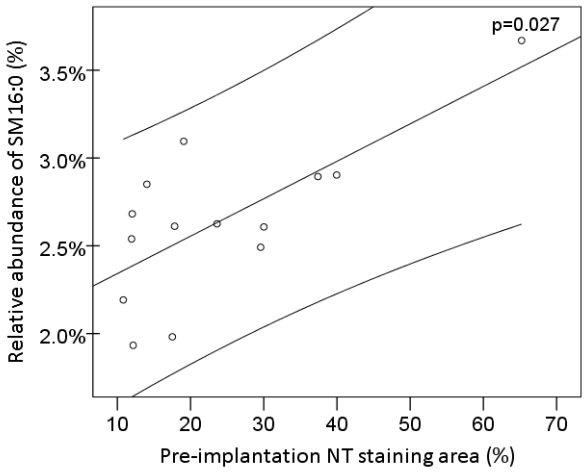


Figure 5-13: Nitrotyrosine staining and plasma sphingomyelin.
The association between NT staining in pre-implantation lung biopsies and plasma SM16:0 at 12 hours after transplantation. X-axis– Area of lung tissue stained for NT in pre-implantation biopsies (%). Y-axis – relative abundance of SM16:0 (%). Linear regression lines with 95% confidence intervals are illustrated. Two-tailed p-values are reported from Spearman's rank test. N=14

Whilst 4-HNE was not associated with and changes in airway phospholipids, pre-implantation staining of 4-HNE was weakly associated with changes in the relative abundance of six plasma phospholipids: SM16:0 (Spearman's rank coefficient 0.470 p=0.049, R^2=0.096), PC16:0/16:1 (Spearman's rank coefficient -0.478, p=0.045, R^2=0.195), PC16:0/18:2 (Spearman's rank coefficient 0.554, p=0.017, R^2=0.414), PC16:0/22:6 (Spearman's rank coefficient 0.542, p=0.020, R^2=0.175), PC18:1/20:4 (Spearman's rank coefficient -0.542, p=0.017, R^2=0.212), and PC18:0/20:3 (Spearman's rank coefficient -0.618, p=0.006, R^2=0.175).

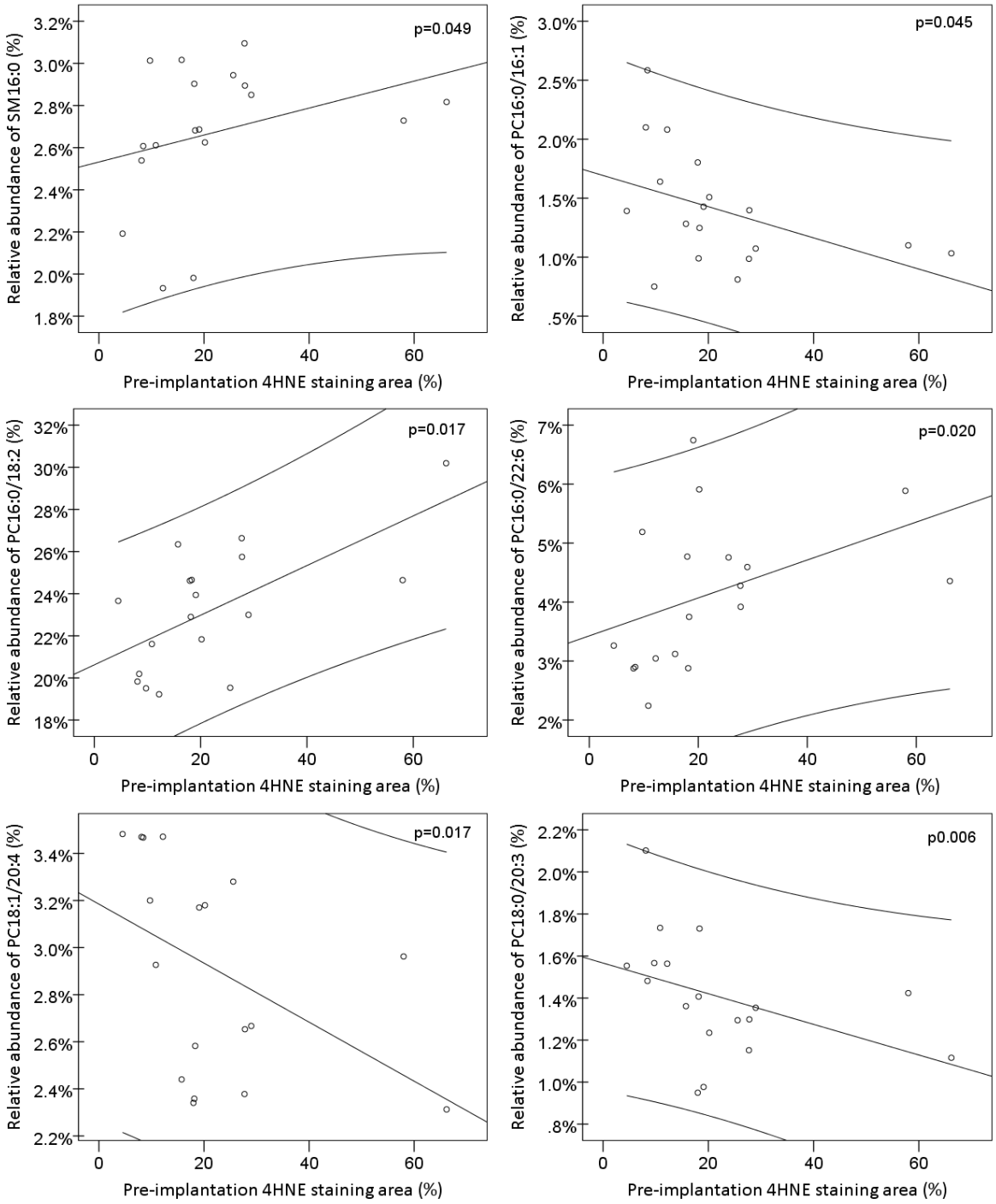


Figure 5-14: Pre-implantation 4-HNE staining and the relative abundance of plasma phospholipids. The association between 4-HNE staining in pre-implantation lung biopsies and D9-incorporation into plasma phospholipid species sampled at 12 hours after transplantation. X-axes all panels – Area of lung tissue stained for 4-HNE in pre-implantation biopsies (%). Y-axis (top left)– Relative abundance of plasma SM16:0 at 12 hours (%). Y-axis (top right – Relative abundance of plasma PC16:0/16:1 at 12 hours (%). Y-axis (middle left) – Relative abundance of plasma PC16:0/18:2 at 12 hours (%). Y-axis (middle right) – Relative abundance of plasma PC16:0/22:6 at 12 hours (%). Y-axis (bottom left) – Relative abundance of plasma PC18:1/20:4 at 12 hours (%). Y-axis (bottom right) – Relative abundance of plasma PC18:0/20:3 at 12 hours (%) Linear regression lines with 95% confidence intervals are illustrated for each panel. Two-tailed p-values are reported from Spearman's rank test. N=18

5.3.3.4 Key findings – association with plasma phospholipids

- Postoperative nitrites were negatively associated with plasma levels of SM20:0 and PC16:0/18:1, but not with deuterium enrichment of those species
- Postoperative thiols were weakly associated with deuterium enrichment of PC16:0/22:6
- Tissue markers of oxido-nitrosative stress were weakly associated with several phospholipid species

5.4 Discussion

5.4.1 Summary of key findings

- There was no evidence of an association between perioperative oxido-nitrosative stress and airway DPPC in lung transplantation
- There was no consistent association between deuterium labelling across phospholipid species and the selected indices of oxido-nitrosative stress
    - Therefore, there was minimal evidence that oxido-nitrosative stress directly disrupts Kennedy pathway in lung transplantation
- There were some weak associations between markers of oxido-nitrosative stress and several phospholipids

This study has not found evidence to support the hypotheses that perioperative changes in NO metabolism and oxidative stress are associated with changes in airway DPPC or PC flux through the Kennedy pathway in lung transplantation. However, this study has identified multiple weak associations between markers of oxido-nitrosative stress that were associated with many other phospholipids which are of uncertain significance. Oxido-nitrosative stress could affect perioperative phospholipid metabolism in lung transplantation through Kennedy pathway independent mechanisms.

5.4.2 Perioperative changes in NO metabolites and oxidative stress

Numerous studies have explored multiple markers of oxidative stress in lung transplantation, and these include isoprostanes, isofurans, malondialdehyde, oxidized glutathione, carbon monoxide, myeloperoxidases, haem oxygenase-1 (HO-1), lipid peroxides, nitrites, nitrates, and nitric oxide (NO); [46,158,160,323-326]. This study has endeavoured to add to this existing body of work, with an attempt to identify an association between the selected markers of oxidative stress and surfactant phospholipid metabolism.

5.4.2.1 NO Metabolites

NO mediated oxidative/nitrosative stress may contribute to the development of the ARDS[321], and increased plasma nitrate and nitrite concentration are also associated with septic shock in the general intensive care population[327]. Elevated levels of NO metabolites (nitrite and nitrate) have been seen in both the airways and circulation of lung transplant recipients, and probably reflect ongoing airway inflammation[319,325]. Changes in NO metabolism are expected following IRI (ischemia reperfusion injury)[328], are disrupted in human lung transplantation[159,325], and are associated with increasing indices of oxidative stress and lipid peroxidation[329]. Given this previously established link between NO metabolism, and lipid peroxidation, this concept has been explored in further detail by assessing their impact on phospholipid metabolism in lung transplantation.

Previous data from healthy control subjects showed total NOx (combined nitrates and nitrites) levels of 29.2µM (95%CI 21.9-34.4µM)[36], with similar values reported in other studies with healthy volunteers[330,331]; levels [37]which are comparable to those seen in our preoperative lung transplant recipients - Table 5-2. However, lung transplantation was associated with a prompt increase in both plasma nitrate and nitrite concentrations. These results echo the similar findings of increased nitrates and nitrites in ARDS and other critically ill cohorts[169,327].

Elevated NO metabolites have previously been demonstrated in the plasma of stable lung transplant recipients, and were associated with increased airway inflammation[325], but this study is the first to show that plasma NO metabolites are elevated within the hours of lung transplantation. Ischemic tissues will accumulate precursors of NO synthesis, and increased amounts of nitrates and nitrites are predictable following ischemia[328]; these data suggest that this is also the case in clinical lung transplantation - Table 5-3. Changes in the nitrate:nitrite ratio have also been associated with lipid peroxidation[329], but this ratio was unchanged in the immediate perioperative period for this study cohort.

The observed increases in NO metabolites after transplantation could have several possible explanations. Airway inflammation can result in increased NO production through increased activity of nitric oxide synthase (NOS) enzymes, typically inducible nitric oxide synthase (iNOS)[169,332]; NO is then oxidized into nitrates and nitrites[333]. In states of oxidative stress, $O_2^{\bullet-}$ and NO will react to form peroxynitrite that will eventually degrade into nitrite (and hydroxide ions)[169,334,335]. An alternative explanation would be that the injured lung is less able to utilise or clear NO metabolites[38]. Reduced utilisation of NO metabolites would seem like a less likely explanation in this cohort, as the NOS-independent pathway of NO synthesis from nitrite reduction is typically enhanced in hypoxic and acidic states[333,336].

NO is an important regulator of vascular tone in the pulmonary vessels, and suppressed NO metabolites have been described in patients with pulmonary artery hypertension. As pulmonary artery hypertension is seen in some patients waiting for lung transplantation[337], it could also be hypothesized that the increase in NO metabolites seen in transplantation reflects the replacement of diseased pulmonary vasculature with healthy organs.

Nitrosative stress could have an adverse effect on other components of surfactant. Protein nitration will impair lipid binding and aggregation mediated by surfactant proteins, and could also

theoretically contribute to surfactant dysfunction in lung transplantation[338,339]. The links between NO metabolites and surfactant phospholipids are discussed in more detail later.

5.4.2.2 Free Thiols

Lung transplantation was associated with an increase in free thiols during perioperative lung transplantation. This observation was unexpected, as free thiol depletion is seen in multiple diseases associated with states of elevated oxidative stress[315,340].

Free thiols are adjusted for protein concentration, because proteins are the major source of free thiols in human plasma[322]. Transplantation was associated with an immediate fall in plasma protein (Table 5-3); intraoperative redistribution and haemodilution are the most likely explanation for this observation during transplantation[341]. Albumin is the major source of thiols in the plasma, and the exogenous administration of albumin or other proteins could have contributed to the unexpected climb in thiols seen during transplantation[322]; the potential confounders are discussed in section 5.4.5.1.

All preoperative lung transplant recipients suffer from end-stage (and otherwise terminal) respiratory disease, and so elevated preoperative plasma indices of oxidative stress could be expected. Whilst the process of lung transplantation would be expected to generate profound oxidative stress [158], it could be difficult to detect a fall in free thiols if preoperative levels were already depleted by the oxidative stress associated with end-stage respiratory disease. This explanation is less likely, as preoperative levels of free thiols were still higher than those seen in other cohorts, like renal transplant recipients and heart failure patients[315,342].

The more likely explanation for this unexpected finding is that this reflects a methodological failing of the study (see section 5.4.5.1). The definitive explanation for this unexpected observation is not immediately apparent from this pilot dataset, and so this will require continued investigation in

larger studies with improved data collection on potential confounders (like exposure to exogenous proteins).

5.4.2.3 Tissue Markers

Immunohistochemistry combined with confocal microscopy has been attempted to both evaluate the location and attempt semi-quantitative analyses of multiple markers of oxidative stress, nitrosative stress, and lipid peroxidation. Primary antibodies for all of the tissue markers produced diffuse patterns of staining across the whole of the alveolus, and were not obviously confined to any one cell type (e.g. type 2 pneumocytes). Co-localisation of the primary antibodies with epithelial markers was not attempted, but arguably was not necessary given the diffuse pattern of staining with the primary antibodies. Assuming that this diffuse staining of the alveolus was not an artefact, subsequent studies could use homogenised biopsy samples of lung parenchyma with alternative quantitative techniques to assess for HO-1, NT, and 4-HNE.

No intraoperative changes were seen in tissue markers of oxido-nitrosative stress (HO-1, NT, and 4HNE). Lung biopsies were only taken a few hours apart from each other, and that may not have provided sufficient time to detect perioperative changes in tissue markers of oxidative stress.

Many RNS can nitrate tyrosine residues[343], and nitrites form NT through neutrophil derived myeloperoxidase[344,345]; therefore, the lack of association between nitrites and NT staining in this cohort is unexpected. This finding raises two possibilities; lung transplantation has disrupted this NT-myeloperoxidase pathway, or the more likely explanation is that this reflects an experimental failure due to methodological limitations (see section 5.4.5.2).

Clinical outcomes

Depleted free thiols have been associated reduced survival rates in patients with heart failure, which is thought to reflect their impaired redox status[315]. In a study of 695 stable renal transplant recipients followed over a year, elevated free thiols were associated with improved graft and patient survival[342], yet no such observations were seen in this cohort or perioperative lung transplantation.

However, this was a pilot study primarily designed to evaluate the impact of transplantation on phospholipid biology, and was not designed to demonstrate associations with clinical outcomes. As previously discussed, limitations in the methodology may have also affected the ability to detect this signal in this cohort of lung transplant recipients.

5.4.3 Airway phospholipids

Whilst lung transplantation was associated with perioperative changes in airway DPPC, NO metabolites, and free thiols (Table 5-3 and Chapter 3); this study has failed to demonstrate evidence of a link between these three variables.

Previous in vitro cell culture experiments have suggested that increasing oxidative stress can cause surfactant dysfunction through up-regulated protease-mediated cleavage of phosphocholine cytidylyltransferase and impaired phospholipid synthesis[163,164]. If it were the case, increasing oxidative stress should have affected deuterium labelling of all PC species synthesised by the Kennedy pathway. However, this study has not found any consistent evidence of a link between oxido-nitrosative stress and PC flux through the Kennedy pathway in perioperative lung transplant recipients. These data would not support the hypothesis that oxido-nitrosative stress causes surfactant dysfunction through up-regulated protease-mediated cleavage of phosphocholine cytidylyltransferase.

Oxidative stress could still exert an indirect effect on surfactant function, without changing DPPC content. Whilst this chapter has not demonstrated any significant association between markers of oxidative stress and the surfactant phospholipid species DPPC, oxidative stress markers were weakly associated with changes in several other species. Fatty acids containing double bonds are particularly susceptible to reactions with free radicals, and the lack of fatty acid double bonds in the fully saturated DPPC could offer relative protection against lipid peroxidation[346]. Oxidised phospholipid species in proximity to DPPC can have a profound impact on surface tension, by

altering the structural dynamics and organisation of DPPC with a layer of fluid[347,348]. Whilst unsaturated phospholipid species have less impact on alveolar surface tension, these species facilitate the alveolar adsorption of other surfactant phospholipid species like DPPC, and so disruption to the unsaturated PC component of surfactant may also have an indirect (and potentially detrimental) impact on alveolar surface tension[119]. Disruption of surfactant proteins have not been studied in this cohort, but they are an essential component of intracellular surfactant packaging and facilitate alveolar distribution of phospholipids[231]. These surfactant proteins are also vulnerable to the effects of oxidative stress, undergoing structural and functional changes when exposed to free radicals[312].

Some oxidatively truncated PC species can act as analogues of lipid mediators (e.g. platelet activating factor - PAF) and exert a wide range of inflammatory effects[227,349,350]. In addition to these truncated species, lipid peroxidation will also generate a range of toxic aldehydes that can also drive the inflammatory response[351,352]. Aldehyde formation from lipid peroxidation is dependent on the configuration of double bonds in the acyl chain moiety, and 4-HNE is largely derived from lipid peroxidation of ω-6 series PUFA species[351]. However, this study has not identified evidence of a link between 4-HNE and perioperative changes in airway phospholipids. Alternative measures of lipid peroxidation are discussed in section 5.4.5.3.

Pre-implantation tissue staining for nitrotyrosine was associated with changes in several airway sphingomyelin species. Sphingomyelin has been implicated as a marker of endothelial barrier function in ARDS[353] and of graft performance during assessment on ex vivo lung perfusion (EVLP) [354]. Sphingomyelin species are typically found in the cell membrane of most eukaryotic cells, and this observation could reflect a link between nitrosative stress and the release of cell membrane phospholipids[355].

It is expected that some polyunsaturated phospholipids would be more susceptible to the effects of oxidative stress than saturated species[351]. This chapter has identified several unsaturated

phospholipids that may have been affected by oxidative stress in lung transplantation, but further focused investigations with alternative indices of oxidative stress are required. In this study NO metabolites and markers of oxidative/nitrosative stress were weakly associated with changes in several phospholipid species after lung transplantation, but the impact of these changes on surfactant function is uncertain and would require further investigation. Nonetheless, these results suggest that there could be merit in examining individual species of phospholipids when attempting to assess the impact of oxidative stress, instead of narrowly focussing on a few individual species like DPPC or global measure of total phospholipid content.

5.4.4 Plasma phospholipids

Free thiols after lung transplantation were positively associated with PC16:0/22:6 flux; a lipid species containing docosahexaenoic acid (DHA). Earlier ex vivo lung experiments have already confirmed that PC16:0/22:6 is synthesised by the lung and secreted into the circulation - Figure 4-9. The significance of this modest in vivo association between DHA PC metabolism and a marker of oxidative stress is questionable, especially in light of the methodological limitations that were highlighted in sections 5.4.2.3 and 5.4.5.2. Thiols are protective in vivo[315], presumably through the thioredoxin and glutathione antioxidant systems[316]. The large number of double bonds make DHA susceptible to lipid peroxidation, theoretically less DHA would be available when antioxidant defences are depleted and lipid peroxidation is unopposed. However, this hypothesis is unlikely as similar trends were not seen with the many other polyunsaturated phospholipids that were detected with ESI-MS/MS. PC16:0/22:6 contains DHA, which is a precursor for proresolving lipid mediators called resolvins[288]. Both DHA and DHA-derived resolvins can attenuate the inflammatory effects of oxidative stress within in vitro models of lung injury[356]; but this weak association between free thiols and PC16:0/22:6 would need to be confirmed with repeat studies, using alternative markers of oxidative stress.

The relative abundance of postoperative plasma sphingomyelin species was associated with plasma nitrites and tissue staining for nitrotyrosine (with a weak association noted between plasma SM16:0 and tissue staining of 4-HNE). Sphingomyelin species have been highlighted as important in ARDS and EVLP[353,354], and so this link with indices of oxido-nitrosative stress could be worthy of continued investigation.

Whilst tissue staining with 4-HNE was not associated with any changes in 12-hour airway samples, but it was associated with changes in six different plasma phospholipids. Some of these phospholipids (PC16:0/18:2, PC18:1/20:4, and PC18:0/20:3 – Figure 5-14) could contain moieties with ω-6 series PUFA that preferentially give rise to 4-HNE during lipid peroxidation[351]. The precise configuration of double bonds cannot be determined with ESI-MS/MS, and future studies attempting to evaluate the links between ω-6 series PUFA and 4-HNE formation could consider alternative mass spectrometry techniques to separate such isomers.

5.4.5 Limitations of experimental techniques

5.4.5.1 Free thiols

The intraoperative rise in free thiols was unexpected, as discussed in section 5.4.2.2. A time-course analysis of the trends in free thiols could have helped to determine the optimal time to sample free thiols and assess for perioperative changes.

Proteins are the major source of free thiols in blood, and so correction for the plasma protein is required[315]. Blood products, like fresh frozen plasma,[357] or priming solutions for cardiopulmonary bypass (CPB)[358] are potential intraoperative sources of protein that could have affected the measurements of free thiols, and could have resulted in these unexpected findings. Unfortunately, data on CPB priming solutions and intraoperative blood products were not collected, and so adjustment for these potential confounders is not possible. The potential confounders make it very difficult to draw any firm conclusions from the thiol data, but this study has identified a number of

methodological limitations that should be considered if future attempts are made to analyse free thiols in perioperative lung transplantation.

5.4.5.2 Semi-quantitative Immunohistochemistry imaging

Automated quantitative immunohistochemistry image analysis offers a more objective approach when compared against operator dependant scoring based judgments of images[264]. Whilst the thresholding methods utilised have been previously been described in the literature, there is still no standardised approach for the quantitative assessment of immunolabelled images[359]. Images were screened by the author for technical adequacy before analysis, and this subjective assessment could introduce a source of bias into the dataset. In an attempt to control for any bias in the subjective assessment for adequacy, the author remained blinded to the origins of the images before the analysis was completed.

Further work is required to validate the techniques described in this chapter by comparing these results with alternative methods of measuring HO-1, 4-HNE, and NT. Elevated nitrotyrosine levels were found in the bronchoalveolar lavage of patients with ARDS with a gas chromatography mass spectrometry technique[360], and this method could be better suited to address these issues.

As lung biopsies were only taken at the discretion of the consultant transplant surgeon, relatively few perioperative tissue samples pairs were available for analysis. The logistical difficulties in obtaining lung tissue substantially reduced the available number of biopsy pairs, and further increases probability of a type II error, in a cohort that was already of a small size[361].

The lack of an externally validated analytical technique combined with a very small number of biopsies make it very difficult to firmly accept or reject any hypothesis on basis of the immunohistochemistry imaging data. However, some weak associations were detected between NT, sphingomyelin species and polyunsaturated PC species; these associations should probably be

confirmed with another validated measure of NT, such as gas chromatography combined with mass spectrometry[360].

5.4.5.3 Markers of oxido-nitrosative stress

Numerous markers of oxidative stress have been evaluated in lung transplantation (section 5.4.2). However, the range of biomarkers of oxidative stress that have been used in other conditions is vast[362]. This study has not demonstrated a link between PC flux through the Kennedy pathway and the markers of oxido-nitrosative stress that have been presented in this chapter, but alternative markers of oxido-nitrosative stress could have had more success; only further studies could address this potential study limitation. If lipid peroxidation is the primary mechanism of deranged phospholipid metabolism during increased oxido-nitrosative stress in lung transplantation, then this could be quantified with direct measurements of oxidatively truncated PC species or nitrated phospholipids[363,364].

5.4.5.4 Statistical analyses

The small patient numbers will increase the risk of type 2 errors, and so it difficult to conclude that oxidative stress markers have no link with DPPC metabolism. Furthermore, the assessment of a large range of phospholipids species presents an increased risk of type 1 errors. The risk of type 2 errors becomes most apparent when examining the tissue markers of oxidative stress, when there are relatively few biopsies available for analysis.

The numerous associations between the measured tissue markers and perioperative changes in phospholipids suggest that there is merit in the presented techniques. However, the risk of type 1 errors with these large lipidomic datasets have already been discussed in previous chapters, and could potentially result in a spurious association between phospholipid measurements and tissue markers – see sections 2.6.4 & 3.4.6.5.

The objective of this pilot study is to identify potential links between oxidative stress and phospholipid biology in lung transplantation, and present those candidate phospholipids as candidates for future directed studies. Uncorrected p-values are presented for this pilot dataset and the increased risk of type 1 errors is acknowledged, with the expectation that further investigations will be required to confirm these findings (previously discussed in sections 2.6.4 & 3.4.6.5). These results should be viewed as a pilot data set that provides evidence supporting further investigation into the impact of oxidative stress on individual phospholipid species.

5.4.5.5 Lipidomic profiling

Apart from DPPC, phospholipids have not been selected for analyses against markers of oxidative and nitrosative stress; instead, a systematic analysis was performed of all measured phospholipids. A targeted study of oxidatively truncated phospholipid species against other markers of oxidative stress would have been desirable. Previous studies based on ESI-MS/MS have identified 29 lipids that probably represented these truncated phospholipid species, and would be a logical target for investigation[363]. Especially as oxidatively truncated PC species that can act as analogues of lipid mediators and exert a wide range of inflammatory effects[227,349,350], further study of these species could be of interest. Whilst information on those truncated phospholipids might exist in the raw data from the spectra generated from this study, the analytical software used to process mass spectra was unable to identify most of these species (section 2.6.2). Revisiting the raw mass spectra in the future with an alternative approach to data analysis could provide more information on this topic. Alternative mass spectrometry techniques could also provide insights into the effects of oxido-nitrosative stress on pulmonary phospholipid biology in lung transplantation. Novel techniques based on hydrophilic interaction liquid chromatography coupled to ESI-MS/MS can directly measure nitro phospholipids that have been formed under conditions of nitrosative stress[364], and could be used to explore this concept if future studies were contemplated.

The general limitations of the study design and lipidomic profiling techniques have already been discussed in section 3.4.6. As described in section 2.2.2, this was designed as a pilot study with a small patient cohort that was not powered to identify any specific clinical endpoint; therefore, there is an increased risk of type 2 errors[361]. Instead, this pilot dataset has attempted to identify phospholipids that could be of interest for future studies, apply novel analytical techniques, and identify mechanisms that will lead to perioperative changes in phospholipid biology.

5.4.6 Conclusions

In vitro experiments have suggested that surfactant dysfunction could be caused by oxidative stress mediated degradation of phosphocholine cytidylyltransferase, the rate-limiting enzyme in the Kennedy pathway of phospholipid synthesis[163,164]. However, the data presented in this chapter have not identified any link between DPPC flux through the Kennedy pathway and the measured indices of oxidative and nitrosative stress. Instead this pilot dataset has identified several other phospholipid species that were weakly associated with perioperative indices of oxidative and nitrosative stress in lung transplantation. Oxidative inactivation of the rate limiting step in the Kennedy pathway would have affected deuterium labelling of all PC species produced by that pathway, and not only a few isolated PC species; this could have three different explanations.

1. Oxidative stress affects phospholipid metabolism after they have been synthesised by the Kennedy pathway
    a. Land's acyl remodelling pathway
    b. Preferential lipid peroxidation of unsaturated phospholipids
    c. Altered phospholipid recycling by the macrophages and AT2 cells
2. Oxidative stress affects metabolic pathways that are independent of the Kennedy pathway
    a. The hepatic phosphatidylethanolamine N-methyltransferase (PEMT)
3. Experimental limitations of the study

Numerous experimental limitations have been discussed in section 5.4.5, and include the measurements of oxido-nitrosative stress, potential confounders, phospholipid selection, and a limited sample size; these limitations should be considered if further study into this topic is contemplated.

Lipid peroxidation of polyunsaturated phospholipids has been well described in the lite rature[311], and could be another explanation. Oxidation of polyunsaturated species could play an important role in inflammation and exert an indirect impact on the physical properties of surfactant, but these effects will require further study. Links between oxido-nitrosative stress and polyunsaturated PC species might be of interest as a target for future investigation. Tissue staining for nitrotyrosine and plasma NO metabolites were associated with some changes in perioperative phospholipids. These species could be candidates for further study into the impact of oxido-nitrosative stress on pulmonary phospholipid biology.

Previous chapters have used deuterium isotope labelling to highlight the lungs central role in phospholipid metabolism, and the important clinical impact of disrupted phospholipid flux after lung transplantation. Stable isotope labelling has not provided evidence of any direct link between perioperative oxido-nitrosative stress and Kennedy pathway activity. Metabolism of some phospholipid species were associated with perioperative changes in NO metabolism and oxidative stress, suggesting that oxido-nitrosative stress could disrupt phospholipid metabolism after lung transplantation though Kennedy pathway independent mechanisms. However, this hypothesis will still require continued investigation with other validated techniques to confirm any metabolic or clinical effects. This pilot dataset has identified several phospholipids for future targeted studies into the links between perioperative NO metabolism, oxidative stress, and phospholipid metabolism.

6 Discussion

6.1 Summary of key findings

Lung injury associated with transplantation has a profoundly detrimental impact on postoperative survival[1,29]. Phenotyping surfactant dysfunction in perioperative lung transplant recipients could identify patients at risk of developing graft dysfunction, and represents an opportunity to develop novel diagnostic markers and investigate new therapeutic targets. This study has demonstrated the perioperative variability in Kennedy pathway dependant phospholipid metabolism, and shown that this variability is associated with graft function after lung transplantation. This is not restricted to the metabolism of surfactant phospholipids (like DPPC), as changes in the metabolism of polyunsaturated PC species (like those containing arachidonic acid) are also associated with graft function. The true value of measuring phospholipid kinetics remains to be seen, but has the potential to identify novel biomarkers and therapeutic targets in lung transplantation.

The complementary ex vivo research model has been demonstrated to be a viable platform for pulmonary phospholipid research. Our data from the EVLP platform suggest that Kennedy pathway preservation could act as one of the protective mechanisms behind clinical normothermic ex vivo lung perfusion, and demonstrate basal phospholipid secretion into the circulation by the pulmonary epithelium.

Translating insights into pulmonary phospholipid biology from both lung transplant recipients and the human ex vivo model into the wider population could theoretically benefit patients with ARDS outside of the lung transplantation programme, and this hypothesis warrants continued research. This chapter will provide a brief commentary and overview on the methodology and key results presented in this thesis.

6.1.1 Clinical study

- Lung transplantation results in an immediate and severe decrease of surface active phospholipids, like DPPC, in the airways
- Stable isotope labelling suggests that changes in Kennedy's CDP-choline pathway are associated with poor outcomes following lung transplantation
- Changes in DPPC flux are associated with mid-term graft function, whereas static measures of DPPC content are not
- Perioperative changes in PUFA-PC flux are also predictors of mid-term graft function
- Indices of oxidative stress are associated with perioperative changes in PUFA PC species

6.1.2 Ex vivo study

- The human ex vivo platform has provided supporting evidence of basolateral phospholipid secretion by the pulmonary epithelium, that is different to the apical secretion of surfactant phospholipids into the airways
- Lungs synthesise phospholipids containing PUFA species that are secreted into the circulation
- Normothermic ex vivo lung perfusion protects the Kennedy CDP-choline pathway of surfactant phospholipid synthesis during ex vivo transport and assessment.

6.2 Dynamic lipidomics

Lipidomics based on ESI-MS/MS allows the rapid analysis of hundreds of lipid species[204], and this thesis presents the first application of the technology in perioperative lung transplantation. ESI-MS/MS has allowed airway phospholipid identification with unprecedented molecular specificity in perioperative lung transplantation. Whilst isotopic labelling of PC synthesis dates back 60 years to Kennedy's first description of his eponymous pathway[126], many similar labelling strategies have been used to characterise other metabolic pathways that determine a particular phenotype; utilising a so-called fluxomic approach[365]. Combined metabolomic and fluxomic approaches have been used in animal models to quantify the effects of dietary modification on lipid kinetics by illustrating the up-regulation of de novo lipogenesis in mice on low-fat diets; this homeostatic concept was demonstrated through the incorporation of deuterium labels in the absence of differences in static lipid concentrations[366]. Similarly, this clinical study has shown that deuterium incorporation into surfactant phospholipids is associated with poor graft function, even if static measures of surfactant phospholipids do not share those associations; implying that dynamic measurements of phospholipid flux could provide clinically significant information that cannot be found from static measurements alone. Beyond the measurement of static quantities of phospholipid at various time-points, isotopic labelling of the Kennedy pathway can provide in vivo data on lipid flux and allow the depiction of a dynamic lipidomic profile[249,251]. The utility of this dynamic lipidomic technique has already been demonstrated in patients with ARDS and neonatal RDS[137,367]. This pilot dataset offers further evidence to support the continued investigation into the nascent field of dynamic lipidomics in translational critical care research.

Like other shotgun ESI-MS/MS lipidomic studies, a notable limitation of the technique is the difficulty in identifying the exact location of double bonds in polyunsaturated fatty acids and the discrimination between certain isomers. The location of double bonds in PUFA species is relevant

when considering the subsequent metabolism into lipid mediators, as ω-3 fatty acids are a key substrate for the synthesis of many specialized pro-resolving mediators[368,369]. Localising PUFA double bonds and further structural discrimination between isomers could be addressed in future studies with the use of reversed phase liquid chromatography for candidate species of interest[251].

Large volumes of lipid kinetic data on hundreds of species derived from multiple patients over many time points introduces significant challenges for statistical analysis[370]. As lipidomics continues to develop, the complementary bioinformatic approaches will also have to evolve. Clustering analyses are frequently employed for high-dimensional data-sets that are encountered in lipidomics (and in other fields of '-omics' research)[371]. Topological data analysis (TDA) is another clustering tool used generate hypotheses from large datasets[268], and its potential lipidomic applications have been briefly explored in this thesis (section 3.3.3). TDA offers a novel approach for dealing with the large and complex lipidomic datasets generated by ESI-MS/MS, and could offer an alternative method for visualising temporal changes in the lipidome. Here, TDA has been used to illustrate those temporal changes in airway phospholipid profiles as recipients recover from transplantation, and to help identify species of interest for future studies; continued investigation into the application of these clustering platforms in lipidomics is justified.

6.3 Clinical study

This clinical study has suggested that Kennedy CDP-choline pathway disruption is associated with poorer outcomes in clinical lung transplantation. This observation could shed light onto the pathogenesis of perioperative lung injury in transplantation, and has potentially identified candidate phospholipids for further investigation as novel disease markers and therapeutic targets. This pilot dataset presents a detailed analysis of surfactant disruption and phospholipid kinetics in perioperative lung transplantation that could serve as the foundation for future studies in the field.

6.3.1 Insights into perioperative lung injury

Postoperative lung function is of crucial importance following lung transplantation, as it is the defining feature of CLAD – the biggest cause of death in lung transplant patients [22]. Therefore, any association between phospholipid metabolism and poor graft function would be of great clinical importance.

PC species represent 68% of surfactant phospholipid content, and these phospholipids exert the greatest impact on alveolar surface tension; particularly saturated species like DPPC[74,97,121,122]. Lung transplantation is associated with a marked reduction of surfactant phospholipid species in the airway, specifically DPPC. DPPC levels in the airways continued to fall for several days in transplant recipients who required prolonged invasive respiratory support, before a trend for recovery was seen – section 3.3.2.2. Over half of DPPC is synthesised through remodelling pathways that are dependent on the action of PLA_2 (Phospholipase A_2)[122], and so future studies into disruption of those remodelling pathways in lung transplantation could also investigate perioperative changes in PLA_2. Indeed, it has already been shown that PLA_2 contributes to the pathogenesis of acute lung injury in the general ICU population, presumably by either surfactant hydrolysis and remodelling, the cytotoxic effects of lysoPC on the alveolar epithelium, or by stimulating the inflammatory response [136,372-374]. Changes in PLA_2 have been implicated in the pathogenesis of many inflammatory conditions, including septic shock and multi-organ dysfunction syndrome; presumably through the release of PUFA inflammatory mediator precursors [375-379].

Clustering analyses suggest that there is a relative increase in the proportion of PUFA-PC species immediately after surgery, before saturated PC species slowly start to recover and predominate in the airways. The novel application of stable isotopic labelling of the Kennedy pathway has provided data on phospholipid flux that has shown associations with important clinical outcomes after

transplantation; patients with evidence of increased perioperative DPPC turnover have impaired graft function for months after surgery (section 3.3.4).

Exogenous surfactant replacement therapy has been a source of great disappointment for adult intensivists who had hoped to translate its successes in neonatal RDS into adult lung injury[180,181]. Phenotyping surfactant dysfunction in adults with acute lung injury could identify subsets of patients who might benefit exogenous surfactant replacement and allow for a more personalised therapeutic strategy[74,184]. This study has shown that isotopic labelling of lung transplant recipients can identify a subset of lung transplant recipients who might be at risk of poor graft function, and exogenous surfactant replacement for these individuals might represent one targeted therapeutic strategy to improve outcomes. Whilst initial use of surfactant replacement therapy has shown promise in lung transplantation[5,6,185-189], caution is required to ensure that the failures of previous adult trials in ARDS are not repeated. Stable isotopic labelling in perioperative lung transplantation has been demonstrated as a viable technique to phenotype pulmonary phospholipid biology; a technique that could theoretically identify recipients who might gain benefit from exogenous surfactant therapy[74,184].

The strong association between PUFA-PC flux and graft function is particularly interesting, when considering the biological significance of these molecules (section 3.3.4). Fatty acids from cell membrane PC species that are liberated by PLA_2 (phospholipase A_2) are a major source of PUFA species like arachidonic acid, which is a crucial substrate for the cyclooxygenase and lipoxygenase pathways that synthesise a wide range of inflammatory mediators[210]. Recipients with a high-turnover of PC species containing arachidonic acid in their airways had reduced graft function after lung transplantation. It is possible that stable isotope labelling of these phospholipids could serve as a sensitive indicator of inflammatory mediator metabolism in lung transplantation. The association

between arachidonic acid PC flux and impaired graft function supports further investigation of inflammatory mediators that are derived from arachidonic acid and other PUFA species; typically assessed with a combined high performance liquid chromatography and tandem mass spectrometry technique[308].

Whilst PC phospholipids are a major source of PUFA species that are precursors for a wide range of inflammatory mediators, other phospholipid classes like phosphatidylethanolamine (PE) and phosphatidylinositol (PI) also probably serve as reservoirs of arachidonic acid for the synthesis into inflammatory mediators[12]. To complete a truly comprehensive catalogue of the perioperative lipidome would require inclusion of all phospholipid classes, as well as the neutral lipids and glycolipids. Whilst the other phospholipid classes are less abundant in human airways, they also play important supporting roles in airway surfactant. Phosphatidylglycerol (PG) and other phospholipid classes may also indirectly reduce surface tension by facilitating alveolar adsorption of surfactant along the alveolar wall; and whilst these other phospholipid classes might be less abundant, they are still worthy of further investigation[119]. Collecting data on PG and PI class phospholipids is achieved with ESI-MS/MS precursor scans for product ions with m/z 153 (for glycerophosphate fragments) and m/z 241 (inositol phosphate fragments) respectively (m/z – mass/charge ratio)[380]; preliminary scans have already been performed for these phospholipid classes for inclusion in future studies, but the inclusion of these preliminary data was beyond the scope of this thesis.

A causative association between oxidative stress, nitrosative stress, and phospholipid metabolism is difficult to prove based on the data presented in this study. Weak associations have been demonstrated between indices of oxidative stress and NO metabolites, and perioperative changes in several PUFA species, and it is difficult to ascribe biological or clinical significance to these weak associations. Perioperative changes in DPPC were not associated with NO metabolites or oxidative stress (section 5.3.2) and this is probably the result of one of the following explanations. Saturated

fatty acids in surfactant phospholipids are relatively resistant to lipid peroxidation due to their lack of double bonds, and are thus less susceptible to oxidative stress or changes in NO metabolites. In contrast, weak associations were seen between indices of oxidative/nitrosative stress and perioperative changes in PUFA species with many double bonds. Reactive oxygen species will induce arachidonic acid metabolism in animal models of ARDS[381], and so the complex interactions between oxidative stress and perioperative changes in PUFA species probably extend beyond their number of double bonds and susceptibility to lipid peroxidation. PLA_2 enzymes are thought to mobilise free arachidonic acid from PC species for eicosanoid synthesis in the presence of oxidative stress[382]. Oxidative stress may also inhibit the reacylation step of Land's remodelling pathway that could also decrease the amount of arachidonic acid that is esterified in PC species[383,384]. This study supports the further investigation of the effects of oxidative stress in perioperative lung transplantation, but instead of focussing on the impact on pulmonary surfactant phospholipids, it might be more fruitful to focus studies on the impact on PUFA species (such as arachidonic acid), their metabolites, and associated enzymes (like PLA_2 and LPCAT1).

Conditions associated with oxidative stress are typically associated with reduced levels of free thiols[315,340]. The perioperative increase in serum free thiols demonstrated in section 5.3.1.2 was unexpected. Possible explanations for this unexpected finding are reviewed in section 5.4.5.1, and probably reflect limitations with the methodology. The first step to address this unexpected finding would be to see if this observation is repeatable in another cohort of perioperative lung transplant recipients, whilst controlling for potential confounders as described in section 5.4.5.1.

This study has largely focused on surfactant lipids, but the protein component is also essential for normal surfactant function[114,115]. Surfactant Protein D (SP-D) is a plasma marker of PGD that is associated with reduced 90-day survival rates in lung transplantation. Preliminary data generated from this cohort (in collaboration with others) has found that patients with reduced levels of airway

SP-D tend to have impaired postoperative oxygenation, prolonged postoperative ventilation, and impaired graft function at 3 months[385]. Whilst detailed analyses of surfactant proteins were beyond the scope of this thesis, future work should endeavour to characterise any links in the perioperative disruption for both surfactant phospholipids and proteins.

Aqueous fractions extracted from plasma samples taken from deuterium labelled transplant recipients could yield further insights into choline metabolism that have not been explored in this thesis, as they have in similar research into the neonatal respiratory distress syndrome (RDS)[152]. Archived aqueous extracts from this cohort could still be utilised for future analyses into the interaction with associated proteins and aqueous-phase metabolites, and would represent a logical follow-on study from this thesis. This 'systems biology' approach to the integration of genomics, proteomics, and metabolomics has been proposed for the study of other respiratory diseases such as asthma[386,387]. Similar approaches are currently being utilised in the study of CLAD, but such an integrated systems biology approach to the research of PGD has not yet taken place in lung transplantation[388,389]. Surfactant remodelling of PUFA phospholipids into saturated species via the Lands cycle illustrates the need for a systems biology approach to explore the interactions between lipids and proteins, and gain a more comprehensive understanding of the mechanisms behind surfactant dysfunction after lung transplantation.

In summary, this pilot data-set from perioperative lung transplant recipients supports further research into lipidomic flux; and these future studies would benefit from a detailed analysis of PUFA derived inflammatory mediators, and integration with complementary proteomic and metabolite data.

6.3.2 Lung transplantation – a human model of ARDS?

Lung transplant recipients are a unique cohort of critically ill patients who are subjected to the most severe pulmonary ischemia reperfusion injury (IRI). There are many variables that are unique to

transplanted lungs that are not seen in ARDS in the wider ICU population, like the effects of the bronchial anastomosis (and routine sacrifice of the bronchial circulation), donor brain death, donor-graft immune interactions, and donor size matching[35,56,390]. Despite these unique considerations in lung transplantation, PGD and ARDS probably share more similarities than differences; as evidenced by their identical clinical, physiological, radiological, and histopathological presentations[2,37-40]. It is because of these many similarities that most treatments for PGD have been extrapolated from ARDS[2,56], and so it stands to reason that insights obtained from perioperative lung transplantation could also be extrapolated back into the general population with ARDS.

Research in the critically ill is logistically and ethically challenging because of difficulties in obtaining patient consent, timely enrolment, and heightened concerns of patient safety[391]. The inherent challenges of research into conditions that present as an emergency (like ARDS) makes it particularly difficult to study the illness in its early stages[392]. Many of these barriers to research in ARDS have been avoided in this cohort of perioperative lung transplantation. Potential lung transplant recipients can be fully consented before surgery, and it is possible to time sampling and interventions at the earliest stages of injury, or possibly before lung injury has become apparent.

Other human in vivo models of lung injury have studied patient groups at risk of developing lung injury, like those undergoing one-lung ventilation (OLV) or cardiopulmonary bypass (CPB). However, OLV usually causes sub-clinical injury, and the incidence of ARDS after CPB (~2%) is considerably lower than the incidence of PGD after lung transplantation[57]. As PGD may affect 25% of lung transplant recipients[28], a high-throughput transplant centre could reliably identify reasonable numbers of motivated patients at high risk of developing severe lung injury who are willing to participate in clinical research; as evidenced by the successful recruitment in this this study. This study has also demonstrated the safety and feasibility of stable isotopic labelling, frequent sampling from the airways, and the collection of contemporaneous lung biopsies from perioperative lung transplant recipients.

The clinical study has shown that increased deuterium PC enrichment is associated with poor clinical outcomes. Similarly, ARDS is associated with increased deuterium PC enrichment, when compared to healthy controls in previously published studies[137]. A comparable pattern of lipidomic flux could reinforce the position of PGD in lung transplantation as a model of ARDS. A more detailed comparison of the lipidomic datasets for both patient cohorts was beyond the scope of this thesis, but could help to establish PGD after lung transplantation as a model for ARDS research.

This study's successful recruitment of patients at high risk of developing lung injury, combined with many inherent similarities between PGD and ARDS would justify further efforts to establish clinical lung transplantation as a human model of ARDS.

6.4 Ex vivo study

Labelling of the Kennedy pathway has confirmed the activity of a major source of PC synthesis in ex vivo lung perfusion, and could represent one of the mechanisms that protects the graft during clinical EVLP in lung transplantation. This pilot lipidomic dataset offers supporting evidence for the previously proposed theory of basolateral phospholipid secretion by the pulmonary epithelium[10,11], and may serve as the foundation for future studies into ex vivo phospholipid biology.

6.4.1 Graft selection and protection in clinical EVLP

Ex vivo lung perfusion has the potential to expand the donor pool of organs for transplantation by allowing further assessment of 'high risk' marginal organs and possibly the assessment of donors after cardiac death[393]. The ability to use EVLP to recondition marginal organs or prevent the development of PGD remains to be seen and is the matter of ongoing trials[86].

Ventilation dependant surfactant phospholipid release is abolished when the lungs are cooled below 13°C, as is routinely the case during organ retrieval without EVLP[309]. This study proves that the

Kennedy CDP-choline pathway remains active during normothermic EVLP, with continued release of newly synthesised surfactant phospholipids into the airspace. Therefore, it could be hypothesized that preservation of Kennedy pathway dependant phospholipid synthesis is one of the protective mechanisms associated with clinical EVLP. As previously discussed, the correction of surfactant deficiency in EVLP can improve post-implantation graft performance in an experimental animal model of transplantation[89], and combined with these data presented suggest that the ex vivo pulmonary surfactant system is a potential therapeutic target that warrants further investigation.

Airway DPPC was reduced in ex vivo organs when compared to healthy controls[7], but not to the same extent that was seen in vivo after lung transplantation. This interesting observation requires further study, as it could imply that in vivo pulmonary reperfusion is more damaging to airway DPPC than ex vivo pulmonary reperfusion. This observation would imply either that normothermic EVLP offers some form of protection to the pulmonary surfactant system that is not seen in vivo, or that the reduction in airway DPPC after lung transplantation is not the sole effect of rewarming and re-oxygenating the organs. Labelling of the Kennedy pathway in lung transplant recipients has suggested that increased DPPC flux is associated with poor outcomes, and so a post-implantation fall in DPPC could reflect changes in that Kennedy CDP-choline pathway. However, this observation does not exclude the possibility of coexisting pathological changes to Land's remodelling cycle. In ARDS there is an increase in airway lysoPC species in relation to saturated PC species[55], which could reflect pathological or deficient surfactant remodelling by PLA_2 or LPCAT1[124,125,394-397]. Investigating the integrity of Land's remodelling cycle in parallel to the Kennedy pathway could be addressed in future studies through an assessment of PLA_2 and LPCAT1 activity.

It is difficult to make any quantitative comparisons of Kennedy pathway activity between the available ex vivo and in vivo data because of the different experimental conditions, so it is not possible to say if there is greater Kennedy pathway activity within ex vivo or in vivo lungs. However,

this pilot dataset may guide the design of future studies to address this hypothesis, and would support further investigation into the surfactant protective mechanisms of EVLP.

Metabolomic profiling in clinical EVLP has been used to identify novel biomarkers of PGD after transplantation. For example, the appearance of the cell membrane sphingomyelin species SM16:0 in the perfusate has been highlighted as a potential candidate[354]. This study has found that the perfusate of rejected ex vivo organs had a higher content of surfactant phospholipid species like DPPC, and PC16:0/14:0, presumably reflecting increased leak of the alveolar contents into the circulation. If EVLP was unable to protect or recondition the potential graft, it could be hypothesized that lipidomic profiling of the perfusate could help screen marginal organs for transplantation. This hypothesis could be addressed in future studies with a greater number of organs that would be adequately powered to identify novel biomarkers, and this pilot dataset could also assist in the design of those studies.

6.4.2 Insights into pulmonary phospholipid biology

Whilst it is accepted that the lungs secrete saturated phospholipids into the airspace to form a layer of pulmonary surfactant, these ex vivo modelling experiments have shown for the first time in man that the lungs also preferentially release PC species containing polyunsaturated fatty acids (PUFA) into the circulation. Arachidonic acid is one such PUFA species that is a precursor for numerous inflammatory mediators, contributes to leukocyte activation, and has been implicated in the development of PGD after lung transplantation[12-14]. Pulmonary PUFA secretion may contribute to the systemic inflammatory response seen with ARDS, and its role in PGD and perioperative lung transplantation should also be studied[398].

The liver is a major source of circulating phospholipids, especially polyunsaturated PC species [277]. The extent to which the lungs depend on the liver for phospholipid metabolism was previously unknown, yet these data suggest that the lungs can synthesise a diverse range of phospholipid species in isolation from the hepatic PEMT (Phosphatidylethanolamine N-methyltransferase) pathway, including those polyunsaturated PC species that are actively and preferentially released into the circulation by the lung. The function of these polyunsaturated PC species that are secreted by the lungs into the circulation remains to be seen, and certainly warrants further investigation. The role of polyunsaturated PC as a substrate for lipid mediator synthesis has been previously discussed; perhaps the pulmonary secretion of polyunsaturated PC reflects the synthesis of those inflammatory mediators and may even contribute to the systemic inflammatory response that is frequently seen with lung injury and VALI termed biotrauma[54]. As previously discussed, combining future lipidomic studies with a detailed analysis of those PUFA-derived inflammatory mediators could help to answer some of these important questions.

Any insights into the surfactant-protective mechanisms of EVLP that could be translated into *in vivo* lung injury would certainly be of great benefit to both lung transplant recipients with PGD and the wider population suffering with ARDS, and warrants continued investigation.

6.4.3 Human ex vivo lung perfusion – a model for phospholipid research?

In vitro models may fail to reproduce the mechanical architecture of the lung that is needed for normal phospholipid metabolism, and the full diversity of phospholipids seen in man [101 191 192]. Discoveries in animal models of acute lung injury frequently fail to translate into human ARDS, and are limited by interspecies variation in pulmonary phospholipid biology [201].

The ex vivo study of whole human lungs has the potential to provide an excellent approximation of human ALI[399,400], without being restrained by the important safety and practical restrictions of conducting in vivo research in humans[57].

In establishing their human EVLP model of acute lung injury, Matthay's group have quantified ex vivo alveolar fluid clearance (using radiolabelled albumin and wet:dry ratios), epithelial markers of injury (such as RAGE – receptor for advanced glycation end products) and associated inflammatory cytokine responses (IL-1β, TNFα, and IL-8)[401,402]. After characterising the human ex vivo model, they were able to investigate responses to injury (bacterial endotoxin) and therapeutic interventions (β_2-agonists and mesenchymal stem cells)[88,400,401]. Similarly, if human EVLP is to be established as a platform for phospholipid research, the first step must include the definition of the model's lipidomic profile, before proceeding to injury modelling and therapeutic interventions that target pulmonary phospholipid metabolism. This pilot dataset represents a small part of that essential first step towards establishing human EVLP as a platform for pulmonary phospholipid research; by offering a first description of the models lipidome and establishing the ex vivo integrity of Kennedy's CDP-choline pathway. Animal models of lung injury have used a multitude of insults that include the use of gastric acid, injurious positive pressure ventilation, and infection[57]; any of these approaches could be brought into future studies on the impact of lung injury on the human EVLP lipidome. Keshavjee's group have already investigated the therapeutic effects of exogenous surfactant replacement in their animal EVLP model of gastric acid aspiration[89], and a similar study into human EVLP could be investigated once the platform has been established. Continued work with animal EVLP in parallel to human EVLP will still be required to facilitate model development, given the relative scarcity of human lungs for research[403].

The systems biology approach that strives to understand protein-lipid-metabolite interactions in vivo[386,387] will probably be required as human EVLP research is developed. The importance of surfactant protein-lipid interactions in the airways has already been discussed earlier in section 1.2.2. However, proteins are essential for normal lipid homeostasis on both sides of the alveolar interface, as apolipoproteins are vital for lipid transport in both the circulation and the lymphatics[305].

Future studies could explore the changes in EVLP phospholipid secretion into circulation in the absence and presence of those dedicated apolipoproteins in the perfusate. The choice of perfusate in clinical EVLP has been the source of much debate, and there is continuing disagreement on the optimal solution for ex vivo support in clinical lung transplantation[404]. Perfusates were not standardised in this study because of logistical constraints, and could potentially account for some of the variability in ex vivo lipidomic profiles seen in this pilot dataset. However, once the human EVLP phospholipid research platform has become established, additional resources could permit a systematic investigation into the impact of different perfusates on phospholipid biology in future studies.

It has previously been proposed in section 1.2.11 that the gold standard model for research into pulmonary phospholipid biology would have the following attributes:

1. Faithfully replicates the physical effects of breathing
2. Maintains a preserved alveolar & vascular architecture
3. Maintains cell-cell interactions that would be expected in the lungs
4. Capable of producing a comparable range of phospholipids expected in man
5. Can reliably reproduce injury in a predictable pattern
6. Replicates the challenges in drug delivery encountered in adult human lungs

Replicating the effects of positive pressure ventilation used on ICU in human EVLP is straightforward, as similar ventilators are used in the model. The secretion of distinctly different phospholipid profiles into the circulation and the airways (section 4.3.3.3) would support the concept that the alveolar and vascular architecture are preserved in human EVLP; this would also have to be the case if human EVLP were to succeed in clinical lung transplantation. The diverse range of phospholipids measured in the human EVLP model might imply that transcellular biosynthesis of phospholipids is

preserved, but further work in defining the model is required to say that the model can produce the complete range of phospholipids seen in man. This thesis has only presented data on PC species, and further evaluation of the other major lipid classes should be undertaken. The ability to experimentally injure pulmonary phospholipid biology in the human ex vivo model remains to be proven, and this work will be required before undertaking therapeutic studies in human EVLP.

The interest in clinical EVLP in lung transplantation continues to grow[405], and as increasing numbers of organs are placed on EVLP a unique opportunity is presented to study human pulmonary phospholipid biology with unrivalled detail.

6.5 Conclusions

Lipidomic profiling with ESI-MS/MS is a viable technique that enables surfactant phenotyping with unprecedented molecular specificity in lung transplantation. Stable isotopic labelling of the Kennedy pathway provides data on lipid flux that is associated with graft function, and this link should be studied in further detail.

Ex vivo lung perfusion is a viable model of pulmonary phospholipid biology, and has already provided valuable insights into the role of the alveolar epithelium; specifically, its role in basolateral phospholipid secretion. The novel application of dynamic lipidomic profiling in both perioperative lung transplantation and EVLP has generated an interesting pilot dataset that would support further investigation in the field.

7 Appendices

7.1 Appendix: Publications and presentations

Results from chapter 3 were presented at the Annual Meeting for the International Society of Heart and Lung Transplantation in Washington DC, USA – April 2016[406].

Results from chapter 4 were presented at the American Thoracic Society Annual meeting in San Francisco 2016, and at the Annual meeting for the International Society of Heart Lung Transplantation in Washington DC 2016 & San Diego 2017[407,408].

Results from chapters 3 and 4 were presented at the Intensive Care Society Sate of The Art Meeting in Liverpool 2017, and shortlisted as a finalist for the Gold Medal Prize.

A retrospective review was performed on lung transplantation before starting the clinical study, and the data is presented in Appendix 7.1[56].

7.2 Appendix: Mechanical ventilation after lung transplantation

A preliminary retrospective review was performed before starting the clinical study, to generate background data on transplant activity, duration of ventilation, incidence of lung injury in the trust. This novel dataset was published as an original peer-reviewed manuscript, and it is attached in this appendix. Copyright for reproduction retained with author, and permission not required from publisher (Elsevier).

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Mechanical ventilation after lung transplantation☆,☆☆

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ABSTRACT

Introduction: To explore the hypothesis that early ventilation strategies influence clinical outcomes in lung transplantation, we have examined our routine ventilation practices in terms of tidal volumes (Vt) and inflation pressures.
Methods: A total of 124 bilateral lung transplants between 2010 and 2013 were retrospectively assigned to low (<6 mL/kg), medium (6-8 mL/kg), and high (>8 mL/kg) Vt groups based on ventilation characteristics during the first 6 hours after surgery. Those same 124 patients were also stratified to low-pressure (<25 cm H_2O) and high-pressure (≥25 cm H_2O) groups.
Results: Eighty percent of patients were ventilated using pressure control mode. Low, medium, and high Vt were applied to 10%, 43%, and 47% of patients, respectively. After correcting for patients requiring extracorporeal support, there was no difference in short-term to midterm outcomes among the different Vt groups. Low inflation pressures were applied to 61% of patients, who had a shorter length of intensive care unit stay (5 vs 12 days; $P = .012$), higher forced expiratory volume in 1 second at 3 months (77.8% vs 60.3%; $P < .001$), and increased 6-month survival rate (95% vs 77%; $P = .008$).
Conclusion: Low Vt ventilation has not been fully adopted in our practice. Ventilation with higher inflation pressures, but not Vt, was significantly associated with poorer outcomes after lung transplantation.
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1. Introduction

Lung transplantation is associated with a spectrum of lung injury, resulting from multiple insults sustained in the donor, in transit, and in the recipient. Contributing factors may include donor brain injury [1,2], the injurious effects of mechanical ventilation in the donor, superimposed with the subsequent effects of ischemia and reperfusion injury [3,4]. There are multiple perioperative factors within the recipient that could also contribute to the risk of lung injury, which may include fluid overload [5,6], right ventricular dysfunction, cardiopulmonary bypass [7], one-lung ventilation [8], volutrauma, and biotrauma [9]. Innovations in surgical and perioperative care have allowed continual advances in outcomes after lung transplantation. However, primary graft dysfunction (PGD), infection, and rejection remain as important contributors to mortality after lung transplantation. Primary graft dysfunction is the most common postoperative complication, occurring in 25% of recipients [10,11]. The treatment of PGD varies with the severity of lung injury, with general supportive care in low-grade forms. Grade 3 (severe) PGD often necessitates perioperative extracorporeal support to assist gas exchange, allow the reparative processes to occur, and facilitate recovery. Given the high risk of PGD after lung transplantation, it may be beneficial to use protective ventilation strategies perioperatively to avoid volutrauma/barotrauma.

Despite the strong body of evidence from clinical studies on ventilation in the general intensive care unit (ICU) population, limited information is available regarding the influence of these trials on the usual clinical practice of mechanical ventilation after lung transplantation or the potential impact on PGD. In addition, there are no international practice guidelines or consensus statements for the perioperative management of lung transplantation, and anesthetic management is not currently standardized [12].

Lung protective ventilation strategies using lower tidal volumes (Vt) have been beneficial in various clinical settings outside of transplantation. Low Vt ventilation reduces mortality in patients with acute respiratory distress syndrome (ARDS) [13] and may reduce the risk of developing lung injury in patients with healthy and uninjured lungs

☆ Previous presentations: Part of this work has been presented as a poster at the European Respiratory Society's annual congress in Barcelona, Spain, on September 7 to 11, 2013, and the European Respiratory Society congress in Munich, Germany, on September 6 to 10, 2014.

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[14]. Higher positive end-expiratory pressure (PEEP) levels also appear to be beneficial in ARDS [15], but optimum PEEP levels have not been defined in lung transplant recipients.

Before organ retrieval for lung transplantation, ventilation with higher Vt is an independent risk factor for the development of lung injury in organ donors with brain injuries. Furthermore, protective ventilation strategies in donors (low Vt, appropriate PEEP, and alveolar recruitment maneuvers) may increase the number of transplantable organs [2,16,17].

There is strong experimental evidence showing that ventilation strategies influence the development of lung injury after ischemia and reperfusion. Small animal models of ischemia and reperfusion injury after lung transplantation found that only 3 hours of injurious mechanical ventilation (higher Vt with low PEEP) resulted in lower oxygenation indices and greater measures of elastance, cytokines, and morphological injury when compared to protective ventilation (lower Vt with appropriately titrated PEEP) [18]. These experiments clearly suggest that lung protective strategies immediately after implantation of the lung and in the early postoperative period are of major importance. Yet, despite these experimental results, minimal clinical information is available on mechanical ventilation in lung transplant recipients. In particular, there are no studies directly observing perioperative ventilation techniques in human lung transplantation, and the influence of different ventilation strategies on clinical outcomes after human lung transplantation currently remains unknown. Inflation pressures could also present additional risks to the transplant recipient, with specific postoperative concerns such as pneumothoraces and anastomotic complications, but these risks have not been quantified in clinical studies and there is no consensus on target inflation pressures. It remains unclear whether low Vt or plateau/peak pressures are the key factors or if both should be limited in an "ultra-protective" strategy in lung transplant patients [19,20].

Despite the results from the ARDSNet trial and other studies, protective ventilation has not been universally translated into clinical practice [21]. We hypothesized that lung protective ventilation strategies are not routinely used in clinical lung transplantation and that ventilation of transplant recipients with traditionally higher Vt or pressures in the early reperfusion period will negatively influence lung function and the patient journey in the ICU. To test these hypotheses, we have analyzed the experience of a large-volume UK lung transplant unit at Harefield Hospital.

2. Methods

The current study was approved by the Research Office within the Royal Brompton and Harefield Foundation NHS Trust. We conducted a single-center retrospective review of all patients who underwent bilateral sequential single-lung transplantation (BSSLTx) at Harefield Hospital between June 2010 and April 2013, based on all available data from our electronic notes system.

Inclusion criteria

- Bilateral sequential single-lung transplant recipients

Exclusion criteria

- Single-lung transplant recipients
- Patients extubated in theaters

Clinical data were recorded for all patients, with a focus on ventilation mode, tidal volume, airway pressures, pulmonary compliance, and blood gas analyses. Ventilation data were recorded hourly for the first 6 postoperative hours after surgery. Based on these early ventilation parameters, patients were stratified to low (<6 mL/kg), medium (6-8 mL/kg), and high (>8 mL/kg) tidal volume groups. The same patients were also assigned to low (<25 cm H_2O) and high (≥25 cm H_2O) inflation pressure groups. To test that these early ventilation strategies influenced subsequent clinical progress, the following outcome data were documented: development of PGD, inflammatory markers, requirement for vasoactive and renal support, the duration of mechanical ventilation, ICU length of stay (LOS), lung function (forced expiratory volume in 1 second [FEV_1], expressed as a percentage of predicted values after 3 months), and the 6-month survival rate. Grade 3 PGD was diagnosed in accordance with internationally established criteria and was defined by the presence of diffuse interstitial infiltrates on the chest radiograph combined with a ratio of arterial oxygen partial pressure to fractional inspired oxygen (P/F) ratio less than 200 m mHg within the first 72 hours after transplantation [22]. Chest radiographs were considered compatible with PGD when agreed upon by 2 chest physicians.

2.1. Statistics

All clinical information was anonymized before statistical analyses were performed with SigmaPlot 12 (Systat Software Inc. Chicago, Il. USA). Variables were assessed for normality with the Shapiro-Wilk test. All normally distributed data have been highlighted and reported as mean values with the SE (±SEM); group comparisons were performed with either 1-way analysis of variance (ANOVA) or *t* tests. All other nonparametric data are expressed as median values with the 25% to 75% interquartile range (IQR); group comparisons were performed with either ANOVA on ranks or Mann-Whitney *U* tests. Correlations between variables were assessed with Spearman rank correlation, and outcomes from contingency tables were assessed with χ^2 test. Two-tailed *P* values are reported and considered significant when less than .05.

3. Results

3.1. Patient characteristics and perioperative details

Table 1 summarizes patient characteristics stratified by their ventilation parameters. We have focused on both the Vt and inflation pressures used within the first 6 hours after surgery.

Basic patient demographics, operative techniques, and underlying diseases between the various groups were mostly well matched, but there were several clinically important and statistically significant differences between them. The "<6 mL/kg" group had significant differences in the indication for transplant (fewer patients with chronic obstructive pulmonary disease [COPD] and a greater proportion with pulmonary vascular disease) and exhibited lower pulmonary compliance, and more than half of this group received extracorporeal life support (ECLS) within the first 6 hours. While examining the patients by their inflation pressures, there were significant differences between the groups with regard to blood transfusion requirements, the need for early ECLS, the average pulmonary compliance, and the lowest P/F ratio within the first 6 hours.

In our hospital, 2 main surgical techniques are used for BSSLTx: the clamshell incision and the sternum-sparring minimally invasive approach using anterolateral thoracotomies. There was no significant difference in the surgical techniques used between the various ventilation groups. In addition, neither surgical approach was associated with a need for greater inflation pressures within the first 6 hours (minimally invasive 23 cm H_2O vs clamshell 24 cm H_2O; $P = .165$) or greater Vt (minimally invasive 8.0 mL/kg vs clamshell 7.9 mL/kg; $P = .875$).

3.2. Ventilation strategies

3.2.1. Mode of ventilation

A total of 124 cases of BSSLTx were reviewed. Fig. 1 demonstrates that the preferred ventilation strategy immediately after BSSLTx was pressure-controlled ventilation (PCV), and a smaller proportion of patients were ventilated with synchronized intermittent mandatory ventilation + pressure support. Most patients were extubated after 2 days, but a small proportion of patients required noninvasive ventilation after extubation. Eight patients (6%) received "early"

Table 1
Patient demographics

Recipient details	Tidal volume							Inflation pressure					All patients	
	Low Vt		Medium Vt		High Vt			Low pressure		High pressure				
	<6 mL/kg		6-8 mL/kg		>8 mL/kg		P	<25 cm H_2O		≥25 cm H_2O		P		
No. of patients	13		53		58			76		48			124	
Age (y)	29	26-48	49	32-55	45	29-53	.075	47	27-55	45	29-54	.646	46	
Sex							.053					.995		
Male	5	38%	31	58%	21	36%		33	43%	20	42%		52	42%
Female	8	62%	22	42%	37	64%		43	57%	28	58%		72	58%
Primary indication for transplantation							<.001*					.075		
COPD/emphysema	1	8%	27	51%	27	47%		40	53%	15	31%		55	44%
CF/bronchiectasis	6	46%	19	36%	27	47%		30	39%	22	46%		52	42%
Diffuse interstitial lung disease	2	15%	6	11%	2	3%		3	4%	7	15%		10	8%
Pulmonary vascular disease	3	23%	0	0%	2	3%		2	3%	3	6%		5	4%
Bronchiolitis obliterans	1	8%	1	2%	0	0%		1	1%	1	2%		2	2%
Surgical approach							.728					.860		
Clamshell	8	62%	38	72%	42	72%		54	71%	34	71%		88	71%
Minimally invasive	5	38%	15	28%	16	28%		22	29%	14	29%		36	29%
Intraoperative cardiopulmonary bypass							.745					.187		
On-pump	11	85%	40	75%	46	79%		56	74%	41	85%		97	78%
Off-pump	2	15%	13	25%	12	21%		20	26%	7	15%		27	22%
Intraoperative blood products														
Blood (U)	10	1-15	3	1-5	3	1-4	.138	3	1-4	4	2-8	.011*	3	
Fresh frozen plasma (U)	4	2-4	2	0-4	2	0-3	.072	2	0-2	2	0-4	.064	2	
Platelets (U)	1	0-3	1	0-1	1	0-1	.491	1	0-1	1	0-2	.054	1	
Pulmonary physiology (over the first 6 h)														
Pulmonary compliance (mL/cm H_2O)	17	11-25	29	24-35	31	26-35	<.001*	34	28-39	24	20-27	<.001*	29	
Lowest P/F ratio (mm Hg)	228	127-407	242	155-308	264	191-320	.609	263	200-329	216	146-307	.031*	247	
$Paco_2$ (mm Hg)	41	34-47	47	43-52	43	38-49	.003*	44	41-49	46	38-52	.583	44	
pH	7.35	7.26-7.40	7.32	7.29-7.38	7.37	7.31-7.40	.078	7.36	7.30-7.39	7.32	7.28-7.40	.214	7.35	
Systolic PA pressure (mm Hg)	24	23-37	36	30.3-40	33	28-38.3	.090	34	28-38	35	28-41	.199	34	
Early extracorporeal support														
Within first 6 h	7	54%	1	2%	1	2%	<.001*	2	3%	7	15%	.032*	9	7%

The cohort was grouped by the Vt and the inflation pressures used within the first 6 hours after surgery. For inflation pressures, the peak airway pressure was used for patients on PCV, but the plateau pressure was used for those on a volume-controlled mode of ventilation. Normality was assessed with the Shapiro-Wilk test, and all nonparametric data are reported as median values with the 25% to 75% IQR. Statistical significance was tested with ANOVA on ranks for tidal volume groups and Mann-Whitney *U* test for inflation pressure groups. Outcomes from contingency tables were assessed with χ^2 test.

* *P* values less than the 5% significance level.

extracorporeal membrane oxygenation, and 1 patient received early extracorporeal CO_2 removal within the first 6 hours; all 9 received concurrent ventilation with PCV.

3.2.2. Tidal volumes

Fig. 2 describes the spectrum of Vt used during the first 6 hours of ICU stay, with the corresponding pressures used in those patients to achieve those volumes. Considering all lung transplant recipients, patients typically received a Vt that was greater than the recommended 6 mL/kg of the recipients' ideal body weight (RIBW) with a median Vt of 7.91 mL/kg. Only 13 patients (10%) received a Vt that was less than 6 mL/kg, 53 patients (43%) received a Vt of 6 to 8 mL/kg, and the remaining 58 patients (47%) received a Vt of greater than 8 mL/kg. More than half (54%) of the patients who received Vt less than 6 mL/kg ideal

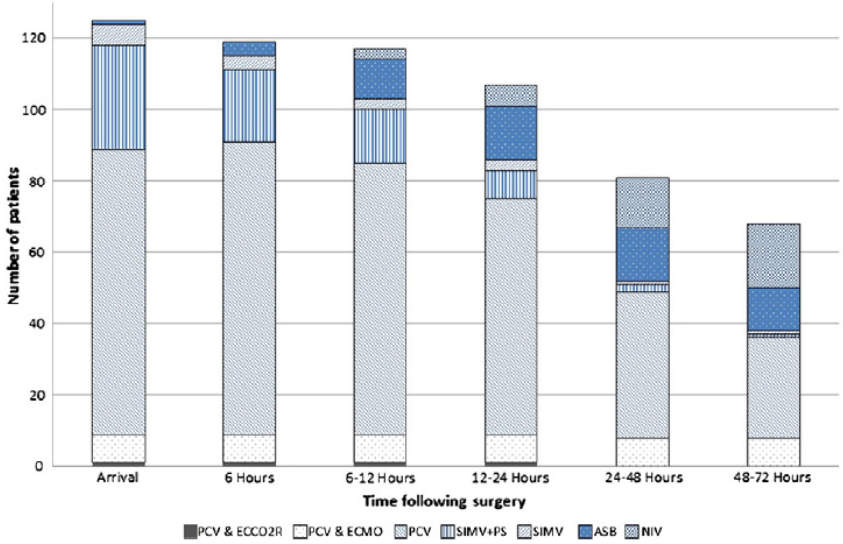


Fig. 1. Ventilation modes used after lung transplantation. $ECCO_2R$ indicates extracorporeal carbon dioxide removal; ECMO, extracorporeal membrane oxygenation; PS, pressure support; SIMV, synchronized intermittent mandatory ventilation; ASB, assisted spontaneous breathing; NIV, noninvasive ventilation.

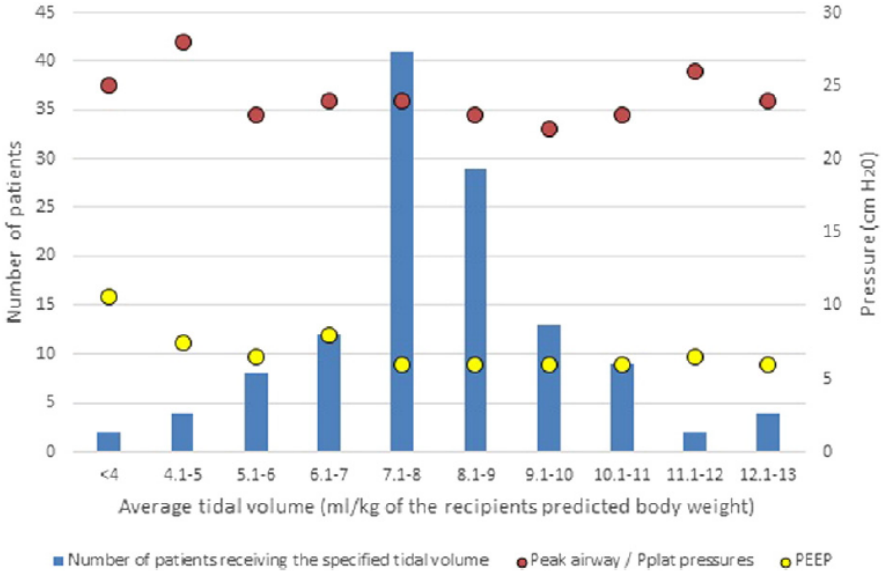


Fig. 2. Tidal volumes used after transplantation. The distribution of Vt used in the first 6 hours after lung transplantation and the median pressures used to achieve those Vt. Tidal volumes are expressed as milliliters per kilogram of the RIBW. The peak airway pressure was used for patients on PCV, but the plateau pressure was used for those on a volume-controlled mode of ventilation.

body weight (IBW) did so with the assistance of extracorporeal respiratory support.

Taking body weights into consideration, the donors' IBW was 1.81 kg greater (IQR, −1.81 kg to +6.34 kg) than the RIBW. Expressing postoperative Vt in terms of the donors' IBW (as opposed to the RIBW) only resulted in an average difference of 0.2 mL/kg. However, the difference was as much as 2.8 mL/kg in some transplant recipients.

3.2.3. Positive end-expiratory pressure and inflation pressure

The range of airway pressures used in transplant recipients over the first 6 hours is described in Fig. 3; the median PEEP was 6 cm H_2O, the median inflation pressure was 24 cm H_2O, and the median driving pressure was 17 cm H_2O.

3.3. Association between early ventilation strategies and outcomes

For each group described in Table 1, we collected data on the patients' organ support requirements, pulmonary physiology, ICU LOS, mortality, and postoperative lung function. Table 2 reports selected clinical outcome measures that characterize patient recovery for each group.

3.3.1. Tidal volumes

Tidal volumes had no association with inflammatory markers or vasoactive support requirements (in terms of noradrenaline administration). Other vasoactive agents occasionally included adrenaline, milrinone, dobutamine, and vasopressin, but their use did not differ significantly between the tidal volume groups (data not shown). The duration of mechanical ventilation, ICU LOS, survival, and postoperative lung function were also similar among low, medium, and high tidal volume groups. Grade 3 PGD and renal failure requiring hemofiltration occurred with greater frequency in the low tidal volume group. The 6-month survival tended to be lower in the low tidal volume group, but this tendency was also lost when patients on early ECLS were excluded (Fig. 4A). However, the low Vt group had a disproportionate number of patients receiving ECLS, and when these patients were excluded from the analysis, there was no statistically significant difference in hemofiltration requirements between the Vt groups (Table 2).

As an alternative statistical analysis, we have stratified our patients into 2 tidal volume groups by dividing the study cohort across the median tidal volume, giving us a "high Vt group" (>8 mL/kg) and a "low Vt group" (≤8 mL/kg). This analysis also showed no statistically significant differences in the duration of ventilation ($P = .531$), incidence of hemofiltration ($P = .881$), ICU LOS ($P = .46$), lung function at 3 months ($P = .331$), or midterm survival ($P = .506$).

3.3.2. Inflation pressures

When outcomes were stratified according to the pressure component of early mechanical ventilation, higher inflation pressures were associated with prolonged mechanical ventilation, impaired lung function, increased requirement for renal replacement therapy and increased ICU LOS, and increased mortality. These associations also remained statistically significant, after all patients receiving early ECLS were excluded from the analysis (Table 2, and survival in Fig. 4B). There was no significant difference in inflammatory markers and vasoactive support between the low and high pressure groups.

3.3.3. Accounting for the impact of patients on ECLS

A total of 13 patients (10%) required some form of ECLS at any time during their ICU recovery, of whom 7 patients (54%) survived to 6 months. In contrast, 102 survived to 6 months out of the 111 patients (92%) who did not require ECLS ($P < .001$).

3.3.4. Correlation between ventilation and outcomes

Correlation analyses (Spearman rank correlation) were performed to further explore the relationship between specific ventilation parameters and clinical outcomes (after excluding any confounding effects from patients on ECLS). We found no association between early Vt and the subsequent development of lung injury within the first 72 hours (as reflected by the P/F ratio; $R = 0.0081$; $P = .932$), the duration of mechanical ventilation ($R = 0.0837$; $P = .375$), the ICU LOS ($R = 0.0851$; $P = .368$), or postoperative lung function ($R = -0.0661$; $P = .505$; Fig. 5B). However, early inflation pressures (within the first 6 hours of surgery) were associated with lung injury within the first 72 hours ($R = -0.354$; $P = .0001$), the duration of mechanical ventilation ($R = 0.337$; $P = .0002$), and the ICU LOS ($R = 0.295$; $P = .0012$). Early inflation pressures also had a strong and highly significant association with lung function at 3 months ($R = -0.415$; $P = .00001$; Fig. 5A), and so, the relationship between ventilation and postoperative lung function was explored in greater detail. Higher inflation pressures were used in less compliant lungs ($R = -0.689$; $P = .0000002$) with lower P/F ratios ($R = -0.360$; $P = .0009$) (Fig. 5C and D), so we also

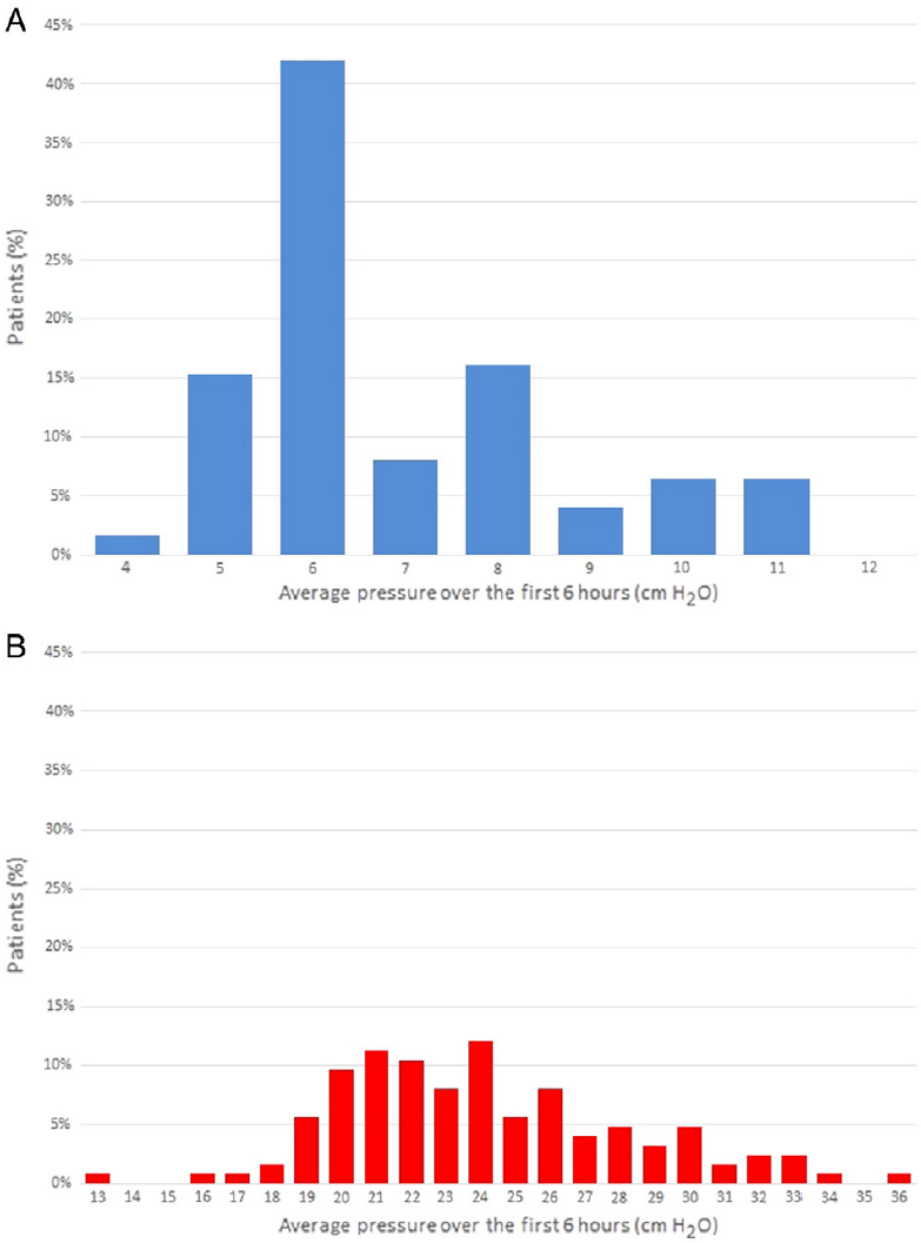


Fig. 3. The range of ventilation pressures used after lung transplantation. A, The median PEEP used over the first 6 hours after transplantation. B, The median inflation pressure used over the first 6 hours after transplantation. The peak airway pressure was used for patients on PCV, but the plateau pressure was used for those on a volume-controlled mode of ventilation.

evaluated if the effect could be explained by relationships between early compliance characteristics or by early defects in gas exchange.

The P/F ratio was significantly associated with the FEV_1 at 3 months, but the strength of the association was weak with an *R* value of only 0.196. The average pulmonary compliance over the first 6 hours held a stronger association with lung postoperative FEV_1 with an *R* value of 0.361 (Fig. 5E and F).

4. Discussion

This report provides new insights into current ventilation practices in lung transplantation and indicates that the ARDSNet recommendations on low tidal volume ventilation have not been fully adopted in our daily care. Our retrospective analysis suggests that initial ventilation with low, medium, or high tidal volume ventilation had no association with patient outcomes. However, high-pressure ventilation was associated with poorer physiological and clinical outcomes and reduced lung function.

4.1. Ventilation practices in lung transplantation

It is accepted that protective Vt of 6 mL/kg IBW can reduce mortality from ARDS [13] and may improve clinical outcomes in the general ICU population without preexisting lung injury [14]. However, despite considerable research into ventilator-induced lung injury within the general ICU population, the optimal strategy for ventilating transplanted lungs has not been defined.

Our review of mechanical ventilation at Harefield Hospital suggests that most lung transplant patients are ventilated with larger Vt than recommended by the ARDSNet trial. Indeed, only 10% of our patients received the recommended 6 mL/kg tidal volume, but most of these patients also received postoperative ECLS. It has been suggested that ECLS could be used to provide "enhanced" protective ventilation in milder forms of lung injury [23]; however, at our center, ECLS is primarily used as a recue therapy in very severe cases of PGD after lung transplantation. Most of the patients who received early postoperative ECLS in this study cohort had

Table 2
Association between ventilation strategy and clinical outcomes

Recipient outcomes	Tidal volume							Inflation pressure					All patients	
	Low Vt		Medium Vt		High Vt			Low pressure		High pressure				
	<6 mL/kg		6-8 mL/kg		>8 mL/kg		P	<25 cm H_2O		≥25 cm H_2O		P		
No. of patients (all recipients)	13		53		58			76		48			124	
Postoperative respiratory support														
Hours on ventilator during ICU stay	193	14-858	42	22-235	51	19-360	.599	32	16-230	147	24-624	.003*	45	
Late extracorporeal support (>6 h)	7	54%	3	6%	1	2%	<.001*	2	3%	9	19%	.006*		
Grade 3 PGD within the first 72 h	8	62%	20	38%	16	28%	.062	21	28%	23	48%	.035*	43	35%
Postoperative inflammatory markers (first 72 h)														
Peak C-reactive protein (mg/L)	157.5	95-195	189	135-225	184	137-233	.229	180	137-224	187	119-231	.971	182	
Peak white cell count ($\times 10^9$ cells/L)	17.4	12.0-29.9	22.7	18.0-28.4	24.6	19.1-33.0	.369	21.5	18.5-26.9	26.7	17.3-34.2	.083	23.6	
Postoperative vasopressor support (first 72 h)														
Total noradrenaline dose (μg/kg/72 h)	292.5	29.6-587	175.9	76.1-372.2	214.6	89.7-440.0	.630	187.6	91.3-3294	225.6	47.4-523.7	.431	203.17	
Postoperative renal support														
Required hemofiltration during ICU stay	8	62%	15	28%	16	28%	.047*	16	21%	23	48%	.003*	39	31%
LOS														
ICU LOS (d)	18	4-38	7	4-19	8	4-24	.420	5	4-19	12	4-36	.012*	7	
Midterm clinical outcomes														
FEV_1 at 3 mo (% of predicted)[a]	65.5%	±6.4%	74.8%	±2.9%	70.3%	±3.1%	.395	77.8%	±2.4	60.3%	±3.1	<.0001*	68.9%	
Alive at 6 mo (%)	10	77%	48	91%	51	88%	.401	72	95%	37	77%	.008*	109	88%
No. of patients (excluding early ECLS)	6		52		57			74		41			115	
Postoperative respiratory support														
Hours on ventilator during ICU stay	19	5-1187	38	22-226	33	18-360	.528	32	16-223	70	24-480	.017*	34	
Postoperative renal support														
Required hemofiltration during ICU stay	2	33%	14	27%	16	28%	.945	15	20%	17	41%	.027*	32	28%
LOS														
ICU LOS (d)	7	3-53	7	4-19	7	4-23	.723	5	4-19	10	4-27	.047*	7	
Midterm clinical outcomes														
FEV_1 at 3 mo (% of predicted value)[a]	68.6%	±8.9%	75.0%	±3.0%	70.2%	±3.2%	.514	78.3%	±2.4%	59.9%	±3.3%	<.0001*	72.3%	
Alive at 6 mo	6	100%	48	92%	50	88%	.514	70	95%	33	80%	.04*	103	90%

Patients were divided into the same ventilation tidal volume/inflation pressure groups that were used in Table 1. For inflation pressures, the peak airway pressure was used for patients on PCV, but the plateau pressure was used for those on a volume-controlled mode of ventilation. Normality was assessed with the Shapiro-Wilk test. Parametric data have been highlighted and reported as the mean value with the SE (±SEM); nonparametric data are reported as median values with the 25% to 75% IQR. Statistical significance was tested with ANOVA on ranks for tidal volume groups and Mann-Whitney *U* test for inflation pressure groups. Outcomes from contingency tables were assessed with χ^2 test. Those same ventilation groups were then reanalyzed using the same methods after all patients receiving ECLS had been excluded.

[a] Parametric FEV_1 data were assessed with ANOVA or *t* tests accordingly.

* $P < .05$.

evidence of established lung injury and were given smaller Vt via PCV in allografts with very poor pulmonary compliance, as a part of our "lung-rest" strategy.

Many lung transplant recipients (43%) received "moderate" Vt of 6 to 8 mL/kg, and approximately 47% received Vt greater than 8 mL/kg. Although these Vt cannot be considered protective, they are comparable to those used in many European ICUs [21] and are substantially lower than the Vt of 12 mL/kg that were classed as "traditional ventilation" in the control arm of the ARDSNet trial [13]. It is also notable that these volumes were achieved without excessive inflation pressures

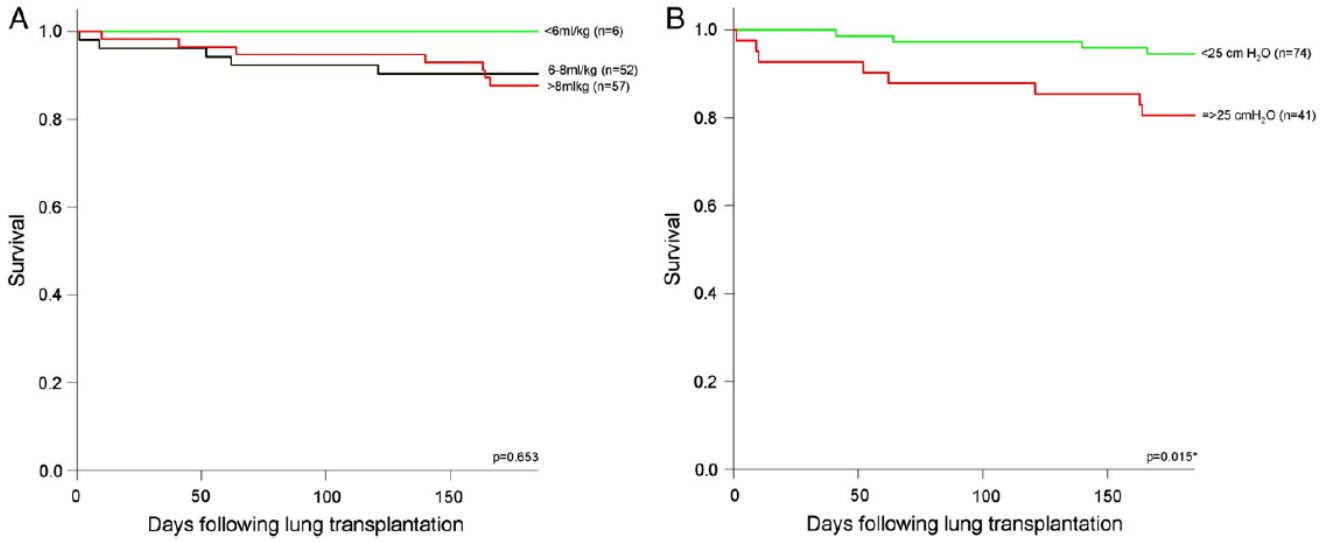


Fig. 4. Kaplan-Meier plot. A, Six-month survival in lung transplant recipients, stratified by the average Vt used in the first 6 hours after surgery. B, Six-month survival in lung transplant recipients, stratified by the average inflation pressures used in the first 6 hours after surgery.

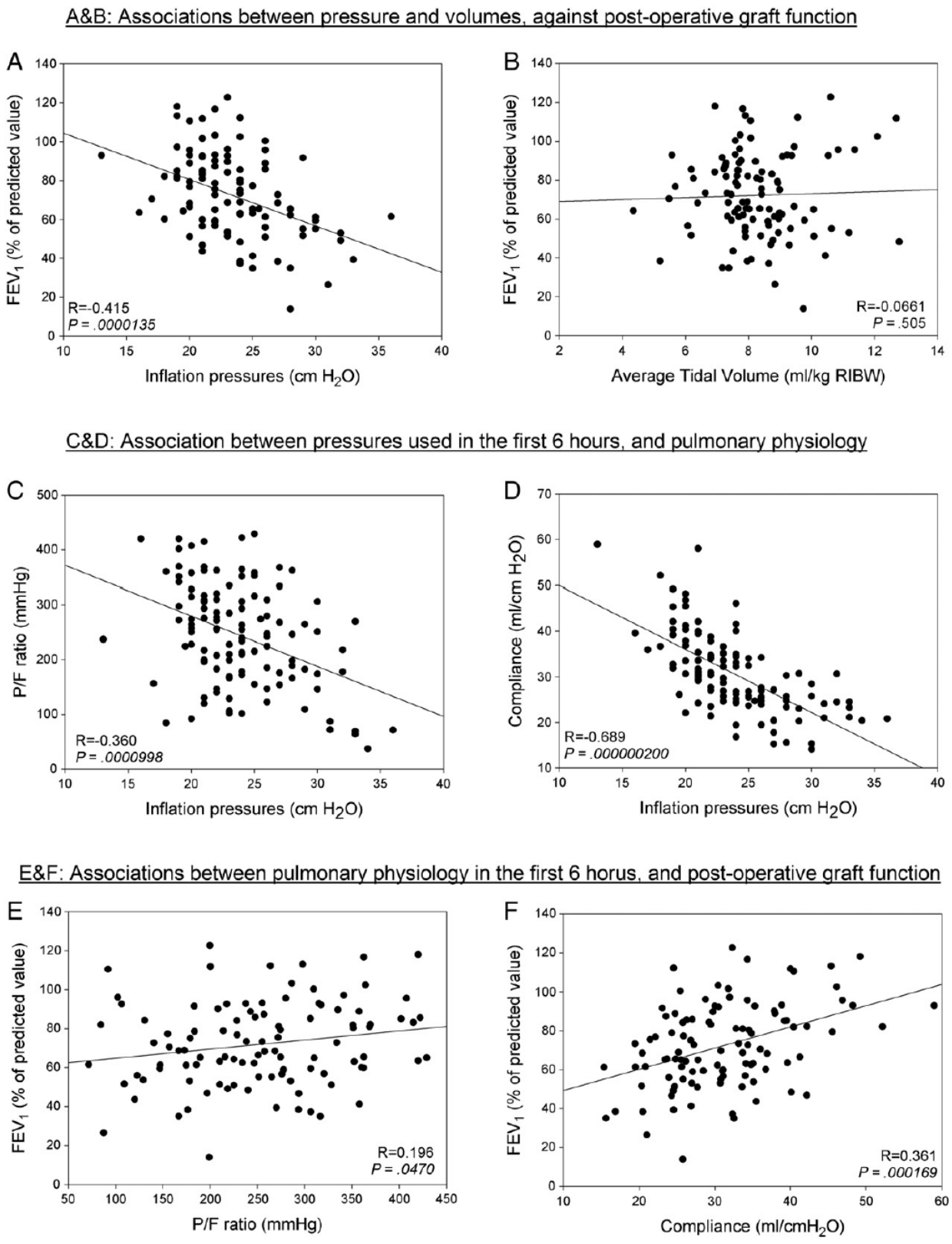


Fig. 5. Correlation analyses. A, The relationship between the median inflation pressures used in the first 6 hours after transplantation, and the FEV_1 at 3 months after surgery. B, The relationship between the median Vt used in the first 6 hours after transplantation and the FEV_1 at 3 months after surgery. C, The relationship between the inflation pressures used in the first 6 hours after transplantation and the corresponding P/F ratio for that same period. D, The relationship between the inflation pressures used in the first 6 hours after transplantation and pulmonary compliance within the first 6 hours. E, The relationship between the lowest recorded P/F ratio in the first 6 hours after transplantation and the subsequent FEV_1 at 3 months. F, The relationship between the median pulmonary compliance within the first 6 hours and the subsequent FEV_1 at 3 months. For the inflation pressures, the peak airway pressure was used for patients on PCV, but the plateau pressure was used for those on a volume-controlled mode of ventilation. All analyses have been performed with Spearman rank correlation.
*$P < .05$.

and in the presence of moderate levels of PEEP, which may have limited volutrauma, overstretch, and overall biotrauma. Indeed, this hypothesis would be supported by recently published work by Amato et al [24], which suggested that lower driving pressure may play an important role in limiting the development of lung injury within the general ICU population.

Uniquely in lung transplantation, Vt can be defined by either the recipient or the donor body weight. A recent survey of international practice demonstrated that most transplant centers do not consider the donor body weight while optimizing the ventilator settings, and this is mirrored in our own practices. Although the donor height and weight are available to the ICU team, the recipients' measurements are usually considered in our posttransplant care.

Surveys on postoperative ventilation strategies have reported preferential use of volume-controlled modes over PCV [25]. However, PCV has potential clinical benefits, and attitudes toward more widespread use are changing [26]. Compared to volume-controlled ventilation, PCV may result in lower peak airway pressures, more homogenous gas exchange, and reduced regional overdistension [27]. A recent international survey reported that PCV is the preferred mode of ventilation after lung transplantation, which is mirrored by our own practice [28].

The effects of PEEP after lung transplantation are unknown but may have additional significance in this patient cohort. The bronchial circulation is lost during BSSLTx, and so, the bronchial anastomoses become dependent on collateral retrograde blood flow from the pulmonary circulation [29]. Paradoxically, a canine model of lung transplantation suggested that PEEP actually resulted in increased bronchial blood flow [30]. The optimal levels of PEEP in clinical lung transplantation will require further investigation.

4.2. Association with outcomes and complications

To our knowledge, the only other clinical report that explored protective ventilation strategies in lung transplant recipients and their associated outcomes was by Currey et al [31]. This Australian group applied protective Vt (with other interventions) in 53 lung transplant recipients and compared outcomes to historical controls. Their protective strategy appeared to reduce the severity of PGD, but there was no impact on other clinical outcome measures. Beyond the recognition of our ventilation practices, this study has also explored the hypothesis that ventilation practices influence the patient journey of the transplant recipient on the ICU and beyond. In our transplant cohort, there was no association between initial Vt and subsequent clinical outcomes in terms of development of PGD, respiratory support, inflammatory response, midterm lung function, or survival. Unfortunately, we only had a small number of patients in the less than 6 mL/kg group, and the patients were not comparable to the other groups in terms of clinical severity due to the high frequency of extracorporeal membrane oxygenation. The medium and higher tidal volume groups were both more comparable and sizeable, but Vt greater than 8 mL/kg were not associated with adverse clinical outcomes when compared to the 6 to 8 mL/kg Vt group. In contrast, lower early inflation pressures were strongly associated with shorter ICU LOS, improved postoperative lung function, and improved 6-month survival rates. There are several potential mechanisms that could explain our observation that inflation pressures (but not Vt) are associated with outcomes after transplantation. Physicians may have used inappropriately high inflation pressures in PCV modes that might have inflicted lung injury with consequences that adversely affected clinical outcomes. However, correlation analyses between inflation pressures, gas exchange, compliance, and lung function favor the alternative explanation that poor compliance in early lung injury necessitated the use of higher inflation pressures, and this preexisting lung injury subsequently influenced clinical outcomes.

We realize that underlying diagnoses were statistically different in the stratified groups, with a higher representation of COPD patients in the low-pressure group. Therefore, we cannot rule out that patient outcomes were related to recipient diagnoses in this study [32]. The increased chest size in COPD (in contrast with the contracted chest of interstitial lung disease) can facilitate the use of "oversized" lungs, possibly allowing ventilation with larger Vt and lower peak/plateau pressures [33,34]. Therefore, recipient diagnoses could have affected both pulmonary mechanics and ventilation as well as subsequent clinical outcomes [32].

4.3. Limitations

We acknowledge the limitations of this study. First, it is a single-center retrospective study comprising small numbers of complex patients in heterogeneous groups. A larger study cohort could allow covariate analyses to address this heterogeneity, and hopefully, this could be addressed in future multicenter trials. Second, management was based on individual consultant preferences rather than a comprehensive institutionalized policy. Third, ICU stay may not reflect readiness to discharge, as ward occupancy may limit patient transfer. In addition, we relied on routine clinical measures of inflammation rather than molecular indices of innate immunity such as chemokines and cytokines.

4.4. Concluding remarks

To date, no study has established the influence of ventilation strategies on clinical outcomes in lung transplantation. Recommendations for low tidal volume ventilation in this cohort have been extrapolated from animal studies and results in clinical trials involving unrelated patients [28,35]. In this regard, our observational study fills an important gap, by describing contemporary critical care in a leading UK transplant center. It demonstrates that recommended Vt are only used in the minority of patients and that there is an increasing trend for the use of PCV.

The associations between ventilation strategy and subsequent clinical outcomes in our cohort would suggest that the inflation pressures are particularly important in lung transplant recipients during the early perioperative period. Adequately powered prospective studies involving multiple centers are required to confirm this observation.

Author contributions

Louit Thakuria	Designed study, collected data, data analyses, wrote manuscript.
Rosada Davey	Collected data, contributed to writing of manuscript
Rosalba Romano	Collected data, contributed to writing of manuscript
Martin R Carby	Contributed to writing of manuscript
Sundeep Kaul	Contributed to writing of manuscript
Mark J Griffiths	Contributed to writing of manuscript, student supervisor
André R Simon	Contributed to writing of manuscript
Anna K Reed	Collected data, contributed to writing of manuscript, student supervisor
Nandor Marczin	Designed study, data analyses, wrote manuscript, and senior supervising author

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7.3 Appendix: Research Ethics Committee Approval Letter – Clinical study

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16 August 2013

Dr Anna Reed
Consultant
Royal Brompton and Harefield NHS Trust
Transplant
Harefield Hospital. Hill end Road,
Harefield
UB9 6JH

Dear Dr Reed

Study title:	**A Perioperative study of lung transplantation and acute respiratory failure.**
REC reference:	**13/LO/1052**
IRAS project ID:	**125520**

The Research Ethics Committee reviewed the above application at the meeting held on 05 August 2013.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator on nrescommittee.london-riverside@nhs.net.

Ethical opinion

The Committee agreed that this study could be issued a favourable opinion (no conditions) and that there was no need to invite the researcher in for a discussion unless the researcher had questions. The Coordinator informed the researcher of the Committee's decision and stated that the Committee had no questions it needed to ask. The researcher was invited into the meeting but decided not to attend as he had no questions to ask.

Decision: Favourable Opinion (no conditions)

The members of the Committee present gave a favourable ethical opinion of the above research

on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter		19 June 2013
Investigator CV		
Letter of invitation to participant	1.0	17 June 2013
Other: CV: Louit Thakuria		
Other: CV: Dr Griffiths		
Other: CV: Dr Marczin		
Participant Consent Form	1.2	22 May 2013

Participant Information Sheet	1.0	22 May 2013
Protocol	1.8	13 May 2013
REC application		17 June 2013

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Dr Julian Collinson declared an interest in knowing the Dr Mark Griffiths, the Academic Supervisor for this study. The Committee agreed that Dr Collinson could remain in the room and take part in the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/LO/1052	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

Pp

Dr Sabita Uthaya
Chair

Email: nrescommittee.london-riverside@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Mr Patrik Pettersson, Royal Brompton and Harefield NHS Foundation Trust*

NRES Committee London - Riverside

Attendance at Committee meeting on 05 August 2013

Committee Members:

Name	*Profession*	*Present*	*Notes*	
Dr Mark Atkins	Microbiologist	No		
Ms Anna Bischler	Pharmacist	Yes		
Mr John Clifford	Lawyer	No		
Dr Julian Collinson	Consultant Cardiologist	Yes		
Ms Stephanie Ellis	Former Civil Servant	Yes		
Dr Matthew Hyde	Research Scientist	Yes		
Dr Margaret Jones	General Practitioner	Yes		
Ms Alexandra Mancini	Modern Matron	Yes		
Ms Fanny Mitchell	Retired	Yes		
Dr Karen Phekoo	Researcher	No		
Mr Kamen Shoylev	Lawyer	Yes		
Ms Fiona Slomovic	Advocacy and Mediation Consultant	No		
Mrs Dinah Smith	Head Teacher	Yes		
Dr Sabita Uthaya	Consultant Neonatologist	Yes		
Ms Julia Williams	Senior Producer	No		
Dr Daniel Wood	Clinical Psychologist	Yes		

Also in attendance:

Name	*Position (or reason for attending)*	
Miss Tina Cavaliere	Coordinator	

7.4 Appendix: NHS Trust Permission for research – Clinical study

Royal Brompton & Harefield NHS
NHS Foundation Trust

Royal Brompton & Harefield NHS Foundation Trust
Research Office
Chelsea Wing
Sydney Street
SW3 6NP
www.rbht.nhs.uk

Direct Line: 020 7 351 8121 ext. 2610
Email: a.cooper@rbht.nhs.uk

23 August 2013

Dr Anna Reed
Consultant Transplant Medicine
Harefield Hospital
Middlesex
Uxbridge
UB9 6JH

Dear Dr Reed,

Project Title: A Perioperative study of lung transplantation and acute respiratory failure.
R&D Ref: 2013LS001H
REC Ref: 13/LO/0152
CSP Ref: n/a
Study Sponsor: Royal Brompton & Harefield NHS Foundation Trust

Notification of RB&HFT NHS Management Permission for Research

Thank you for registering the above study with the Research Office. I am pleased to inform you that your study now has NHS Management Permission (previously know as R&D approval) and can commence at Royal Brompton & Harefield NHS Foundation Trust (RB&HFT).

NHS management permission for the above research study is granted on the basis that the study will be conducted as described in the protocol and in accordance with the supporting documentation submitted (listed below), and on the understanding that the study is conducted in accordance with the principles set out in the Research Governance Framework for Health and Social Care (April 2005, 2nd Edition, Department of Health (DoH)) and RB&HFT Policies and procedures.

Documents Reviewed	Version number	Date
Protocol	1.8	13May2013
Patient Information Sheet (PIS)	1.0	22May2013
Informed Consent Form (ICF)	1.2	22May2013

Study Amendments

It is the responsibility of the Principal Investigator (PI) to notify the Research Office of **all** study amendments or changes to the status of the projects including study suspension or premature termination.

Royal Brompton & Harefield NHS
NHS Foundation Trust

Safety Reporting

The research Sponsor, or the Chief Investigator (CI) or the local Principal Investigator (PI) at a research site, may take appropriate Urgent Safety Measures in order to protect research participants against any immediate hazard to their health or safety. The Research Office should be notified of such measures within the same time frame of notifying the REC. The notification should include reasons why the measures were taken and the plan for further action.

All **patient related incidents**, including study-related Adverse Events/Reactions (AE/Rs), must be reported internally by the study team in line with the Trust's Adverse Incident Management and Reporting Policy *via* the Quality and Safety Department database **Datix** and marked "**research-related**".

In addition, **all** SAE/Rs must be reported to the study Sponsor and the main REC in line with the approved research protocol.

Audit

Please note the Trust is required to monitor research to ensure compliance with the Research Governance Framework and other legal and regulatory requirements. This responsibility is delegated to the Research Office and will be achieved by random audit of research projects ongoing in the Trust in accordance with RB&HFT Audit SOP.

Yours sincerely

Dr Angela Cooper
Associate Director of Research

7.5 Appendix: Patient invitation letter

Royal Brompton & Harefield NHS
NHS Foundation Trust

Transplant Unit

Harefield Hospital

Hill End Road

Harefield

Middlesex

UB9 6JH

Tel: 01895 823737 ext: 5070

Dear ……..,

Name numbers

Address

RE: A perioperative study of lung transplantation and acute respiratory failure

We would like to inform you about a research study at Harefield Hospital. We are looking for patients on the lung transplant waiting list to volunteer for our study, and we would be most grateful if you will consider taking part. We plan to explore how patients recover from lung transplantation, in the hope to improve the care for other patients in the future. If you are interested, we have enclosed a patient information sheet that will explain the study in greater detail.

If you would like to participate in our study, you will have the opportunity to discuss the study in person with a member of the study team. When you come into hospital for surgery, a member of the study team will go through the patient information sheet with you and ask you to sign a consent form before you go to theatre. If you are interested in this research study, you do not need to do anything else at this time.

The research team would be delighted to answer any questions about this study. If you would like to discuss the study in greater detail before you come to hospital, a member of the team can call you at home.

Participation in medical research is entirely voluntary, and there will be no impact in your care if you decide not to participate. Thank you for taking the time to read about our ongoing research.

Yours sincerely



Dr Anna Reed

Primary Investigator

Consultant Respiratory & Transplant Physician

7.6 Appendix: Expression of interest form

Royal Brompton & Harefield NHS
NHS Foundation Trust

Transplant Unit
Harefield Hospital
Hill End Road
Harefield
Middlesex
UB9 6JH

Tel: 01895 823737 ext: 5070
Fax: 01895 828975

Dear Lung Transplant Research Team,

RE: A perioperative study of lung transplantation and acute respiratory failure

☐ I have read the patient information sheet. I have no further questions at this time.

……or……

☐ I have read the patient information sheet, but I would like more information on the study before I come into hospital.
Please call me on this telephone number:……..

The most convenient time to call me is

Monday AM ☐	Tuesday AM ☐	Wednesday AM ☐	Thursday AM ☐	Friday AM ☐
Monday PM ☐	Tuesday PM ☐	Wednesday PM ☐	Thursday PM ☐	Friday PM ☐

Signed:……

Date:……

Patient Name:……..

7.7 Appendix: Patient information sheet

Royal Brompton & Harefield NHS
NHS Foundation Trust
Harefield Hospital
Hill End Road
Harefield
Middlesex
UB9 6JH
01895 823737

PATIENT INFORMATION SHEET

A perioperative study of lung transplantation and acute respiratory failure

Principal Investigator
Dr Anna Reed
Consultant in Transplant Medicine

Study Coordinator
Dr Louit Thakuria
Clinical Research Fellow

Invitation

We would like to invite you to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. We thank you for your interest and taking the time to read this.

What is the purpose of the study?

Lung transplantation is a complex procedure used to treat advanced respiratory disease. It can take a while after the transplantation procedure for the inflammation in your new lungs to settle down and for the organs to start working properly. This period of recovery usually takes place on the Intensive Care Unit where your breathing can be supported with a ventilator while you are asleep. The purpose of this study is to examine why some transplanted lungs take a while to start working properly, so we can develop new ways to monitor patients' recovery. The information that we will gain from this study might also lead to new ways to treat lungs that fail to work properly after transplantation.
This critical period of recovery after surgery can be a difficult time for some patients and we would like to learn more about this process so that we can give future patients the best possible outcome following lung transplantation.

Why have I been invited?

You are being asked to participate in this research study because you are on the active waiting list for lung transplantation and you have been identified as a potential candidate. This study will involve a total of 40 participants.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form to show you have agreed to take part. You may choose not to participate in this study or you may leave the study at any time without giving a reason. This would not affect the standard of care you receive.

What will happen to me if I take part?
The study will be conducted at Harefield Hospital. A member of the team will go through this information sheet with you when you are called in for lung transplantation. If you have not done so already, you will be asked to sign a form that confirms your consent to participate in the study. From this point on, there will be minimal changes to the remainder of your care, and lung transplantation will proceed as agreed with the clinicians. We will start collecting samples after the anaesthetist has put you to sleep for your operation. After your surgery has been completed, we will continue to perform tests while you are sleeping to monitor the recovery of your new lungs. Once you wake up, the remainder care will continue as normal.

The first 10 patients enrolled into this study will be entered into group 1, the subsequent 30 patients will be entered into group 2:

Group 1
We will simply collect some samples while you sleep so that we can learn more about transplantation. The details for sample collection is listed below (see page 3). There will be no other changes to your care.

Group 2
After you have received you lung transplant, you will receive an infusion of choline (see below) over 3 hours while you are sleeping on the ICU. We will then continue to collect samples as you sleep. There will be no other changes to your care.

What is a choline infusion?
Choline is an essential nutrient that is required in your daily dietary intake for you to remain healthy. Amongst other functions, it is used by the lungs to create a protective fluid called surfactant. The choline infusion will help act as a marker of lung metabolism, and tell us when your new lungs restart normal surfactant production after transplantation.
We will measure the recovery of surfactant production by measuring the amount of choline in the samples collected from your new lungs.

The infusion contains a completely safe, non-toxic, non-radioactive version of this essential nutrient that will have no other effects on your body. It is so safe, that the same choline infusion regimen is currently given to critically-ill babies in a similar study on the paediatric ICU.

If you are in group 2, you will receive a one-off choline infusion after surgery. It will not interact with your other medicines and you should not experience any side effects from the infusion. You will not need any extra venflons, needles, lines, or cannulae to receive this infusion, as we will utilize the existing lines inserted during your operation.

How and when will samples be collected?
Sample collection will begin after the anaesthetist has put you to sleep for the operation. It is likely that once the lung transplant surgery has been completed that

you will return to the Intensive Care Unit (ICU). Patients are initially kept asleep while they are recovering from lung transplantation, and all patients will routinely have many tests to monitor their recovery. Most of the tests performed in this study are simply a part of everyday clinical care for patients on the ICU, and it is likely that you will have little or no awareness of sample collection. We will collect the following samples from you in theatres and the ICU:

- Blood
  - It is routine practice for all patients to have blood tests taken every day on the ICU. Blood is usually taken from indwelling cannulae called central lines that are inserted while you are sleeping. Central lines allow us to collect blood samples without repeatedly piercing the skin with needles. Therefore it is likely that you will not even notice when blood samples are taken. In addition to the routine blood samples taken by the clinical team, we will take an additional 10mls of blood every day (which is about one tablespoon of blood). The amount of additional blood that is taken for research will have no clinical effects on your health.
    - Urine
  - It is routine practice for a catheter to be inserted into your bladder while you are in theatre. The catheter will drain urine from your bladder while you are sleeping, and it is usually removed once you are able to get out of bed. Urine samples are taken from a bag connected to the catheter, and it is likely that you will not notice when they are collected.
- Exhaled breath
  - Samples of your exhaled breath will be taken while you are breathing with the ventilator. When you are breathing into a ventilator, it is routine practice for the machines to analyse your exhaled breath to ensure that you are breathing properly. We will collect additional samples using equipment connected to the ventilator tubing. It is a totally non-invasive test and it is likely that you will not notice sample collection.
    - Endotracheal and endobronchial aspirates
  - Nursing staff will regularly clear out secretions from your lungs by suctioning the excess sputum from within your breathing tube. The procedure is usually done many times every day to prevent the development of chest infections. Sometimes, 10-20mls (1-2 tablespoons) of sterile salty water is flushed through the tubing to help loosen the secretions from your chest. These secretions are usually discarded once they are cleared from your chest, but we will save the fluid for analysis in the laboratory.
    - Bronchoaveolar lavage fluid
  - It is routine practice for the doctors to look inside your lungs with a camera after your transplant surgery. Patients are usually asleep during this test and it is often done to wash out your lungs from the inside (this is called a Bronchoalveolar lavage). Once your doctors have completed their tests, we will collect any extra fluid for our study.

    - Biopsy material
  - During the operation, the Transplant surgeon may decide to take a small sample of the new lungs for analysis under the microscope, this is called a biopsy Surplus biopsy material will be retained for further research
    - Ultrasound scans

- We will perform ultrasound scans of your muscles while you recover from surgery. We will focus on the muscles in your chest that are used for breathing, and also on the muscles in your legs. Theses scans will allows us to explore the impact of transplant surgery on your muscle strength. We will perform an ultrasound scan after surgery, and then again before you are discharged from the Intensive Care Unit (ICU). If you need to stay for longer on the ICU, we will perform additional ultrasound scans at weekly intervals until you come off the breathing machine. Ultrasound scans are completely safe, and do not use any form of radiation. To perform an ultrasound scan, you will feel a smooth plastic probe pressed against your skin for a few minutes. We will also apply a small amount of jelly to the skin to help us gain better pictures.
  - Physiological measurements
- You will be connected to many different machines while you are sleeping on the ICU that will allow the clinicians to monitor your vital signs and measure how well your lungs are working. These machines have been a part of everyday clinical care for many years, and will provide your doctors will valuable information on your recovery. As a part of this study, we will assist the ICU team with these measurements and make a note of these recordings. You will only be connected to machines that are routinely available for clinical monitoring.

All of these samples will be collected during the operation, and while your lungs recover from the surgery. Once you have started to breathe on your own without the assistance of the ventilator all sample collection will stop.

What will happen to the samples after the study has been finished?

We will make every effort to ensure that any remaining biological samples that we collect from you do not go to waste. On completion of the study, surplus material will be transferred to a research tissue bank. Leftover samples will be stored for future use in other ethically approved research projects. All retained samples will be anonymised, so it will be impossible for any other researchers to identify you from the samples that you have donated.

Ongoing research may also reveal new areas for study on your stored samples in the years to come. We will continue to use your samples in collaboration with other international experts and industry leaders from across the globe.

Will I be reimbursed for my expenses?

You will not incur and additional expenses by participating in this study, and therefore financial reimbursement is not available.

What will I have to do?

You will not have to undertake any additional study visits as part of this research, you simply need to undergo lung transplantation as planned.

This study will not impose any additional restrictions to your lifestyle following lung transplantation

What are we testing for?

We will use our samples to identify markers of lung injury. We hope that these markers will allow us to develop new tests to monitor patients who are recovering from lung transplantation. We are also trying to find out how your new lungs will produce the protective “surfactant” fluid after the transplantation. This information

will help us to gain a greater understanding of acute lung injury following lung transplantation.

What are the possible disadvantages and risks of taking part?
There are no additional risks from participating in this study. Sample collection is a part of essential care for all patients undergoing lung transplantation. In most instances, we will simply collect surplus samples for research purposes.

What are the side effects of any treatment received when taking part?
You should not experience any additional side effects or discomfort by participating in this study. The choline infusion is totally safe, non-radioactive, non-toxic and without any known side-effects. It is so safe, that the same choline infusion regimen is currently given to critically-ill babies in a similar study on the paediatric ICU.

Are there any benefits to me if I participate in the study?
If you participate in this study, research staff will monitor your recovery from surgery very closely, in addition to all of the routine monitoring that you will receive from the clinical staff. If researchers identify anything of concern during your post-operative recovery, the information will be shared immediately with the clinical team.
The information that we will gather from this study may provide benefits to lung transplant recipients in the future and improve their outcomes following surgery.

What happens when the research study stops?
At the end of the research, your care will continue as usual.

Are there any reasons why I should not participate in the study?
No, this is an observational study. All that we are doing is collecting surplus samples and monitoring your recovery. Participation will have no adverse impact on your recovery.

What if I do not want to carry on with the study?
If you do not want to take part in this study, you will receive standard care as determined by your doctor. Your participation in this study is voluntary and you may withdraw from the study at any time without prejudice to your future medical care. Should you decide to withdraw from the study for any reason, you are asked to contact Dr Thakuria or Dr Reed immediately. Should your participation in the study be terminated, regardless of the reason, you will not suffer any penalties or loss of benefits to which you are otherwise entitled.

What if there is a problem?
The research team wants know if you have any concerns or criticisms on how you have been treated while assisting us with this study. All feedback will be greatly appreciated, and will help to ensure that we continue to conduct our research to the highest possible standards.

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions *(Dr A Reed)*. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure: Patient Advice and Liaison Service (PALS) 01895 826 572

Harm
In the unlikely event that something does go wrong and you are harmed during the research as a result of someone's negligence then you may have grounds for legal action for compensation against The Royal Brompton and Harefield NHS Foundation Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you. NHS indemnity does not offer no-fault compensation i.e. for non-negligent harm, and NHS bodies are unable to agree in advance to pay compensation for non-negligent harm.

Will my taking part in the study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name, address and personal details removed so that you cannot be recognised from it.

Will my doctor be informed of my involvement?
Your Transplant Consultants will be informed that you are participating in the study and kept informed of your medical progress. Information on your recovery will be shared with members of the transplant team if it is felt that it will help with your on-going care. It will not be necessary to routinely inform your GP, as the transplant physicians will take on the responsibility of your care while you recover from surgery.

What will happen to the results of the research study?
Your medical records will be made available for review by the study investigators and regulatory authorities (who periodically check that the studies are being carried out correctly). The information in these records will be kept confidential but on rare occasions the law may require disclosure to third parties. At the end of the project all the research results are gathered together and analysed. The researchers have a professional responsibility to publish their findings, however your identity will not be revealed. Most research is published in the medical press – if you are interested in knowing the overall results of the study, ask the researchers about this. You are entitled to see any results or information about you under the Freedom of Information Act.

Who is organising and funding the research?
The Royal Brompton and Harefield NHS Foundation Trust is sponsoring the research and paying for this research to be carried out. The doctors conducting the research are not being paid for including you in the study.

Who has reviewed the study?
All research in the NHS is looked at by an Independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by London – Riverside Research Ethics Committee and local R&D Offices

Contact for Further Information
If you would like any further information about the study, either now or at time during the course of the study, please ask a member of the Research Team at:

Dr Louit Thakuria

Clinical Research Fellow and study coordinator, or
Dr Anna Reed
Consultant Transplant Physician and Primary Investigator

Department of Transplant Medicine
Harefield Hospital
C/O Alison Leng ext:5070

Thank you for taking the time to consider this study. If you do choose to participate, you will be given a copy of this information sheet to keep and also a copy of the consent form that you will be asked to sign.

7.8 Appendix: Patient Consent Form

Royal Brompton & Harefield NHS
NHS Foundation Trust
Harefield Hospital
Hill End Road
Harefield
Middlesex
UB9 6JH
01895 823737

CONSENT FORM

A PERIOPERATIVE STUDY OF LUNG TRANSPLANTATION AND ACUTE RESPIRATORY FAILURE

Primary Investigator: Dr A Reed - Consultant Transplant Physician

Patient Details
Name:
DOB:
Hospital number:

Please initial box if you agree

1. I confirm that I have read and understand the information sheet dated 22/5/13 (version 1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust. I give permission for these individuals to have access to my records, where it is relevant to my taking part in this research. ☐

4. I agree for my samples to be retained by research staff so that they may be used for other ethically approved studies in the future. Samples may be analysed in collaboration with other international experts in the field. ☐

5. I agree to take part in the above research ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of witness (if patient is unable to sign)	Date	Signature
_____	_____	_____
Name of researcher	Date	Signature

When completed: 1 for patient, 1 for researcher site file, 1 (original) to be kept in medical notes

7.9 Appendix: Imaging data entry form

CXR report form

xx/xxxxxx

Study ID Number	Date Time transplantation completed
1	01/01/2001 00:00

Arrival - Diffuse Interstitial Infiltrates	Arrival - Primary diagnosis

Arrival - Report

24 hours - Diffuse Interstitial Infiltrates	24 hours - Primary diagnosis

24 hours - Report

48 hours - Diffuse Interstitial Infiltrates	48 hours - Primary diagnosis

48 hours - Report

72 hours - Diffuse Interstitial Infiltrates	72 hours - Primary diagnosis

72 Hours - Report

7.10 Appendix: Research Ethics Committee Approval Letter – Ex vivo study

NHS
Health Research Authority
NRES Committee London - Brent
80 London Road
Skipton House
London
SE1 6LH

Telephone: 020 7972 2552

28 February 2014

Dr Anna Reed
Consultant
Royal Brompton and Harefield NHS Trust
Transplant
Harefield Hospital. Hill end Road,
Harefield
UB9 6JH

Dear Dr Reed

Study title:	**Ex vivo transplant platforms for the research of lung injury**
REC reference:	**14/LO/0326**
IRAS project ID:	**128103**

The Research Ethics Committee reviewed the above application at the meeting held on 24 February 2014. Thank you for attending to discuss the application.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager Ms Julie Kidd, Juliekidd@nhs.net.

Ethical opinion

a. The Chair confirmed with the researchers that the NHS consent form is in regular use within the NHS for participants with regard to organ donation, and pointed out the critical section of the form where participants tick yes to donating organs prior to transplantation for research.
b. The Chair asked why consent was required for ten years as this was part of a PhD student study. Dr Thakuria explained that other authors are involved in the study so the research may go on after he finishes his PhD, in addition to it being set as the protocol objective. You added that further grant applications would be made once more data is collected, but at present funding is for three years.

c. The Chair asked if you were only looking for generic approval to harvest organs to use for future research. You said that other authors were conducting studies at Southampton Hospital and their main aim was to establish a platform for on-going ex vivo research.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter		06 February 2014
Investigator CV		
Other: Student CV Dr Louit Thakuria		16 August 2013
Other: CV for Academic Supervisor Anna Reed		18 June 2013
Other: CV for Dr Mark Griffiths		22 June 2013
Other: CV Dr Nandor Marczin		14 August 2013
Participant Consent Form	K	04 November 2013
Protocol	1	05 February 2013
REC application		07 February 2014
Referees or other scientific critique report		06 January 2014
Referees or other scientific critique report		04 February 2014

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol

- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

14/LO/0326	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely
PP



Dr John Keen
Chair

Email: Juliekidd@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Mr Patrik Pettersson, Royal Brompton and Harefield NHS Foundation Trust*

7.11 Appendix: NHS Trust permission for research – Ex vivo study

Royal Brompton & Harefield NHS
NHS Foundation Trust

Royal Brompton & Harefield NHS Foundation Trust
Research Office
Chelsea Wing
Sydney Street
SW3 6NP
www.rbht.nhs.uk

Direct Line: 020 7 351 8121 ext. 2610
Email: a.cooper@rbht.nhs.uk

09 April 2014

Dr Anna Reed
Consultant
Harefield Hospital
Royal Brompton & Harefield NHS Foundation Trust
Sydney Street
London SW3 6NP

Dear Dr Reed,

Project Title: Ex vivo transplant platforms for the research of lung injury
R&D Ref: 2013LS002H
REC Ref: 14/LO/0326
CSP Ref: N/A
Study Sponsor: Royal Brompton & Harefield NHS Foundation Trust

Notification of RB&HFT NHS Management Permission for Research

Thank you for registering the above study with the Research Office. I am pleased to inform you that your study now has NHS Management Permission (previously know as R&D approval) and can commence at Royal Brompton & Harefield NHS Foundation Trust (RB&HFT).

NHS management permission for the above research study is granted on the basis that the study will be conducted as described in the protocol and in accordance with the supporting documentation submitted (listed below), and on the understanding that the study is conducted in accordance with the principles set out in the Research Governance Framework for Health and Social Care (April 2005, 2nd Edition, Department of Health (DoH)) and RB&HFT Policies and procedures.

Documents Reviewed	Version number	Date
Protocol	1	05/02/2013
Patient Information Sheet & Consent Form (Consent - Solid Organ and Tissue Donation - FORM FRM4281/3.1)	K	04/11/2013

Study Amendments

It is the responsibility of the Principal Investigator (PI) to notify the Research Office of **all** study amendments or changes to the status of the projects including study suspension or premature termination.

Safety Reporting

The research Sponsor, or the Chief Investigator (CI) or the local Principal Investigator (PI) at a research site, may take appropriate Urgent Safety Measures in order to protect research participants against any immediate hazard to their health or safety. The Research Office should be notified of such measures within the same time frame of notifying the REC. The notification should include reasons why the measures were taken and the plan for further action.

All **patient related incidents**, including study-related Adverse Events/Reactions (AE/Rs), must be reported internally by the study team in line with the Trust's Adverse Incident Management and Reporting Policy *via* the Quality and Safety Department database **Datix** and marked "**research-related**".

In addition, **all** SAE/Rs must be reported to the study Sponsor and the main REC in line with the approved research protocol.

Audit

Please note the Trust is required to monitor research to ensure compliance with the Research Governance Framework and other legal and regulatory requirements. This responsibility is delegated to the Research Office and will be achieved by random audit of research projects ongoing in the Trust in accordance with RB&HFT Audit SOP.

Yours sincerely

PP Rabia Pebbrie

Dr Angela Cooper
Associate Director of Research

7.12 Appendix: NHSBT Permission for research – Ex vivo study

NHS
Blood and Transplant

Yorkshire Organ Donation Services Team
NHSBT
Bridle Path
Seacroft
Leeds
LS15 7TW

Tel: 0300 1230912
Fax: 0113 2155973

Dr Anna Reed
Consultant Transplant Physician
The Royal Brompton and Harefield NHS Foundation Trust
Harefield Hospital
Hill End Road
Harefield
UB9 6JH

a.reed@rbht.nhs.uk

Dear Dr Reed,

RE Study: **Ex vivo transplant platforms used to explore the pathogenesis of acute lung injury.**

I am writing to thank you for contacting Christian Brailsford, our Research Manager and for submitting your research proposal to the Organ Donation and Transplantation Directorate of NHSBT.

We thoroughly reviewed your proposal at our recent Organ Donor and Transplantation Clinical Audit and Risk Effectiveness meeting and I am pleased to inform you that we are able to lend our support to your study at the current time, pending any other outstanding approvals required. Once you have received confirmation of these approvals, could you send copies to Christian? Could you also confirm with Christian when your study is ready to go live?

Christian will be in contact with you to discuss the operational aspects of your study. This will include how the Specialist Nurses–Organ Donation (SN-ODs) will be able to work with you in facilitating the study and any other logistical requirements and a suggestion around the geographical recruitment area for the study.

If you have any questions or comments about the approval process please direct them to Christian and I wish you well with your study.

yours

Professor James Neuberger
Accociate Medical Director

7.13 Appendix: List of Phospholipids identified by their m/z ratio

mass species	mass species	mass species	mass species	mass species	mass species	mass species
396 LPC10:1a	568 LPC22:6	661 SM14:0a	720 PC16:0a/16:0	771 SM22:1a	829 SM26:0a	916 PC20:4/26:3
398 LPC10:0a	570 LPC22:5	662 PC14:0a/14:1	722 PC22:6/10:0	772 PC18:0a/18:2	830 PC20:4/20:4	918 PC22:5/24:1
410 LPC10:1	572 LPC22:4	663 H2SM14:0a	723 SM18:4	773 SM22:0a	831 H2SM26:0a	920 PC24:0/22:5
412 LPC10:0	574 LPC22:3	664 PC14:0a/14:0	724 PC22:5/10:0	774 PC18:0a/18:1	832 PC22:5/18:2	922 PC26:0/20:4
424 LPC12:1a	576 LPC22:2	666 ox2PC16:0/9:1	725 SM18:3	775 SM22:6	834 PC18:0/22:6	924 PC24:0/22:3
426 LPC12:0a	578 LPC22:1	668 PC18:4/10:1	726 PC14:1/18:3	776 PC18:0a/18:0	836 PC18:0/22:5	926 PC24:0/22:2
438 LPC12:1	580 PC12:0a/10:0	670 PC18:3/10:1	727 SM18:2	777 SM22:5	837 SM26:3	928 PC22:5a/26:3
440 LPC12:0	582 LPC24:6a	672 PC10:0/18:3	728 PC14:1/18:2	778 PC22:5/14:1	838 PC18:0/22:4	930 PC22:5a/26:2
452 LPC14:1a	590 PC12:1/10:1	673 SM14:1	729 SM18:1	779 SM22:4	839 SM26:2	932 PC22:5a/26:1
454 LPC14:0a	592 PC12:0/10:1	674 PC14:1/14:1	730 PC14:0/18:2	780 PC16:0/20:5	840 PC18:0/22:3	934 PC22:5a/26:0
466 LPC14:1	594 PC12:0/10:0	675 SM14:0	731 SM18:0	781 SM22:3	841 SM26:1	936 PC26:0a/22:4
468 LPC14:0	596 LPC24:6	676 PC14:0/14:1	732 PC16:0/16:1	782 PC16:0/20:4	842 PC18:0/22:2	938 PC26:0a/22:3
476 LPC16:3a	603 SM10:1a	677 H2SM14:0	733 H2SM18:0	783 SM22:2	843 SM26:0	940 PC22:6/26:3
478 LPC16:2a	604 PC10:1a/14:1	678 PC14:0/14:0	734 PC16:0/16:0	784 PC18:1/18:2	844 PC20:4a/22:4	942 PC22:5/26:3
480 LPC16:1a	605 SM10:0a	680 PC10:1a/20:5	735 SM20:5a	785 SM22:1	845 H2SM26:0	944 PC22:5/26:2
482 LPC16:0a	606 PC14:1a/10:0	682 PC10:0a/20:5	736 PC14:1a/20:5	786 PC18:0/18:2	846 PC22:3a/20:4	946 PC22:5/26:1
490 LPC16:3	607 H2SM10:0a	683 SM16:3a	737 SM20:4a	787 SM22:0	848 PC20:4a/22:2	948 PC22:5/26:0
492 LPC16:2	608 PC10:0a/14:0	684 PC10:0a/20:4	738 PC20:4a/14:1	788 PC18:0/18:1	850 PC20:3a/22:2	950 PC26:0/22:4
494 LPC16:1	610 ox2PC16:0/5:1	685 SM16:2a	739 SM20:3a	789 SM24:6a	852 PC22:6/20:5	952 PC26:0/22:3
496 LPC16:0	616 LPC26:3a	686 PC12:0a/18:3	740 PC14:0a/20:4	790 PC18:0/18:0	854 PC22:5/20:5	954 PC26:0/22:2
502 LPC18:4a	617 SM10:1	687 SM16:1a	741 SM20:2a	792 PC18:2a/20:4	856 PC22:5/20:4	956 PC24:0/24:1
504 LPC18:3a	618 PC14:1/10:1	688 PC16:1a/14:1	742 PC16:0a/18:3	794 PC18:1a/20:4	858 PC20:4/22:4	958 PC24:0/24:0
506 LPC18:2a	619 SM10:0	689 SM16:0a	743 SM20:1a	796 PC18:0a/20:4	860 PC22:3/20:4	960 PC24:6a/26:0
508 LPC18:1a	620 PC14:1/10:0	690 PC14:0a/16:1	744 PC16:0a/18:2	797 SM24:2a	862 PC20:4/22:2	962 PC24:2a/26:3
510 LPC18:0a	621 H2SM10:0	691 H2SM16:0a	745 SM20:0a	798 PC18:0a/20:3	864 PC20:3/22:2	964 PC26:3a/24:1
516 LPC18:4	622 PC10:0/14:0	692 PC14:0a/16:0	746 PC16:0a/18:1	799 SM24:1a	866 PC22:5a/22:6	966 PC24:0a/26:3
518 LPC18:3	624 ox2PC16:0/6:1	694 PC20:5/10:1	747 H2SM20:0a	800 PC18:0a/20:2	868 PC22:5a/22:5	968 PC26:2a/24:0
520 LPC18:2	628 PC10:1a/16:3	696 PC20:5/10:0	748 PC18:0a/16:0	801 SM24:0a	870 PC22:5a/22:4	970 PC24:0a/26:1
522 LPC18:1	630 LPC26:3	697 SM16:3	749 SM20:5	802 PC20:5/18:3	872 PC22:5a/22:3	972 PC24:0a/26:0
524 LPC18:0	631 SM12:1a	698 PC10:0/20:4	750 PC14:1/20:5	803 SM24:6	874 PC24:0/18:0	974 PC26:0/24:6
528 LPC20:5a	632 PC14:1a/12:1	699 SM16:2	751 SM20:4	804 PC18:2/20:5	876 PC22:3a/22:3	976 PC24:2/26:3
530 LPC20:4a	633 SM12:0a	700 PC12:0/18:3	752 PC14:1/20:4	806 PC16:0/22:6	878 PC22:6/22:6	978 PC24:1/26:3
532 LPC20:3a	634 PC16:0a/10:1	701 SM16:1	753 SM20:3	808 PC18:1/20:4	880 PC22:5/22:6	980 PC24:0/26:3
534 LPC20:2a	635 H2SM12:0a	702 PC14:1/16:1	754 PC14:0/20:4	810 PC18:0/20:4	882 PC22:5/22:5	982 PC24:0/26:2
536 LPC20:1a	636 PC10:0a/16:0	703 SM16:0	755 SM20:2	811 SM24:2	884 PC22:5/22:4	984 PC24:0/26:1
538 LPC20:0a	638 ox2PC16:0/7:1	704 PC14:0/16:1	756 PC16:0/18:3	812 PC18:0/20:3	886 PC22:5/22:3	986 PC24:0/26:0
542 LPC20:5	642 PC16:3/10:1	705 H2SM16:0	757 SM20:1	813 SM24:1	888 PC22:5/22:2	988 PC26:3a/26:3
544 LPC20:4	644 PC16:2/10:1	706 PC16:0/14:0	758 PC16:0/18:2	814 PC18:0/20:2	890 PC22:3/22:3	990 PC26:2a/26:3
546 LPC20:3	645 SM12:1	708 PC22:6a/10:0	759 SM20:0	815 SM24:0	892 PC22:3/22:2	992 PC26:2a/26:2
548 LPC20:2	646 PC14:1/12:1	709 SM18:4a	760 PC16:0/18:1	816 PC20:4a/20:4	894 PC22:2/22:2	994 PC26:2a/26:1
550 LPC20:1	647 SM12:0	710 PC22:5a/10:0	761 SM22:6a	817 H2SM24:0	896 PC18:2/26:1	996 PC26:0a/26:2
552 PC10:0a/10:0	648 PC16:0/10:1	711 SM18:3a	762 PC18:0/16:0	818 PC22:5a/18:2	898 PC26:2/18:0	998 PC26:0a/26:1
554 LPC22:6a	649 H2SM12:0	712 PC14:1a/18:3	763 SM22:5a	820 PC18:1a/22:5	900 PC26:1/18:0	1000 PC26:0a/26:0
556 LPC22:5a	650 PC14:0/12:0	713 SM18:2a	764 PC22:5a/14:1	822 PC18:0a/22:5	902 PC26:0/18:0	1002 PC26:3/26:3
558 LPC22:4a	652 ox2PC16:0/8:1	714 PC14:1a/18:2	765 SM22:4a	823 SM26:3a	904 PC22:5a/24:1	1004 PC26:2/26:3
560 LPC22:3a	654 PC10:1a/18:4	715 SM18:1a	766 PC16:0a/20:5	824 PC18:0a/22:4	906 PC22:5a/24:0	1006 PC26:2/26:2
562 LPC22:2a	656 PC10:1a/18:3	716 PC14:0a/18:2	767 SM22:3a	825 SM26:2a	908 PC26:0a/20:4	1008 PC26:2/26:1
563 SM6:0	658 PC10:0a/18:3	717 SM18:0a	768 PC16:0a/20:4	826 PC20:5/20:5	910 PC24:6/22:4	1010 PC26:0/26:2
564 PC10:0/10:1	659 SM14:1a	718 PC18:0a/14:1	769 SM22:2a	827 SM26:1a	912 PC22:3/24:6	1012 PC26:0/26:1
566 PC10:0/10:0	660 PC14:1a/14:1	719 H2SM18:0a	770 PC18:2a/18:1	828 PC20:5/20:4	914 PC20:5/26:3	

7.14 Appendix: Automated image analysis macro script

```
title = "Louit's lung biopsy IHC image analyser";
msg = "This Macro will analyse all TIFF IHC images within a specified folder. \n Ensure that all images have been saved in the following format: green autofluorescence is saved as channel 2, Red signal for primary antibody is channel 1, and DAPI is channel 3. \n All masks of the tissue area and the primary antibody will be saved into an output folder. \n Press OK to continue";
waitForUser(title, msg);
//open top directory of images
input = getDirectory("Please select the folder where images have been stored");
//select location where images are stored
output = getDirectory("Location for results");
//get list of sub directories
list = getFileList(input);
for (im=0;im<list.length;im++){
full = input + list[im];
open(full);
fn=getTitle();
run("Make Composite");
rename("temp");
run("Smooth");
run("Split Channels");
selectWindow("C2-temp");
run("Multiply...", "value=4");
imageCalculator("Subtract create", "C1-temp","C2-temp");
run("Grays");
rename(fn +" Signal area");
setAutoThreshold("Default dark");
run("Threshold...");
//title = "Set Threshold";
//msg = "set threshold for signal\n press OK to continue";
//waitForUser(title, msg);
run("Analyze Particles...", "size=0-Infinity summarize");
run("Create Mask");
rename(fn + " Mask of Signal");
out = output + "Mask of Signal "+fn;
saveAs("tiff", out);
close();
selectWindow(fn +" Signal area");
close();
run("Merge Channels...", "c2=C2-temp c1=C1-temp c3=C3-temp");
run("Make Composite");
run("RGB Color");
run("8-bit");
rename(fn +" Tissue area");
setAutoThreshold("Huang dark");
run("Threshold...");
run("Analyze Particles...", "size=0-Infinity summarize");
run("Create Mask");
rename(fn + " Mask of total Area ");
out = output + "Mask of total Area "+fn;
saveAs("tiff", out);
close();
selectWindow(fn +" Tissue area");
close();
}
title = "Image analysis is complete";
msg = "All images within the specified input folder have been analysed. \n Ensure that you review all image masks within the output folder, and remember to save all calculated values. \n Press OK to return to FIJI.";
waitForUser(title, msg);
```

7.15 Abbreviations

4-HNE – 4-Hydroxynoneal
AAG – Alkyl Acylglycerol
ABCA1 – Adenosine Triphosphate Binding Cassette A1
ABCA3 – Adenosine Triphosphate Binding Cassette A3
ADP – Adenosine Diphosphate
AECC – American-European Consensus Conference
ARDS – Acute Respiratory Distress Syndrome
AT1 – Type 1 Alveolar Epithelial Cell
AT2 – Type 2 Alveolar Epithelial Cell
AUC – Area Under the Curve
BAL – Bronchoalveolar Lavage
BALF – Bronchoalveolar Lavage Fluid
BBA – Blind Bronchial Aspirates
BHT - Butylated Hydroxytoluene
CDP – Cytidine Diphosphate
CF – Cystic Fibrosis
CLAD – Chronic Lung Allograft Dysfunction
CMP – Cytidine Monophosphate
COPD – Chronic Obstructive Pulmonary Disease
COX – Cyclooxygenase
CTL1 – Choline Transporter-Like Protein 1
D9 – Labelled With 9 Atoms of Deuterium
DAD – Diffuse Alveolar Damage
DAG – Diacylglycerol
DAMP – Damage Associated Molecular Pattern
DAPI – 4',6-Diamidino-2-Phenylindole
DCD – Donation After Cardiac Death
DCM – Dichloromethane
DHA - Docosahexaenoic Acid
DMPA – Dimyristoylglycerophosphate
DMPC – Dimyristoylglycerophosphocholine
DMPE – Dimyristoylglycerophosphoethanolamine
DMPG – Dimyristoylglycerophosphoglycerol
DNA – Deoxyribose Nucleic Acid
DPPC – Dipalmitoylphosphatidylcholine
DTNB – Dithionitrobenzoic Acid
ECMO – Extra Corporeal Membrane Oxygenation
EDTA - Ethylenediaminetetraacetic acid
EPA – Eicosapentaenoic Acid
ER – Endoplasmic Reticulum
ESI-MS/MS – Electrospray Ionisation Tandem Mass Spectrometry
EVLP – Ex Vivo Lung Perfusion
FEV_1 – Forced Expiratory Volume In 1 Second
FIJI – FIJI Is Just ImageJ
FVC – Forced Vital Capacity
HO-1 – Haem Oxygenase-1
HPLC – High Performance Liquid Chromatography
ICU – Intensive Care Unit
IQR – Interquartile Range

IRI – Ischemia-Reperfusion Injury
KS – Kolmogorov – Smirnov
LHPC – Heptadecanoylglycerophosphocholine
LO - Lipoxygenase
LOS – Length Of Stay
LPCAT1 – Lyso PC Acyltransferase-1
M/Z – Mass / Charge Ratio
MALDI-IMS – Matrix-Assisted Laser Desorption Ionization Imaging Mass Spectrometry
MS – Mass Spectrometry
MVB – Microvesicular Bodies
NHS – National Health Service
NHSBT – National Health Service for Blood and Transplantation
NO – Nitric Oxide
NOK – Next of Kin
NOx – NO Metabolites
NRES – National Research Ethics Service
NT – Nitrotyrosine
OCS – Organ Care System
OCT – Optimum Cutting Temperature Compound
P/F Ratio - Pao2/Fio2 Ratio
P184 – A Scan for Precursors of Fragments with A M/Z Of 184
P193 – A Scan for Precursors of Fragments with A M/Z Of 193
PAF - Platelet Activating Factor
PC – Phosphatidylcholine
PDME – Phosphatidyl-Dimethylethanolamine
PE – Phosphatidylethanolamine
PEMT – Phosphatidylethanolamine N-Methyltransferase
PG – Phosphatidylgycerol
PGD – Primary Graft Dysfunction
PI – Phosphatidylinositol
PLA2 – Phospholipase A2
PMME - Phosphatidylmonomethylethanolamine
PP – Pyrophosphate
PS – Phosphatidylserine
PUFA – Polyunsaturated Fatty Acids
RBC – Red Blood Cell
RDS – Respiratory Distress Syndrome
RNS – Reactive Nitrogen Species
ROC – Receiver Operator Characteristics
ROS – Reactive Oxygen Species
SA/LA Ratio – Small Aggregate / Large Aggregate Ratio
S-Adohyc - S-Adenosylhomocysteine
S-Adomet - S-Adenosylmethionine
SM – Sphingomyelin
SP – Surfactant Protein
TDA – Topological Data Analysis
THAM - Tromethamine
TRIS - Tris(Hydroxymethyl)Aminomethane
UNOS – United Network for Organ Sharing
UP-H_2O – Ultrapure Water
VALI – Ventilator Associated Lung Injury

8 References

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